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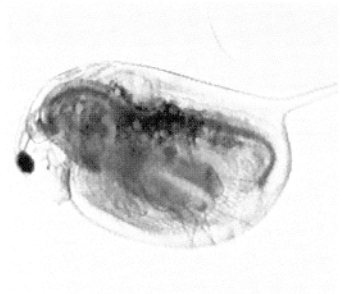

ENVIRONMENTAL
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Biological Test Method: Reference Method for Determining Acute Lethality of Effluents to *Daphnia magna*

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Biological Test Method: Reference Method for Determining Acute Lethality of Effluents to *Daphnia magna*

Method Development and Applications Section
Environmental Technology Centre
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Review Notice

This report has been reviewed by staff of the Environmental Technology Advancement Directorate, Environment Canada, and approved for publication. Mention of trade names or commercial products does not constitute endorsement by Environment Canada. Other products of similar value are available.

Abstract

Explicit standard or reference methods for determining the acute lethal toxicity of effluents to the crustacean "waterflea" Daphnia magna *are described in this report. Specific instructions for performing and reporting acute lethality tests with samples of effluent are given. The guidance provided in the generic methodology report "Acute Lethality Test Using* Daphnia *spp." (EC, 1990a, including its May 1996 Amendments) is built upon herein.*

The present report represents the second edition of Reference Method EPS 1/RM/14, published in July 1990 and amended in May 1996 (EC, 1990b, including its May 1996 Amendments). It supersedes that earlier version, and is to be applied as Environment Canada's current reference method *for determining the acute lethality of effluents to* Daphnia magna.

Methods are given for: (1) a single-concentration test, with full-strength effluent unless otherwise specified; (2) a multi-concentration test to determine the median lethal concentration (LC50); and (3) a test with a reference toxicant. Instructions are included on holding and culturing the daphnid crustaceans, facilities and water supply, handling and storage of samples, preparation of solutions, test conditions, observations to be made, endpoints with methods of calculation, and the use of reference toxicants.

Foreword

This is one of a series of **reference methods** *for measuring and assessing the toxic effect(s) on single species of aquatic or terrestrial organisms, caused by their exposure to samples of test materials or substances under controlled and defined laboratory conditions.*

A **reference method** *is defined herein as a specific biological test method for performing a toxicity test, i.e., a toxicity test method with an explicit set of instructions and conditions which are described precisely in a written document. Unlike other multi-purpose (generic) biological test methods published by Environment Canada, the use of a* **reference method** *is frequently restricted to testing requirements associated with specific regulations.*

Reference methods *are those that have been developed and published by Environment Canada (EC), and are favoured:*

- *for regulatory use in the environmental toxicity laboratories of federal and provincial agencies;*
- *for regulatory testing which is contracted out by Environment Canada or requested from outside agencies or industry;*
- *for incorporation in federal, provincial, or municipal environmental regulations or permits, as a regulatory monitoring requirement; and*
- *as a foundation for the provision of very explicit instructions.*

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Terminology

The following definitions are given in the context of the procedures in this report. Additional definitions in the detailed companion document (EC, 1990a, including its May 1996 Amendments) apply here.

Grammatical Terms

Must is used to express an absolute requirement.
Should is used to state that the specified condition or procedure is recommended and ought to be met if possible.
May is used to mean "is (are) allowed to".
Can is used to mean "is (are) able to".

Technical Terms

Acute means happening within a short period of time, usually taken as ≤48 h for daphnids.

Brood stock is the group of parental animals that is cultured to produce test organisms through reproduction.

Conductivity is a numerical expression of the ability of an aqueous solution to carry an electric current. This ability depends on concentrations of ions in solution, their valence and mobility, and on temperature. Conductivity is normally reported as millisiemens/metre, an SI unit (Système internationales d'unités), or as micromhos/cm (1 mS/m = 10 μmhos/cm).

Control is a treatment in an investigation or study that duplicates all the conditions and factors that might affect the results, except the specific condition that is being studied. In an aquatic toxicity test, the control must duplicate all conditions of the exposure treatment(s), but must contain no test material. The control is used to determine the absence of measurable toxicity due to basic test conditions (e.g., temperature, quality of dilution water, health of test organisms, or effects due to their handling).

Control/dilution water is the water used for diluting the sample of effluent, and for the control test.

Culture as a noun means the mixed age stock of animals that is raised under controlled conditions to produce brood stock through reproduction.

Dechlorinated water is a chlorinated water (usually municipal drinking water) that has been treated to remove chlorine and chlorinated compounds from solution.

Deionized water is water that has been purified to remove ions from solution by passing it through resin columns or a reverse osmosis system.

Dilution water is that which used to dilute a test material, to prepare different concentrations for the toxicity test.

EC50 (median effective concentration) is the concentration of material in water estimated to cause a specified non-lethal or lethal effect in 50% of test organisms. The exposure-time and effect must be specified (e.g., "48-h EC50 for immobilization").

Effluent means any liquid waste (e.g., industrial, municipal) discharged to the aquatic environment.

Ephippium is an egg case that develops inside the postero-dorsal carapace of an adult female daphnid in response to adverse conditions. The eggs within have usually been fertilized through sexual reproduction.

