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

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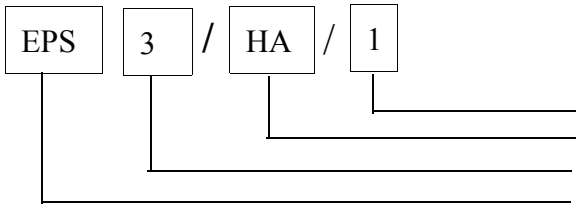

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Biological Test Method:
Test of Reproduction and Survival Using the Cladoceran *Ceriodaphnia dubia*

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Environment Canada
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Review Notice

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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. This second edition of EPS 1/RM/21, published in 2007, supersedes the first edition that was published in 1992. It includes numerous procedural modifications as well as updated guidance and instructions to assist in performing the biological test method.*

General or universal conditions and procedures are outlined for undertaking this chronic toxicity test using a variety of test materials or substances. 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*. Cette deuxième édition du document SPE 1/RM/21, publiée en 2007, remplace la première édition, parue en 1992. Elle comporte de nombreuses modifications procédurales, de même que des indications et des instructions à jour qui faciliteront l'exécution de la méthode d'essai biologique.*

Les conditions et procédures générales ou universelles décrites ici permettent de réaliser des essais de toxicité chronique avec un large éventail de matières ou de substances d'essai. Le document précise d'autres conditions et procédures propres à l'évaluation d'échantillons de substances chimiques, d'effluents, d'élutriats, de lixiviats ou d'eaux réceptrices. Il renferme aussi des instructions sur les conditions et règles d'élevage des organismes d'essai, la préparation des aliments, la manipulation et l'entreposage des échantillons, les exigences en matière d'installations d'essai, les procédures entourant la préparation des solutions d'essai et la mise en route des essais, les conditions prescrites pour les essais, les observations et les mesures pertinentes, les paramètres, les méthodes de calcul et l'utilisation de toxiques de référence.

Foreword

*This is one of a series of **recommended methods** for measuring and assessing the toxic effect(s) on single species of aquatic or terrestrial organisms, caused by their exposure to samples of toxic or potentially toxic substances or materials under controlled and defined laboratory conditions. Recommended methods are those that have been evaluated by Environment Canada (EC), and are favoured:*

- *for use in EC environmental toxicity laboratories;*
- *for testing that 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 because of their acceptability for the needs of programs for environmental protection and management carried out by Environment Canada. These reports are intended to provide guidance and to facilitate the use of consistent, appropriate, and comprehensive procedures for obtaining data on the toxicity to aquatic or terrestrial life of samples of specific test substances or materials destined for or within the environment. Depending on the biological test method(s) chosen and the environmental compartment of concern, substances or materials to be tested for toxicity could include samples of chemical or chemical product, effluent, elutriate, leachate, receiving water, sediment or similar particulate material, or soil or similar particulate material. Appendix G provides a listing of the biological test methods and supporting guidance documents published to date by Environment Canada as part of this series.

Words defined in the Terminology section of this document are italicized when first used in the body of the report according to the definition. Italics are also used as emphasis for these and other words, throughout the report.

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List of Abbreviations and Chemical Formulae

ANOVA	analysis of variance
°C	degree(s) Celsius
$CaCO_3$	calcium carbonate
$CaCl_2$	calcium chloride
$CaSO_4$	calcium sulphate
cm	centimetre(s)
$CoCl_2$	cobalt chloride
$CuCl_2$	copper chloride
d	day(s)
DO	dissolved oxygen (concentration)
EDTA	ethylenediamine tetraacetate ($C_{10}H_{14}O_8N_2$)
$FeCl_3$	ferric chloride
g	gram(s)
g/kg	gram(s) per kilogram
h	hour(s)
H_3BO_3	boric acid
HCl	hydrochloric acid
H_2O	water
ICp	inhibiting concentration for a (specific) percent effect
KCl	potassium chloride
K_2HPO_4	potassium phosphate
L	litre(s)
LC	lethal concentration
LC50	median lethal concentration
LT50	time to 50% mortality (lethality)
LOEC	lowest-observed-effect concentration
m	metre(s)
mg	milligram(s)
mS/m	millisiemens per meter
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
$MnCl_2$	manganous chloride
min	minute(s)
mL	millilitre(s)
mm	millimetre(s)
mS	millisiemen(s)
N	Normal
Na_2EDTA	disodium ethlenediamine tetraacetate
$NaHCO_3$	sodium bicarbonate
NaOH	sodium hydroxide
Na_2MoO_4	sodium molybdenate
$NaNO_3$	sodium nitrate
NOEC	no-observed-effect concentration
SD	standard deviation
sp	species
TM (^{TM})	Trade Mark
µg	microgram(s)
µE	micro Einsteins
µmhos	micromhos
µm	micrometre (s)
YCT	yeast, Cerophyll^{TM} and trout chow
$ZnCl_2$	zinc chloride
$ZnSO_4$	zinc sulphate
>	greater than
<	less than
≥	greater than or equal to
≤	less than or equal to
/	per; alternatively, “or” (e.g., control/dilution water)
±	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 express the possibility that something could exist or happen.

