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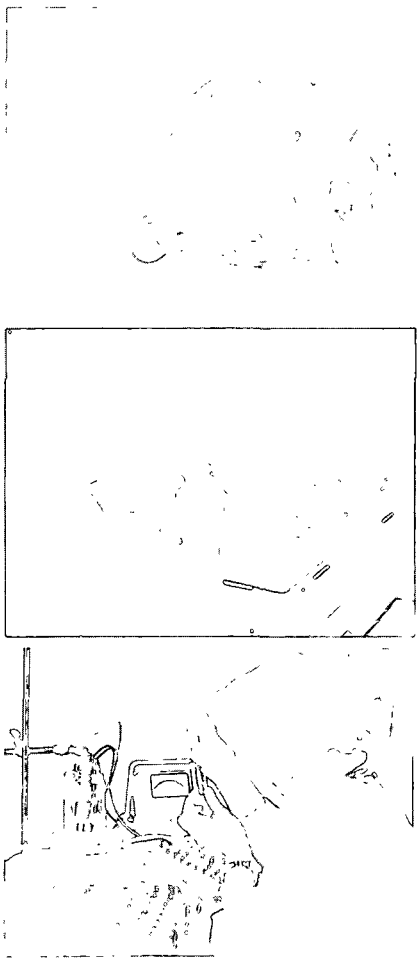

Biological Test Method:

Test of Reproduction and Survival Using the Cladoceran *Ceriodaphnia dubia*

Report EPS 1/RM/21
February 1992

Canada

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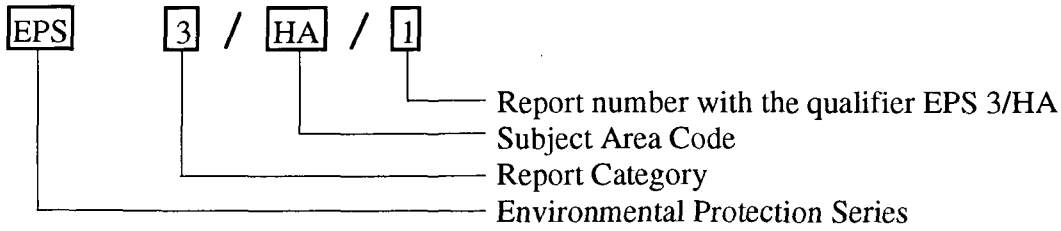

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Readers' Comments

Comments regarding the content of this report should be addressed to:

Gary Sergy
Technology Development Branch
Environment Canada
No. 210, 4999-98 Avenue
Edmonton, Alberta
T6B 2X3

Richard Scroggins
Industrial Programs Branch
Environment Canada
Ottawa, Ontario
K1A 0H3

Cette publication est aussi disponible en français. Pour l'obtenir, s'adresser à:

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Abstract

Methods recommended by Environment Canada for performing chronic three-brood toxicity tests with the freshwater cladoceran, Ceriodaphnia dubia, *are described in this report.*

General or universal conditions and procedures are outlined for undertaking this chronic toxicity test using a variety of test materials. Additional conditions and procedures are stipulated which are specific for assessing samples of chemicals, effluents, elutriates, leachates, or receiving waters. Included are instructions on culturing conditions and requirements, food preparation, sample handling and storage, test facility requirements, procedures for preparing test solutions and test initiation, specified test conditions, appropriate observations and measurements, endpoints, methods of calculation, and the use of reference toxicants.

Résumé

Le présent document expose les méthodes recommandées par Environnement Canada pour l'exécution d'essais de toxicité chronique sur trois couvées du cladocère d'eau douce Ceriodaphnia dubia.

Il présente les conditions et méthodes générales ou universelles permettant de réaliser cet essai de toxicité chronique avec un large éventail de substances. Il précise d'autres conditions et méthodes propres à l'évaluation d'échantillons de produits chimiques, d'effluents, d'élutriats, de lixiviats ou de milieux récepteurs. Le lecteur y trouvera des instructions sur les conditions et les règles d'élevage, la préparation des aliments, la manipulation et le stockage des échantillons, les installations d'essai requises, les méthodes de préparation des solutions d'essai et de mise en route des essais, les conditions prescrites pour les essais, les observations et mesures appropriées, les résultats des essais, les méthodes de calcul et l'utilisation de produits toxiques de référence.

Foreword

This is one of a series of ***recommended methods*** *for measuring and assessing the aquatic biological effects of toxic materials. Recommended methods are those which have been evaluated by Conservation and Protection (C&P), and are favoured:*

- *for use in C&P aquatic toxicity laboratories;*
- *for testing which is contracted out by Environment Canada or requested from outside agencies or industry;*
- *in the absence of more specific instructions, such as are contained in regulations; and*
- *as a foundation for the provision of very explicit instructions as might be required in a regulatory protocol or standard reference method.*

The different types of tests included in this series were selected on the basis of their acceptability for the needs of programs for environmental protection and management carried out by Environment Canada. These documents are intended to provide guidance and to facilitate the use of consistent, appropriate, and comprehensive procedures for obtaining data on toxic effects of samples of chemical, effluent, elutriate, leachate, receiving water or, where appropriate, sediment.

Mention of trade names in this document does not constitute endorsement by Environment Canada; other products with similar value are available.

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List of Abbreviations and Chemical Formulae

°C . . . . . .degree(s) Celsius

$CaCO_3$ . . . . . .calcium carbonate

$CaSO_4$ . . . . . .calcium sulphate

cm . . . . . .centimetre(s)

d . . . . . .day(s)

DO . . . . . .dissolved oxygen (concentration)

g . . . . . .gram(s)

g/kg . . . . . .gram(s) per kilogram

h . . . . . .hour(s)

HCl . . . . . .hydrochloric acid

H_2O . . . . . .water

IC_p . . . . . .inhibiting concentration for a (specified) percentage effect

KCl . . . . . .potassium chloride

L . . . . . .litre(s)

LC . . . . . .lethal concentration

LC_{50} . . . . . .median lethal concentration

LT_{50} . . . . . .time to 50% mortality (lethality)

LOEC . . . . . .lowest-observed-effect concentration

m . . . . . .metre(s)

mg . . . . . .milligram(s)

$MgSO_4$ . . . . . .magnesium sulphate

min . . . . . .minute(s)

mL . . . . . .millilitre(s)

mm . . . . . .millimetre(s)

mS . . . . . .millisiemen(s)

N . . . . . .Normal

$NaHCO_3$ . . . . . .sodium bicarbonate

NaOH . . . . . .sodium hydroxide

NOEC . . . . . .no-observed-effect concentration

P . . . . . .probability

SD . . . . . .standard deviation

sp. . . . . .species

TEC . . . . . .threshold-effect concentration

TM (™) . . . . . .Trade Mark

µg . . . . . .microgram(s)

µm . . . . . .micrometre(s)

YCT . . . . . .yeast, Cerophyll™ and trout chow

> . . . . . .greater than

< . . . . . .less than

≥ . . . . . .greater than or equal to

≤ . . . . . .less than or equal to

± . . . . . .plus or minus

% . . . . . .percentage

‰ . . . . . .parts per thousand

Terminology

Note: all definitions are given in the context of the procedures in this report, and might not be appropriate in another context.

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".

Might is used to mean "could".

General Technical Terms

Acclimation means to become physiologically adjusted to a particular level of one or more environmental factors such as temperature. The term usually refers to controlled laboratory conditions.

Brood means a group or cohort of sibling offspring released from the female during an inter-molt period; i.e., before the carapace is shed by that female during molting.

Compliance means in accordance with governmental permitting or regulatory requirements.

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

Culture, as a noun, means the stock of animals or plants that is raised under defined and controlled conditions in order to produce healthy test organisms. As a verb, it means to carry out this procedure of raising organisms.

as a water flea. Species of daphnids include: *Ceriodaphnia dubia*, *Daphnia magna*, and *Daphnia pulex*.

Dispersant means a chemical substance which reduces the surface tension between water and a hydrophobic substance (e.g., oil), thereby facilitating the dispersal of the hydrophobic material throughout the water as an emulsion.

Emulsifier means a chemical substance that aids the fine mixing (in the form of small droplets) within water, of an otherwise hydrophobic material.

Ephippium is an egg case that develops under the postero-dorsal part of the carapace of a female adult daphnid in response to adverse conditions (e.g., overcrowding, infrequent exchange of culture water, inadequate diet, low temperature, reduced photoperiod). The eggs within are normally fertilized.

First-generation daphnids mean those organisms placed in solutions at the start of the test.

Flocculation is the formation of a light, loose precipitate (i.e., a floc) from a solution.

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, and is expressed as mg/L calcium carbonate or equivalent.

Individual culture means a culture of neonates established from isolated organisms cultured in individual beakers or cups. Neonates from established individual brood animals are then used for toxicity tests.

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.

Mass culture means a culture containing multiple brood organisms (usually 40 to 50) and their young. Neonates from mass cultures serve as a source of brood organisms for individual cultures.

Monitoring is the routine (e.g., daily, weekly, monthly, quarterly) checking of quality, or collection and reporting of information. In the context of this report, it means either the periodic (routine) checking and measurement of certain

information. In the context of this report, it means either the periodic (routine) checking and measurement of certain biological or water-quality variables, or the collection and testing of samples of effluent, elutriate, leachate, or receiving water for toxicity.

Neonate is a newly-born or newly-hatched individual (first-instar daphnid,<24-h old).

Percentage (%) is a concentration expressed in parts per hundred parts. One percentage represents one unit or part of material (e.g., effluent, elutriate, leachate, or receiving water) diluted with water to a total of 100 parts. Concentrations can be prepared on a volume-to-volume or weight-to-weight basis, or less accurately on a weight-to-volume basis, and are expressed as the percentage of test material in the final solution.

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.

Precipitation means the formation of a solid (i.e., precipitate) from a solution.

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

Salinity is 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 (see APHA *et al.*, 1989). It is usually reported in grams per kilogram (g/kg) or parts per thousand (‰).

Turbidity is the extent to which the clarity of water has been reduced by the presence of suspended or other matter that causes light to be scattered and absorbed rather than

transmitted in straight lines through the sample. It is generally expressed in terms of Nephelometric Turbidity Units.

Terms for Test Materials

Chemical is, in this report, any element, compound, formulation, or mixture of a chemical substance that might enter the aquatic environment through spillage, application, or discharge. Examples of chemicals which are applied to the environment are insecticides, herbicides, fungicides, sea lamprey larvicides, and agents for treating oil spills.

Control is a treatment in an investigation or study that duplicates all the conditions and factors that might affect the results of the investigation, except the specific condition that is being studied. In an aquatic toxicity test, the control must duplicate all the 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., quality of the dilution water, health, or handling of test organisms).

Control/dilution water is the water used for diluting the test material, or for the control test, or both.

Culture medium is the water used for culturing *C. dubia*.

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 solutions by passing it through resin columns or a reverse osmosis system.

Dilution water is the water used to dilute a test material in order to prepare different concentrations for the various toxicity test treatments.

Distilled water is water that has been passed through a distillation apparatus of borosilicate glass or other material, to remove impurities.

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

Elutriate is an aqueous solution obtained after adding water to a solid material (e.g., sediment, tailings, drilling mud, dredge spoil), shaking the mixture, then centrifuging or filtering it or decanting the supernatant.

Leachate is water or wastewater that has percolated through a column of soil or solid waste within the environment.

Receiving water is surface water (e.g., in a stream, river, or lake) that has received a discharged waste, or else is about to receive such a waste (e.g., it is just upstream from the discharge point). Further descriptive information must be provided to indicate which meaning is intended.

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

Reference toxicant is a standard chemical used to measure the sensitivity of the test organisms in order 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 for that chemical obtained by the laboratory.

Stock solution is a concentrated aqueous solution of the material to be tested. Measured volumes of a stock solution are added to dilution water in order to prepare the required strengths of test solutions.

Upstream water is surface water (e.g., in a stream, river, or lake), that is not influenced by the effluent (or other test material), by virtue of being removed from it in a direction against the current or sufficiently far across the current.

Wastewater is a general term which includes effluents, leachates, and elutriates.

Toxicity Terms

Acute means within a short period (seconds, minutes, hours, or a few days) in relation to the life span of the test organism.

Acute lethality, acutely lethal mean causing the death of the test organisms within a short period of exposure to a test material, usually 48 h for daphnids.

Chronic means occurring during a relatively long-term period of exposure, usually a significant portion of the life span of the organism such as 10% or more. For tests with cladocerans, chronic is typically defined as continuing until three broods are produced.

Chronic toxicity implies long-term effects that are related to changes in such things as: metabolism, growth, reproduction, survival, or ability to survive.

Chronic value is the geometric mean of the NOEC and LOEC in tests which have a chronic exposure. See also *TEC* as the recommended term.

Endpoint means the variables (i.e., time, reaction of the organisms, etc.) that indicate the termination of a test, and also means the measurement(s) or value(s) derived, that characterize the results of the test (NOEC, LC_{50}, ICp, etc.).

IC_p is the inhibiting concentration for a (specified) percentage effect. It represents a point estimate of the concentration of test material that causes a designated percentage impairment in a quantitative biological function such as reproductive success. For example, an IC25 could be the concentration estimated to cause a 25% reduction in mean number of young produced, relative to the number produced by control animals. This term should be used for any toxicological test which measures a change in rate, such as reproduction, growth, or respiration. (The term EC_{50} or median effective concentration is limited to quantal measurements, i.e., number of individuals which show a particular effect.)

LC_{50} is the median lethal concentration, i.e., the concentration of test material in water that is estimated to be lethal to 50% of the test organisms. The LC_{50} and its 95% confidence limits are usually derived by statistical analysis of mortalities in several test concentrations, after a fixed period of exposure. The duration of exposure must be specified (e.g., seven-day LC_{50}).

Lethal means causing death by direct action. Death of daphnids is defined as the cessation of all visible signs of movement or

other activity, including antennae, antennule, postabdomen and heartbeat, as observed through a microscope.

LOEC is the lowest-observed-effect concentration. This is the lowest concentration of a test material to which organisms are exposed, that causes adverse effects on the organism, which are detected by the observer and are statistically significant. For example, the LOEC might be the lowest concentration at which the number of live young produced per adult daphnid differed significantly from that in the control.

LT_{50} is the time (period of exposure) estimated to cause 50% mortality in a group of first-generation daphnids held in a particular test solution. The value is estimated graphically since there is no standard mathematical or computer technique in common use (see Appendix F).

NOEC is the no-observed-effect concentration. This is the highest concentration of a test material to which organisms are exposed, that does not cause any observed and statistically significant adverse effects on the organism. For example, the NOEC might be the highest test concentration at which an observed variable such as number of live young produced per adult daphnid does not differ significantly from that in the control. NOEC customarily refers to sublethal effects, and to the most sensitive effect unless otherwise specified.

Static describes toxicity tests in which test solutions are not renewed during the test.

Static renewal describes a toxicity test in which test solutions are renewed (replaced) periodically during the test, usually at the beginning of each 24-h period of testing. Synonymous terms are "semi-static","static replacement", and "batch replacement".

TEC is the threshold-effect concentration. It is calculated as the geometric mean of NOEC and LOEC. *Chronic value* or *subchronic value* are alternative terms that might be appropriate depending on the duration of exposure in the test.

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

Toxicity Identification Evaluation describes a systematic sample pre-treatment (e.g., pH adjustment, filtration, aeration), followed by tests for toxicity. This evaluation is used to

identify the agent(s) that are primarily responsible for toxicity in a complex mixture. The toxicity test can be lethal or sublethal.

Toxicity test is a determination of the effect of a material on a group of selected organisms, under defined conditions. An aquatic toxicity test usually measures either (a) the proportions of organisms affected *(quantal)*, or (b) the degree of effect shown *(graded or quantitative)*, after exposure to specific concentrations of chemical, effluent, elutriate, leachate, or receiving water.

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Members of the Inter-Governmental Aquatic Toxicity Group (IGATG, Appendix A) participated actively in the development and review of this report and are thanked accordingly. Special acknowledgement is made of the many technical contributions provided by the IGATG subcommittee members (S. Abernethy[i], G. Craig[ii], K. Day[iii], T. Kovacs[iv], T. Norberg-King[v], R. Scroggins[vi], G. Sergy[vi], and G. Westlake[i]) responsible for initial and final review. The laboratory testing support of Environment Canada (Appendix B) is also acknowledged.

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i Ontario Ministry of Environment, Rexdale, Ontario.
ii Beak Consultants Ltd., Brampton, Ontario.
iii National Water Research Institute, Burlington, Ontario.
iv Pulp & Paper Research Institute of Canada, Pointe Claire, PQ.
v U.S. Environmental Protection Agency, Duluth, Minnesota.
vi Environment Canada

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Section 1

Introduction

1.1 Background

Aquatic toxicity tests are used within Canada and elsewhere to measure, predict, and control the discharge of materials that might be harmful to indigenous aquatic life. Recognizing that no single test method or test organism can be expected to satisfy a comprehensive approach to environmental conservation and protection, the Inter-Governmental Aquatic Toxicity Group (Appendix A) recently proposed a set of aquatic toxicity tests which would be broadly acceptable, and would measure different toxic effects using organisms representing different trophic levels and taxonomic groups (Sergy, 1987). A chronic toxicity test, using a daphnid species (i.e., a freshwater microcrustacean invertebrate from the family Daphniidae), was one of several aquatic toxicity tests which was selected to be standardized sufficiently to help meet Environment Canada's testing requirements.

Universal procedures for conducting three-brood chronic toxicity tests with the cladoceran *Ceriodaphnia dubia* are described in this report. Also presented are specific sets of test conditions and procedures, required or recommended when using this chronic toxicity test for evaluating different types of materials (namely samples of chemicals, effluents, elutriates, leachates, or receiving waters) (see Figure 1). Those procedures and conditions relevant to the conduct of a test are delineated and, as appropriate, discussed in explanatory footnotes.

In formulating these procedures, an attempt was made to balance scientific, practical, and cost considerations, and to ensure that the results will be accurate and precise enough for the majority of situations in which they will be applied. The authors assume that the user has a certain degree of familiarity with aquatic toxicity tests. Explicit instructions that might be required in a regulatory protocol are not provided, although this report is intended to serve as a guidance document useful for that and other applications.

1.2 Species Description and Historical Use in Tests

Daphnids are freshwater microcrustaceans, commonly referred to as water fleas, belonging to the Order Cladocera. Cladocerans from the family Daphniidae, which includes *Daphnia* sp. and *Ceriodaphnia* sp., are ubiquitous in temperate fresh waters (Berner, 1986). Both genera are abundant in lakes, ponds, and quiescent sections of streams and rivers throughout North America (Pennak, 1978). Within such habitats, these cladocerans are ecologically-important species since they are among the major groups converting phytoplankton and bacteria into animal protein (Carpenter *et al.*, 1985), and form a significant portion of the diet of numerous fish species including young salmonids.

The selection of daphnids for routine use in toxicity testing by Canadian laboratories is appropriate for a number of reasons.