Hardness is the concentration of cations in water that will react with a sodium soap to precipitate an insoluble residue. In general, hardness is a measure of the concentration of calcium and magnesium ions in water, expressed as mg/L calcium carbonate.

Immobility is defined as the inability to swim during 15 seconds following gentle agitation of the test solution, even if antennal movement is still present.

LC50 (median lethal concentration) is the concentration of material (in this case, effluent) in water that is estimated to be lethal to 50% of the test organisms. The LC50 and its 95% confidence limits are usually derived by statistical analysis of percent mortalities in several test concentrations, after a fixed period of exposure. The duration of exposure must be specified (e.g., 48-h LC50).

Lethal means causing death by direct action. Death of daphnids is defined here as the cessation of all visible signs of movement or activity, including second antennae, abdominal legs, and heartbeat as observed through a microscope.

Lux is a unit of illumination based on units per square metre. One lux = 0.0929 foot-candles and one foot-candle = 10.76 lux.

Neonate is a daphnid newly released from the mother ("born") (i.e., a first-instar daphnid, 24 hours old or less).

pH is the negative logarithm of the activity of hydrogen ions in gram equivalents per litre. The pH value expresses the degree or intensity of both acidic and alkaline reactions on a scale from 0 to 14, with 7 representing neutrality, numbers less then 7 signifying increasingly greater acidic reactions, and numbers greater than 7 indicating increasingly basic or alkaline reactions.

Photoperiod is the duration of illumination and darkness within a 24-h day.

Pretreatment means, in this report, treatment of a sample or dilution thereof, before exposure of daphnids.

Reconstituted water is high purity deionized or glass-distilled water to which reagent-grade chemicals have been added. The resultant synthetic fresh water should be free from contaminants and have the desired pH, alkalinity, and hardness characteristics.

Reference method refers to a specific biological test method for performing a toxicity test, i.e., a toxicity test method with an explicit set of instructions and conditions which are described precisely in a written document. Unlike other multipurpose (generic) biological test methods published by Environment Canada, the use of a *reference method* is frequently restricted to testing requirements associated with specific regulations; or testing to access whether there has been a violation of the General Provisions of the Canadian Fisheries Act.

Reference toxicant is a standard chemical used to measure the sensitivity of the test organisms to establish confidence in the toxicity data obtained for a test material. In most instances, a toxicity test with a reference toxicant is performed to assess the sensitivity of the organisms at the time the test material is evaluated, and the precision of results obtained by the laboratory for that chemical.

Salinity means the total amount of solid substance, in grams, dissolved in 1 kg of water. It is determined after all carbonates have been converted to oxides, all bromide and iodide have been replaced by chloride, and all organic matter has been oxidized. Salinity can also be measured directly using a salinity/conductivity meter or other means (APHA *et al.*, 1998). It is usually reported in grams per kilogram or parts per thousand (‰).

Sublethal means detrimental to the daphnid, but below the level that directly causes death within the test period.

Toxicity is the inherent potential or capacity of a material to cause adverse effect(s) on daphnids or other living organisms. The effect(s) could be lethal or sublethal.

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The following persons contributed to the May 1996 Amendments to the first edition of this *reference method* (EC, 1990b), which are now incorporated (with changes, as deemed appropriate) in this second edition: B. Bayer (Manitoba Environment, Winnipeg); K. Doe (Environmental Conservation Service [ECS], Moncton); G. Elliott (EPS, Edmonton); P. Jackman (ECS, St. John's); J. Miller (EPS, Ottawa); D. Poirier (Ontario Ministry of Environment, Rexdale); G. van Aggelen (ECS, North Vancouver); R. Watts (ECS, North Vancouver); and G. Westlake (Ontario Ministry of Environment, Rexdale).

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Introduction

This report replaces the first edition of Environment Canada's *reference method* EPS 1/RM/14 (EC, 1990b), which was published in July 1990 and amended in May 1996. Testing instructions, guidance, and wording in this (second) edition of EPS 1/RM/14 remain the same as in the amended first edition except for the changes or additions included here, which were considered by Environment Canada to be necessary or prudent improvements.

The procedures for an acute lethality test with *Daphnia magna*, as specified by Canadian governments involved in pollution monitoring and control of industrial or municipal effluents, are given in this report. The procedures have their roots in methods for testing fish, published earlier by Environment Canada (e.g., EPS, 1980). This *reference method* should be used in conjunction with a more extensive report which gives supporting rationale and additional details (EC, 1990a, including its 1996 Amendments).

Many components of procedures in this report are similar to Canadian provincial methods (B.C. MOE, 1988; Poirier *et al.,* 1988; BNQ, 1990), United States methods (USEPA, 1982; 1985a; 1985b; Plotkin and Ram, 1983; ASTM, 1984; APHA *et al.,* 1998; Greene *et al.,* 1988), or international techniques (The Netherlands, 1980; BHSC, 1982; OECD, 1981; ISO, 1982; IGATG, 1986). The contribution of the above-mentioned methods to all parts of the present report is acknowledged, and they are recommended as sources of supporting information. Procedures stipulated in this *reference method* should, however, be taken as the definitive ones for regulatory purposes.