Technical Terms

Acclimation is physiological adjustment to a particular level of one or more environmental factors such as temperature. The term usually refers to the adjustment 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. The presence of two or more neonates in any test chamber, during any given day of the test, constitutes a brood.

Brood organism refers to a healthy adult (female) daphnid that produces and releases multiple broods of live neonates.

Compliance means in accordance with governmental regulations or requirements for issuing a permit.

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 measured at 25 °C, and is 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 organisms raised in the laboratory under defined and controlled conditions through one or more generations, to produce healthy test organisms. As a verb, it means to carry out the procedure of raising healthy test organisms from one or more generations, under defined and controlled conditions.

Daphnid is a freshwater microcrustacean invertebrate, commonly known 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 substance or 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 substance or 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 means 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 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 or substance (e.g., chemical, 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 substance or 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 some or all of the dissolved components of a solution.

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

Protocol is an explicit set of procedures for a test, formally agreed upon by the parties involved, and described precisely in a written document.

Reference method refers to a specific protocol for performing a toxicity test, i.e., a biological test method with an explicit set of test procedures and conditions, formally agreed upon by the parties involved and described precisely in a written document. Unlike other multi-purpose (generic) biological test methods published by Environment Canada, the use of a *reference method* is frequently restricted to testing requirements associated with specific regulations.

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, 2005). 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 or Substances

Chemical is, in this report, any element, compound, formulation, or mixture of a 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 toxicity tests, the control must duplicate all the conditions of the exposure treatment(s), but must contain no contaminated test material or substance. The control is used as a check for the absence of toxicity due to basic test conditions (e.g., quality of the dilution water, health of test organisms, or effects due to their handling).

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

Material is the *substance* or substances from which something is made. A material would have more or less uniform characteristics. Effluent, leachate, elutriate, or surface water are materials. Usually, the material would contain several or many substances.

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 or substance. 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 or substance is evaluated, and the precision of results for that chemical obtained by the laboratory.

Reference toxicity test is a test conducted using a reference toxicant in conjunction with a definitive toxicity test using a particular test material or substance, to appraise the sensitivity of the organisms and the precision and reliability of results obtained by the laboratory for that reference chemical at the time the test material or substance is evaluated. Deviations outside an established normal range indicate that the sensitivity of the test organisms, and the performance and precision of the test, are suspect.

Stock solution is a concentrated aqueous solution of the substance or 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.

Substance is a particular kind of material having more or less uniform properties. The word *substance* has a narrower scope than *material*, and might refer to a particular chemical (e.g., an element) or chemical product.

Upstream water is surface water (e.g., in a stream, river, or lake), that is not influenced by the effluent (or other test material or substance), 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.

Statistical and Toxicological Terms

Acute means within a short period of exposure (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 substance or 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.

Endpoint means the measurement(s) or value(s) that characterize the results of a test (e.g., LC50, IC25). It also means the response of the test organisms that is measured (e.g., death, or number of progeny produced).

Geometric mean is the mean of repeated measurements, calculated on a logarithmic basis. It has the advantage that extreme values do not have as great an influence on the mean as is the case for an arithmetic mean. The *geometric mean* can be calculated as the n^{th} root of the product of the "n" values, and it can also be calculated as the antilogarithm of the mean of the logarithms of the "n" values.