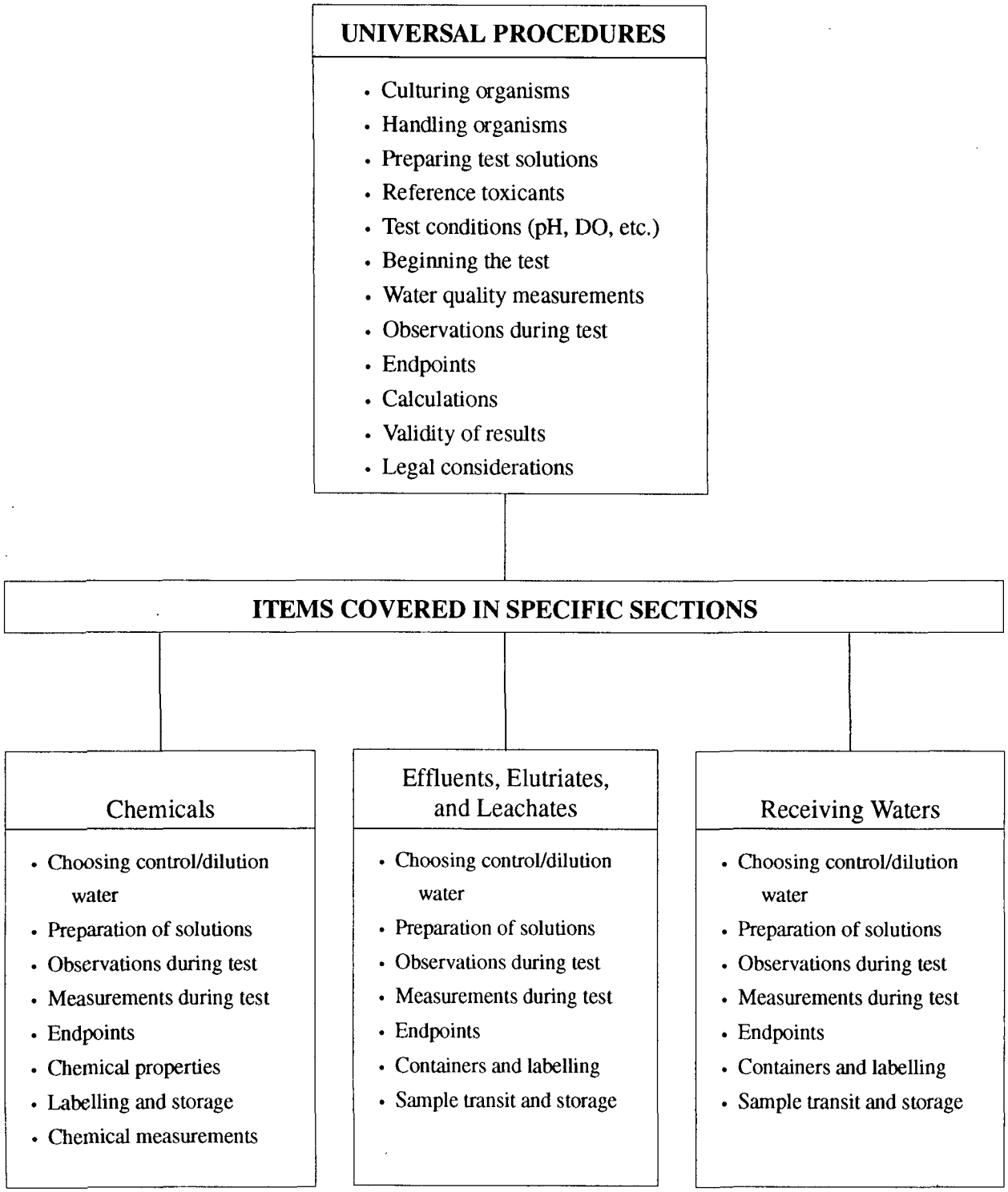

Figure 1 **Diagram of Approach Taken in Delineating Test Conditions and Procedures Appropriate for Various Types of Materials**

- Daphnids are broadly distributed in Canadian freshwater bodies and are present throughout a wide range of habitats.

- These organisms are an important link in many aquatic food chains and a significant source of food for small fish.

- Daphnids have a relatively short life cycle and can be cultured in the laboratory.

- Daphnids are sensitive to a broad range of aquatic contaminants, and are widely used as test organisms for evaluating the acute or chronic toxicity of chemicals or effluents.

- The small size of daphnids requires only small volumes of test and dilution water, leading to ease of sampling and transporting wastewater and receiving-water samples.

The larger *Daphnia* spp. (i.e., *D. pulex* and *D. magna*) have been used for acute (48-h) toxicity tests with effluents or chemicals for many years, and standardized procedures are now available for conducting acute lethality tests using these species (Environment Canada, 1990a). *Daphnia* spp. (in particular, *D. magna*) have also been used for chronic (life-cycle) tests with chemicals and wastewaters (IGATG, 1986), although such tests are labour-intensive and might require 14 to 21 days for their completion. A three-brood chronic toxicity test using *Ceriodaphnia dubia* can normally be completed within 7 ± 1 days, thus reducing costs and sample volumes appreciably. Since its inception (Mount and Norberg, 1984), this test has become popular in Canada and the United States, and is now in prominent use within Canada at a number of private, provincial, and federal (see Appendix B) laboratories engaged in aquatic toxicity tests. A number of studies comparing the findings of three-brood *C. dubia* tests with field surveys have demonstrated excellent correlations of test results for specific effluents with their ecological impacts (Mount *et al.*, 1984, 1985, 1986; Mount and Norberg-King, 1986; Norberg-King and Mount, 1986; Eagleson *et al.*, 1990).

A seven-day, three-brood, static-renewal life-cycle test using the cladoceran *Ceriodaphnia* sp. (initially *C. reticulata*) was developed in the early 1980s by the U.S. Environmental Protection Agency (Mount and Norberg, 1984). In 1985, the test method (using *C. dubia*) was published by U.S. EPA as one of three short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms (U.S. EPA, 1985a). A revised method for undertaking this test, which incorporates greater descriptive details, an improved diet, and modified and expanded test endpoints and methods for their calculation, has been published (U.S. EPA, 1989). The American Society for Testing and Materials has also prepared a standard guide for conducting three-brood, static-renewal toxicity tests with *C. dubia* (ASTM, 1989). Additional documents which describe procedures and conditions for undertaking this test are reviewed in Appendix C.

Researchers familiar with the U.S. EPA (1985a, 1989) test methods for performing chronic toxicity tests with *C. dubia* have examined the influence on test results of a number of test conditions including temperature (McNaught and Mount, 1985), culture history and health (Keating, 1985; Cooney and DeGraeve, 1986; Cowgill, 1987), food type and ration (Cooney and DeGraeve, 1986; Cowgill, 1987; DeGraeve and Cooney, 1987; Cooney *et al.*, 1988;

Cowgill *et al.*, 1988; Melville and Richert, 1989), water quality (Cooney and DeGraeve, 1986; Cowgill, 1987; DeGraeve and Cooney, 1987; Cooney *et al.*, 1988; Melville and Richert, 1989; Keating *et al.*, 1989), and test-container type and volume (Melville and Richert, 1989; Cowgill and Milazzo, 1989). The precision of the U.S. EPA (1985a) test method has also been assessed in intra- and inter-laboratory studies (DeGraeve *et al.*, 1989). The findings of these studies have been considered in developing the present report.

The purpose of this report is to provide a "standardized" Canadian methodology for undertaking tests for the chronic toxicity of various materials using *Ceriodaphnia dubia*. Whereas the application of other published methods (see Appendix C) for performing this test might have been restricted to certain types of materials, this report is intended for use in evaluating the chronic toxicity of chemicals, effluents, leachates, elutriates, or receiving waters. The generic conditions and procedures herein are largely those developed by the U.S. EPA (1989), with the incorporation of useful test modifications and additions obtained from ASTM (1989) and elsewhere.

This method is intended for use with freshwater-acclimated *C. dubia*, with fresh water as the dilution and control water, and with effluents, leachates, or elutriates that are essentially fresh water (i.e., salinity ≤10 g/kg) or saline but destined for discharge to fresh water. Its application may be varied but includes instances where the impact or potential impact of materials on the freshwater environment is under investigation. Other tests, using other species acclimated to seawater, may be used to assess the impact or potential impact of materials in estuarine or marine environments, or to evaluate wastewaters having a salinity >10 g/kg which are destined for estuarine/marine discharge.

Section 2

Test Organisms

2.1 Species

The microcrustacean cladoceran *Ceriodaphnia dubia* (family Daphniidae) is to be used as the test species (see Figure 2). This species has been considered synonymous with *C. affinis*, and the designation *C. dubia* has taxonomic precedence (Berner, 1986). Certain features of the adult female (length to 0.9 mm, height 0.6 times length) distinguish this species from related organisms. In particular, the postabdomen is moderately long and wide (about twice as long as wide), with a slight midpoint inflection and seven or eight anal denticles. The postabdominal claw is moderately curved with the three subdivisions of the lateral setules (teeth) being of similar size (Figure 2).

2.2 Life Stage

Neonate daphnids, less than 24 h old and all within 8 h of the same age, are to be used to begin the test (U.S. EPA, 1989). For greater standardization, it is desirable although not always practical to use neonates less than 12 h old and all within 4 h of the same age (ASTM, 1989). These neonates should be taken from individual cultures (i.e., brood cultures set up exclusively for obtaining neonates for tests) (Subsection 2.4.1), and should meet the requirements specified in Subsection 2.4.11.

2.3 Source

Cultures of *Ceriodaphnia dubia* are available from government and private laboratories engaged in toxicity testing. Advice concerning sources of daphnids can be obtained by contacting a regional Environmental Protection office (Appendix B). Very few organisms (e.g., 10 to 20 neonates) are required to start a culture. These can be transported in a 1-L bottle filled with culture water and containing food (Section 2.4).

Species taxonomy must be confirmed microscopically (Berner, 1986; U.S. EPA, 1989) upon initiation of cultures using organisms from outside sources[a]. Periodic taxonomic checks of the laboratory's culture are also advisable to verify the test species. When starting cultures using organisms from an outside source, it is desirable to use a single individual, which is sacrificed after producing young, embedded, prepared on a permanent microscope slide (U.S. EPA, 1989), and identified to species.

2.4 Culturing

2.4.1 General

Recommended or required conditions and procedures for culturing daphnids are discussed here and summarized in Table 1. These are intended to allow some degree of inter-laboratory flexibility while standardizing those conditions which, if

[a] Initial *Ceriodaphnia* cultures established in the U.S. EPA laboratories at Duluth showed a progressive transition with time from *C. reticulata* to *C. dubia*. A morphological variant of *C. dubia* has also been identified in certain U.S. EPA cultures (Berner, 1986).

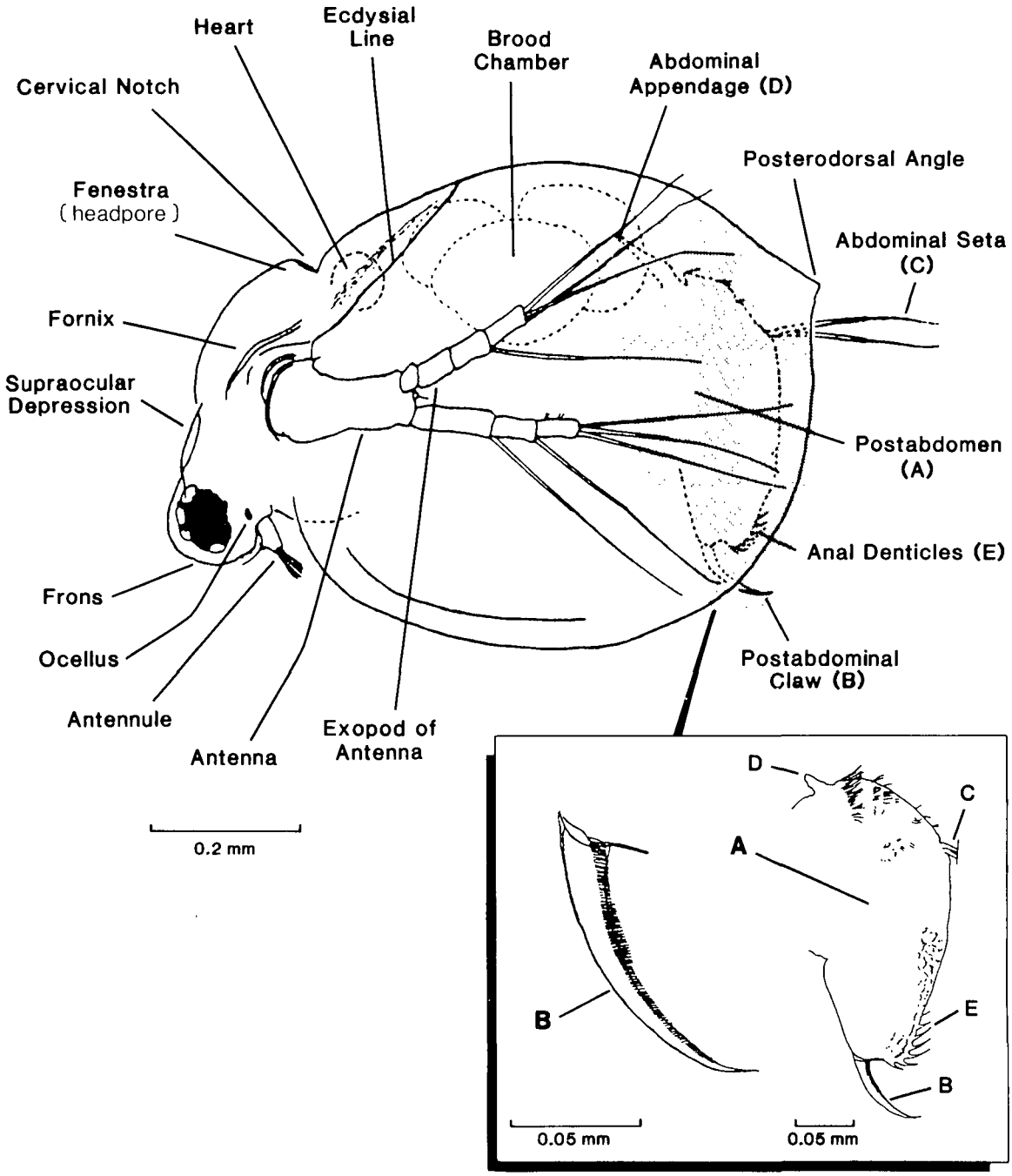

Figure 2 **Anatomy of Female *Ceriodaphnia dubia***
(from Berner, 1986)

Table 1 Checklist of Recommended Conditions and Procedures for Culturing *Ceriodaphnia dubia*

Source of daphnids	- biological supply house or government laboratory; species confirmed by microscopic examination
Culture medium	- uncontaminated ground, surface, dechlorinated municipal water, or reconstituted water; water replaced ≥2 (mass culture) or ≥3 (individual culture) times per week
Temperature	- within the range 25 ±1°C
Oxygen/aeration	- culture medium aerated before use as required to provide 90 to 100% saturation with O_2; no aeration of cultures
pH	- within the range 6.0 to 8.5
Hardness	- within the range ±20% of that of control/dilution water for ≥2 generations of daphnids preceding test organisms
Lighting	- "cool white" fluorescent, ≤600 lux at water surface, 16 ±1 h light : 8 ±1 h dark
Feeding	- yeast, Cerophyll™ and trout chow (YCT) plus algae is recommended
Handling	- minimal, by pipetting
Health criteria	- to be suitable for tests, individual cultures to have ≤20% mortality of brood organisms and an average of ≥15 young produced during week before test, with ≥6 young produced by a brood organism in previous brood; no ephippia produced in culture

uncontrolled, might affect the health and performance of the test organism.

A training video and supplemental report was prepared recently by the U.S. Environmental Protection Agency which illustrates and describes conditions and procedures now used by the Environmental Research Laboratory at Duluth, Minnesota for culturing *C. dubia* (Norberg-King, 1989). This reference source, as well as a video depicting their test method, is now available within Canada and can be obtained for viewing by contacting a regional office of Environment Canada (see Appendix B).

All organisms used in a test must be from the same culture. Cultures should be started at least three weeks before the brood animals are needed, in order to ensure their acclimation to laboratory conditions and an adequate supply of neonates for the test. Longer acclimation periods are desirable (Cowgill *et al.*, 1985).

Mass cultures should be established and maintained to ensure a supply of neonates for individual cultures. These cultures can be started by adding 10 to 20 neonates per litre of culture water. As overcrowding produces stress and ultimately ephippia, densities as low as 10 adults/3 L have been recommended (Cowgill, 1989). Higher densities in mass cultures could prove acceptable provided that the water is changed and the young removed on a frequent, routine basis (e.g., daily or every second day). As a minimum, brood organisms should be transferred to new culture water at least twice a week for two weeks, after which the adults are discarded, and the culture re-started with neonates in fresh culture water. At each renewal, the number of surviving brood organisms should be determined and recorded, and their offspring and the old medium discarded[b]. Maintenance of multiple (e.g., ≥4) mass cultures in separate vessels and of differing age (0 to 2 weeks) is advisable to guard against unanticipated problems[c]. Neonates from mass cultures are not to be used in toxicity tests.

Individual cultures (i.e., those from a single brood-organism) are required to provide test organisms. To initiate these cultures, one neonate, taken from a mass culture, is placed in each of a series of 30-mL capacity cups, beakers or test tubes (Subsection 2.4.2) containing 15 mL of culture water. Brood organisms should be transferred to new culture water at least three times per week (typically on Monday, Wednesday, and Friday) and preferably daily. Young produced from the first two broods should be discarded. Those produced from the third and subsequent broods may be used for toxicity tests provided that the adults are ≤14 days of age (Cowgill, 1989; U.S. EPA, 1989). To provide cultures of overlapping ages, new cultures are started weekly using adults which produce at least six young in their third or subsequent broods.

2.4.2 Facilities and Apparatus

Daphnids are to be cultured in a controlled-temperature laboratory facility (constant-temperature room, incubator, or recirculating water bath). The culture area should be well ventilated and the air supply free of odours and dust. Ideally, the culturing facility should be isolated from the test facility to reduce the possibility of culture contamination by test materials. Cultures should also be isolated from regions of the laboratory where stock or test solutions are prepared, effluent or other test material is stored, or equipment is cleaned.

Vessels and accessories contacting the organisms and culture media must be nontoxic. Glass, type 316 stainless steel, nylon, and perfluorocarbon plastics (e.g., Teflon™) should be used whenever possible to minimize leaching and sorption (ASTM, 1989). Materials such as copper, brass, galvanized metal, lead, and natural rubber must not come in contact with culture vessels or media, nor with test samples, test vessels, dilution water or test solutions.

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[b] If the culture water is not replaced at frequent, regular intervals and if the population density is not reduced, a population crash or the production of male and/or ephippia will likely occur.

[c] The use of multiple cultures will provide protection against loss of the entire population due to accidents or population "crashes" in one or more vessels.

Items made of material other than those previously mentioned should not be used unless it has been shown that their use does not adversely affect the survival or reproduction of *C. dubia*. All culture vessels and accessories should be thoroughly cleaned (APHA *et al.*, 1989; ASTM, 1989) and rinsed with culture water between uses. New glass beakers used as culture or test vessels must be cleaned and acid-soaked before use. Each culture vessel should be covered with glass or transparent Plexiglas™ to exclude dust and minimize evaporation.

Glass beakers (1 or 2 L) or other suitable containers (e.g., aquaria, wide-mouthed glass jars) may be used as vessels for mass cultures. If rigid plastics are used for this purpose, they should be soaked in uncontaminated non-chlorinated water for several days before use, and rinsed with culture water. Glass beakers used for mass or individual cultures should be rinsed thoroughly with culture medium (Subsection 2.4.4) before use.