The "waterflea", *Daphnia magna*, a small freshwater crustacean of the Order Cladocera is to be used as the test organism when performing this *reference method*. This daphnid is found in ponds and lakes of North America including western Canada, and is often an important component of aquatic communities. Daphnids are sensitive to a broad range of aquatic contaminants, and are used in toxicity tests internationally. They have the advantages of small size, short life cycles (which allow rapid tests), and relative ease of culture in laboratories.

Three basic procedures are described. One uses a single concentration of effluent (full strength unless otherwise specified) and a control, as would be suitable for a pass/fail test. A second procedure estimates the median lethal concentration (LC50), or if necessary, the median effective concentration for immobilization (EC50) (i.e., it determines the degree of toxicity using several concentrations of effluent including full strength). A third procedure is a multi-concentration test with a reference toxicant, to assess the sensitivity of the test organisms to a standard toxicant and the precision of the data produced by the laboratory for that chemical.

This test is to be used with effluents containing fresh water or having a salinity of ≤10‰, defined as conductivity ≤1550 mS/m at a temperature of 20°C (1 mS/m = 10 micromhos/cm). Saline (>10‰) effluents discharging into fresh water should also be tested using this reference method and *D. magna* cultured in fresh water. Saline (>10‰) effluents discharging directly to estuarine or marine receiving waters should be tested with a species authorized by the regional Environment Canada laboratory (see Appendix) and acclimated to seawater of similar salinity to that of the effluent.

Culturing Organisms

Specific requirements for culturing daphnids are given here, with further explanation in the more detailed document (EC, 1990a).

Culturing techniques vary (USEPA, 1982; 1985a; ASTM, 1984; Greene *et al.,* 1988; Poirier *et al.,* 1988) and the success of an approach may be judged by the criteria at the end of Section 2.1.

Neonate *Daphnia magna* must be used as test organisms.

2.1 Maintaining Cultures

Glass aquaria, wide-mouthed jars, or beakers are recommended as culture vessels, each loosely covered to exclude dust and reduce evaporation. Vessels and all accessories contacting the organisms, water, or culture media must be made of nontoxic materials (e.g., glass, stainless steel, Nalgene™ , porcelain, polyethylene).

Water in each culture vessel should be almost completely replaced, at least weekly. The population of daphnids should be thinned at this time to twenty or fewer animals per litre.

Adult daphnids can be handled by gentle pouring from one container to another or by careful pipetting or siphoning. Neonates are susceptible to air entrapment, and minimal handling should be practiced, with a disposable glass pipette cut off and fire-polished to provide a 5-mm opening. The tip of the pipette should be under the surface when daphnids are released. Transfers should be quick, with minimal carryover of "old" water to the new container.

At least one species of green alga must be used for feeding daphnids, although it is strongly recommended that a mixture of at least two species be used. One or more species of green alga with a diatom is beneficial; algae must be grown in a suitable culture medium (EC, 1990a). Additionally, a supplement such as yeast, Cerophyll™, and trout chow (see EC, 1990a; Appendix C) may be used.

The health of the daphnid brood stock is judged by the following health criteria which must be met if neonates from the brood stock are to be used in toxicity tests.

- ephippia must not be present in the brood stock;
- age at delivery of first brood must be ≤ 12 days;
- females 2 to 5 weeks old must deliver an average of 15 or more neonates per brood; and
- no more than 25 % of brood stock can die during the seven-day period before a test is initiated.

To monitor these health indices, an individual daphnid, or group of daphnids, representative of the brood stock, may be placed in a separate glass container(s) and maintained under the same conditions as the brood stock. This "health monitoring" daphnid(s) must be:

- the same age as the brood stock and of known age;
- a member of the same brood or broods used to create the brood stock;
- cultured under similar loading conditions and feeding rates as the brood stock; and

- maintained for as long as the brood stock is being used to supply neonates as test organisms.

Neonates used in a specific test must be traceable back to a specific health monitoring daphnid(s) and to the brood stock it represents. If there is no traceability, it cannot be assured that the health criteria pertaining to the specific test organisms used in that test were met.

The results of the negative test control and the findings of a test with a reference toxicant (Section 7) give further indication of suitability of the daphnid culture for testing.

2.2 Water

Water to be used for culturing daphnids and as control/dilution water may be natural or reconstituted. For natural water, uncontaminated groundwater, surface water, or dechlorinated municipal drinking water may be used provided that water hardness is within the 80 to 250 mg/L range. For reconstituted water, moderately hard water (i.e., hardness 80 to 100 mg/L) is to be used.

If dechlorinated municipal drinking water is to be used as culture/control/dilution water, it must be free of any harmful concentration of chlorine or chlorinated compounds upon daphnid exposure. The target value for total residual chlorine in cultures, and for that in control/dilution water within test vessels is $\leq$0.002 mg/L (see EC, 1990a).

If moderately hard (80 to 100 mg/L) reconstituted water is required, it may be prepared using any proven formulae that provide cultures of daphnids which meet the health criteria previously specified (see EC, 1990a). The pH of this reconstituted water must be in the 7.0 to 8.0 range; values of 7.4 to 7.8 are common (EC, 1990a). The addition of selenium and Vitamin B_{12} to reconstituted water used for culturing organisms is necessary (see EC, 1990a). These nutrients may also be added to natural water, but only if they are required to assure that the health criteria are met.

