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Biological Test Method:

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

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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, and the guidance provided in the generic methodology document "Acute Lethality Test Using* Daphnia *spp". is built upon (Environment Canada, 1990a).*

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 (LC_{50}); 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.

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Terminology

The following definitions are given in the context of this report, and additional definitions in the detailed companion document (Environment Canada, 1990a) apply here.

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

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

Control is a treatment in an investigation that duplicates all the factors that might affect results, except the specific condition being studied. In toxicity tests, the control must duplicate all conditions in the exposure treatment(s), but must contain no test material. The control is used to check for toxicity due to basic conditions such as quality of dilution water, or health and handling of organisms.

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

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

Deionized water has been passed through resin columns to remove ions from solution and thereby purify it.

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

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

EC_{50} (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 EC_{50} for immobilization").

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 used to mean Total Hardness, the sum of calcium and magnesium concentrations, both expressed as calcium carbonate in milligrams per litre (mg/L).

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.

LC_{50} (median lethal concentration) is the concentration of material (in this case effluent) in water that is estimated to be lethal to 50% of test organisms after a defined period of exposure (e.g., 48-h LC_{50}).

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 than 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.

Pre-treatment means, in this report, treatment of a sample or dilution thereof, prior to exposure of daphnids.

Reconstituted water is deionized or glass-distilled water with reagent-grade chemicals added. The resultant synthetic fresh water is intended to be free of contaminants and have desired characteristics of pH and hardness.

Reference toxicant means a standard chemical used to assess the sensitivity of organisms and the validity of measurements of effluent toxicity.

Salinity means the total amount of solid material, in grams, dissolved in 1 kg of seawater. 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.*, 1989). It is usually reported in parts per thousand (‰).

Sublethal means detrimental to an organism, but below the level which directly causes death within the test period.

Toxicity means the inherent potential or capacity of a material to cause adverse effects in living organisms.

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Section 1

Introduction

The procedures for an acute lethality test with *Daphnia magna*, specified by the Canadian government in pollution control regulations for various categories of industry, are given in this report. The procedures have their roots in methods for testing fish, published earlier by Environment Canada (e.g., EPS, 1980). The present report should be used in conjunction with a more extensive report which gives supporting rationale and additional details (Environment Canada, 1990a).

Many components of procedures in this report are similar to Canadian provincial methods (B.C. MOE, 1988; Poirier *et al.*, 1988; BNQ, 1990), U.S. methods (U.S. EPA, 1982; 1985a;b; Plotkin and Ram, 1983; ASTM, 1984; APHA *et al.*, 1989; Greene *et al.*, 1988), or international techniques (The Netherlands 1980; BHSC, 1982; OECD, 1981; ISO, 1982; IGATG, 1986). The contribution of those methods to all parts of the present report is acknowledged, and they are recommended as sources of supporting rationale. Procedures stipulated in this report should, however, be taken as the definitive ones for regulatory purposes.

The organism to be tested is the "waterflea" *Daphnia magna*, a small freshwater crustacean of the Order Cladocera. 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 (LC_{50}), or if necessary the median effective concentration for immobilization (EC_{50}) (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.

This test is to be used with effluents containing fresh water or having a salinity of $\leq$ 10 ‰, defined as conductivity $\leq$ 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 effluents discharging directly to seawater 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.

Section 2

Culturing Organisms

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

Culturing techniques vary (U.S. EPA, 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 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 practised, 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. It is strongly recommended that a mixture of at least two species be used. Additionally, a supplement such as yeast, Cerophyll™ and trout chow (see Appendix C of Environment Canada, 1990a) may be used. For the mixture of algae, one or more species of green alga with a diatom is beneficial; algae must be grown in a suitable culture medium (Environment Canada, 1990a).

Success of the daphnid culture may be judged by the following health criteria which must be met if the cultures are to be used in toxicity tests. Constant check should be maintained on the first item, and frequent checks on the next two.

- ephippia must not be present in the culture;
- age at delivery of first brood must be $\leq$ 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 should die during the seven-day period prior to a test, assuming a culture of mixed ages.