Vessels most commonly used for individual cultures and as test containers are 30-mL capacity clear plastic cups (e.g., medicine cups, or deep cups used for salad dressing by the airline industry) or 30-mL borosilicate glass beakers, although larger or smaller (≥20 mL) vessels may be used. Small glass test tubes with slip-on caps (e.g., Ka-put™) may also be used. Pieces of Styrofoam™ insulation board, 50 × 30 × 2.5 cm, drilled to hold six rows of 10 cups or beakers, are suitable for holding culture/test cups or beakers (U.S. EPA, 1989); other rack or supporting devices may also be used.

2.4.3 Lighting

Organisms being cultured should be illuminated, using a daily photoperiod of 16 ± 1 h light and 8 ± 1 h dark[d]. Cool-white fluorescent or alternate light skewed towards the blue end of the spectrum (colour-rendering index ≥90) is suitable (Buikema, 1973). Light intensities must not exceed 600 lux at the water surface.

2.4.4 Culture Water

Sources of water for culturing *C. dubia* can be an uncontaminated supply of groundwater, surface water*, dechlorinated municipal drinking water, a sample of "upstream" receiving water* taken from a waterbody to be tested, dilute mineral water (e.g., 20% Perrier™ water, 80% deionized water; U.S. EPA, 1989), or reconstituted water adjusted to the desired hardness and pH (see Subsection 2.4.8). The choice of water used as culture medium can depend upon the test material (e.g., receiving-water sample) and control/dilution water, as water with similar or identical characteristics should be used for both culturing and testing (unless test objectives dictate otherwise).

The characteristics of the water used for culturing organisms (Subsection 2.4.1) should be uniform. The culture water should consistently support good survival, growth, and reproduction of daphnids (see Subsection 2.4.11). A given batch of culture water (or control/dilution water) should not

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[d] A long (16-h) daily light cycle stimulates asexual reproduction of daphnids (required for the test), whereas short light periods can stimulate sexual reproduction (Buikema *et al.*, 1980).

* If surface water is used, it should be filtered through a fine-mesh net (60 µm) to remove potential predators and competitors of *C. dubia*.

be stored for more than 14 days[e]. The container should be kept covered, and the water protected from light.

Reconstituted water may be used for procedures requiring a standardized culture/control/dilution water, or if a suitable supply of uncontaminated natural water is not available. Some inherent problems using reconstituted water have been identified[f] (DeGraeve and Cooney, 1987; Melville and Richert, 1989; Keating *et al.*, 1989), although these can be largely overcome provided that adequate quantities of trace nutrients (notably selenium, zinc, and vitamin B_{12}) and a well-balanced diet are present for the organisms (Cooney *et al.*, 1988; Cowgill, 1989; Keating *et al.*, 1989). If reconstituted water is used, addition of 2 to 5 µg of selenium and 1 to 2 µg of crystalline vitamin B_{12} per litre of culture water is recommended (Keating, 1985; ASTM, 1989; Cowgill, 1989). Guidance for preparing reconstituted water with a desired hardness is given in Subsection 2.4.8.

If municipal drinking water is to be used for culturing *C. dubia* (and as control and dilution water), extremely effective dechlorination must be assured, because daphnids are very sensitive to chlorine. A target value for total residual chlorine in dechlorinated municipal water, recommended for the protection of freshwater aquatic life, is ≤0.002 mg/L (CCREM, 1987). The use of activated carbon (bone charcoal) filters and subsequent ultraviolet radiation (Armstrong and Scott, 1974) is suitable for this purpose. As alternatives, municipal water could be autoclaved, or held in reservoirs and aerated strongly for several days after carbon filtration.

Monitoring and assessment of culture-water (and control/dilution-water) quality parameters such as hardness, alkalinity, residual chlorine (if municipal water), pH, total organic carbon, specific conductivity, suspended solids, dissolved oxygen, total dissolved gases, temperature, ammonia nitrogen, nitrite, metals and pesticides, should be performed as frequently as necessary to document water quality. For each method used, the detection limit should be appreciably (e.g., 3 to 10 times) below either (a) the concentration in the water, or (b) the lowest concentration that has been shown to adversely affect the survival and reproduction of *C. dubia* (ASTM, 1989).

Culture water must not be supersaturated with gases. In situations where gas supersaturation within the water supply is a valid concern (e.g., air-saturated cold or cool water heated to 25°C in a closed or semi-closed vessel), total gas pressure within water supplies should be frequently checked (Bouck, 1982). Remedial measures (e.g., passing through aeration columns

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[e] Prolonged storage of control or control/dilution water can result in microbial growth and the problems associated with it.

[f] Certain researchers (DeGraeve and Cooney, 1987; Cooney *et al.*, 1988; Melville and Richert, 1989; Keating *et al.*, 1989) have reported periodic incidences of unacceptable survival and reproduction rates for *C. dubia*, cultured using reconstituted water prepared according to U.S. EPA (1989) using either the formula given in Table 2 or an alternative (U.S. EPA, 1989) using mineral water. In some instances, these problems were not attributable to diet deficiencies or lack of essential trace elements. It has been speculated (Cooney *et al.*, 1988) that unidentified contaminants in the makeup (distilled or deionized) water might account for the (occasional) unexplained problems associated with using reconstituted water.

before use, or vigorous aeration in an open reservoir) must be taken if dissolved gases exceed 100% saturation. It is not a simple matter to completely remove supersaturation, and frequent checking should be done if the problem is known or suspected to exist. Water temperature, dissolved oxygen, and pH should be monitored for each culture, preferably daily.

2.4.5 Temperature

When *C. dubia* are brought into the laboratory, the transport water should be replaced gradually with culture water (Subsection 2.4.4) over a period of ≥2 days. Water temperature should be changed at a rate not exceeding 3°C/day until the desired temperature is reached. *Ceriodaphnia* should be cultured at a temperature of 25 ± 1°C. If cultures are maintained outside this temperature range, temperature should be adjusted gradually (≤3°C/day) to within the range 25 ± 1°C, and held there for a minimum of two weeks before the test is initiated. Temperature in the culture vessels should be periodically checked and compared with that in the constant-temperature room, water bath or incubator to ensure that the organisms are being cultured within the desired temperature range.

2.4.6 Dissolved Oxygen

Water to be used as culture medium should be aerated vigorously just before use, to ensure its saturation with oxygen and to prevent its supersaturation with gases. Its dissolved oxygen content should be measured at this time to confirm that a value within the range 90 to 100% saturation has been attained. The aeration of culture vessels is not required provided that cultures are maintained as indicated in Subsection 2.4.1.

2.4.7 pH

The pH of the culture medium should be within the range 6.0 to 8.5. Values for pH within the range 7.0 to 8.5 are preferred.

2.4.8 Hardness

Unlike certain daphnid species, *C. dubia* can be cultured successfully (to meet health criteria identified in Subsection 2.4.11) in soft or hard water (ASTM, 1989). Notwithstanding, marked differences in hardness (and alkalinity) between culture and control/dilution water could cause osmotic stress. Accordingly, *C. dubia* should be cultured in water with similar or identical hardness and alkalinity to that which will be used in tests as the control/dilution water[g]. Organisms used in tests should be derived from two or more prior generations cultured from birth in water with a hardness within a range ±20% of that of the control/dilution water (Section 3.4).

Some tests (e.g., those with samples of receiving water, or those intending inter-laboratory comparison of results) might require the use of reconstituted water to achieve a desired water hardness (see Sections 4.1 and 5.3). Formulae for preparing reconstituted water of a desired hardness (and pH) are given in Table 2 (U.S. EPA, 1989); other suitable formulae

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[g] Culture water could be reconstituted water of the same source and formulation as that to be used in the test for the control and dilutions, or a natural water with hardness adjusted to within a range ±20% of that of the control/dilution water. Any greater differences in hardness (and/or alkalinity) between culture and control/dilution water could result in erroneous test results due to osmotic stress on the organisms. For most waters, adjustment for hardness differences should also adjust for differences in alkalinity. Separate adjustment for hardness and alkalinity is generally impractical.

are also available (e.g., ISO, 1982). Preparations from commercial mineral waters can also provide suitable reconstituted water, for example a mixture of 20% Perrier™ and 80% deionized water yields a satisfactory moderately hard water (U.S. EPA, 1989). Alternatively, the laboratory supply of uncontaminated ground, surface, or dechlorinated municipal water may be adjusted to the desired hardness by dilution with deionized or distilled water (if too hard) or by the addition of the required quantity of reconstituted hard water or the appropriate ratio and amount of salts (if too soft).

2.4.9 Feeding

Daily feeding is required during culturing (and testing) of *C. dubia*.[h] The food used should be sufficient and suitable to maintain the test organisms in a nutritional state that will support growth, survival, and reproduction, and achieve the health criteria specified in Subsection 2.4.11. Various combinations of yeast, Cerophyll™ and

Table 2 Preparation of Reconstituted Water of a Desired Hardness (from U.S. EPA, 1985a)[1]

	Reagent Added [2,3] (mg/L)				Final Water Quality	
Water Type	$NaHCO_3$	$CaSO_4$[4]	$MgSO_4$	KCl	Hardness[5]	pH[6]
very soft	12.0	7.5	7.5	0.5	10 to 13	6.4 to 6.8
soft	48.0	30.0	30.0	2.0	40 to 48	7.2 to 7.6
moderately hard	96.0	60.0	60.0	4.0	80 to 100	7.4 to 7.8
hard	192.0	120.0	120.0	8.0	160 to 180	7.6 to 8.0
very hard	384.0	240.0	240.0	16.0	280 to 320	8.0 to 8.4

1 Reconstituted waters of a desired hardness may also be prepared using mineral water (e.g., Perrier™), diluted with deionized water. For instance, a mixture of 20% Perrier water and 80% deionized water will provide a suitable moderately hard reconstituted water (U.S. EPA, 1989).

2 Add reagent-grade chemicals to distilled or deionized water. Addition of 2 to 5 µg of selenium and 1 to 2 µg of crystalline vitamin B_{12} per litre is recommended. Reconstituted water should be aerated vigorously in a clean vessel for at least 24 h prior to use.

3 A time-saving procedure is to prepare stock solutions of $NaHCO_3$, $MgSO_4$, and KCl in deionized water. Details are available from J.M. Lazorchak or P.A. Lewis, U.S. EPA, Environmental Monitoring Systems Laboratory, Quality Assurance Research Division, 3411 Church Street, Cincinnati, Ohio, 45244.

4 $CaSO_4 \cdot 2H_2O$

5 Expressed in mg/L as $CaCO_3$.

6 Approximate pH after aerating for 24 h.

h Organisms could be stressed by less frequent feedings, resulting in low numbers of young, large numbers of males, and/or ephippial females (U.S. EPA, 1989).

trout chow* (YCT), if provided along with unicellular algae (most frequently *Selenastrum capricornutum*)[i], will provide suitable nutrition if fed daily (Cooney *et al.*, 1988; ASTM, 1989; Cowgill, 1989; U.S. EPA, 1989). A mixed algal diet, usually a green alga (*Ankistrodesmus convolutus* or *S. capricornutum*) and a freshwater diatom (*Nitzchia frustulum*) appears to sustain healthier animals than unialgal diets (Cowgill, 1989). Other food sources have also been used with success (Anon., 1989).

The U.S. EPA (1989) recommends that *C. dubia* routinely be fed YCT and algae in order to assure good nutrition and provide greater standardization of culture (and test) conditions. Formulae for preparing this food are given in Appendix D**. Final choice of ration and feeding regime is left to the discretion of the individual laboratory, and should be based on experience and success in meeting the health criteria specified for cultured organisms (Subsection 2.4.11).

If the YCT-algal diet is used, mass cultures should be fed at the rate of 7 mL YCT and 7 mL algae concentration per litre culture. Individual cultures should be fed at the rate of 0.1 mL YCT and 0.1 mL algae concentrate per 15-mL culture (U.S. EPA, 1989). Food should be added to fresh culture medium immediately before or after the transfer of organisms. Algal concentrate and YCT must be thoroughly mixed by shaking before dispensing. If the YCT is stored frozen, aliquots thawed for use must be stored in a refrigerator (not re-frozen). Unused portions of unfrozen or thawed YCT must be discarded after two weeks. Unused portions of algal concentrate are to be stored in the refrigerator and discarded after one month.

2.4.10 Handling Organisms

Handling and transfer of *C. dubia* should be minimal and physical shock to culture vessels must be avoided. Organisms should be transferred from one container to another using a smooth glass pipette. A disposable pipette with the delivery end cut off and fire polished to provide an opening of approximately 2 mm is ideal for this purpose (U.S. EPA, 1985a). The tip of the pipette should be kept under the surface of the water when the daphnids are released.

Organisms that are dropped or injured or touch dry surfaces during handling must be discarded. The amount of solution carry-over during transfer of organisms should be restricted to that necessary to facilitate the transfer.

2.4.11 Health Criteria

Individual brood cultures of *C. dubia* to be used in toxicity tests should meet the

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* Researchers at U.S. EPA's Environmental Monitoring Systems Laboratory in Cincinnati, Ohio recommend using the commercially available tropical fish food *Tetra-min*™ as a suitable substitute for the commercially available trout chow (J.M. Lazorchak and P.A. Lewis, pers. comm., 1991).

i Other algae used as a food for *Ceriodaphnia* include *Ankistrodesmus convolutus, A. falcatus, Chlamydomonas reinhardtii* and *Scenedesmus* sp. (Cooney *et al.*, 1988; NWRI, 1988; ASTM, 1989; Cowgill, 1989). Sources of algal cultures include laboratories engaged in aquatic toxicity testing, commercial biological supply houses, and the University of Toronto Culture Collection (Dept. of Botany, University of Toronto, Toronto, Ontario, M5S 1A4. Telephone (416) 978-3641, Fax (416) 978-5878. Delivery time is about a week and there is a small fee).

** From U.S. EPA (1989).

following health criteria (ASTM, 1989; U.S. EPA, 1989):

- No more than 20% of the brood organisms in individual cultures should die within the seven-day period prior to testing.
- At least six young should be produced by the brood organism in its previous brood.
- Brood organisms in individual cultures must produce an average of at least 15 young per adult within the seven-day period before testing.
- Ephippia must not be present in the culture.

A further indication of the health of the culture and its suitability for use in a toxicity test is provided by the test for daphnid sensitivity to a reference toxicant (see Section 4.6).

Test System

3.1 Facilities and Apparatus

The test may be performed in a water bath, environmental chamber, or equivalent facility with good temperature control (25 ± 1°C). This facility should be well ventilated, and isolated from physical disturbances that could affect the test organisms. The test facility should also be isolated from that where daphnids are cultured. Dust and fumes within the test and culturing facilities should be minimized.

Construction materials and any equipment that may contact the test solutions or control/dilution water should not contain any substances that can be leached into the solutions or increase sorption of test material (see Subsection 2.4.2). The laboratory must have the instruments to measure the basic water quality variables (temperature, conductivity, dissolved oxygen, pH) and must be prepared to undertake prompt and accurate analysis of other variables such as: hardness, alkalinity, ammonia, and residual chlorine.

3.2 Lighting

Lighting conditions to which test organisms are subjected should be the same as those defined in Subsection 2.4.3. The photoperiod (16 ± 1 h light:8 ± 1 h dark) is to be timed to coincide with that to which the organisms have been acclimated.

3.3 Test Vessels

Vessels used most frequently for this test are 30-mL capacity plastic cups or glass beakers. Smaller (≥20 mL) or larger-capacity clear plastic cups, glass beakers, or glass test tubes may also be used. Supporting boards or racks suitable for holding large numbers of small test vessels (e.g., six rows of ten test vessels per board) are recommended for use (see Subsection 2.4.2). Sheets of glass should be used to cover test vessels[j].

3.4 Control/Dilution Water

The choice of control/dilution water depends on a number of variables including the test material and intent (see Sections 5 to 7), the hardness of the solution(s) to be tested, and the hardness and type of water in which the test organisms have been cultured (Subsection 2.4.4). Accordingly, control/dilution water may be uncontaminated groundwater or surface water from a stream, river, or lake; dechlorinated municipal water from an uncontaminated source[k]; reconstituted water of a desired pH and hardness (see Subsection 2.4.8); or a sample of receiving water collected upstream of the influence of the contaminant source, or adjacent to the source, but removed from it.

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[j] Transparent covers will allow the illumination of test organisms while minimizing evaporation of test solutions and reducing their contamination.

[k] The addition of thiosulphate or other chemicals to dilution water in order to remove residual chlorine is not recommended. Such chemical(s) could alter toxicity.

If surface water is to be used as control/dilution water, this water should be filtered through a 60-µm plankton net to assure the absence of undesirable organisms (U.S. EPA, 1989). If receiving water is to be used, conditions for its collection, transport, and storage should be as described in Section 6.1.

Ideally, the quality of the culture and control/dilution waters should be identical or essentially the same. Notwithstanding, the purpose of the test (e.g., evaluation of receiving waters for toxicity) or problems of practicality, logistics, or cost could lead to the selection of a control/dilution water that is not the same as the culture medium. The hardness (or anticipated hardness, based upon previous analysis of this water source) of the intended control/dilution water should be known before the test is initiated. In instances where the hardness of control/dilution water differs from that of the culture water by greater than ±20% of this value, new individual cultures should be started using either the control/dilution water or reconstituted water adjusted to within this range. A minimum of two generations of brood organisms preceding the neonates to be used for the test should be acclimated to this water (Subsection 2.4.8).

Section 4

Universal Test Procedures

Procedures described in this section apply to all the tests of chemicals and wastewaters described in Sections 5, 6 and 7. All aspects of the test system described in the preceding Section 3 must be incorporated into these universal test procedures.

A summary checklist in Table 3 gives recommended universal procedures for performing three-brood renewal toxicity tests with *Ceriodaphnia dubia*, and also procedures for testing specific types of materials.

4.1 Preparing Test Solutions

All test vessels, measurement and stirring devices, and daphnid-transfer apparatus must be thoroughly cleaned and rinsed in accordance with good laboratory procedures. Suitable cleaning procedures are given by U.S. EPA (1989). Control/dilution water should be used as the final rinse water.

Reconstituted water with the desired hardness (Subsection 2.4.8) may be prepared for use as the dilution and control water. Table 2 provides guidance concerning types and quantities of reagent-grade chemicals to be added to distilled or deionized water in order to prepare control/dilution (or culture) water of a specific hardness, alkalinity, and pH. The use of "moderately hard" reconstituted water (80 to 100 mg $CaCO_3$/L) is recommended for tests requiring a high degree of standardization and intercomparability of test results[1]. Freshly prepared reconstituted water should be aerated vigorously in a nontoxic vessel for at least 24 h before use (U.S. EPA, 1989).

Uncontaminated groundwater, natural surface water, or dechlorinated municipal water may also be adjusted to a desired hardness and used as the dilution and control water. Such waters may be diluted with deionized water (if too hard) or increased in hardness by addition of the appropriate ratio and amount of reagent-grade chemicals (Table 2).

The characteristics of the control/dilution water used daily throughout the test period should be uniform. Uniformity is improved if a sample of control/dilution water sufficient to complete the test is stored, and aliquots used for the daily renewal of test solutions (Section 4.3). A 10-L volume is adequate for the daily replacement of all test solutions (assuming ten replicate 15-mL volumes of each of five test concentrations plus a control) and for the required chemical analyses.