The culture/control/dilution water supply must consistently support good survival, growth, and reproduction of daphnids. Chemical quality of the laboratory's water supply should be measured as often as necessary to document variation. This should include at least hardness, pH, conductivity and dissolved oxygen as well as total dissolved gases, as appropriate. The water must not be supersaturated with gases. Any supersaturation should be remedied (see EC, 1990a). A more detailed chemical analysis could be carried out periodically on other items listed by EC (1990a).

2.3 Physicochemical Conditions

Light intensity should be within the range of 400 to 800 lux at the water surface, and the spectrum should be skewed towards the blue end (colour rendering index $\geq$90). Cool white fluorescent lights are suitable. Photoperiod must be a 16 ± 1 h light:8 ± 1 h dark cycle.

Water temperature in the cultures must be 20 ± 2°C for at least two weeks before animals are used in tests.

Dissolved oxygen in cultures should not fall below 60% of air saturation; this should be maintained by gentle aeration of each culture if necessary. The compressed air supplied to the culture should be filtered and free of oil, dust, and odours.

The pH of water used for culturing and as control/dilution water should be within the 6.0 to 8.5 range (preferably 6.5 to 8.5).

Temperature, oxygen, pH and presence or absence of ephippia should be monitored in each brood stock culture vessel, preferably daily.

Weekly or more frequent monitoring of culture/control/dilution water is also recommended for water hardness, and for total residual chlorine if municipal drinking water is used as the water source.

Facilities

Tests must be performed in a separate laboratory with a section walled or curtained off for control of lighting, or in an environmental chamber. This facility must be isolated from any physical disturbance and dust and fumes should be minimized.

Test vessels must be large enough that the loading density does not exceed one daphnid per 15 mL of solution. Borosilicate glass beakers (150- or 250-mL) or glass screw-top test tubes work well; bags made of high quality, inert, nontoxic plastic (e.g., polyethylene or polypropylene; Whirl-Pak™) may also be used but must not be re-used. The vessels and all other material and equipment that might contact the test solutions or control/dilution water must not contain leachable substances, nor should they sorb toxicants from the test solution. All containers and apparatus must be thoroughly cleaned and rinsed with control/dilution water or de-ionized water in accordance with good laboratory procedures.

General Procedure for Determining Acute Lethality of Effluent

4.1 Sample Labelling, Transport, and Storage

Sample-volume requirements depend on numbers of daphnids exposed to each test solution, loading-density requirements, test concentrations, and the use of replicates. Sample volumes of 2 L or more (depending on chemical-analytical requirements) are normally required for either single-concentration tests or determination of an LC50.

Containers for storage and transportation of effluent samples must be of nontoxic material (e.g., glass, polyethylene, or polypropylene). The containers must be new or thoroughly cleaned and dried, and should be rinsed with uncontaminated water, then with the sample to be collected. They should be filled with sample to exclude air and then sealed (e.g., using a snap-on lid if the sample container is a pail). Labelling must include at least sample type, source, date and time of collection, and name of sampler(s).

Samples must be kept from freezing. During transport, samples should be kept dark, with a temperature from 1 to 8°C if more than two days are spent in transit. Upon the receipt of sample(s) at the laboratory, the temperature of the effluent in each sample should be measured and recorded. That portion of each sample to be used in the toxicity test must be adjusted to 20 ± 2°C, before the toxicity test can be started.

To enable the toxicity test to be started on the day that the sample is received in the laboratory, temperature adjustment of the effluent sample(s) may be done quickly (see Section 4.3). Alternatively, the laboratory may choose to store the sample in the dark at 4 ± 2°C for a brief period (e.g., over the weekend, if the sample arrived on a Friday afternoon). Using this option, the sample must be stored in full, sealed containers which are held in the dark within a refrigerated facility. A third option is to hold the sample overnight within a facility adjusted to the test temperature (i.e., 20 ± 2°C), in which instance the test must be started the next day. If a sample is warmed or cooled at 20 ± 2°C overnight, it must be kept in one or more full, sealed containers during that time.

Testing of samples should commence as soon as possible after collection. The test should begin within three days and must commence no later than five days after termination of sampling. The contents of each sample container must be agitated thoroughly just before pouring aliquots for preparing solutions. Sub-samples (i.e., aliquots of a sample divided between two or more containers) must be combined.

4.2 Test Conditions

This is a 48-h static test (i.e., there is no replacement of solutions). Density of daphnids in solutions must not exceed one animal per 15 mL. Daphnids are not fed during the test. The test is not valid if mortality in the control exceeds 10% or if >10% of control organisms show overt, stressed behaviour (e.g., immobility).

The test must be conducted at 20 ± 2°C and with a solution hardness of ≥25 mg/L. During the test, solutions are not aerated nor is pH adjusted. Lighting must be the same as that defined for culturing in Section 2.3. Photoperiod (a light : dark cycle of 16 ± 1 h : 8 ± 1 h) must coincide with the timing which prevailed during culturing.