The findings of a test with a reference toxicant (Section 7) give further indication of suitability of the culture for toxicity tests.

2.2 *Water*

Water to be used for culturing daphnids and as control/dilution water may be natural or reconstituted. For natural water, uncontaminated ground-, surface, or dechlorinated municipal 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 water is to be used as culture/control/dilution water, it must be free of any harmful concentration of chlorine upon daphnid exposure. The target value for total residual chlorine in cultures, and for that in control/dilution water within test vessels is ≤ 0.002 mg/L (see Environment Canada, 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 Environment Canada, 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 (Environment Canada, 1990a). The addition of selenium and Vitamin B_{12} to reconstituted water used for culturing organisms is necessary (see Environment Canada, 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 must consistently support good survival, growth, and reproduction of daphnids. Chemical measurements should be made as often as necessary to document variation (at least hardness, pH, conductivity, dissolved oxygen, and total dissolved gases). The water must not be supersaturated with gases. Any supersaturation should be remedied (see Environment Canada, 1990a). A more detailed chemical analysis could be carried out periodically on other items listed by Environment Canada (1990a).

2.3 *Physicochemical Conditions*

Light intensity should not exceed 800 lux at the water surface, and the spectrum should be skewed towards the blue end (colour rendering index ≥ 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 culture vessel, preferably daily; weekly or more frequent monitoring is recommended for water hardness, and for total residual chlorine if municipal water is used.

Section 3

Facilities

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

Test vessels must be large enough that the loading density does not exceed one daphnid per 15 millilitres of solution. Borosilicate glass beakers (150- or 250-mL) or glass screw-top test tubes are good; bags made of inert nontoxic plastic (e.g., polyethylene or polypropylene; Whirl-Pak™) may also be used but must not be re-used. It is recommended that the test vessels be covered, and if volatile components are suspected in the effluent, the vessels must be closed and there should be little air space. The vessels and all other material and equipment that may 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 deionized water in accordance with good laboratory procedures.

Section 4

General Procedures 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 2L or more (depending on chemical-analytical requirements) are normally required for either single-concentration tests or determination of an LC_{50}.

Containers for storage and transportation of effluent samples must be of nontoxic material, for example: 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 be sealed. Labelling must include at least sample type, source, date and time of collection, and name of sampler(s).

Samples should be kept from freezing. During transport, samples should be kept dark, with a temperature from 1 to 8 °C (preferably 4 ± 2 °C) if more than two days are spent in transit. Upon arrival at the laboratory, samples may be adjusted immediately or overnight to 20 ± 2 °C, then testing commenced. If stored at the test facility, samples must be kept dark and cool (8 °C or less, preferably 4 ± 2 °C).

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. Samples must be agitated thoroughly just before pouring aliquots for preparing solutions. Sub-samples (a sample divided between two or more containers) must be combined.

4.2 Test Conditions

This is a 48-h static test (i.e., with 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 ± 1h : 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 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 (Mount and Anderson-Carnahan, 1988).

4.3 Preparing Test Solutions

Adjustment of the effluent sample and control/dilution water to 20 ± 2 °C must be done if temperature is outside that range.

If it is possible that the effluent sample may be very low in hardness, then that must be measured before preparing solutions. If the sample has a hardness < 25 mg/L, it must be adjusted to a hardness of 25 ± 5 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 : 1.265 : 1.0 : 0.0667 [see formula for reconstituted water, Table 2 in Environment Canada (1990a)]. For each desired incremental increase of 5 mg/L of hardness, add the following to each litre of effluent: 5.11 mg of $NaHCO_3$; 4.04 mg of $CaSO_4 \cdot 2H_2O$; 3.19 mg of $MgSO_4$; and 0.213 mg of KCl.

Control/dilution water is to be the same as that used for culturing (Section 2.2). If 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.