Homoscedasticity refers herein to data showing homogeneity of the residuals within a scatter plot. This term applies when the variability of the residuals does not change significantly with that of the independent variable (i.e., the test concentrations or treatment levels). When performing statistical analyses and assessing residuals (e.g., using Levene's test), for test data demonstrating homoscedasticity (i.e., homogeneity of residuals), there is no significant difference in the variance of residuals across concentrations or treatment levels.

Hormesis is an effect in which low concentrations of the test material or substance act as a stimulant for performance of the test organisms compared to that for the control organisms (i.e., performance in one or more low concentrations is enhanced and "better" than that in the control treatment). At higher concentrations, deleterious effects are seen.

ICp is the inhibiting concentration for a (specified) percent effect. It represents a point estimate of the concentration of test substance or material that causes a designated percent 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 EC50 or median effective concentration is limited to quantal measurements, i.e., number of individuals which show a particular effect.)

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

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 or substance 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.

LT50 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 or substance 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.

Normality (or *normal distribution*) refers to a symmetric, bell-shaped array of observations. The array relates frequency of occurrence to the magnitude of the item being measured. In a *normal distribution*, most observations will cluster near the mean value, with progressively fewer observations toward the extremes of the range of values. The normal distribution plays a central role in statistical theory because of its mathematical properties. It is also central in biological sciences because many biological phenomena follow the same pattern. Many statistical tests assume that data are normally distributed, and therefore it can be necessary to test whether that is true for a given set of data.

Precision refers to the closeness of repeated measurements of the same quantity to each other, i.e., the degree to which data generated from repeated measurements are the same. It describes the degree of certainty around a result, or the tightness of a statistically derived endpoint such as an ICp.

Quantal is an adjective, as in quantal data, quantal test, etc. A quantal effect is one for which each test organism either shows the effect of interest or does not show it. For example, an animal might either live or die, or it might develop normally or abnormally. Quantal effects are typically expressed as numerical counts or percentages thereof.

Quantitative is an adjective, as in quantitative data, quantitative test, etc. A quantitative effect is one in which the measured effect can take any whole or fractional value on a numerical scale. An example would be the number of progeny produced, or the weight attained by individual organisms at the end of a test.

Replicate (*treatment*, *test vessel*) refers to a single test chamber containing a prescribed number of organisms in either one concentration of the test material or substance, or in the control or reference treatment(s). A *replicate* of a treatment must be an independent test unit; therefore, any transfer of organisms or test material from one test chamber to another would invalidate a statistical analysis based on the replication.

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".

Sublethal (*toxicity*) means detrimental to the organism, but below the concentration or level of contamination that directly causes death within the test period.

Toxic means poisonous. A toxic chemical or material can cause adverse effects on living organisms, if present in sufficient amount at the right location. *Toxic* is an adjective or adverb, and should not be used as a noun; whereas *toxicant* is a legitimate noun.

Toxicant is a toxic substance or material.

Toxicity is the inherent potential or capacity of a substance or material to cause adverse effects on living organisms. These effects could be lethal or sublethal.

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

Toxicology is a branch of science that studies the toxicity of substances, materials, or conditions. There is no limitation on the use of various scientific disciplines, field or laboratory tools, or studies at various levels of organization, whether molecular, single species, populations, or communities. Applied toxicology would normally have a goal of defining the limits of safety of chemical or other agents.

Treatment is, in general, an intervention or procedure whose effect is to be measured. More specifically, in toxicity testing, it is a condition or procedure applied to the test organisms by an investigator, with the intention of measuring the effects on those organisms. The treatment could be a specific concentration of a potentially toxic material or substance. Alternatively, a treatment might be a particular test material (e.g., a particular sample of effluent, elutriate, leachate, receiving water, or control water).

Warning chart is a graph used to follow changes over time in the endpoints for a reference toxicant. The date of the test is on the horizontal axis and the effect-concentration is plotted on the vertical logarithmic scale.

Warning limit is plus or minus two standard deviations, calculated on a logarithmic basis, from the historic geometric mean of the endpoints from toxicity tests with a reference toxicant.