The test must be conducted without adjustment of sample or solution pH. However, if it is desired to understand the extent to which extremes in solution or sample pH (e.g., outside the range of 6.0 to 8.5) may contribute to acute lethality, a parallel (pH-adjusted) test may be used. If both pH-adjusted and non-adjusted tests are run, definitive results should be those derived from the non-adjusted test. Rationale and procedural details regarding pH adjustment of effluent samples are provided in Environment Canada (1990a). Adjustment of pH is also one of a number of "Toxicity Identification Evaluation" (TIE) techniques for characterizing the cause of sample toxicity (USEPA, 1991).

4.3 Preparing Test Solutions

Adjustment of the effluent sample and control/dilution water to 20 ± 2°C must be done if the temperature is outside that range. The sample may be cooled using a cold-water bath or immersion cooler made of nontoxic material, or warmed using a hot-water bath. Samples or test solutions must not be heated by immersion heaters.

As it is possible that the effluent sample might be very low in hardness (i.e., <25 mg/L), sample conductivity must be measured after warming to room temperature but before any dilutions are made. If sample conductivity is ≤100 µmhos/cm, sample hardness must be measured before starting the test. If this analysis confirms that sample hardness is <25 mg/L, the sample must be adjusted to a hardness of 25 to 30 mg/L by adding sodium bicarbonate ($NaHCO_3$), calcium sulphate ($CaSO_4 \cdot 2H_2O$), magnesium sulphate ($MgSO_4$), and potassium chloride (KCl), in the ratio 1.6 : 1.0 : 1.0 : 0.0667 (see EC, 1990a; Table 2 - formula for reconstituted water). For each desired incremental increase of 5 mg/L of hardness, add the following to each litre of effluent: 6.0 mg of $NaHCO_3$; 3.75 mg of $CaSO_4 \cdot 2H_2O$; 3.75 mg of $MgSO_4$; and 0.25 mg of KCl.

Control/dilution water is to be the same as that used for culturing (Section 2.2). If the temperature of this water is adjusted upwards, supersaturation with gases must be avoided. It must have an oxygen content within the range 90 to 100% air saturation, achieved if necessary by vigorous aeration with oil-free compressed air passed through air stones or glass diffusers.

Each test vessel including the control(s) must contain an identical volume of solution during the test. Test vessels should be rinsed with control/dilution water before use. Test solutions are made up just before their use, in sufficient volumes for convenient sampling and initial chemical measurements. Solutions must be well mixed with a glass rod, Teflon™ stir bar, or other non-reactive device. All test beakers or test tubes, measurement devices, stirring equipment, and daphnid-transfer vessels must be thoroughly cleaned and rinsed in accordance with standard operational procedures.

Dissolved oxygen must be measured in the sample just before beginning the test. If dissolved oxygen is between 40 % and 100%, the sample must not be pre-aerated. If (and only if) oxygen in the sample is <40% or >100% of air saturation, then the sample must be pre-aerated (i.e., aerated before daphnid exposure) for a period not exceeding 30 minutes, at a rate within the range of 25 to 50 mL/min·L. Any pre-aeration of sample should be provided by bubbling compressed air through a clean silica-glass air diffuser or disposable glass pipette (EC, 1990a). Aeration of the sample is then to be stopped, test solutions prepared, organisms introduced, and the test initiated immediately, regardless of whether 40 to 100% saturation was achieved in the sample. Therefore, during the 48-hour duration of the test, there is no aeration of test solutions or control.

It is recommended that test vessels be uncovered or loosely covered. If volatiles are suspected, and it is desirable to understand the effect of

these compounds on toxicity, a parallel test should be conducted concurrently using capped vessels. The duplicate set of capped vessels must be closed and there should be little air space above the test solution. If both uncapped and capped tests are run in parallel, definitive results should be those derived from the uncapped test.

4.4 Beginning the Test

One or more control solutions must be prepared and included as part of each test conducted on each sample. The multiple use of a control solution and its daphnids for more than one toxicity test and/or more than one effluent sample is unacceptable.

Each test vessel must be clearly coded or labelled as to concentration, date, and time of start. If a multi-concentration test is being performed (Section 6), the concentrations should be positioned at random.

Neonates are to be used for the test. Adult females bearing embryos in their brood pouches can be removed from the brood stock which has met the required health criteria, less than 24 h before the test. These females can be transferred to clean glass beakers (400 mL to 1L) containing control/dilution water and an inoculum of prepared food at the concentration used in culturing. Water must be adjusted to 20 ± 2°C and 90 to 100% air saturation with dissolved oxygen before adults are added. Stocking density in the beakers should be approximately 20 adults/L or less. Alternatively, neonates can be removed from the brood stock the day before the test. Neonates found in the brood stock on the following day are less than 24 hours old and therefore may be used for the toxicity test.

Experience has shown that using adult females 2 to 5 weeks of age, avoids the inappropriate use of both young and senescent females. Separate culture vessels may be stocked periodically with neonates, which at age 2 to 5 weeks will have become neonate-producing adults with offspring available for testing.

To begin the test, equal numbers of neonates must be introduced into each concentration including each control solution. At least 10 daphnids per concentration are required for an LC50 test and at least 30 daphnids per concentration, divided among at least three replicates are required for a single concentration test. Loading density must not exceed one individual per 15 mL. Neonates should be handled as described in Section 2.1; floating and injured ones should be replaced immediately after transfer.