The control/dilution water is to be adjusted to the test temperature (25 ± 1°C) before use. This water must not be supersaturated with excess gases (see Subsection 2.4.4). Before it is used, the control/dilution water should have a dissolved oxygen content 90 to 100% of the air-saturation value. As necessary, the

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[1] U.S. EPA (1989) recommends the use of moderately hard (80 to 100 mg/L as $CaCO_3$) reconstituted water as culture and control/dilution water for tests intended to estimate the chronic toxicity of effluent samples. Preparation of moderately hard reconstituted water using dilute mineral water (e.g., 20 % Perrier™ water; U.S. EPA, 1989) is desirable since it could be less deficient in essential trace elements.

Table 3 Checklist of Recommended Test Conditions and Procedures for Three-Brood Chronic Toxicity Tests with *Ceriodaphnia dubia*

Universal	
Test type	- static renewal (at least once daily)
Test duration	- until 60% of control organisms have three broods (normally 7 ± 1 days)
Control/dilution water	- uncontaminated ground, surface, or dechlorinated municipal water, or reconstituted water; moderately hard reconstituted water if a high degree of standardization is desired; upstream receiving water to assess toxic impact at a specific location; dissolved oxygen (DO) 90 to 100% saturation at time of use; hardness within range ±20% of value for culture medium
Test vessel	- normally 30-mL capacity plastic cup, glass beaker, or glass test tube, containing ≥15 mL of test or control solution
Organisms	- neonates (<24 h old and within 8 h of the same age) of *Ceriodaphnia dubia*; one neonate per test vessel; ≥10 neonates per test treatment; equal number of neonates among treatments
Temperature	- 25 ± 1°C, daily mean and limits
DO/aeration	- no aeration except in special instances; DO 40 to 100% saturation throughout the test
pH	- no adjustment if pH of test solution is within the range 6.0 to 8.5; a second (pH-adjusted) test might be required or appropriate if pH of sample/solution is beyond this range
Lighting	- "cool white" fluorescent, ≤600 lux at surface, 16 ± 1 h light : 8 ± 1 h dark
Feeding	- daily, with 0.1 mL YCT and 0.1 mL algal suspension (or suitable alternate diet) added to each test vessel
Measurements	- temperature, pH, and DO, at least at beginning and end (before renewal) of each 24-h exposure, in representative concentrations; conductivity at least at start of 24-h periods; hardness of control and (as a minimum) highest test concentration, at least before starting the test
Observations	-daily, for deaths and numbers of live neonates produced
Endpoints	- mortality and reproduction; NOEC/LOEC and/or ICp for multi-concentration tests; if appropriate, LC_{50} at selected time

Reference toxicant	- one or more of sodium chloride, phenol, or zinc sulphate; standard test for NOEC/LOEC and/or ICp, within 14 days of definitive test
Test validity	- Invalid if control mortality >20% and/or if an average of <15 live young produced per surviving female in the control solution(s)
Chemicals	
Solvents	- to be used only in special circumstances; maximum concentration, 0.1 mL/L.
Concentration	- desirable to measure at least at the beginning and end of the renewal period on the first and last days of the test, in high, medium, and low strengths and control(s)
Control/dilution water	- as specified and/or depends on intent; reconstituted for a high degree of standardization; receiving water if concerned with local toxic impact; otherwise, uncontaminated laboratory water
Effluents, Elutriates, and Leachates	
Sample requirement	- for off-site tests, a minimum of three samples are collected or prepared (elutriates), and used as indicated in Section 6.1; for on-site tests, samples are collected daily, and used within 24 h; volumes of 1 L (single-concentration test) or 2 L (multiple-strength test) are adequate
Transport and storage	- if warm (>7°C), cool to 1 to 7°C with ice or frozen gel packs; transport at 1 to 7°C (preferably 4 ± 2°C) using frozen gel packs as necessary; Sample must not freeze during transit; store in the dark at 1 to 7°C (preferably 4 ± 2°C); use in testing should begin within 24 h and must start within 72 h of sampling/extraction
Control/dilution water	- as specified and/or depends on intent; laboratory water, reconstituted water or "upstream" receiving water for monitoring and compliance
High solids	- second test with filtered sample is an option to assess the effects of solids
Receiving water	
Sample requirement	- as for effluents, leachates, and elutriates
Transport and storage	- as for effluents, leachates, and elutriates
Control/dilution water	- as specified and/or depends on intent; if studying local impact, use "upstream" receiving water as control/dilution water

required volume of control/dilution water should be aerated vigorously (oil-free compressed air passed through air stones) immediately before use, and its dissolved oxygen content checked to confirm that 90 to 100% saturation has been achieved.

The test concentrations and numbers of test solutions to be prepared will depend on the purpose of the test. Regulatory or monitoring tests of wastewaters or receiving waters could, in some instances, involve the preparation of only one test concentration (e.g., 100% sample) plus a control (see Sections 6 and 7). For tests intended to estimate the NOEC/LOEC and/or ICp, at least five test concentrations plus a control solution (100% control/dilution water) are to be prepared*. An appropriate geometric dilution series, in which each successive concentration is about 50% of the previous one (e.g., 100, 50, 25, 12.5, 6.3, etc.), may be used. Test concentrations may be selected from other appropriate dilution series (see Appendix E). If a high rate of mortality is observed within the initial 2 h of the test, additional dilutions can be added. A dilution factor as low as 30% (e.g., concentrations 100, 30, 9, etc.) is not recommended for routine use because of poor precision of the estimate of toxicity; however, it might be used if there is considerable uncertainty about the range of concentrations likely to be toxic.

When water other than that in which the organisms have been cultured is used as dilution and control water, a second control solution should be prepared using the culture water. Upstream water cannot be used as control/dilution water if it is toxic according to the criteria of the test for which it was intended[m]. In such cases, the culture water should be used as the control water and for all dilutions.

For a given test, the same control/dilution water is to be used for preparing the control and all test concentrations. Each test solution must be mixed well using a glass rod, Teflon™ stir bar or other device. The temperature, dissolved oxygen, and pH of each test solution should be checked upon mixing.

Sample/solution temperature should be adjusted as required to attain an acceptable value for each solution (25 ± 1°C). If (and only if) the measured dissolved oxygen concentration at this time in one or more test solutions is <40% or >100% of air saturation, all test solutions should be pre-aerated (prior to daphnid exposure). To achieve this, bubbles of oil-free compressed air should be dispensed through a disposable glass pipette. Bubble size should be in the 1- to 3- mm range. Any pre-aeration of solutions should be at a minimal rate effective for aeration of the particular vessel and volume of fluid being used. Duration of

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* More than five concentrations may be necessary, especially if the purpose or the test includes determination of LC_{50}(s). A preliminary range-finding test may be conducted before starting the definitive test. A range-finder normally covers a broader concentration range, and is frequently terminated in 24h or less.

[m] The use of water other than upstream receiving water as dilution and control water will not enable the natural substances or other contaminants within the receiving water to show any effect on the toxicity of the test material. For instance, natural chelating agents such as humic or fulvic acids in the receiving water could bind with the test material and reduce its toxic impact. Conversely, the presence of contaminants in upstream water could increase the toxicity determined for the test material if it was diluted with that water.

pre-aeration should be the lesser of 20 minutes and attaining 40% saturation in the highest test concentration (or 100% saturation, if supersaturation is evident). Any pre-aeration of test solutions should normally be discontinued following this period and the test initiated, regardless of whether 40 to 100% saturation was achieved in all test solutions (see Subsection 4.4.1).

Adjustment of sample/solution pH might be necessary (see Subsection 4.4.2). Solutions of hydrochloric acid (HCl) or sodium hydroxide (NaOH) at strengths ≤1 *N* should normally be used for all pH adjustments. Some situations (e.g., effluent samples with highly-buffered pH) could require the use of higher strengths of acid or base.

Abernethy and Westlake (1989) provide useful guidelines for adjusting pH. Aliquots of test solutions or samples receiving pH-adjustment[n] should be allowed to equilibrate after each incremental addition of acid or base. The amount of time required for equilibration will depend on the buffering capacity of the solution/sample. For effluent samples, a period of 30 to 60 minutes is recommended for pH adjustment (Abernethy and Westlake, 1989). Once daphnid exposure is initiated, the pH of each test solution is monitored (Subsection 4.5) but not adjusted.

4.2 Beginning the Test

Once the test solutions have been prepared and any permitted and/or required adjustments made for temperature, pH, dissolved oxygen, and solids content (see Sections 4.1 and 6.4), the test should be initiated. In instances where the influence of sample/solution hardness on toxicity is of concern, water hardness should be measured in at least the control, low and high test concentrations. These initial measurements should be made on larger volumes of solutions made up in beakers, after any pH adjustments have been made and just before their use to fill the test vessels. With the exception of special investigations[o], no attempt should be made to adjust the hardness of samples or test solutions.

Test solutions, consisting of ten replicates of each test concentration including the control water(s), should be randomly assigned to a position on a test board, using a template (U.S. EPA, 1989) or a table of random numbers. If a template is used, it should be one of several available (to prevent the same ordering for each test). Once a numbered position for each test solution has been

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[n] If the test material is being diluted and the toxicity of one or more dilutions to daphnids is being studied, it is preferable to adjust the pH of each test solution rather than that of the (undiluted) test material. Addition of acid or base to a sample of undiluted effluent, elutriate, or leachate can significantly alter the ionized/non-ionized form of some toxicants (e.g., ammonia, resin acids), and can destroy the integrity of the test sample.

Tests with chemicals or samples of effluent, leachate, or elutriate requiring pH adjustment usually require the separate adjustment of each test solution (including the control). Those with samples of receiving water normally adjust an aliquot of the diluted sample, prior to preparing the test concentrations.

[o] Alteration of the hardness of the sample or test solution by the addition of the appropriate ratio and amount of salts (Table 2) could be undertaken in special situations (e.g., second test) where sample/solution hardness is appreciably lower than that of the culture/control/dilution water and the investigator wishes to assess the influence of this difference on toxicity. Reduction of sample or solution hardness could not be achieved without its dilution (e.g., with deionized water) or chemical treatment, neither of which is acceptable.

assigned, an identical measured volume (≥15 mL) of each solution should be added to each of the ten replicate test vessels. Thereafter, the ten test vessels are transferred to the assigned (same number) positions on the board. This process is repeated for each of the remaining test solutions.

Neonate daphnids used in tests must be <24 h old and within 8 h of the same age; it would be desirable if the neonates were <12 h old and within 6 h of the same age. The neonates should come from individual cultures which satisfy the requirements indicated in Subsection 2.4.1 and health criteria given in Subsection 2.4.11. For multi-concentration tests, ten brood cups/beakers*, each with eight or more young, are identified on a brood board for use in setting up the test. To begin the test, one neonate from the first brood cup is transferred to each of the six test vessels in the first row on the test board (each board normally holds ten rows and six columns of test vessels). A second brood cup is chosen at random, and one neonate from this cup transferred to each of the six test vessels in the second row. This procedure is repeated until each of the 60 test vessels** contains one neonate (U.S. EPA, 1989).

The appropriate volumes of food (e.g., 0.1 mL YCT and 0.1 mL algae if diet outlined in Appendix D is used) are to be added to each test solution immediately before or after the introduction of a single test organism (Subsection 2.4.9). If neonates selected from individual cultures for the test are held in separate cups or beakers for more than 1 h before transfer to test solutions, they should also be fed during this transitional period.

4.3 Renewing Test Solutions

Each test solution must be renewed at least once daily[p]. Replacement solutions, including fresh inocula of food, should be prepared and added to a separate test board (same ordering sequence) as described in Sections 4.1 and 4.2. The first-generation daphnid must be transferred to the respective new solution (Section 2.4.10) and any live progeny counted, recorded and discarded. Dead neonates may be discarded without counting (U.S. EPA, 1989), although records of numbers dead or non-viable could prove useful for Toxicity Identification Evaluations (Mount and Anderson-Carnahan, 1988) or other research investigations. The used solutions should be chemically analyzed (Section 4.5) and discarded, or stored if additional chemical determinations are required (Section 5.4).

4.4 Test Conditions

The test is a static-renewal one, i.e., replacement of solutions at intervals of ≤24 h throughout the test.

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* Small glass test tubes, held in racks, may be substituted for cups or beakers held on brood boards (see Subsection 2.4.2).

** Depending on the nature of the test or test objectives, this number could be as few as 20 (i.e., 10 control vessels and 10 test vessels at a single concentration) or could exceed 60 in multiples of ten (more than six test concentrations including control).

[p] Solutions are usually renewed at 24-h intervals. Tests with volatile or unstable materials could require solution replacement at more frequent intervals (e.g., every 8 or 12 h).

Tests are initiated using a single neonate organism per 15-mL volume of test solution in each of ten replicate test vessels.

Daphnids are fed daily throughout the test. Food type and ration should be identical to that provided individual cultures and as described in Subsection 2.4.9.

The test should be conducted at a daily mean temperature of 25 ± 1°C.

Test solutions are not to be aerated.

The test is continued until at least 60% of the first-generation daphnids in the control solutions have produced three broods[q]. At 25 ± 1°C, this should occur within 7 ± 1 days. If ≥60% of the first-generation adults in the control solutions have not produced three broods by day nine, at this temperature, the test is invalid and should be terminated. Additionally, the test is not valid if mortality of first-generation test organisms in the control water exceeds 20% and/or reproduction in the controls averages <15 live young per surviving adult[r].

4.4.1 Dissolved Oxygen

The use of oxygen-saturated control/dilution water (Section 4.1) and daily renewal of test solutions will, in most instances, prevent dissolved oxygen levels in test solutions from becoming depressed to the extent that they stress the test organisms and influence the test results. The concentration of dissolved oxygen in each test vessel should be between 40 and 100% of saturation (i.e., 3.3 to 8.2 mg/L at 25°C) at all times during the test (ASTM, 1989). In those instances where the test material has a considerable oxygen demand and high concentrations (e.g., 100% effluent, leachate, or elutriate) are being tested, more frequent renewal of test solutions could be required to maintain an acceptable (≥40% saturation) DO level. Alternatively, the objective of the test might require this oxygen demand to be included as part of the measurement of sample toxicity, in which case the conventional renewal frequency (once/24 h) would normally be applied[p].

In certain cases (usually experimental), the investigators might wish to aerate oxygen-deficient test solutions, or prepare additional control solutions deficient in dissolved oxygen in order to examine the influence of this parameter on daphnid survival and reproduction rates (ASTM, 1989). Any aeration of solutions prior to ("pre-aeration") or during the test should be at a minimal rate effective for aeration of the particular vessel and volume of fluid being used. For this purpose, oil-free compressed air should be dispensed through a disposable glass pipette, with bubble size 1 to 3 mm. Duration of pre-aeration should be the lesser of 20 minutes and attaining 40% saturation

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[q] A test should be terminated earlier if the mortality rate for the first-generation control daphnids is >20%, or if ephippia are evident in controls.

[r] If test results are rendered invalid due to unacceptably low survival or reproduction rates for the control daphnids, the performance of the culture should be examined by checking their reproductive output (Subsection 2.4.11). A search should be made for contaminants in the control/dilution water and for nutritional deficiencies or other problems associated with the culture or the test. The search should be continued until control performance is acceptable.

A round-robin test with ten laboratories showed that average mortality of first-generation controls was only 2%. The overall mean production of young was 20 per female, with a range of averages from the various laboratories of 13 to 31 young/female (Anderson and Norberg-King, 1991).

in the highest test concentration (or 100% saturation, if supersaturation is evident). Aeration during and/or prior to testing must be reported (Section 8).

4.4.2 pH

Toxicity tests should normally be carried out without adjustment of pH. In instances where the chemical, wastewater, or receiving-water sample causes the pH of any test solution to be outside the range 6.0 to 8.5, and it is desired to assess toxic chemicals rather than the lethal or modifying effects of pH, then the pH of the test solutions or sample should be adjusted before use, or a second (pH-adjusted) test conducted concurrently using a portion of the same sample. For this (second) test, the initial pH of the sample, or of each test solution (see footnote "n", Section 4.1), could (depending on the test objectives) be neutralized (adjusted to pH 7.0) or adjusted to within ±0.5 pH units of that of the control/dilution water, before daphnid exposure. Another acceptable approach for this second test is to adjust each test solution (including the control) to pH 6.0 to 6.5 (if test sample has pH or causes such a pH) or to pH 8.0 to 8.5 (if sample has/causes pH >8.5).

If the purpose of the toxicity test is to gain an understanding of the nature of the toxicants in an effluent, elutriate, leachate, or receiving-water sample, pH adjustment is frequently used in combination with a number of other treatment techniques (e.g., oxidation, filtration, air stripping, addition of chelating agent) for characterizing sample toxicity. Mount and Anderson-Carnahan (1988) list pH adjustment as one of nine "Toxicity Identification Evaluation" (TIE) techniques which, when performed with an acutely toxic aqueous sample, provide the investigator with a useful method for assessing the physical/chemical nature of the toxicant(s) and their susceptibility to detoxification.

4.5 Test Observations and Measurements

The daphnid(s) in each test vessel must be observed daily (i.e., at 24-h intervals) during the test. Observation is improved if each test vessel is temporarily illuminated from the side or from below by placing it on a light box or by other means. A black background is also beneficial, and might be combined with advantageous lighting by having one light at the side and one underneath.

For test solutions that are opaque due to colour or suspended solids, the test solution should be transferred temporarily to a shallow dish (e.g., Petri™ dish) to assist in observations of daphnid survival and numbers of live young produced. Control solution(s) are to receive identical treatment. Surviving first-generation daphnids (i.e., those introduced to test solutions at the start of the test) should be transferred to fresh test solutions in 30-mL cups or beakers as soon as these observations are completed (see Sections 3.3 and 4.3).

The death of any first-generation daphnid is to be recorded upon observation. Death is indicated by lack of movement of the body, appendages and heart as observed through a dissecting stereo-microscope or other magnifying device[s]. Each live first-generation daphnid is to be transferred

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[s] With narcotic toxicants, daphnids might be completely immobile and the heart rate might slow to 1 or 2 beats per minute. In such a case, beating of the heart becomes the final criterion of death.

to its respective new test solution (see Subsection 2.4.10 and Section 4.3) immediately thereafter.

The number of live neonates produced by each first-generation daphnid must be counted and recorded during each daily observation. These young are discarded after counting. Any dead young observed should be discarded; counting of dead neonates is normally not required.[t]

Temperature must be monitored throughout the test. As a minimum, temperature must be measured at the beginning and end of each 24-h period of exposure (i.e., in fresh solutions and those to be discarded) in at least the high, medium, and low test concentrations and in the control(s). If temperature records are based on measurements other than in the test vessels (e.g., in a water bath, incubator, or controlled-temperature room within the vicinity of the test vessels), the relationship between these readings and temperatures within the vessels must be established. Continuous recordings or daily measurement of the maximum and minimum temperature are acceptable options.

Dissolved oxygen and pH must be measured at the beginning and end of each 24-h period of exposure (i.e., in fresh solutions and those to be discarded) in at least the high, medium, and low test concentrations, and in the control(s). For convenience, readings may be made using composites of the ten replicate solutions, or in one replicate from each treatment monitored.

Hardness of the control/dilution water and, as a minimum, the highest test concentration[u], should be measured before beginning the test (see Sections 3.4, 4.1, and 4.2). It might also be worthwhile to determine the alkalinity of these solutions.

As a check on test concentrations, it is recommended that conductivity be measured in each newly-prepared test solution, before dispensing it to the test vessels. Monitoring the conductivity of selected test solutions (e.g., the high, medium, and low test concentrations, and the control) at the beginning and end of their use might be desirable for certain test materials[v].