Acknowledgements

The first edition of this report, printed in February 1992 and amended in November 1997, was co-authored by D. McLeay (McLeay Associates Ltd., West Vancouver, B.C.) and J. Sprague (Sprague Associates Ltd., Guelph, Ontario). It was based on pre-existing reports describing a chronic (three-brood) daphnid (*Ceriodaphnia dubia*) test for survival and reproduction that was prepared in the U.S.A. (USEPA, 1985a; USEPA, 1989; Norberg-King, 1989; USEPA, 1994). Messrs. G. Sergy and R. Scroggins (Environmental Protection Service, Environment Canada) acted as Scientific Authorities and provided technical input and guidance throughout the work. Members of the Inter-Governmental Environmental Toxicity Group (IGETG, Appendix A) participated actively in the development and review of the first edition of this report and are thanked accordingly. The laboratory testing support of Environment Canada (Appendix B) is also acknowledged.

This (second) edition was prepared by D. McLeay (McLeay Environmental Ltd., Victoria, B.C.), with assistance and guidance from L. Taylor (Manager, Method Development and Applications Section), R. Scroggins (Chief, Biological Methods Division), and L. Van der Vliet (Method Development and Applications Section) of the Environmental Science and Technology Centre, Environment Canada, Ottawa, Ontario. The second edition incorporates the November 1997 Amendments (with modification as appropriate), and includes numerous updates such as the use of regression analyses for quantitative endpoint data. Numerous comments and suggestions for change to the first edition, which were forwarded to Environment Canada's Method Development and Applications Section by Canadian laboratory personnel performing this chronic toxicity test method, were considered when preparing this second edition of Report EPS 1/RM/21. Procedural differences in the fourth edition of USEPA's *C. dubia* survival-and-reproduction test method (Section 13 in USEPA 2002), relative to their third edition of this biological test method (USEPA, 1994), were also considered when preparing the current edition of EPS 1/RM/21.

Introduction

1.1 Background

Aquatic *toxicity tests* are used within Canada and elsewhere to measure, predict, and control the discharge of *substances* or *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 Environmental 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. The first edition of this biological test method was published in February 1992 as Report EPS 1/RM/21, and amended in November 1997. The current (second) edition includes numerous procedural improvements, updated and more explicit guidance, and instructions for the use of revised statistics (i.e., regression analyses) when calculating the test *endpoint* for reproductive effects.

Universal procedures for conducting three-*brood* chronic toxicity tests with the cladoceran *Ceriodaphnia dubia* are described in this second edition. Also presented are specific sets of test conditions and procedures, required or recommended when using this chronic toxicity test for evaluating different types of substances or materials (namely, samples of one or more *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 *Ceriodaphina* 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 cladaocerans 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