Besides positioning the test vessels at random within the testing facility, the order of adding daphnids to each test solution should also be at random. One or two daphnids should be introduced sequentially to each test solution, including the control solution(s), until 10 daphnids have been placed in each.

4.5 Observations and Measurements

Colour, turbidity, odour, and floating or settling solids in the sample should be noted at the start of the test. The appearance of test solutions should also be noted, and any obvious changes during the test should be recorded.

Measurements of dissolved oxygen, pH, and temperature must be made in each test solution including the control(s), at the start and end of the test as a minimum. Final measurements should be done after biological observations are complete. Conductivity of each test solution must be measured at the start of the test as a minimum.

The hardness of the controls and 100% effluent solutions must be measured at the start of the test. These initial measurements are to be made

on the larger volumes of solutions made up in beakers, after adjustments have been made and just before they are used to fill the actual test vessels (Section 4.3).

General observations on narcotization, unusual movement, or other behaviour should be made when starting the exposure. Numbers of dead daphnids in each vessel are recorded at the end of the test (48 h). Death is defined here as the lack of all movement of the antennae, other appendages and heart, as observed through a dissecting microscope.

With some narcotic toxicants, daphnids may become completely immobile and the heart rate may slow to 1 to 2 beats/min. In such a case, beating of the heart becomes the final criterion of death. If such narcosis is suspected, but careful observation of the heart cannot be made, numbers of daphnids immobilized at 48 h should be recorded. Immobilization is defined here as the inability to swim during a 15-second time period following gentle agitation of the solution, even if the antennae are still moving.

Observations of daphnids are aided by a black background or by illumination from the side or below. Opaque solutions and corresponding control solutions may be poured into Petri™ plates for observation, but only at the end of the test (EC, 1990a). If necessary, solutions may be poured through netting at the end of the test, and the daphnids re-suspended in water for observation.

Procedure for a Single-concentration Test to Determine the Mortality Rate at 48 Hours

All conditions, procedures, and facilities specified in Sections 1, 2, 3, 4, 7, and 8 apply to this procedure.

This procedure uses one concentration of effluent, 100 % unless otherwise specified, plus a control. The use of replicate solutions (i.e., minimum of three replicates and 30 daphnids for the 100 % concentration and for the control solutions) is required for this test, to provide greater confidence in the test results and their interpretation.

The endpoint for this test is percentage mortality at 48 h, reported for each replicate of effluent and control ($n \geq 10$). If immobilization is the sole biological effect observed (Section 4.5), the test endpoint is percentage of immobilization at 48 h, reported for each replicate.

For the purpose of providing a single value representing percent mortality (and/or percent immobilization) for each of the effluent and control treatments, the mortality (and/or immobility) data for the three replicates are to be combined. The test is invalidated if >10 % of the control daphnids (combined data) exhibit overt stressed behaviour (e.g., immobility) and/or mortality, or if >2 of the control animals in any single test vessel exhibit either of these responses.

Procedure for a Multi-Concentration Test to Determine the 48-h LC50

All conditions, procedures, and facilities specified in Sections 1, 2, 3, 4, 7, and 8 apply to this procedure.

At least five concentrations of effluent plus a control (dilution water only) must be used in tests to estimate an LC50. The highest concentration must be full-strength (100 %) effluent, and each successive concentration must have at least 50 % of the strength of the next higher one. A geometric (logarithmic) series is beneficial (e.g., percentage concentrations such as: 100, 50, 25, 12.5, and 6.3). Concentrations may be based on other proportions or on standard dilution series (see EC, 1990a; Appendix D). At least ten daphnids must be exposed to each test concentration, including the undiluted (100 %) concentration and the control.

Since this LC50 test must include full-strength (100 %) effluent as the highest concentration, the single-concentration endpoint of percent mortality in 100 % effluent at 48 h (see Section 5) can also be determined from the results of this test.

For these multi-concentration tests, calculate the 48-h LC50 and its 95 % confidence limits, and report them, along with the method of calculation. Computer programs for calculating LC50 and confidence limits are available (EC, 1990a) and should be used. A recommended program is available from Environment Canada (address in Appendix) for copying onto a user-supplied diskette, through the courtesy of C.E. Stephan (Stephan, 1977). A check of the computer-derived LC50 should be made by examining a plot on logarithmic-probability scales, of percent mortalities at 48 h for the various concentrations (see EC, 1990a).

If death cannot be confirmed, a 48-h EC50 for immobilization (Section 4.5) should be reported rather than an LC50. The statistical procedure for estimating an EC50 and its confidence limits is the same as for an LC50 except for the different criterion of effect.

Replicates of each concentration may be used but are not required. If replicates are used, their data are combined for calculating the LC50. The precision of the estimate of LC50 increases with the number of organisms exposed to each test concentration, although the accuracy of this estimate is not necessarily improved.

The test is invalidated if >10% of the control daphnids (combined data if replicates are used) exhibit overt stressed behaviour (e.g., immobility) and/or mortality, or if >2 of the control animals in any single test vessel exhibit either of these responses.