4.6 Test Endpoints and Calculations

The endpoints for chronic (three-brood) toxicity tests using *Ceriodaphnia dubia* are based on the adverse effects of test materials on daphnid survival and reproduction. There are two biological endpoints to the test, the first being based on increased mortality of the first-generation daphnids. The other endpoint is based on the reduction in the number of live neonates produced by each first-generation daphnid during the test period (7 ± 1 days). In both cases, the adverse effect is assessed by statistical

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[t] Young which are partially or fully developed might be released or might die in the test solution during the interval between their release and observation. These organisms are not to be included in calculations of number of (live) young produced by first-generation daphnids during the test (ASTM, 1989; U.S. EPA, 1989). Such information could, however, be useful for Toxicity Identification Evaluations (Mount and Anderson-Carnahan, 1988) or other research investigations.

[u] In tests with effluents, leachates, elutriates, and receiving-water samples, this will normally be the undiluted sample.

[v] Changes in conductivity of solutions during the test are indicative of chemical alterations (e.g., ionization, degradation).

comparison with data from the controls. The more sensitive of the two effects (normally, reduced reproductive success) is taken as the definitive indication of toxicity.

Various endpoints can be calculated from these data, and the rationale and methods of calculation are discussed in detail in U.S. EPA (1989). No-observed-effect concentrations and LOECs may be derived statistically by the hypothesis-testing approach, and this is recommended as a primary technique. The ICp may be calculated as a point-estimate of the inhibiting concentration for a specified percentage effect, and can be useful as an additional or substitute primary technique[w] (Norberg-King, 1988; U.S. EPA, 1989). Advice should be sought from a statistician in carrying out the analyses of results.

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[w] An interpolated point estimate called the percentage inhibiting concentration (ICp,) is a useful primary endpoint, as this statistic provides an estimation of a single concentration causing a specified effect, rather than the pair of concentrations represented by NOEC and LOEC. A major disadvantage of the NOEC/LOEC is that no variance can be calculated, and so no confidence limits can be given for the estimates. Nor can confidence limits be calculated for the TEC, i.e., the *threshold-effect concentration* which is the geometric mean of NOEC and LOEC. An alternative is to estimate the concentration(s) causing 20%, 25%, and/or 50% reductions in number of neonates produced per first-generation daphnid (i.e., the IC20, IC25, and/or IC50). In most cases, the IC20 or IC25 would be closer to the NOEC, whereas there is more statistical precision surrounding the IC50 and thus greater confidence in this point estimate. The ICp could be read from a graph which shows percentage reduction of number of young against the logarithm of test concentration. Such a graph should be plotted to provide a visual assessment of the nature of the data, and to check any mathematical estimates. A given ICp can be estimated by an assumption-free *linear interpolation* method, using a computer program called BOOTSTRP (Norberg-King, 1988; Appendix J of U.S., EPA, 1989). An up-to-date version of this program can be obtained by contacting Environment Canada (see Appendix B).

One or more percentage inhibiting concentrations (ICps) can be generated, together with the 95% confidence limits. These endpoint calculations are also useful to compare the toxicity of different samples of wastewaters or chemicals. In such comparions, the use of hypothesis testing based on NOEC/LOEC would be illogical, since it would not be possible to apply a test for significant difference between the NOECs/LOECs for the different samples (nor between the TECs). It is possible, however, to apply statistical tests with ICps since each one has confidence limits associated with it. Accordingly, ICps have advantages as primary endpoints to be used in addition to the NOEC/LOEC (and TEC) or to replace those values.

[x] The methods of TOXSTAT (Gulley *et al.*, 1989) are not detailed here since the instructions are best followed in the written description that accompanies the programs on computer diskette. An up-to-date version of TOXSTAT can be obtained by contacting Environment Canada (see Appendix B). Briefly, data are tested for normality by the Shapiro-Wilks test, and for homogeneity by Bartlett's test. If the data do not meet the requirements, it might be possible to transform them with logarithms or arc-sine to meet the requirements. It should be realized that the transformation might reduce the sensitivity of the analysis and the ability of the toxicicty test to detect differences.

If the data are regular or can be made so by suitable transformations, an analysis of variance is carried out. That is followed by Dunnett's test, a multiple-comparison test which tests each concentration for significant difference from the control. If there are unequal numbers of replicates, the Bonferroni t-test is substituted. As mentioned in Section 4.6, Williams' test is recommended as a better choice for comparing effects since it incorporates and uses some information about the gradation in concentration, rather than ignoring that information as is the case with Dunnett's test. Williams' test is available in TOXSTAT (Gulley *et al.*, 1989).

If a set of data cannot meet the requirements for normality or homogeneity, and cannot be transformed to do so, there are non-parametric tests provided in TOXSTAT which may be substituted (*Steel's many-one rank test* or the *Wilcoxon rank sum test* in the case of unequal replicates). Those non-parametric options may be used, and are powerful tools for data that are not normally distributed. The non-parametric tests are less powerful than parametric tests when used on normally distributed data, and in that situation they might fail to detect real differences in effect, i.e., an underestimate of sublethal toxicity could result.

For mortality, the NOEC/LOEC and/or ICp are determined from the number of first-generation daphnids that died in each replicate of the control and the various concentrations of wastewater or chemical. If there is complete mortality in all replicates at a given concentration, that concentration is excluded from the analysis to determine the NOEC/LOEC and/or ICp for mortality. If NOEC and LOEC are to be determined, the procedures are those given in the computer program TOXSTAT[x] (Gulley *et al.*, 1989). These start with a check of normality and homogeneity of data and provide suitable tests of significance for particular types of distribution, and also for sets of data in which the numbers of replicates are unequal because of accidental loss or other cause. The comparison of mortality in each test concentration with mortality in the control has customarily been done (U.S. EPA, 1989) by means of Dunnett's Test, a multiple-comparison procedure. However, that test ignores information since the magnitude of the concentration is not used, and therefore is not a particularly powerful way of discriminating effects (Masters *et al.*, 1991). Williams' test is also available in TOXSTAT and is designed to be sensitive to a response due to increasing concentration of toxicant (Gulley *et al.*, 1989). Williams' test is recommended as an alternative to Dunnett's test.

An LC_{50} might sometimes be calculated in multiple-concentration tests, for a defined exposure (e.g., 2, 4, or 7 days). If necessary, the range of test concentrations should be expanded upwards to ensure greater than 50% mortality in at least one concentration, so that the LC_{50} can be estimated. Appropriate methods for calculating the LC_{50} and 95% confidence limits are given in Appendix F.

The NOEC/LOEC and/or ICp for reproduction are determined from the number of live neonates produced in replicates of each test concentration. If NOEC and LOEC are calculated, the numbers are compared with those for the control by the TOXSTAT procedures outlined above for mortality. In this case also, a more sensitive analysis might be obtained by using Williams' test. If a female dies before producing young, a value of zero is assigned for number of neonates. Similarly, if a female dies during the test after producing young, the actual number of young is used in the analysis. Thus the comparison of reproduction actually incorporates both mortality of the first-generation daphnids and the reproductive rate (U.S. EPA, 1989). The lowest NOEC/LOEC, whether for first-generation mortality or reproductive success, is taken as the result of the test.

If NOEC and LOEC are determined, geometric average is often calculated for the convenience of having one number rather than two. Such a value may be used and reported, recognizing that it represents an arbitrary estimate of an effect-threshold that might lie anywhere in the range between the LOEC and NOEC. The calculated value of the geometric mean is governed by whatever concentrations the investigator happened to select for the test. No confidence limits can be estimated for the geometric mean, as is the case also for NOEC and LOEC. It is recommended that the geometric mean of NOEC and LOEC should be called the *TEC*, signifying *threshold-effect concentration*. The use of "threshold" is intended in the dictionary sense of "point at which an effect begins to be produced". That term is parallel to the terms EC_{50}, NOEC, and LOEC, and would be appropriate for any designated effect, whether lethal or sublethal, chronic or subchronic. The geometric mean of NOEC

and LOEC is often called the *chronic value* in the United States, but is often misused by applying it to results that are not derived from a true chronic exposure, i.e., a significant portion of the lifetime of the test organism. The term *subchronic value* is also sometimes applied to the mean from tests that are less than chronic.

The inhibiting concentration for a percentage reduction in number of young can also be used as a primary criterion of sublethal effect. An IC20 or IC25 appears to be currently favoured as a primary criterion of effect (i.e., 20% or 25% reduction in number of young compared to the control). Such an analysis could begin with a plot of percentage reduction of number of young against the logarithm of test concentration, with IC20 or IC25 read off. The graph would also serve as a check against results from mathematical computations. A straightforward *linear interpolation* method provides a mathematical estimate of ICp (Appendix J of U.S. EPA, 1989).

In a single-concentration test, a t-test is normally the appropriate method of comparing the data from the test concentration with those of the control, and the procedure for a t-test can be taken from any statistics textbook. An effect of the test material is accepted if mortality is significantly higher, or daphnid reproduction significantly lower, than the same statistics for the control. Requirements for homogeneity of variance and normality (Appendix H of U.S. EPA, 1989; Gulley *et al.*, 1989) must be satisfied before using the standard t-test. If the data do not satisfy the requirements, a non-parametric test could be selected with advice from a statistician; no particular test appears to have become standard practice as yet.

In some cases, the test-groups might not represent various concentrations of a single effluent or chemical, but a set of different samples, such as full-strength effluents from different industries or surface waters from different places. It might be desired to test not only whether each sample is different from the control, but also whether the samples are different from each other. That can be done by one option in the statistical program TOXSTAT[x] (Tukey's test). Such sets of tests should report the results of each sample tested, not as the NOEC/LOEC, but as the number of live neonates produced per first-generation daphnid as a percentage of the control(s), and whether that number was significantly different from the control(s).

4.7 Reference Toxicant

The routine use of a reference toxicant or toxicants is required to assess, under standardized test conditions, the relative sensitivity of the culture of *C. dubia* and the precision and reliability of data produced by the laboratory for that/those reference toxicant(s). Daphnid sensitivity to the reference toxicant(s) should be evaluated within 14 days before or after the toxicity test or during it. Ideally, the same stock of brood animals should be used for tests on both the reference toxicant and sample.

The criteria used in selecting the appropriate reference toxicants for this test included the following:

- chemical readily available in pure form;
- stable (long) shelf life of chemical;
- highly soluble in water;
- stable in aqueous solution;

- minimal hazard posed to user;
- easily analyzed with precision;
- good dose-effect curve for *Ceriodaphnia dubia*;
- known influence of pH on toxicity of chemical to test organism; and
- known influence of water hardness on toxicity of chemical to *Ceriodaphnia dubia*.

One or more of the following three chemicals (reagent-grade) are recommended for use as reference toxicants for this test: sodium chloride; zinc sulphate; phenol. Daphnid sensitivity should be evaluated by standard tests following the methods given in this document, to determine the NOEC/LOEC and/or ICp for one or all of these chemicals. The tests should use the control/dilution water that is customary at the laboratory, or moderately-hard reconstituted water if a greater degree of standardization is required[y].

A warning chart (Environment Canada, 1990b) should be prepared and updated for each reference toxicant used. Successive NOECs or ICps are plotted on this chart and examined to determine whether the results are within ± 2 SD of values obtained in previous tests. The geometric mean NOEC or ICp, together with its upper and lower warning limits (±2 SD, calculated on a geometric [logarithmic] basis)* are recalculated with each successive NOEC or ICp until the statistics stabilize (U.S. EPA, 1989; Environment Canada, 1990b).

If a particular NOEC or ICp falls outside the warning limits, the sensitivity of the neonates and the test system are suspect. Inasmuch as this might occur 5% of the time due to chance alone, an outlying value does not necessarily mean that the sensitivity of the population of daphnids or the precision of the toxicity data produced by the laboratory are in question. Rather, it provides a warning that this might be the case. A thorough check of the health of the culture (Subsection 2.4.11) together with all culturing and test conditions is required at this time. Depending on the findings, it could be necessary to commence the acclimation of new cultures of daphnids for possible use in tests with reference toxicants and/or test materials.

Use of warning limits does not necessarily indicate that a laboratory is generating consistent results. A laboratory that produced extremely variable data for a reference toxicant would have wide warning limits; a new datum point could be within the warning limits but still represent undesirable variation in results obtained in tests. A coefficient of variation of 20% or 30% is tentatively suggested as a limit by Environment Canada (1990b), and that seems to be a reasonable range. However, establishing a limit for allowable variation of results for testing reference toxicants would require more data on the reproduciblity that can be achieved in Canadian laboratories for

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[y] Moderately hard reconstituted water (Table 2) should be used for a greater degree of standardization, particularly if comparison with the results from other laboratories is desired (U.S. EPA, 1989). The test laboratory might prefer to use another water source (e.g., uncontaminated surface or groundwater) as the control/dilution water for routine reference toxicant tests. This is satisfactory provided that periodic (e.g., quarterly) tests are performed using moderately hard reconstituted water.

* If the NOECs or ICps fail to show a lognormal distribution, an arithmetic mean and SD may prove more suitable.

the test of reproduction and survival using *Ceriodaphnia dubia.*

Stock solutions of phenol and sodium chloride should be made up on the day of use. Concentration of sodium chloride should be expressed as the weight of the total salt (NaCl) in the water (g/L). Zinc sulphate (usually $ZnSO_4 \cdot 7H_2O$, molecular weight 4.398 times that of the zinc) should be used for preparing stock solutions of zinc, which should be acidic (pH 3 to 4). Acidic zinc solutions may be used when prepared, or stored in the dark at 4 ± 2°C for several weeks before use. Concentration of zinc should be expressed as mg Zn^{++}/L.

Concentrations of reference toxicant in all stock solutions should be measured chemically by appropriate methods (e.g., APHA *et al.*, 1989). Upon preparation of the test solutions, aliquots should be taken from at least the control, low, middle, and high concentrations, and analyzed directly or stored for future analysis should the NOEC or ICp be atypical (outside warning limits). If stored, sample aliquots must be held in the dark at 4 ± 2°C. Zinc solutions should be preserved before storage (APHA *et al.*, 1989). Stored aliquots requiring chemical measurement should be analyzed promptly upon completion of the toxicity test. It is desirable to measure concentrations in the same solutions at the end of the test, after completing biological observations. Calculations of NOEC or ICp should be based on the geometric average measured concentrations if they are appreciably (i.e., ≥20%) different from nominal ones and if the accuracy of the chemical analyses is reliable.

4.8 Legal Considerations

Care must be taken to ensure that samples collected and tested with a view to prosecution will be admissible in court. For this purpose, legal samples must be: representative of the material being sampled; uncontaminated by foreign substances; identifiable as to date, time and location of origin; clearly documented as to the chain of continuity; and analyzed as soon as possible after collection. Persons responsible for conducting the test and reporting the findings must maintain continuity of evidence for court proceedings (McCaffrey, 1979), and ensure the integrity of the test results.

Section 5

Specific Procedures for Testing Chemicals

This section gives particular instructions for testing chemicals, additional to the procedures in Section 4.

5.1 Properties, Labelling, and Storage of Sample

Information should be obtained on the properties of the chemical to be tested, including water solubility, vapour pressure, chemical stability, dissociation constants, n-octanol:water partition coefficient, and biodegradability. Data-sheets on safety aspects of the material should be consulted, if available. If solubility in water is in doubt or problematic, acceptable procedures used previously for preparing aqueous solutions of the chemical should be obtained and reported. Other available information such as structural formula, degree of purity, nature and percentage of significant impurities and additives, handling precautions, and estimates of toxicity to humans, should be obtained and recorded[z]. An acceptable analytical method for the chemical in water at concentrations intended for the test should also be known, together with data indicating the precision and accuracy of the analysis.

An estimate of the lowest concentration of test material that is acutely lethal to *C. dubia* is useful in predicting chemical concentrations appropriate for the chronic toxicity test. The results of a 48-h static LC_{50} (see Section 4.6 and Appendix F), conducted at 25 ± 1°C using the control/dilution water intended for the chronic test, will provide this information. Neonate daphnids, cultured under conditions similar or identical to those used for organisms to be employed in the chronic test, should be used to measure the acute (48 h) lethality of the test chemical. Other test conditions and procedures should be as similar as possible to those used in the chronic test.

Chemical containers must be sealed and coded or labelled (e.g., chemical name, supplier, date received) upon receipt. Storage conditions (e.g., temperature, protection from light) are frequently dictated by the nature of the chemical. Standard operating procedures for chemical handling and storage should be followed.

5.2 Preparing Test Solutions

Test solutions of the chemical may be prepared either by adding pre-weighed (analytical balance) quantities of chemical to control/dilution water as required to give the nominal strengths to be tested[aa], or by adding measured volumes of a stock solution. If the latter is used, the concentration and stability of the test material in the stock solution should be determined before beginning the test. Stock solutions subject to photolysis

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[z] Knowledge of the properties of the chemical will assist in determining any special precautions and requirements necessary while handling and testing it (e.g., testing in a specially vented facility, need for solvent). Information regarding chemical solubility and stability in fresh water will also be useful in interpreting test results.

[aa] This approach is normally used only for preparing high concentrations or large volumes of test solutions. Otherwise, greater accuracy can be achieved by preparing a stock solution.

should be shielded from light. Unstable stock solutions must be prepared daily or as frequently as is necessary to maintain consistent chemical concentrations for each renewal of test solutions.

Stock solutions should be prepared by dissolving the chemical in control/dilution water. For chemicals that do not dissolve readily in water, stock solutions may be prepared using the generator-column technique (Billington *et al.*, 1988; Shiu *et al.*, 1988) or, less desirably, by ultrasonic dispersion[bb]. Organic solvents, emulsifiers, or dispersants should not be used to increase solubility except in cases where those substances might be formulated with the test chemical for its normal commercial purposes. If used, an additional control solution should be prepared containing the same concentration of solubilizing agent as in the most concentrated solution of the test chemical. Such agents should be used sparingly and should not exceed 0.1 mL/L in any test solution. If solvents are used, the following are preferred (U.S. EPA, 1985b; ASTM, 1989): triethylene glycol, dimethyl formamide, methanol, ethanol, and acetone.

5.3 Control/Dilution Water

For normal intra-laboratory assessment of chemical toxicity, control/dilution water may be reconstituted water or the laboratory supply of uncontaminated ground, surface, or dechlorinated municipal water used routinely for culturing *C. dubia*. In instances where the toxic effect of a chemical on a particular receiving water is to be appraised, sample(s) of the receiving water could be taken from a place that was isolated from influences of the chemical, and used as the control/dilution water[m,cc,dd]. Examples of such situations would include appraisals of the toxic effect of chemical spills or intentional applications of chemical (e.g., spraying of a pesticide) on a waterbody. The laboratory supply of natural water or reconstituted water might also be used for this purpose, especially where the collection and use of receiving water is impractical. Normal laboratory water might also be appropriate for use in other instances (e.g.,

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[bb] Ultrasonic dispersion is not a preferred technique, since the ultrasonics might disperse some of the toxic chemical as an emulsion or as fine droplets, and daphnids might take in the droplets selectively, by their filtering activities. Additionally, ultrasonic dispersion can result in variations in the biological availability of the chemical and thus in its toxicity, due to the production of droplets differing in size and uniformity. Droplets could also migrate towards the surface during the test.

[cc] Contaminants already in the receiving water could add toxicity to that of the chemical or wastewater under investigation. In such instances, uncontaminated dilution water (natural, reconstituted, or dechlorinated municipal) would give a more accurate estimate of the toxicity of the test material, but not necessarily of its total impact at the site of interest.