Test vessels should be rinsed with control/dilution water just 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 at least the highest test concentration (normally 100% effluent) when it is prepared. If (and only if) oxygen in the highest test concentration is <40% or >100% of air saturation, then pre-aeration (i.e., prior to daphnid exposure) of all test solutions including the control(s) must be commenced at a rate ≤ 7.5 mL/min · L^{-1} (Environment Canada, 1990a). This period of aeration must be restricted to the lesser of 120 minutes and attaining 40% saturation in the highest test concentration (or 100% saturation, if supersaturation is evident). Aeration of each test solution is then to be stopped, test organisms introduced, and the test initiated immediately, regardless of whether 40 to 100% saturation was achieved in all test solutions. Any pre-aeration of test solutions should be provided by bubbling compressed air through a clean silica-glass air diffuser or disposable glass pipette (Environment Canada, 1990a). Bubble size should range from 1 to 3 mm.

4.4 Beginning the Test

Each test vessel must be clearly coded or labelled with concentration, date and time of start, and the concentrations should preferably be positioned at random.

Neonates are to be used for the test. Adult females bearing embryos in their brood

pouches are removed from the stock cultures, 24 h before the test. These females are transferred to clean glass beakers (400 mL to 1 L) containing control/dilution water and an inoculum of prepared food at the concentration used in culturing. Water must have been adjusted to 20 ± 2 °C and 90 to 100% air-saturation with dissolved oxygen before adding the adults; it should not be aerated. Stocking density in the beakers should be approximately 20 adults/L or less. Young found on the following day are used for the toxicity test.

It is desirable but not absolutely required to use females 2 to 4 weeks of age, to avoid both young and senescent females. Separate culture vessels may be stocked periodically with neonates, which at age 2 to 4 weeks will have become neonate-producing adults with offspring available for testing.

To begin the test, equal numbers of neonates are to be introduced into each solution and the control, at least ten per treatment, without exceeding the density of one individual per 15 mL. The order of adding daphnids to vessels should be randomized beforehand. Neonates should be handled as described in Section 2.1; floating and injured ones should be replaced immediately after transfer.

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 and pH must be made in each test concentration including the control(s), at the start of the test and at the end when biological observations are complete. The conductivity of each test solution should be measured at the start of the test as a minimum. Temperature should be measured daily in representative test solutions.

The hardness of control and 100% effluent solutions is measured at the start of the test. These initial measurements are made on larger volumes of solutions made up in beakers, after adjustments have been made and just before their use 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. At the end of the test (48h), numbers of dead daphnids in each vessel should be recorded. 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 or 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 (Environment Canada, 1990a). If necessary, solutions may be poured through netting at the end of the test, and the daphnids re-suspended in water for observation.

Section 5

Procedure for Testing a Single Concentration of Effluent

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. It uses a minimum of six vessels to test three replicate effluent solutions and three replicate control solutions (i.e., triplicates of each), with at least ten daphnids per triplicate.

The end point for this test is percent 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 end point is percentage of immobilization at 48 h, reported for each replicate. Effect on 50% of the individuals is commonly used, and is associated with greater precision of measurement than percentages such as 20% or 80% which are closer to the extremes of the distribution of effects.

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.

Section 6

Procedure for Determining an LC_{50} for Effluent

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 LC_{50}. The highest concentration must be full-strength 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 Appendix D of Environment Canada, 1990a).

For these multi-concentration tests, calculate the 48-h LC_{50} and its 95% confidence limits, and report them, along with the method of calculation. Computer programs for calculating LC_{50} and confidence limits are available (Environment Canada, 1990a) and should be used. A recommended program is available for copying onto a user-supplied disk through courtesy of C.E. Stephan (Stephan, 1977), from Environment Canada (address in Appendix). A check of the computer-derived LC_{50} should be made by examining a plot on logarithmic-probability scales, of percent mortalities at 48 h for the various concentrations (see Environment Canada, 1990a).

If death cannot be confirmed, a 48-h EC_{50} for immobilization (Section 4.5) should be reported rather than an LC_{50}. The statistical procedure for estimating an EC_{50} and its confidence limits is the same as for an LC_{50} 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 LC_{50}. The precision of the estimate of LC_{50} increases with more organisms, but not necessarily its accuracy.

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.