Procedure for Tests with a Reference Toxicant

A reference toxicant must be used to assess the relative sensitivity of the population of daphnids used in the toxicity test and the precision and reliability of data produced by the laboratory personnel for that reference toxicant under standardized test conditions. The selected reference chemical(s) must be tested within 14 days of when an effluent is tested. The procedures and conditions to be followed are to be identical to those in Section 4 and as described in Environment Canada (1990a, c), except that a reference chemical is measured out and tested, instead of an effluent. The culture/control/dilution water used routinely in effluent tests (Section 2.2) must also be used for the reference toxicant.

One or more of sodium chloride (NaCl), potassium dichromate ($K_2Cr_2O_7$), or zinc sulphate ($ZnSO_4 \cdot 7H_2O$) are recommended for use as reference toxicants. The 48-h LC50 should be determined for the reference toxicant(s) used and expressed as mg/L based on NaCl, chromium, or zinc (EC, 1990a). Stock solutions should be made up on the day of use or stored in the dark. Potassium dichromate should be stored in glass-stoppered bottles, and zinc should be stored at pH 3 to 4.

Concentrations of reference toxicant in all stock solutions should be measured chemically using appropriate methods (APHA *et al.,* 1998). Upon preparation of the test solutions, aliquots are to be taken from the control, low, middle, and high concentrations (as a minimum), and analyzed directly or stored for future analysis should the LC50 be atypical (i.e., outside warning limits). If stored, sample aliquots must be held in the dark at 4 ± 2°C. Zinc solutions should be acidified before storage. Stored aliquots requiring chemical measurement should be analyzed promptly upon completion of the toxicity test.

It is desirable, but not required, to measure concentrations in the same solutions at the end of the test after completing biological observations. Calculations of LC50 should be based on measured concentrations if they are appreciably different (i.e., ≥20%) from nominal ones.

Once sufficient data are available (EC 1990c), a warning chart which plots values for LC50 must be prepared and continually updated for each reference toxicant used. The warning chart should plot logarithm of concentration on the vertical axis against date of the test or test number on the horizontal axis. Each new LC50 for the reference toxicant should be compared with the established warning limits of the chart; the LC50 is acceptable if it falls within the warning limits. All calculations of mean and standard deviation must be made on the basis of log(LC50). The mean of log(LC50), together with its upper and lower warning limits (±2 SD) as calculated by using the available values of log(LC50), are recalculated with each successive LC50 until the statistics stabilize (USEPA, 1985a; EC, 1990c).

The warning chart may be constructed by simply plotting mean and ±2 SD as the logarithms, or if desired, by converting them to arithmetic values and plotting LC50 and ±2 SD on a logarithmic scale of concentration. If a particular LC50 fell outside the warning limits, the sensitivity of the organisms and the performance and precision of the test would be suspect. A check of all culturing and test conditions is required under these circumstances. Depending on the findings, further acclimation and re-evaluation of the culture with one or more reference

toxicants should be undertaken, or a new culture of daphnids should be established for use in subsequent toxicity tests with effluent and reference toxicant(s).

Reporting Requirements

The following is a summary of reporting and record-keeping requirements associated with this *reference method*. Further details or explanation can be found within previous sections of this method.

Unless otherwise specified by Environment Canada, all items listed in Section 8.1 must be reported to Environment Canada for each toxicity test that is initiated. The information is to be provided in accordance with pertinent regulations, and in a manner and format specified by Environment Canada* (i.e., manual or electronic, transmission mode, form and content).

Information additional to that in Section 8.1, such as that required by or distinctive to a regulation, or information that is necessary to clarify reporting and data assessment, may also be specified by Environment Canada.

Unless otherwise specified by Environment Canada, those items listed under Section 8.2 must be recorded and held on file for a period of five years. This information is to be provided as and when requested by Environment Canada. It will be required on a less frequent basis, such as during an audit or investigation.

8.1 Data to be Reported

8.1.1 Effluent

- name and location of operation generating the effluent;
- date and time of sampling;
- type of sample (e.g., "whole effluent from plant", "final mill effluent", "discharge from emergency spill lagoon", "leachate");
- brief description of sampling point;
- sampling method (e.g., "grab", "batch", "24-h composite with sub-samples at 1-h intervals"); and
- person collecting sample.

8.1.2 Test Facilities and Conditions

- test type and method (e.g., "single-concentration test", method as specified in the second (December 2000) edition of EPS 1/RM/14);
- indication of any deviation from one or more "must" requirements delineated in Sections 2 to 7 of this report, including details as to the deviation;
- name and city of testing laboratory;
- species of test organism;
- date and time for start of definitive test;
- person(s) performing the test and verifying the results;
- the pH, temperature, dissolved oxygen, and conductivity of unadjusted, undiluted effluent, just before preparing test solutions;
- confirmation that no adjustment of sample or solution pH occurred; indication of procedure used for any pH adjustment if both pH-adjusted and non-adjusted tests were run (see Section 4.2);

* Contact an office listed in the Appendix for details.

- indication of any adjustment of hardness of effluent sample and, if adjusted, measurements of sample hardness before and after adjustment;

- indication of any aeration of sample (rate, time) before introduction of daphnids;

- concentrations and volumes tested, including controls, and indication of any replication;

- measurements of dissolved oxygen, pH, and temperature determined for each test solution including control(s) at the beginning and end of the test, as well as conductivity of each test solution at the start of the test; hardness measurements on 100% effluent and control solutions at start of test;

- for brood stocks: estimates of time to first brood; average number of neonates per brood (i.e., second and all subsequent broods); and percent mortality during the seven-day period prior to a test; and

- number of neonates per test vessel and millilitres of solution per daphnid.