If the intent of the test is to determine the effect of a specific chemical or wastewater on a specific receiving water, it does not matter if that receiving water modifies sample toxicity by the presence of, for instance, humic substances or additional toxicants. In the case of added toxicity from the receiving water, it is appropriate to include in the test, as a minimum, a second control of culture water and, as a maximum, another series of concentrations using culture water as diluent.

[dd] An alternative (compromise) to using receiving water as dilution and control water is to adjust the pH and hardness of the laboratory water supply (or reconstituted water) to that of the receiving water. Depending on the situation, the adjustment could be to those values measured at a particular time, or to seasonal means.

preliminary or intra-laboratory assessment of chemical toxicity).

If a sample of upstream receiving water is to be used as dilution and control water, a separate control solution should be prepared using the culture water (Subsection 2.4.4). The survival and reproduction rates for *C. dubia* held in ten replicate solutions of culture water should be compared to those for test organisms held in the ten replicate solutions of receiving water[m,ee]. The sample of upstream water is unsuitable for use as the control or as dilution water if mortalities of first-generation daphnids exceed 20% or if fewer than 15 neonates per surviving adult are produced during the test (see Section 4.4). Test conditions and procedures for evaluating each control solution should be identical and as described in Section 4.

If a high degree of standardization is required (for instance, if the toxicity of a chemical is to be determined and compared at a number of test facilities), reconstituted water of specified hardness should be used for all dilutions and as the control water[ff]. The use of moderately hard (80 to 100 mg/L) water is recommended for such purposes[l] (see Section 4.1). If hardness and other qualities of the dilution water are expected to affect the toxicity of the test chemical, and the intent of the study is to assess the degree to which dilution water might influence chemical toxicity, a series of tests could be run with different reconstituted waters (Table 2) and/or natural waters.

5.4 *Test Observations and Measurements*

In addition to the observations on toxicity described in Section 4.5, there are certain additional observations and measurements to be made during tests with chemicals.

During preparation of solutions and at each of the prescribed observation times during the test, each solution should be examined for evidence of chemical presence and change (e.g., odour, colour, opacity, precipitation, or flocculation of chemical). Any observations should be recorded.

It is desirable and recommended that test solutions be analyzed to determine the concentrations of chemicals to which *C. dubia* are exposed[gg]. If chemicals are to be measured, sample aliquots should be taken from at least the high, medium, and low test concentrations, and the control(s). As a minimum, separate analyses should be performed with samples taken at the beginning and end of the renewal period on the first and last days of the test (ASTM, 1989). Samples from the old (used) test

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ee A comparison of daphnid survival and reproduction rates in the culture water versus the receiving-water sample collected upstream might distinguish demonstrable toxic responses attributable to contaminants within the upstream water.

ff Since the hardness, pH, and other characteristics of the control/dilution water can markedly influence the toxicity of the test material, the use of a standard reconstituted water (i.e., moderately hard water, 80 to 100 mg/L as $CaCO_3$,) might provide results that could be compared in a meaningful way with results from other laboratories.

gg Such analyses need not be undertaken in all instances, due to analytical limitations, cost, or previous technical data indicating chemical stability in solution under conditions similar to those in the test. Chemical analyses are particularly advisable if (U.S. EPA, 1985b): the test material is volatile, insoluble, or precipitates out of solution; the test chemical is known to sorb the material(s) from which the test vessels are constructed; the test solutions are aerated. Some situations (e.g., testing of pesticides for purposes of registration) could require the measurement of chemical concentrations in test solutions.

solutions should be obtained by pooling the replicates from each treatment.

All samples should be preserved, stored, and analyzed according to proven methods with acceptable detection limits for determining the concentration of the particular chemical in aqueous solution. Toxicity results for any tests in which concentrations are measured should be calculated and expressed in terms of those measured concentrations, unless there is good reason to believe that the chemical measurements are not accurate. In making calculations, each test solution should be characterized by the geometric average measured concentration to which organisms are exposed.

5.5 Test Endpoints and Calculations

The endpoint for tests performed with chemicals will usually be the NOEC/LOEC and/or ICp for survival and reproductive success, i.e., one or more of the primary endpoints described in Section 4.6.

If a solvent control is used, the test is rendered invalid if mortality in this control (or in the untreated control water) exceeds 20%, and/or if the reproduction of neonates in either control averages less than 15 live young per surviving adult (Section 4.4).

Section 6

Specific Procedures for Testing Effluent, Elutriate, and Leachate Samples

This section gives particular instructions for the collection, preparation, and testing of effluents, elutriates, and leachates, in addition to the procedures listed in Section 4.

6.1 Sample Collection, Labelling, Transport, and Storage

Containers for transportation and storage of samples of effluent, elutriate, or leachate must be made of nontoxic material. Glass or Teflon™-coated containers are preferred as they are inert and reduce sorption of chemicals. Polyethylene or polypropylene containers manufactured for transporting drinking water are less desirable but may also be used for sample transport and storage. The containers must either be new or thoroughly cleaned, and rinsed with uncontaminated water. They should also be rinsed with the sample to be collected. Containers should be filled to minimize any remaining air space.

Most tests with effluent, elutriate, or leachate will be performed "off-site" in a controlled laboratory facility. Effluent or leachate samples taken for off-site testing should be collected on three discrete occasions separated by intervals of two to three days. The sampling should be scheduled to provide fresh effluent or leachate for the initial, third, and fifth test days[hh]. Where possible, fresh aliquots of sample elutriate should also be prepared and delivered to the test facility to enable their use according to this schedule[ii]. In those instances where the testing of effluent or leachate samples is performed on-site in controlled facilities (e.g., within portable or industrial laboratories), samples should be collected daily and used within 24 h for each daily replacement of test solutions (U.S. EPA, 1989).

A 2-L sample is adequate for an off-site multiple-concentration test (e.g., using test concentrations of 100, 50, 25, 12.5, 6.3, 3.2, 1.6%) and the associated routine sample analysis. Lesser amounts are required for single-concentration tests (Section 4.6). Upon collection, each sample container must be filled, sealed, and labelled or coded. Labelling should include at least sample type, source, date and time of collection, and name of sampler(s). Unlabelled or uncoded containers arriving at the laboratory should not be tested. Nor should samples arriving in partially filled or unsealed containers be routinely tested, since volatile toxicants escape into the air space. However, if it is known that volatility is not a factor, such samples might be tested at the discretion of the investigator.

[hh] The first sample would be used for test initiation (day 1) and for solution renewal on day 2. The second sample would be used for solution renewal on days 3 and 4. The third sample would be used for solution renewal on days 5,6, and 7 (U.S. EPA, 1989).

[ii] The storage of samples of elutriate for an extended period for use throughout a seven-day static-renewal test is undesirable due to concerns with respect to sample stability.

Testing of effluents and leachates should start as soon as possible after collection. Whenever possible, testing should begin within 24 h, and must commence no later than 72 h after sampling. Samples collected for extraction and subsequent testing of the elutriate should also be tested as soon as possible and no later than ten days following their receipt. Testing of elutriates should commence within 72 h of preparation or as specified in a regulation or protocol.

All samples of effluent or leachate should be kept cool (1 to 7°C, preferably 4 ± 2°C) throughout their period of transport and storage. Upon collection, warm (>7°C) samples should be cooled to 1 to 7°C with ice or frozen gel packs. As necessary, gel packs or other means of refrigeration should be used to assure that sample temperature remains within 1 to 7°C during transit. Samples must not freeze during transport.

Upon arrival at the laboratory, an aliquot of effluent or leachate required at that time may be adjusted immediately or overnight to 25°C, and used in the test. The remaining portion(s) of sample required for subsequent solution renewals should be stored in darkness in sealed containers at 1 to 7°C and preferably at 4 ± 2°C.

Unless otherwise specified, temperature conditions during transportation and storage of elutriates, as well as samples intended for aqueous extraction and subsequent testing of the elutriate, should be as previously indicated.

6.2 *Preparing Test Solutions*

Samples in the collection containers must be agitated thoroughly just before pouring, to ensure the re-suspension of settleable solids. Sub-samples (i.e., a sample divided between two or more containers) must be mixed together and the composited sample (or a portion thereof) returned to the sub-sample containers and stored (Section 6.1) until used.

Samples that might contain small organisms which could attack or compete with the test organisms should be filtered through a 60-µm plankton net before use (U.S. EPA, 1989). In instances where concern exists regarding the effect of this filtration on sample toxicity[ii], a second (concurrent) test should be conducted using portions of the unfiltered sample.

6.3 *Control/Dilution Water*

Tests conducted with samples of effluent or leachate for monitoring and regulatory compliance purposes should use, as the control/dilution water, either the natural or reconstituted water that is used for culturing the daphnids, or a sample of the receiving water (see footnote "m", Section 4.1, and footnotes "cc" and"dd", Section 5.3). Since results could be different for the three sources of water, the objectives of the test must be decided before a choice is made. Difficulties and costs associated with the collection and shipment of receiving-water samples for use as control/dilution water should also be considered.

The use of receiving water as the control/dilution water might be desirable in certain instances where site-specific information is required regarding the potential toxic impact of an effluent, leachate, or elutriate on a particular receiving water (see footnotes "cc" and"dd", Section 5.3). An important example of such a situation would be testing for sublethal

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[ii] Sample filtration might remove suspended or settleable solids that are representative of the test material and which could modify its toxicity to the test organisms.

effect at the edge of a mixing zone, under site-specific regulatory requirements. Conditions for the collection, transport, and storage of such receiving-water samples should be as described in Section 6.1.

If a sample of upstream receiving water is to be used as dilution and control water, a separate control solution should be prepared using the culture water. Test conditions and procedures for evaluating each control solution should be identical and as described in Sections 4 and 5.3.

Tests requiring a high degree of standardization may be undertaken using reconstituted water of a specified hardness (Table 2) as the control/dilution water (see footnote "ff", Section 5.3). Situations where such use is appropriate include investigative studies intended to interrelate toxicity data for various effluent, leachate or elutriate types and sources, derived from a number of test facilities or from a single facility where water quality is variable. In such instances, it is desirable to minimize any modifying influence due to (differing) dilution-water chemistry.

Moderately hard (80 to 100 mg/L) reconstituted water is recommended if only one type of synthetic water is to be used as control/dilution water (U.S. EPA, 1989). Tests examining the influence of receiving-water chemistry on the chronic toxicity of effluent, leachate, or elutriate could be replicated using a series of reconstituted (Table 2) and/or natural waters differing in hardness and other characteristics.

6.4 Test Conditions

Samples of effluent, leachate, or elutriate are normally not filtered or agitated during the test. However, the presence of high concentrations of suspended inorganic or organic solids in a sample might be harmful to filter-feeding daphnids (Robinson, 1957; Arruda *et al.*, 1983; McCabe and O'Brien, 1983; Hall and Hall, 1989). High concentrations of biological solids in certain types of treated effluent could also contribute to sample toxicity due to ammonia and/or nitrite production (Servizi and Gordon, 1986). An additional test should be conducted if concern exists about a contribution to toxicity by elevated concentrations of suspended or settleable solids in samples of effluent, elutriate, or leachate, and if the intent of the study is to quantify the degree to which sample solids contribute to toxicity. The second test should use a portion of the sample, treated by filtering or decanting to remove solids, but procedures should be otherwise identical.

6.5 Test Observations and Measurements

Daphnid survival and reproductive success must be observed and recorded at 24-h intervals throughout the test period, according to Section 4.5.

Colour, turbidity, odour, and homogeneity (i.e., presence of floatable material or settleable solids) of the effluent, leachate, or elutriate sample should be observed at time of preparation of test solutions. Precipitation, flocculation, colour change, odour, or other reactions upon dilution with water should be recorded, as should any changes in appearance of test solutions during the test period (e.g., foaming, settling, flocculation, increase or decrease in turbidity, colour change).

For effluent samples with appreciable solids content, it is desirable to measure

total suspended and settleable solids (APHA *et al.*, 1989) upon receipt, as part of the overall description of the effluent, and as sample characteristics that might influence the results of the toxicity test.

6.6 Test Endpoints and Calculations

Tests for monitoring and compliance with regulatory requirements should normally include, as a minimum, ten replicate solutions of the undiluted sample (or a specified dilution) and ten replicate control solutions. Depending on the specified regulatory requirements, tests for compliance might use a single concentration (100% wastewater unless otherwise specified), might determine the NOEC/LOEC and/or ICp, or might determine the LC_{50} at 7 ± 1 days or other exposure time, if mortality is severe (see Section 4.6).

Toxicity tests conducted for other purposes (e.g., determination of in-plant sources of chronic toxicity, treatment effectiveness, effects of process changes or receiving-water characteristics on chronic toxicity, inter-laboratory investigations) could, depending on the study objectives, be single-concentration tests (100% or an appropriate dilution, plus a control), or multiple-concentration tests. Single-concentration tests are often cost-effective for determining the presence or absence of measurable toxicity or as a method for screening a large number of samples for relative toxicity. Endpoints for these tests would again depend on the objectives of the undertaking, but could include arbitrary "pass" or "fail" ratings, or percentage mortality and number of neonates produced in the wastewater and control solutions at 7 ± 1 days (Section 4.6). Multiple-concentration tests of effluent, leachate, or elutriate samples should be performed and the appropriate endpoints (e.g., LC_{50}, NOEC, LOEC, ICp) calculated in instances where chronic toxicity is anticipated and the test objective is to define the highest concentration of wastewater that is not demonstrably harmful to the test organism when exposure is prolonged.

Section 7

Specific Procedures for Testing Receiving-water Samples

Instructions for testing samples of receiving waters, additional to those provided in Section 4, are given here.

7.1 Sample Collection, Labelling, Transport, and Storage

Procedures for the collection, labelling, transportation, and storage of samples of receiving waters should be as described in Section 6.1. Testing of samples should commence as soon as possible after collection, preferably within 24 h, and no later than 72 h after sampling.

7.2 Preparing Test Solutions

Samples in the collection container(s) should be agitated before pouring to ensure their homogeneity. Compositing of sub-samples should be as described in Section 6.2.

Each receiving-water sample should be filtered through a 60-µm plankton net before use, to enable the removal of potential predators or competitors of *C. dubia*. A second (unfiltered) test could be conducted if there is concern about changes in toxicity due to filtration[ii].

7.3 Control/Dilution Water

For receiving-water samples collected in the vicinity of a wastewater discharge, chemical spill, or other point-source of possible contamination, "upstream" water may be sampled concurrently and used as control water and diluent for the downstream sample(see footnote "cc" and "dd", Section 5.3 and "m" Section 4.1). This control/dilution water should be collected as close as possible to the contaminant source(s) of concern, but upstream of the zone of influence or outside it. Such surface water should be filtered to remove organisms, as described in Section 6.2.

If "upstream" water is used as control/dilution water, a separate control solution should be prepared using the laboratory water that is normally used for culturing *C. dubia*. Test conditions and procedures for preparing and evaluating each control solution should be identical, and as described in Sections 4.1 and 5.3.

Logistic constraints, expected toxic effects or other site-specific practicalities might prevent or rule against the use of upstream water as the control/dilution water. In such cases, an alternate source of uncontaminated water should be used as the culture water (Subsection 2.4.4), control water, and for all dilutions (see footnote "dd", Section 5.3). The water selected for this purpose should have a hardness value similar to that of the test water(s). This may be achieved by selecting or preparing an uncontaminated water source (natural or reconstituted) with the desired hardness, and culturing brood and test organisms in the appropriate water prior to the test (Section 3.4).

7.4 Test Observations and Measurements

Observations and measurements of test samples and solutions for colour, turbidity, foaming, precipitation, etc. should be made

as described in Section 6.5, both during the preparation of test solutions and subsequently during the tests. These are in addition to the primary observations of test organisms described in Section 4.5.

7.5 Test Endpoints and Calculations

Endpoints for tests with samples of receiving water should be consistent with the options and approaches identified in Sections 4.6 and 6.6.

Testing of each receiving-water sample should include a minimum of ten replicate solutions of the undiluted test water and ten replicate control solutions. Endpoints for tests with receiving-water samples would normally be restricted to data on chronic survival and reproduction, obtained for *C. dubia* exposed to full-strength receiving water for 7 ± 1 days (Section 4.6).

If toxicity of receiving-water samples is likely, and information is desired concerning the degree of dilution necessary to permit normal survival and reproductive success of daphnids, a full test to determine NOEC/LOEC and/or ICp should be conducted as outlined in Section 4, with one or more undiluted (100% sample) concentrations as the highest concentration in the series tested.

Certain sets of tests might use a series of samples such as surface waters from a number of locations, each tested at full strength only. Statistical testing and reporting of results for such tests should follow the procedures outlined in Section 4.6.

Section 8

Reporting Requirements

The test report should describe the materials and methods used, as well as the results. The reader should be able to establish from the report whether the conditions and procedures rendered the results acceptable for the use intended.

Procedures and conditions that are common to a series of ongoing tests (e.g., routine toxicity tests for monitoring or compliance purposes) and consistent with specifications in this report may be referred to by citation or by attaching a general report which outlines standard laboratory practice. Where choices exist, the approach selected should be specified. The general report should convey the procedural information included in Sections 8.2 to 8.6. An individual test report giving the findings should contain the information indicated in Sections 8.1 and 8.7. Specific monitoring programs might require selected items (e.g., procedures and results for tests requiring pH and/or hardness adjustments) in the test report. Other details pertinent to the conduct and findings of the test, which are not conveyed by the reports, should be kept on file by the test laboratory, so that the appropriate information can be provided if an audit of the test is required.

8.1 Test Material

- sample type, source, and description (chemical, effluent, elutriate, leachate, or receiving water; sampling location, method and schedule; specifics regarding nature, appearance and properties, volume and/or weight);
- information on labelling or coding of the test material;
- details on manner of sample collection, transport, and storage (e.g., batch, grab, or composite sample, description of container, temperature of sample upon receipt and during storage);
- identification of person(s) collecting and/or providing the sample; and
- dates and times for sample collection, receipt at test facility, and start and end of definitive test.

8.2 Test Organisms

- species and source;
- description of culturing conditions and procedures (facilities and apparatus, lighting, water source and quality, water pre-treatment, breeding method including frequency of water exchange and procedure for replacement, methods of handling organisms, temperature range during culturing, age of culture, food type and method of preparation, records of nutritive value and known contaminants in food, ration, and frequency of feeding);
- estimated percentage mortality in individual cultures during seven days preceding test;
- average number of surviving young produced per adult in individual cultures during seven days preceding test;

- number of young produced by brood organisms in previous brood;
- observation of ephippia in the culture; and
- age of test organisms at beginning of test.

8.3 Test Facilities and Apparatus

- name and address of test laboratory;
- name of person(s) performing the test;
- description of systems for regulating light and temperature within test facility;
- description of test vessels and covers (size, shape, and material); and
- description of procedures used to clean or rinse apparatus.

8.4 Control/Dilution Water

- type(s) and source(s) of water used as control and dilution water;
- type and quantity of any chemical(s) added to control or dilution water;
- water sampling, pre-treatment (adjustment of temperature, hardness, pH, de-gassing, aeration rate and duration, etc.) and storage details; and
- measured water-quality variables before and/or at time of commencement of toxicity test.

8.5 Test Method

- brief mention of method used if standard (e.g., as per this document);
- design and description if specialized procedure (e.g., renewal of test solutions at intervals other than daily, or continuous replacement of solutions) or modification of standard method (e.g., aeration of test solutions during the test);
- procedure used in preparing stock and/or test solutions of chemicals;
- any chemical analyses of test solutions and reference to analytical procedure(s) used;
- use of preliminary or range-finding test; and
- frequency and type of observations made during test.