Section 7

Procedure for Tests With a Reference Toxicant

A reference toxicant must be used to assess the relative sensitivity of daphnids and the precision of data produced by the laboratory. The selected reference toxicant(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), 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 LC_{50} should be determined for the reference toxicant(s) used and expressed as mg/L based on NaCl, chromium (Cr^{+++}), or zinc (Zn^{++}) (Environment Canada, 1990a). Stock solutions should be made up on the day of use or stored in the dark, potassium dichromate in glass-stoppered bottles, and zinc at pH 3 to 4.

Concentrations of reference toxicant in all stock solutions should be measured chemically by appropriate methods (APHA *et al.*, 1989). 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 LC_{50} be atypical (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 LC_{50} should be based on measured concentrations if they are appreciably different (i.e., ≥ 20%) from nominal ones.

A warning chart (Environment Canada, 1990b) or similar record must be prepared for each reference toxicant used, and continually updated. Successive LC_{50}s are plotted to determine whether they are within ± 2 SD of the geometric mean (or arithmetic mean, in those instances where data are not normally distributed) of previous LC_{50}s. The geometric mean with its upper and lower warning limits (± 2 SD, still calculated on a logarithmic basis) is recalculated with each successive LC_{50} until the statistics stabilize (U.S. EPA, 1985a; Environment Canada, 1990b). If a particular LC_{50} falls outside the warning limits, sensitivity of the organisms and validity of recent effluent tests are both suspect. A check of all culturing conditions is required under these circumstances. Depending on the findings, further acclimation and re-evaluation (with one or more reference toxicants) of the culture should be undertaken, or a new culture of daphnids established and tested for its suitability in toxicity tests (see Section 2.1).

Section 8

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 completed toxicity test. 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 that which 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");
- person collecting sample; and
- indication if sample was frozen or partially frozen in transit.

8.1.2 Test Facilities and Conditions

- test type and protocol (e.g., "single-concentration test", protocol specified in Reference Method EPS 1/RM/14;

* Contact an office listed in the Appendix for details.

- indication of any differences from method given in this document;
- name and city of testing laboratory;
- species of test organism;
- date and time for start of definitive test;
- persons performing the test and verifying the results;
- the pH, temperature, dissolved oxygen and conductivity of unadjusted, undiluted effluent, just before preparing test solutions;
- 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 test solutions (rate, time) before introduction of daphnids;
- concentrations and volumes tested, including controls, and indication of replication;
- measurements of dissolved oxygen, pH, temperature, and conductivity determined for each test solution (including control(s)) during the test; hardness measurements on 100% effluent and control solutions;
- most recent estimates of time to first brood and average number of neonates per brood; 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 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 LC_{50} and 95% confidence limits in multi-concentration tests, by computer calculation; the 48-h EC_{50} for immobilization and 95% confidence limits, if determined; indication of statistical method (e.g., log probit, moving average) on which result is based; and,
- most recent 48-h LC_{50} (with 95% confidence limits) for reference toxicant(s); chemical(s); date test initiated; historic geometric mean LC_{50}, and warning limits (± 2 SD).

*8.2 Data to be Held on File**

8.2.1 Effluent

- detailed description of sampling point, source and type of effluent;
- type of container(s) and label or code used;

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* To be stored for a five-year period at the test facility and/or the offices of the discharger. Some of this

- volume and/or weight of sample;
- transport and storage conditions (times, temperatures);
- 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 (e.g., suspended solids content, hardness).

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;
- 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 pre-treatment, 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 municipal 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;
- method of obtaining neonates for tests;
- percentage mortality in culture during seven days preceding the test;
- appearance of solutions; any changes during test;
- test concentrations of reference toxicant(s), both nominal and measured; indication of data set used to estimate LC_{50}; 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 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;

- confirmation that graph verified the computer-derived LC_{50}(s); and
- reason if number immobilized is substituted for number dead.

References

APHA *et al.*, "Toxicity Test Methods for Aquatic Organisms", p. 8-1 – 8-143, in: *Standard Methods for the Examination of Water and Wastewater*, 17th ed. American Public Health Association, American Water Works Association, and Water Pollution Control Federation, Washington, D.C., Part 8000, (1989).