8.1.3 Results

- number of dead and/or immobile daphnids in each test solution including the control(s), at 48 h;

- for single-concentration test, number of daphnids dead (or immobilized if death cannot be confirmed) in each of three replicate effluent solutions and in each of three replicate control solutions ($n \geq 10$) at end of test; mean value representing percent dead (or percent immobilized) for each of the effluent and control solutions;

- estimate of 48-h LC50 and 95% confidence limits in multi-concentration tests, if statistically achievable; 48-h EC50 for immobilization and 95% confidence limits, if determined; indication of statistical method (e.g., log-probit, moving average) on which results are based; and

- most recent 48-h LC50 (with 95% confidence limits) for reference toxicant test(s); reference chemical(s); date test initiated; historic geometric mean LC50 and warning limits ($\pm$ 2 SD).

*8.2 Data to be Held on File**

8.2.1 Effluent

- all information (e.g., code, sample description, date/time of sampling) affixed to label(s) on sample container(s);

- volume of sample;

- transport and storage conditions (times; temperature on receipt at laboratory and during storage; indication if sample frozen or partially frozen on arrival);

- appearance and other properties (observations on colour, turbidity, odour, floating or settleable material);

- colour change, precipitation, flocculation, release of volatiles or other changes when making up test solution(s); and

- procedures and results for any chemical analyses performed on the effluent, if available (e.g., suspended solids content, metal concentrations, resin acid content).

8.2.2 Test Facilities and Conditions

- address of testing laboratory;

- description of culturing and test facilities including general layout of each and means of isolation;

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* To be stored for a five-year period at the testing facility and/or the offices of the discharger. Some of this information may be common to a series of tests, and recorded and held on file as a general report.

- source of test species and date obtained;
- usual culturing conditions (containers, location, lighting, temperatures, aeration, frequency of water replacement, maximum density, food type, ration and frequency of feeding);
- brief history of test-specific conditions and procedures for culturing and handling daphnids if different from usual practice;
- source of culture/control/dilution water; usual pretreatment, if any (e.g., procedures for adjustment of temperature, aeration, dechlorination); procedures for preparing and storing reconstituted water, if used; types and concentrations of any nutrients (e.g., selenium, Vitamin B_{12}) added;
- quality (mean and range values) of culture/control/dilution water; to include hardness, pH, conductivity, dissolved oxygen content, and total residual chlorine (if dechlorinated municipal drinking water); preferably also total dissolved gases, alkalinity, solids, organic carbon, colour, mineral ions, metals, ammonia, nitrite, and organophosphorus pesticides;
- systems to regulate light and temperature;
- light source, photoperiod, and past measures of intensity at surface of culture and test vessels;
- description of test vessels (size, shape, and material), covers, and routine cleaning and rinsing procedures for each;
- procedures used to randomize the introduction of daphnids to test vessels;
- method of obtaining neonates for tests;
- method used to monitor health criteria of daphnid brood stocks;
- characteristics of health monitoring daphnid(s) relative to brood stock (i.e., same age as brood stock and of known age; member of the same brood or brood(s) used to create the brood stock; cultured under similar loading conditions; maintained for as long as the brood stock is being used to supply neonates);
- appearance of solutions; any changes during test;
- test concentrations of reference toxicant(s), both nominal and measured; indication of data set used to estimate LC50; and
- any measurements of water quality in test solutions not included in data reported (Section 8.1.2).

8.2.3 Results

- any observations of numbers of daphnid-mortalities (or numbers of immobilized if death cannot be confirmed) not included in data reported (e.g., at 24 h);
- observations of daphnid behaviour and appearance noted and recorded for each test solution during or upon completion of the test;
- any manual plot(s) of data used to verify a computer-derived LC50; and
- reason if number immobilized is substituted for number dead.

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Appendix

Members of the Inter-Governmental Environmental Toxicity Group and Addresses for Environment Canada's Headquarters and Regional Offices

Members of the Inter-Governmental Environmental Toxicity Group (as of December, 2000):

Federal (Environment Canada)

C. Blaise
Centre St. Laurent
Montreal, Québec

M. Bombardier
Environmental Technology Centre
Ottawa, Ontario

U. Borgmann
National Water Research Institute
Burlington, Ontario

J. Bruno
Pacific Environmental Science Centre
North Vancouver, British Columbia

C. Buday
Pacific Environmental Science Centre
North Vancouver, British Columbia

T. Corbin
Atlantic Environmental Science Centre
Moncton, New Brunswick

K. Doe
Atlantic Environmental Science Centre
Moncton, New Brunswick

G. Elliott
Environmental Protection Service
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M. Harwood
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G. van Aggelen (Chairperson)
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R. Watts
Pacific Environmental Science Centre
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Ministère de l'Environnement du Québec
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B. Bayer
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M. Mueller
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* A computer program for calculating LC50s is available for copying onto an IBM-compatible diskette supplied by the user, by contacting the Environmental Toxicology Section at this address.