8.6 Test Conditions

- number, concentration, depth, and volume of each replicate test solution including controls;
- number of organisms per test solution and per 15-mL volume;
- photoperiod, light source, and intensity at surface of test solutions;
- statement concerning aeration (if any, give rate, duration, and manner of application) of test solutions prior to daphnid exposure;
- description of any test solutions adjusted for pH or hardness, including procedure and timing;
- description of source and type of food used during the test and of the feeding method, frequency, and ration;

- conditions and procedures for preparing solutions of reference toxicant(s) and for performing the test and determining NOEC/LOEC and/or IC_p;

- any chemical measurements on test solutions (e.g., hardness, alkalinity, conductivity, suspended solids, concentration of test chemical, contaminant, and/or degradation product); and

- temperature, pH, dissolved oxygen (mg/L and percent saturation), and conductivity as monitored in each test solution; total hardness of control/dilution water and the highest test concentration at the start of the test.

8.7 Test Results

- appearance of test solutions and changes noted during test;

- percentage mortality and number of neonates per first-generation daphnid in each test solution (including the control), as noted during each observation period;

- results for range-finding test (if conducted);

- the NOEC/LOEC and/or ICp for mortality and reproductive success of first-generation daphnids, and the statistical test(s) used to determine them; minimum significant percentage change from the control data that could be detected in the test; any transformation of data that was required;

- any LC_{50} (and 95% confidence limits) determined, and the statistical method used for calculation; and

- the results for toxicity tests with the reference toxicant(s) performed within 14 days of the test, together with the geometric mean value (±2 SD) for the same reference toxicant(s) as derived at the test facility in previous tests.

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Appendix A

Members of the Inter-Governmental Aquatic Toxicity Group

Federal*

P. Wells (Current Chairperson)
EP, Dartmouth, N.S.

R. McCubbin
St. John's, Nfld.

K. Doe
Dartmouth, N.S.

W. Parker
Dartmouth, N.S.

N. Bermingham
Longueuil, Quebec

C. Blaise
Longueuil, Quebec

G. Elliott
Edmonton, Alberta

R. Watts
North Vancouver, B.C.

K. Day
National Water Research Institute
Burlington, Ont.

B. Dutka
National Water Research Institute
Burlington, Ont.

L. Porebski
Office of Waste Management
Ottawa, Ont.

D. MacGregor
Commercial Chemicals Branch
Ottawa, Ont.

R. Scroggins
Industrial Programs Branch
Ottawa, Ont.

G. Sergy
Technology Development Branch
Edmonton, Alberta

E. Watt
Water Quality Branch
Ottawa, Ont.

Provincial

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

G. Westlake
Ont. Ministry of Environment
Rexdale, Ont.

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, B.C.

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

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* Environment Canada, Conservation and Protection, as of March 1991.

Appendix B

Conservation & Protection Regional and Headquarters Offices

Headquarters
351 St. Joseph Boulevard
Place Vincent Massey
Hull, Quebec
K1A OH3

Atlantic Region*
15th Floor, Queen Square
45 Alderney Drive
Dartmouth, Nova Scotia
B2Y 2N6

Quebec Region
1141 Route De L'Eglise
P.O. Box 10100
Sainte Foy, Quebec
G1V 4H5

Ontario Region
25 St. Clair Ave. East, 6th Floor
Toronto, Ontario
M4T 1M2

Western and Northern Region
Room 210, Twin Atria # 2
4999 - 98 Avenue
Edmonton, Alberta
T6B 2X3

Pacific and Yukon Region**
224 Esplanade Street
North Vancouver, British Columbia
V7M 3J7

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* Programs "BOOTSTRP" and TOXSTAT" are available for copying onto a formatted 13-cm IBM-compatible floppy disk supplied by the user, by contacting the Laboratory Division at this address.

** A BASIC computer program for calculating LC50 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.

Appendix C

Procedural Variations for Chronic Toxicity Tests with *Ceriodaphnia* spp., as Described in Canadian and U.S. Methodology Documents*

1. Test Material and Type of Test

Document[a]	Test Material[b]	Test Type	Test Duration
U.S. EPA 1985a	effl., RW	St-R[c]	7 d
Anon. 1986	effl.	St-R	7 d
Battelle 1986[d]	effl., chem.	St-R	7 d
Battelle 1987	effl.	St-R	7 d
Battelle 1988	NI[e]	St-R	7 d
ASTM 1988	effl., chem. (leach., sed., RW)	St-R	7 d[f]
NWRI 1988	effl., leach, sed.	St-R	7 d
Anon. 1989	effl.	St-R	3 broods[f,g]
U.S. EPA 1989	effl., RW	ST-R	3 broods[g,h]

a Full names of agencies are given in reference list.
b effl. = effluent leach = leachate
elut. = elutriate sed. = sediment
chem. = chemical(s) RW = receiving water
c St-R = static renewal.
d Three versions of the Battelle method are given since all are recent, an investigator might encounter any of them, and they indicate a progression of thought.
e NI = Not Indicated.
f Eight days if third brood not produced in seven days.
g Until 60% of control females have three broods.
h Might require more or less than seven days.

* Based on methodology documents available to the authors as of August 1989.

2. Test Organism

Document	Species and Age	Life Stage
U.S. EPA 1985a	*C. dubia*	2 to 24 h, within 4 h[a]
Anon. 1986	*C. dubia/affinis*	2 to 24 h, within 4 h
Battelle 1986	*C. dubia*	<24 h
Battelle 1987	*C. dubia*	<24 h
Battelle 1988	*C. dubia*	<24 h
ASTM 1988	*C. dubia*	<12 h, within 4 h[b]
NWRI 1988	*C. reticulata*	≤2 h
Anon. 1989	*C. dubia*	<24 h, within 8 h[c]
U.S. EPA 1989	*C. dubia*	<24 h, within 8 h

a Released from mother within 4 h of each other.
b From third brood or later, ≥6 to 8 young in previous brood.
c Released from mother within 8 h of each other.

3. Culture and Acclimation Conditions

Document	Water Source	Temperature (°C)	Hardness	Aeration
U.S. EPA 1985a	Rc[a] (NW[b], DW[c])	25 ± 2[d]	80 to 100	if needed, DO ≥5
Anon. 1986	"as in U.S.EPA 1985a"	.....	.....	.....
Battelle 1986	NW	25 ± 2	100	none
Battelle 1987	Rc	25 ± 2	NI	NI
Battelle 1988	Rc[e]	25 ± 2	160 to 180	none
ASTM 1988	match dilution water	25	NI	NI
NWRI 1988	DW (NW)	25 ± 1	NI	NI
Anon. 1989	"as in U.S.EPA 1989"	.....	.....	.....
U.S.EPA 1989	Rc	25 ± 1	80 to 100	NI

a Rc = Reconstituted water.
b NW = Natural surface or groundwater, uncontaminated source.
c DW = Dechlorinated municipal water.
d Range
e With added bacterial inoculum.

4. Lighting Conditions During Culturing

Document	Photoperiod	Intensity	Type	Dawn/Dusk
U.S. EPA 1985a	16L / 8D (or normal lab)	NI	NI	NI
Anon. 1986	"as in U.S. EPA 1985a"			
Battelle 1986	16L / 8D	540 to 1607 lux	Fluor.[a]	NI
Battelle 1987	NI	NI	NI	NI
Battelle 1988	16L / 8D	540 to 1607 lux	Fluor.[a]	NI
ASTM 1988	16L / 8D	≤600 lux	NI	15 to 30 min[b]
NWRI 1988	NI	NI	NI	NI
Anon. 1989	"as in U.S.EPA 1989"			
U.S. EPA 1989	16L / 8D	NI	NI	NI

[a] Fluorescent with colour rendering index ≥90.
[b] Desirable to minimize stress due to abrupt change.

5. Feeding Conditions During Culture and Testing

Document	Food	Feeding of Culture	Feeding During Test (days after start)
U.S. EPA 1985a	YCT[a]	daily	daily
Anon. 1986	"as in U.S. EPA 1985a"		
Battelle 1986	alga[b] + YCT	3 times/wk	0, 2, 4, or 0, 3, 5
Battelle 1987	alga[b] + YCT	3 times/wk	daily
Battelle 1988	alga[b] + YCT	3 times/wk	0, 2, 4, or 0, 3, 5
ASTM 1988	NI[c]	regularly	daily desirable
NWRI 1988	alga[d] + yeast	daily	0, 2, 4
Anon. 1989	brine shrimp[e]	NI	twice daily
U.S. EPA 1989	alga[b] + YCT	daily	daily

[a] YCT = Yeast + Cerophyll™ (dried plant material) + trout chow.
[b] *Selenastrum capricornutum*.
[c] As suitably documented, mixtures of various algae/Y/T/C.
[d] *Scenedesmus* sp.
[e] Newly-hatched nauplii.

6. Test Conditions

Document	Container	Test Volume (mL)	No. of Daphnids /vessel	No. of Replicates
U.S. EPA 1985a	30-mL BSGB[a] or plastic cup	15	1	10
Anon. 1986	"as in U.S. EPA 1985a"	15	1	12
Battelle 1986	30-mL BSGB	15	1	10
Battelle 1987	30-mL BSGB	15	1	10
Battelle 1988	30-mL BSGB	15	1	10
ASTM 1988	30-mL or larger container[b]	≥15	1	≥10
NWRI 1988	30-mL plastic cup or beaker	10	1	4 to 6
Anon. 1989	30 mL (covered)	15	1	≥10
U.S. EPA 1989	30-mL BSGB or plastic cup	15	1	10

[a] borosilicate glass beaker.
[b] glass, 316 stainless steel, or fluorocarbon plastic.

7. Characteristics of Control/Dilution Water

Document	Water Type[a]	Hardness (mg/L)	pH	Minimum DO[b]	Renewal Times (days)
U.S. EPA 1985a	Rc, NW, RW (DW[c])	80 to 100[d]	NI	aerate if low	daily
Anon. 1986	"as in U.S.EPA 1985a"	.....			2, 5
Battelle 1986	as for culture water	.....		NI	2,4 or daily
Battelle 1987	Rc, RW	NI	NI	aerate if low	daily
Battelle 1988	Rc	160 to 180	7.6 to 8.5	NI	2,4 or 2,5
ASTM 1988	Rc[e], NW, RW (DW[c])	NI[e]	NI	90 to 100% sat'n	daily
NWRI 1988	DW	NI	NI	NI	2, 4
Anon. 1989	NW, Rc, ≤20%DMW[f]	NI	NI	aerate ≥24h	daily
U.S. EPA 1989	Rc[g], NW, RW, DW	NI[g]	NI	aerate if low	daily

[a] Rc = Reconstituted water NW = surface or groundwater from uncontaminated source
RW = receiving water DW = dechlorinated municipal water
DMW = diluted mineral water.
[b] Dissolved oxygen.
[c] "To be used as a last resort".
[d] If reconstituted water. Similar to the receiving water if used for dilution.
[e] Prepared according to ASTM standard no. E729. Hard or soft RW may be used. Added selenium and vitamin B_{12} "might be desirable".
[f] Addition of selenium and vitamin B_{12} to dilution water is recommended.
[g] Moderately hard (80 to 100 mg/L) water recommended if reconstituted.

8. Temperature, Aeration, Dissolved Oxygen, and pH Adjustment During Test

Document	Water Temperature (°C)	Aeration	Minimum DO Prior to Test	pH Adjustment
U.S. EPA 1985a	25 ± 1[a]	none[b]	NI ("low")	NI
Anon. 1986	25 ± 1	none?	5.0 mg/L	NI
Battelle 1986	25 ± 2	none	NI	NI
Battelle 1987	25 ± 2	none	NI	yes if ≤6.0, ≥9.0
Battelle 1988	25 ± 2	none	NI	NI
ASTM 1988	25 ± 1	gentle if needed	40% [c]	NI
NWRI 1988	25 ± 1	none	NI	NI
Anon. 1989	25 ± 1	none	NI	NI
U.S. EPA 1989	25 ± 1	none	NI	NI

a Range
b Aerate sample before testing, if required.
c If ≤40% saturation, renew test solution more frequently. The time-weighted mean in each vessel is to be ≥50% saturation.

9. Lighting Conditions During Test

Document	Photoperiod	Intensity	Type	Dawn/Dusk
U.S. EPA 1985a	16L / 8D	ambient (10 to 20 $\mu E/m^2 \cdot s$)	NI	NI
Anon. 1986	16L / 8D	"as in U.S.EPA 1985a" ·····		
Battelle 1986	16L / 8D	540 to 1607 lux	Fl[a]	NI
Battelle 1987	16L / 8D	ambient (323 to 1076 lux)	Fl	NI
Battelle 1988	16L / 8D	540 to 1607 lux	Fl[a]	NI
ASTM 1988	16L / 8D	NI	NI	15 to 30 min[b]
NWRI 1988	NI	NI	NI	NI
Anon. 1989	16L / 8D	"as in U.S. EPA 1989" ·····		
U.S. EPA 1989	16L / 8D	ambient (10 to 20 $\mu E/m^2 \cdot s$)	NI	NI

a Fluorescent with colour rendering index ≥90.
b Desirable to minimize stress due to abrupt change.

10. Monitoring Water Quality During Tests

Document	Variable[a]	At Exposure-Times (days)
U.S.EPA 1985a	DO pH T cond hard alk	daily[b]
Anon. 1986	DO pH T	0 2 5 7
Battelle 1986	DO pH T cond	0 2 4 7, or 0 3 5 7, or daily
Battelle 1987	DO pH T cond hard alk	0 + daily
Battelle 1988	DO pH T cond	0 2 4 7
	hard alk	0 2 4
ASTM 1988	DO pH T cond hard alk	0 + 7 or more often[c]
NWRI 1988	pH	0 7
Anon. 1989	"as in U.S. EPA 1989"	
U.S. EPA 1989	DO pH T	daily[d]
	cond hard alk	in new samples[e]

[a] DO = dissolved oxygen; pH = hydrogen ion conc'n; T = temperature; cond = specific conductivity; hard = total hardness; alk = total alkalinity

[b] DO at beginning and end of day for representative vessels. Hardness and alkalinity for control and high concentration.

[c] DO in used testwater of control, low, medium and high conc'n, at least at start, middle and end of test. Alkalinity and pH at least once in new and used testwater of high conc'n.

[d] DO + pH at beginning and end of day for representative samples.

[e] Measured in each new sample (100%) and the control.

11. Biological Observations During Test

Document	Variable		At Exposure Time (days)
U.S.EPA 1985a	mortality[a],	no. of live young	daily
Anon. 1986	mortality,	no. of live young	2 5 7
Battelle 1986	mortality,	no. of young	2 4 7 or 3 5 7
Battelle 1987	mortality,	no. of young	2 4 7 or 3 5 7
Battelle 1988	mortality,	no. of young	2 4 7 or 3 5 7
ASTM 1988	mortality,	no. of live young, behaviour	daily[b]
	(size of original females)		7 (optional)
NWRI 1988		no. of young	2 4 7
Anon. 1989	mortality,	no. of live young	daily
U.S. EPA 1989	mortality,	no. of live young	daily

[a] Mortality of adult females first placed in vessels, and of young.

[b] Or at least after each of the three broods produced during test.

12. Test Endpoint (at 7 Days Unless Otherwise Noted)

Document	Endpoint(s)	Criterion
U.S. EPA 1985a	NOEC, LOEC[a]	sig. diff. from control in mortality, no. live young[b]
Anon. 1986	pass/fail, mortality	≥20% in any concentration
	pass/fail, no. of young	sig. diff. from control
	NOEC for reduced young	NI
Battelle 1986	NI	mortality, no. young
Battelle 1987	NOEC, mortality, no. young	sig. diff. from control
Battelle 1988	NI	mortality, no. young
ASTM 1988	NOEC, mortality, no. young	sig. diff. from control[c]
	NOEC, final size of adults	sig. diff. from control
NWRI 1988	IC50[a] for reduced young	no. in conc'n/no. in control
Anon. 1989	"as in U.S. EPA 1989"	
U.S. EPA 1989	NOEC, LOEC[a]	sig. diff. from control in mortality, no. live young[b]
	LC_5, LC_{10}, LC_{50}	mortality, female adults[d]
	IC25, IC50[a]	mortality, no. live young[d]

[a] NOEC = No-Observed-Effect Concentration
LOEC = Lowest-Observed-Effect Concentration
IC_{50} = Inhibiting Concentration for 50% reduction in no. of young

[b] No. live young are compared for concentrations without significant mortality compared to control. Average no. is calculated for each female, up to time of death for any that died.

[c] Based on 8-day test if third brood not produced in seven days. Optional criterion is concentration causing specified decrease in performance compared to control.

[d] Determined by point-estimation technique (e.g. probit, moving average, or binomial).

13. Validity of Test

Document	Maximum Allowed Control Mortality	Acceptable Reproduction (no. of young) in Control
U.S.EPA 1985a	NI	NI
Anon. 1986	20%	>15/female in 7 d, excluding mortality effects
Battelle 1986	20%	≥3 broods, or >15/female in 7 d
Battelle 1987	NI	NI
Battelle 1988	NI	NI
ASTM 1988	20%	≥15/female in 3 broods, in ≤8 d, no ephippia
NWRI 1988	NI	NI
Anon. 1989	20%	as in ASTM 1988
U.S. EPA 1989	20%	≥15/surviving female

Appendix D

Procedure for Preparing YCT and Algal Food for *C. dubia* *

Preparing Digested Trout Chow **

1. Preparation of trout chow requires one week. Use starter or No. 1 pellets.

2. Add 5.0 g of trout chow pellets to 1 L of deionized (Milli-Q™ or equivalent) water. Mix well in a blender and pour into a 2-L separatory funnel. Digest prior to use by aerating continuously from the bottom of the vessel for one week at ambient laboratory temperature. Water lost due to evaporation should be replaced during digestion. Because of the offensive odour usually produced during digestion, the vessel should be placed in a fume hood or other isolated, ventilated area.

3. At the end of the digestion period, place in a refrigerator and allow to settle for a minimum of 1 h. Filter the supernatant through a fine mesh screen (e.g. Nitex™, 110 mesh). Combine with equal volumes of supernatant from Cerophyll™ and yeast preparations (see following). The supernatant can be used fresh, or frozen until use. Discard the sediment.

Preparing Yeast

1. Add 5.0 g of dry yeast, such as Fleischmann's™, to 1 L of deionized water.

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

3. Combine the yeast suspension immediately (with no settling) with equal volumes of supernatant from the trout chow and Cerophyll preparations (see following). Discard excess material.

Preparing Cerophyll (Dried, Powdered Cereal Leaves)

1. Place 5.0 g of dried, powdered Cerophyll or cereal leaves*** in a blender. Dried, powdered alfalfa leaves from health food stores have been found to be a satisfactory substitute for cereal leaves.

2. Add 1 L of deionized water.

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* From U.S. EPA (1989).

** Researchers at U.S. EPA's Environmental Monitoring Systems Laboratory in Cincinnati, Ohio recommend using the commercially available tropical fish food "Tetra-min™" as a suitable substitute for trout chow (J.M. Lazorchak and P.A. Lewis, pers. commun., 1991).

*** Available as "Cereal Leaves" from Sigma Chemical Company, P.O. Box 14508, St. Louis, Missouri 63178 (800-325-3010); or as Cerophyll™ from Ward's Natural Science Establishment Inc., P.O. Box 92912, Rochester, New York 14692-9012 (716-359-2502).