ASTM, "Standard Practice for Conducting Static Acute Toxicity Tests on Wastewaters with *Daphnia*", p. 27-39, in: *Annual Book of ASTM Standards*, Amer. Soc. for Testing and Materials, Philadelphia, PA, Vol. 11.01, Designation D 4229 (1984).

B.C. MOE, "Daphnia 48 hr Acute Mortality Bioassay Procedure", B.C. Ministry of Environment and Parks, Environmental Laboratory, Draft document, Vancouver, B.C. (1988).

BHSC " Testing for Acute Toxicity to *Daphnia*", p. 14-18, in: *Methods for the Determination of Ecotoxicity*, British Health and Safety Commission, London, U.K. (1982).

BNQ, "Eaux - Détermination de la toxicité. Méthode avec le microcrustacé *Daphnia magna*", ministère de l'Industrie et du Commerce, Bureau de Normalisation du Québec, Québec (1990).

Environment Canada," Acute Lethality Test Using *Daphnia* spp.", Conservation and Protection, Ottawa, Ontario, Report EPS 1/RM/11 (1990a).

Environment Canada, "Guidance Document for Control of Toxicity Test Precision Using Reference Toxicants", Conservation & Protection, Ottawa, Ontario, Report EPS 1/RM/12 (1990b).

EPS, "Standard Procedure for Testing the Acute Lethality of Liquid Effluents", Environ. Prot. Serv., Environment Canada, Regulations, Codes and Protocols, Ottawa, Ontario, Report EPS 1-WP-80-1, (1980).

Greene, J.C., C.L. Bartels, W.J. Warren-Hicks, B.P. Parkhurst, G.L. Linder, S.A. Peterson, and W.E. Miller, "Protocols for Short Term Toxicity Screening of Hazardous Waste Sites", U.S. Environmental Protection Agency, Corvallis, OR (1988).

IGATG, "Acute Lethality/Immobility Testing Protocols and Procedures for the Water Flea, *Daphnia magna*. Addendum 2, Part C", to: Sergy, G. 1987. Recommendations on Aquatic Biological Tests and Procedures for Environmental Protection, Environment Canada, Edmonton, Alberta (1986).

ISO, "Water Quality -- Determination of the Inhibition of Mobility of *Daphnia magna* Straus (Cladocera, Crustacea)", First edition, International Organization for Standardization, Report No. ISO 6341-1982 (E), Geneva, Switzerland (1982).

Mount, D.I. and L. Anderson-Carnahan, "Methods for Aquatic Toxicity Identification Evaluations, Phase I. Toxicity Characterization Procedures", U.S. Environmental Protection Agency, Duluth, MN, Report EPA-600/3-88/034 (1988).

OECD, "*Daphnia* sp., 14-day Reproduction Test (including an acute immobilization test)", Organization for Economic Cooperation and Development, Document No. 202, Paris, France (1981).

Plotkin, S. and N.M. Ram, "Acute Toxicity Tests: General Description and Materials and Methods Manual, II. *Daphnia*", Univ. of Massachusetts, Dept of Civil Engineering, Report No. Env.E.73-83-4, Amherst, MA (1983).

Poirier, D.G., G.F. Westlake, and S.G. Abernethy, "*Daphnia magna* Acute Lethality Toxicity Test Protocol", Aquatic Toxicity Unit, Water Resources Branch, Ontario Ministry of Environment, Rexdale, Ontario (1988).

Stephan, C.E., "Methods for Calculating an LC_{50}", p. 65-84, in: *Aquatic Toxicology and Hazard Evaluation*, F.L. Mayer and J.L. Hamelink (eds.), Amer. Soc. for Testing and Materials, Philadelphia, PA, ASTM STP 634, (1977).

The Netherlands, " Determination of Toxicity", Chapter 5 in: *Degradability Ecotoxicity and Bio-accumulation. The Determination of the Possible Effects of Chemicals and Wastes on the Aquatic Environment*, Govt. Publishing Office, The Hague, The Netherlands (1980).