3. Mix in a blender at high speed for 5 min, or stir overnight at medium speed on a magnetic stir plate.

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

Preparing Combined YCT Food

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

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

3. Freshly prepared food can be used immediately, or it can be frozen until needed. Thawed food is stored in the refrigerator between feedings, and is used for a maximum of two weeks.

4. It is advisable to measure the dry weight of solids in each batch of YCT before use. The food should contain 1.7 to 1.9 g solids/L. Cultures or test solutions should contain 12 to 13 mg solids/L.

Preparing Algal* (Selenastrum) *Food

A. Algal culture medium

1. Prepare five stock nutrient solutions using reagent-grade chemicals as described in Table D1.

2. Add 1 mL of each stock solution, in the order listed in Table D1, to approximately 900 mL of deionized water. Mix well after each solution is added. Dilute to 1 L, mix well, and adjust the pH to 7.5 ± 0.1, using 0.1*N* NaOH or HCl, as appropriate. The final concentration of macronutrients and micronutrients in the culture medium is given in Table D2.

3. Immediately filter the pH-adjusted medium through a 0.45 µm pore diameter membrane at a vacuum of not more than 380 mm mercury, or at a pressure of not more than one-half atmosphere. Wash the filter with 500 mL deionized water before use.

4. If the filtration is carried out with sterile apparatus, filtered medium can be used immediately, and no further sterilization steps are required before the inoculation of the medium. The medium can also be sterilized by autoclaving after it is placed in the culture vessels.

5. Unused sterile medium should not be stored more than one week before use, because there could be substantial loss of water by evaporation.

B. Establishing and maintaining stock cultures of algae

1. Upon receipt of the "starter" culture (usually about 10 mL), a stock culture is initiated by aseptically transferring 1 mL to each of several 250-mL culture flasks containing 100 mL of algal culture medium (prepared as described). The remainder of the starter culture can be held in reserve for up to six months in a refrigerator (in the dark) at 4°C.

2. The stock cultures are used as a source of algae to initiate "food" cultures for *Ceriodaphnia* toxicity tests. The volume of stock culture maintained at any one time will depend on the amount of algal

food required for the *Ceriodaphnia* cultures and tests. Stock culture volume can be rapidly "scaled up" to several litres, if necessary, using 4-L serum bottles or similar vessels, each containing 3 L of growth medium.

3. Culture temperature is not critical. Stock cultures may be maintained at 20 to 25°C in environmental chambers with cultures of other organisms if the illumination is adequate (continuous "cool-white" fluorescent lighting of approximately 4300 lux (400 foot-candles).

4. Cultures are mixed twice daily by hand.

5. Stock cultures can be held in the refrigerator until used to start "food" cultures, or can be transferred to new medium weekly. One-to-three millilitres of seven-day old algal stock culture, containing approximately 1.5×10^6 cells/mL, are transferred to each 100 mL of fresh culture medium. The inoculum should provide an initial cell density of approximately 10 000 to 30 000 cells/mL in the new stock cultures, and care should be exercised to avoid contamination by other microorganisms.

Table D.1 Nutrient Stock Solutions for Maintaining Stock Cultures of Algae

Nutrient Stock Solution	Compound	Amount dissolved in 500 mL deionized water
1	$MgCl_2 \cdot 6H_2O$	6.08 g
	$CaCl_2 \cdot 2H_2O$	2.20 g
	H_3BO_3	92.8 mg
	$MnCl_2 \cdot 4H_2O$	208.0 mg
	$ZnCl_2$	1.64 mg[a]
	$FeCl_3 \cdot 6H_2O$	79.9 mg
	$CoCl_2 \cdot 6H_2O$	0.714 mg[b]
	$Na_2MoO_4 \cdot 2H_2O$	3.63 mg[c]
	$CuCl_2 \cdot 2H_2O$	0.006 mg[d]
	$Na_2EDTA \cdot 2H_2O$	150.0 mg
2	$NaNO_3$	12.75 g
3	$MgSO_4 \cdot 7H_2O$	7.35 g
4	K_2HPO_4	0.522 g
5	$NaHCO_3$	7.50 g

[a] $ZnCl_2$ Weigh out 164 mg and make up to 100 mL. Add 1 mL of this solution to Stock Solution #1.
[b] $CoCl_2 \cdot 6H_2O$ Weigh out 71.4 mg and make up to 100 mL. Add 1 mL of this solution to Stock Solution #1.
[c] $Na_2MoO_4 \cdot 2H_2O$ Weigh out 36.6 mg and make up to 10 mL. Add 1 mL of this solution to Stock Solution #1.
[d] $CuCl_2 \cdot 2H_2O$ Weigh out 60.0 mg and make up to 1 000 mL. Take 1 mL of this solution and dilute to 10 mL. Take 1 mL of the second dilution and add to Stock Solution #1.

6. Stock cultures should be examined microscopically weekly, at transfer, for microbial contamination. Reserve quantities of culture organisms can be maintained for 6 to 12 months if stored in the dark at 4°C. It is advisable to prepare new stock cultures from "starter" cultures obtained from established outside sources of organisms every 4 to 6 months.

C. Establishing and maintaining "food" cultures of algae

1. "Food" cultures are started seven days prior to use in *Ceriodaphnia* cultures or tests. Approximately 20 mL of seven-day-old algal stock culture (described in the previous paragraph), containing 1.5×10^6 cells/mL, are added to each litre of fresh algal culture medium (i.e., 3 L of medium in a 4-L bottle, or 18 L in a 20-L bottle). The

Table D.2 Final Concentration of Macronutrients and Micronutrients in the Culture Medium

Macronutrient	Concentration (mg/L)	Element	Concentration (mg/L)
$NaNO_3$	25.5	N	4.20
$MgCl_2 \cdot 6H_2O$	12.2	Mg	2.90
$CaCl_2 \cdot 2H_2O$	4.41	Ca	1.20
$MgSO_4 \cdot 7H_2O$	14.7	S	1.91
K_2HPO4	1.04	P	0.186
$NaHCO_3$	15.0	Na	11.0
		K	0.469
		C	2.14

Micronutrient	Concentration (µg/L)	Element	Concentration (µg/L)
H_3BO_3	185.	B	32.5
$MnCl_2 \cdot 4H_2O$	416.	Mn	115.
$ZnCl_2$	3.27	Zn	1.57
$CoCl_2 \cdot 6H_2O$	1.43	Co	0.354
$CuCl_2 \cdot 2H_2O$	0.012	Cu	0.004
$Na_2MoO_4 \cdot 2H_2O$	7.26	Mo	2.88
$FeCl_3 \cdot 6H_2O$	160.	Fe	33.1
$Na_2EDTA \cdot 2H_2O$	300.	—	—

inoculum should provide an initial cell density of approximately 30 000 cells/mL. Aseptic techniques should be used in preparing and maintaining the cultures, and care should be exercised to avoid contamination by other micro-organisms.

2. Food cultures may be maintained at 20 to 25°C in environmental chambers with the algal stock cultures or cultures of other organisms if the illumination is adequate (continuous "cool-white" fluorescent lighting of approximately 4300 lux).

3. Cultures are mixed continuously on a magnetic stir plate (with a medium size stir bar) or in a moderately aerated separatory funnel, or are mixed twice daily by hand. Caution should be exercised to prevent the culture temperature from rising more than 2 to 3°C*.

D. Preparing algal concentrate for use as food for Ceriodaphnia

1. An algal concentrate containing 3.0 to 3.5×10^7 cells/mL is prepared from food cultures by centrifuging the algae with a plankton or bucket-type centrifuge, or by allowing the cultures to settle in a refrigerator for a minimum of 10 days and a maximum of three weeks, and siphoning off the supernatant.

2. The cell density (cells/mL) in the concentrate is measured with an electronic particle counter, microscope and hemocytometer, fluorometer or spectrophotometer, and used to determine the dilution (or further concentration) required to achieve a final cell count of 3.0 to 3.5×10^7/mL.

3. Assuming a cell density of approximately 1.5×10^6 cells/mL in the algal food cultures at seven days, and 100% recovery in the concentration process, a 3-L, seven-to-ten-day culture will provide 4.5×10^9 algal cells. This number of cells will provide approximately 150 mL of algal cell concentrate for use as food (1500 feedings at 0.1 mL/feeding). This is enough algal food for four *Ceriodaphnia* tests.

4. Algal concentrate may be stored in the refrigerator for up to one month.

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* If the cultures are placed on a magnetic stir plate, heat generated by the stirrer might elevate the culture temperature several degrees.

Appendix E

Logarithmic Series of Concentrations Suitable for Use in Toxicity Tests*

Column (Number of concentrations between 100 and 10, or between 10 and 1)**						
1	2	3	4	5	6	7
100	100	100	100	100	100	100
32	46	56	63	68	72	75
10	22	32	40	46	52	56
3.2	10	18	25	32	37	42
1.0	4.6	10	16	22	27	32
	2.2	5.6	10	15	19	24
	1.0	3.2	6.3	10	14	18
		1.8	4.0	6.8	10	13
		1.0	2.5	4.6	7.2	10
			1.6	3.2	5.2	7.5
			1.0	2.2	3.7	5.6
				1.5	2.7	4.2
				1.0	1.9	3.2
					1.4	2.4
					1.0	1.8
						1.3
						1.0

* Modified from Rocchini *et al.* (1982).

** A series of five (or more) successive concentrations may be chosen from a column. Midpoints between concentrations in column (x) are found in column (2x +1). The values listed can represent concentrations expressed as percentage by volume or weight, mg/L, or µg/L. As necessary, values may be multiplied or devided by any power of 10. Column 1 might be used if there was considerable uncertainty about the degree of toxicity. More widely spaced concentrations (differing by a factor >3.2) should not be used. For effluent testing, there is seldom much gain in precision by selecting concentrations from a column to the right of column 3; the finer gradations of columns 4 to 7 might occasionally be useful for testing chemicals that have an abrupt threshold of effect.

Appendix F

Analysis of Mortality to Estimate the Median Lethal Concentration

The three-brood (7 ± 1 day) test for mortality and reproductive success of *Ceriodaphnia dubia* is intended to be a sensitive, chronic, *sublethal* toxicity test. Therefore, the focus of this test is usually on determining the NOEC/LOEC and/or the ICp (see Section 4.6). However, there might be circumstances in which it is desired to estimate the LC_{50} for one or more of the exposure-times from 1 to 7 ± 1 days, so the method of estimating it is given here.

To estimate an LC_{50}, data are combined for all replicates at each concentration. If mortality is not ≥50% in at least one concentration, the LC_{50} cannot be estimated. If there is no mortality at a certain concentration, that information is used in fitting the probit line, being an effect of 0% mortality. However, if successive concentrations yield a series of 0% mortalities, only one such value should be used in estimating the LC_{50}, and that should be the result for the highest concentration, i.e., the one that is "closest to the middle" of the distribution of data. Similarly, if there were a series of successive complete mortalities at the high concentrations in the test, only one value of 100% effect would be used, again the one "closest to the middle", i.e., the effect at the lowest of these concentrations. Use of only one 0% and one 100% effect applies to analyzing the data by computer program or to hand plotting on a graph (see the following). Using additional values of 0% and/or 100% might distort the estimate of LC_{50}.

Various computer programs for calculating LC_{50} and confidence limits are suitable for use. Stephan (1977) developed a program for estimating the LC_{50} which uses probit, moving average, and binomial methods, and adapted it for the IBM-compatible personal computer. This program in the BASIC language is recommended, and is available for copying onto a user-supplied floppy diskette through courtesy of Dr. Charles E. Stephan (U.S. EPA, Duluth, Minn.), from Environment Canada (address in Appendix B). An efficient micro-computer program for probit analysis is also available from J.J. Hubert (1987), and other satisfactory computer and manual methods (APHA *et al.*, 1989; U.S. EPA, 1989) may be used. Programs using the Trimmed Spearman-Karber method (Hamilton *et al.*, 1977) are available for personal computers but are not recommended here because divergent results might be obtained by operators who are unfamiliar with the implications of trimming off ends of the dose-response data.

The recommended program of C.E. Stephan (1977) provides estimates of LC_{50} and confidence limits by each of its three methods, if there are at least two partial mortalities in the set of data. For smooth or regular data, the three estimates will likely be similar (see the following), and values from the probit analysis should be taken as the preferred ones and reported. The binomial estimate might differ somewhat from the others. If the results do not include two partial mortalities, only the binomial

method functions, and it can be used to provide a best estimate of the LC_{50} with conservative (wide) confidence limits.

Any computer-derived LC_{50} should be checked by examining a plot on logarithmic-probability scales, of percentage mortalities at a fixed observation-time (e.g., 96 h) for the various test concentrations (APHA *et al.*, 1989, see example in Figure F1). Any major disparity between the estimated LC_{50} derived from this plot and the computer-derived LC_{50} must be resolved.

In the hypothetical example shown in Figure F.1, ten *Ceriodaphnia* were tested at each of five concentrations (1.8, 3.2, 5.6, 10, and 18 mg/L). Mortalities of 0, 2, 4, 9, and 10 organisms were plotted and a line fitted by eye. The concentration expected to be lethal to half the organisms was read by following across from 50% (broken line) to the intersection with the eye-fitted line, then down to the horizontal axis, where an estimated LC_{50} of 5.6 mg/L was read off.

In fitting a line such as that in Figure F.1, relatively more emphasis should be assigned to points that are near 50% mortality. Logarithmic-probability paper ("log-probit", as in Figure F.1) can be purchased in good technical bookstores, or ordered through them.

Computer programs gave very similar estimates to the graphic one, for the regular data of Figure F.1. The LC_{50}s (and 95% confidence limits) were as follows:

Probit analysis of Hubert (1987):		5.56	(4.28 to 7.21)
Stephan (1977)	probit analysis:	5.58	(4.24 to 7.37)
	moving average:	5.58	(4.24 to 7.33)
	binomial:	6.22	(1.8 to 10)
Spearman-Karber method:	0% trim:	5.64	(4.38 to 7.26)
(Hamilton *et al.*, 1977)	10% trim:	5.73	(4.34 to 7.58)
	20% trim:	5.95	(4.34 to 9.80)

The binomial method did not estimate confidence limits, but selected two concentrations from the test as outer limits of a range within which the true confidence limits would lie.

It is also possible, if desired, to estimate the "time to 50% mortality" (LT_{50}) in a given concentration. A graph similar to Figure F.1 can be plotted using logarithm of time as the horizontal axis. Individual times to death of organisms could be used, but they are seldom available since tests are not inspected continuously. The cumulative percentage mortality at successive inspections is quite satisfactory for plotting, and an eye-fitted line leads to estimates of confidence limits following the steps listed in Litchfield (1949). Data permitting, such LT_{50}s could be estimated from successive records of mortality at 24-h intervals. Observed mortality must be greater than 50% in order to estimate an LT_{50}.

Neither an LT_{50} nor the percentage mortality at short exposure times is a dependable method of judging ultimate toxicity; therefore, comparisons based on those endpoints give only semi-quantitative guidance. It might sometimes be useful, however, to document whether the material being tested is rapidly or slowly lethal. For example, it might give guidance on a question of regulatory allowances for short-term excursions in concentration above a long-term permitted limit. In theory, deriving LT_{50}s instead of an LC_{50} can allow more complete utilization of information from the test, and a time-concentration curve of lethality might provide useful insight for investigating mechanisms of effect (Sprague, 1969; Suter *et al.*, 1987).

Erratum: Biological Test Method:
Test of Reproduction and Survival Using
the Cladoceran *Ceriodaphnia dubia*

Page 73
The following figure was omitted in error:

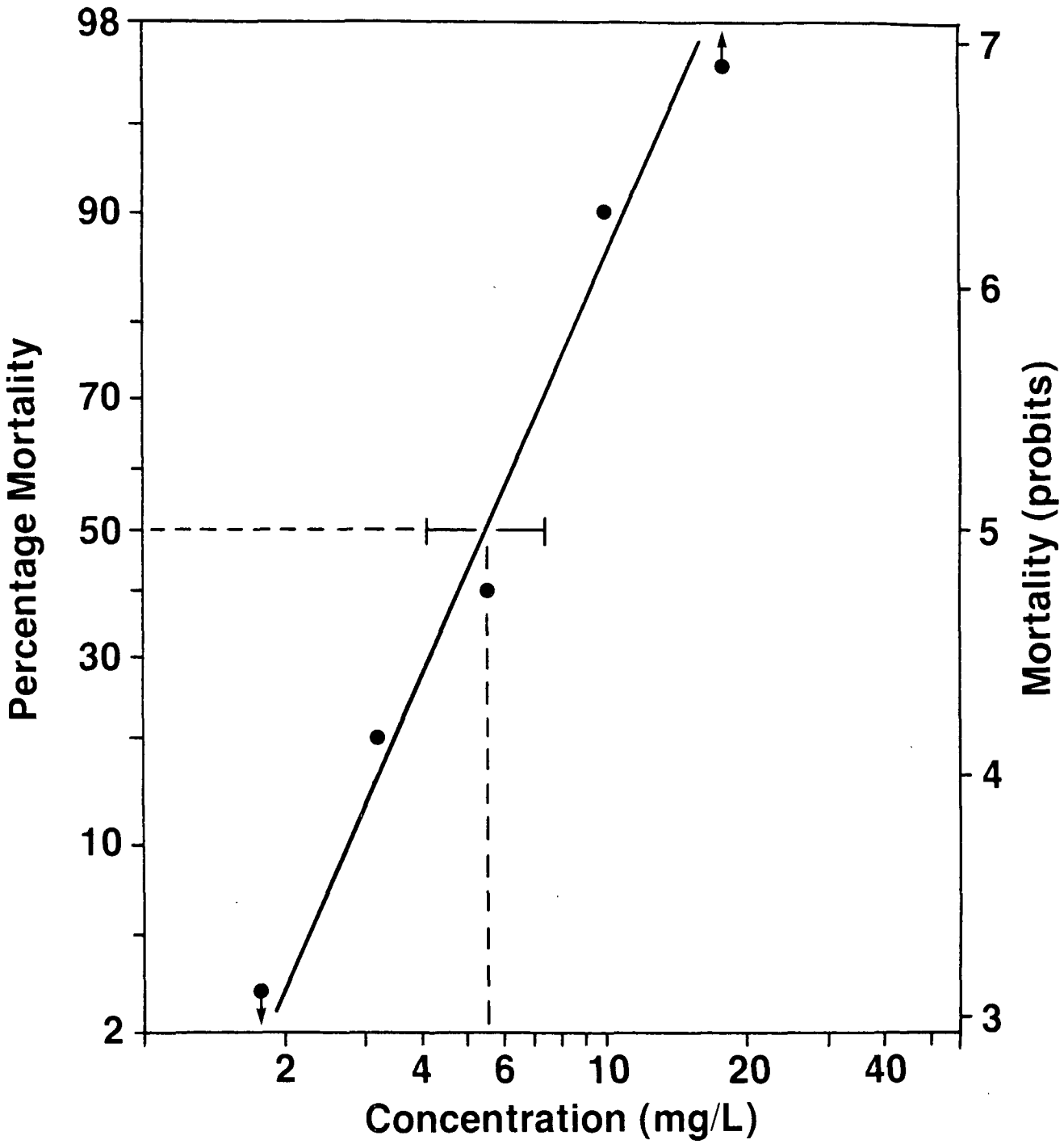

Figure F.1 **Estimating a Median Lethal Concentration by Plotting Mortalities on Logarithmic-probability Paper**