U.S. EPA, "Daphnid Acute Toxicity Test", U.S. Environmental Protection Agency, Office of Toxic Substances, Document EG-1/ES-1, Washington, D.C. (1982).

U.S. EPA, "Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms", W.H. Peltier and C.I. Weber (eds.), U.S. Environmental Protection Agency, Report EPA/600/4-85/013, Cincinnati, OH (1985a).

U.S. EPA, "Acute Toxicity Test for Freshwater Invertebrates", U.S. Environmental Protection Agency, Hazard Evaluation Division, Office of Pesticides Programs, Washington, D.C., Report EPA-540/9-85-005 (1985b).

Appendix

List of Members of the Inter-Governmental Aquatic Toxicity Group and Conservation & Protection Regional and Headquarters' Office Addresses

Members of the Inter-Governmental Aquatic Toxicity Group*

Federal**

P. Wells (Chairperson)
EP, Dartmouth, Nova Scotia

B. Moores
St. John's, Newfoundland

K. Doe
Dartmouth, Nova Scotia

W. Parker
Dartmouth, Nova Scotia

N. Bermingham
Longueuil, Québec

C. Blaise
Longueuil, Québec

G. Elliott
Edmonton, Alberta

R. Watts
North Vancouver, British Columbia

K. Day
National Water Research Institute
Burlington, Ontario

B. Dutka
National Water Research Institute
Burlington, Ontario

C. Kriz
Federal Programs Branch
Ottawa, Ontario

D. MacGregor
Commercial Chemicals Branch
Ottawa, Ontario

P. MacQuarrie
Commercial Chemicals Branch
Ottawa, Ontario

R. Scroggins
Industrial Programs Branch
Ottawa, Ontario

G. Sergy
Technology Development Branch
Edmonton, Alberta

P. Farrington
Water Quality Branch
Ottawa, Ontario

Provincial

C. Bastien
Ministère de l'Environnement du Québec
Sainte Foy, Québec

G. Westlake
Ontario Ministry of Environment
Rexdale, Ontario

W. Young
Manitoba Environment and Public Safety
Winnipeg, Manitoba

K. Lauten
Saskatchewan Environment and Public Safety
Regina, Saskatchewan

J. Somers
Alberta Environment
Vegreville, Alberta

S. Horvath
B.C. Ministry of Environment
Vancouver, British Columbia

G. van Aggelen
B.C. Ministry of Environment
North Vancouver, British Columbia

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* IGATG membership as of July 1990
** Conservation and Protection, Environment Canada

Conservation & Protection Regional and Headquarters' Office Addresses

Headquarters
351 St. Joseph Boulevard
Place Vincent Massey
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Ontario Region
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Western and Northern Region
Room 210, Twin Atria # 2
4999 -98 Avenue
Edmonton, Alberta
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Pacific and Yukon Region*
224 West Esplanade Street
North Vancouver, British Columbia
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* A BASIC computer program for calculating LC_{50}s is available for copying onto a formatted 13-cm IBM-compatible floppy disk supplied by the user, by contacting the Aquatic Toxicity Laboratory at this address.

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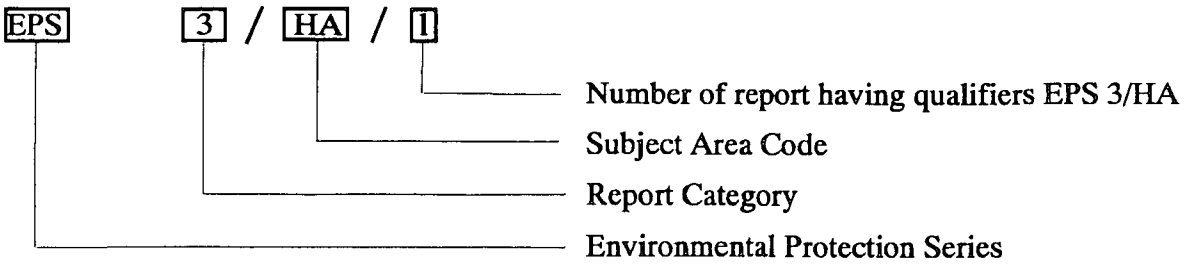

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