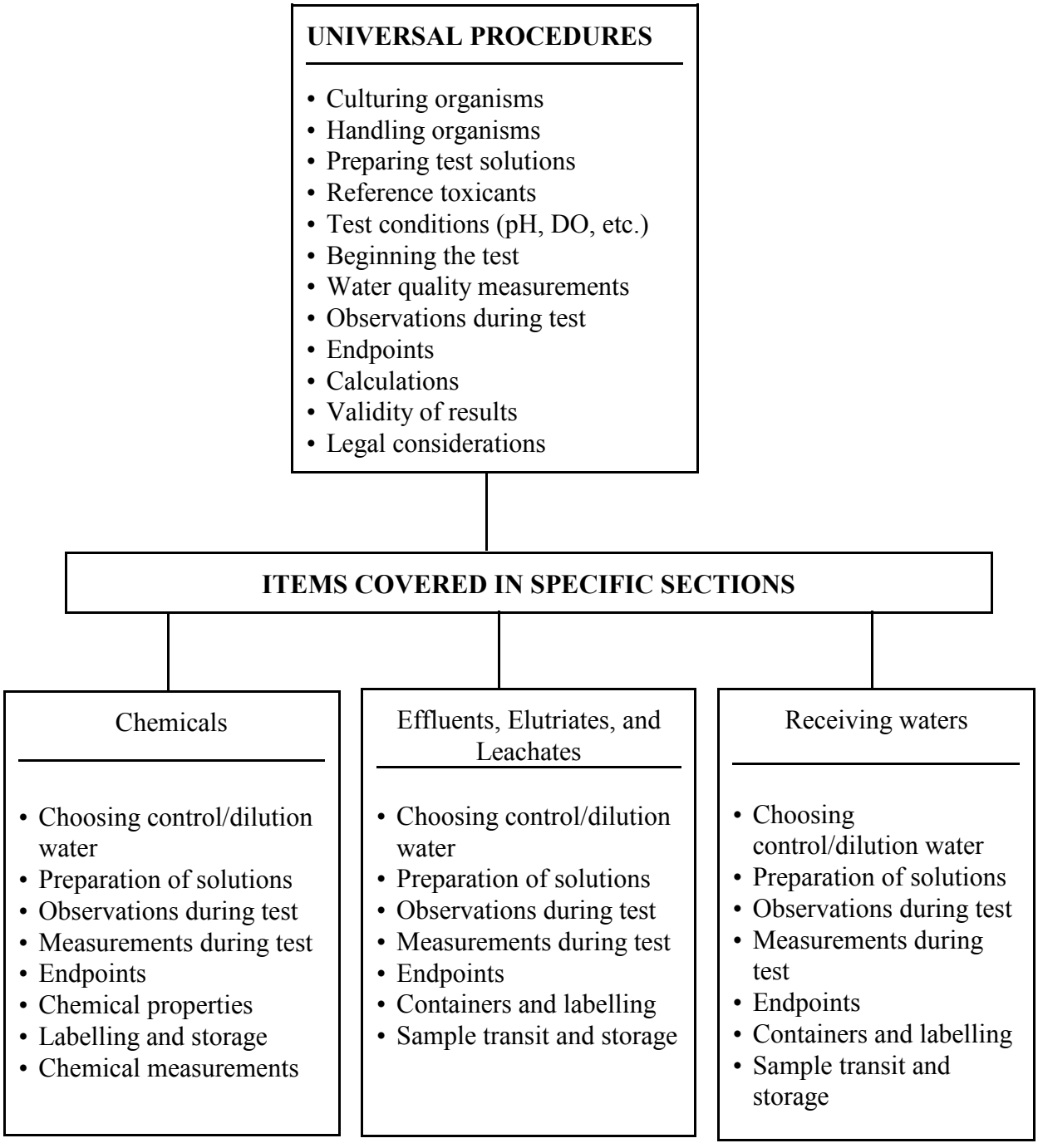

Figure 1 Diagram of Approach Taken in Delineating Test Conditions and Procedures Appropriate for Various Types of Test Materials or Substances

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:

- 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 large *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 5 to 8 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 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 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 USEPA as one of three short–term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms (USEPA, 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, was published by USEPA as second (USEPA, 1989), third (USEPA, 1994), and fourth (USEPA, 2002) editions. 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, 2006). Additional documents which describe procedures and conditions for undertaking this test are reviewed in Appendix C.

Researchers familiar with the USEPA (1985a, 1989, 1994, 2002) test method 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 USEPA (1985a, 1989, 1994, 2002) test method using *C. dubia* 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 or substances 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 substances or 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 USEPA (1989, 1994, 2002), with the incorporation of useful test modifications and additions obtained from ASTM (1989, 2006) 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* $\leq$10 g/kg) or saline but destined for discharge to fresh water. Its application can be varied but includes instances where the impact or potential impact of one or more substances or 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 substances or materials in estuarine or marine environments, or to evaluate wastewaters having a salinity > 10 g/kg which are destined for estuarine/marine discharge.

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 postadominal 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 used in tests must be <24 h old and within 12 h of the same age; it would be desirable if the neonates were <12 h old and within 6 h of the same age. These neonates *should* be taken from *individual cultures* (i.e., brood cultures set up exclusively for obtaining neonates for tests) (Section 2.4.1), and should meet the requirements specified in Section 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; USEPA, 1989, 1994, 2002) upon initiation of cultures using organisms from outside sources[1]. 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 (USEPA, 1989, 1994, 2002), 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 uncontrolled, might affect the health and performance of the test organism.

[1] Initial *Ceriodaphnia* cultures established in the USEPA 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 USEPA cultures (Berner, 1986).

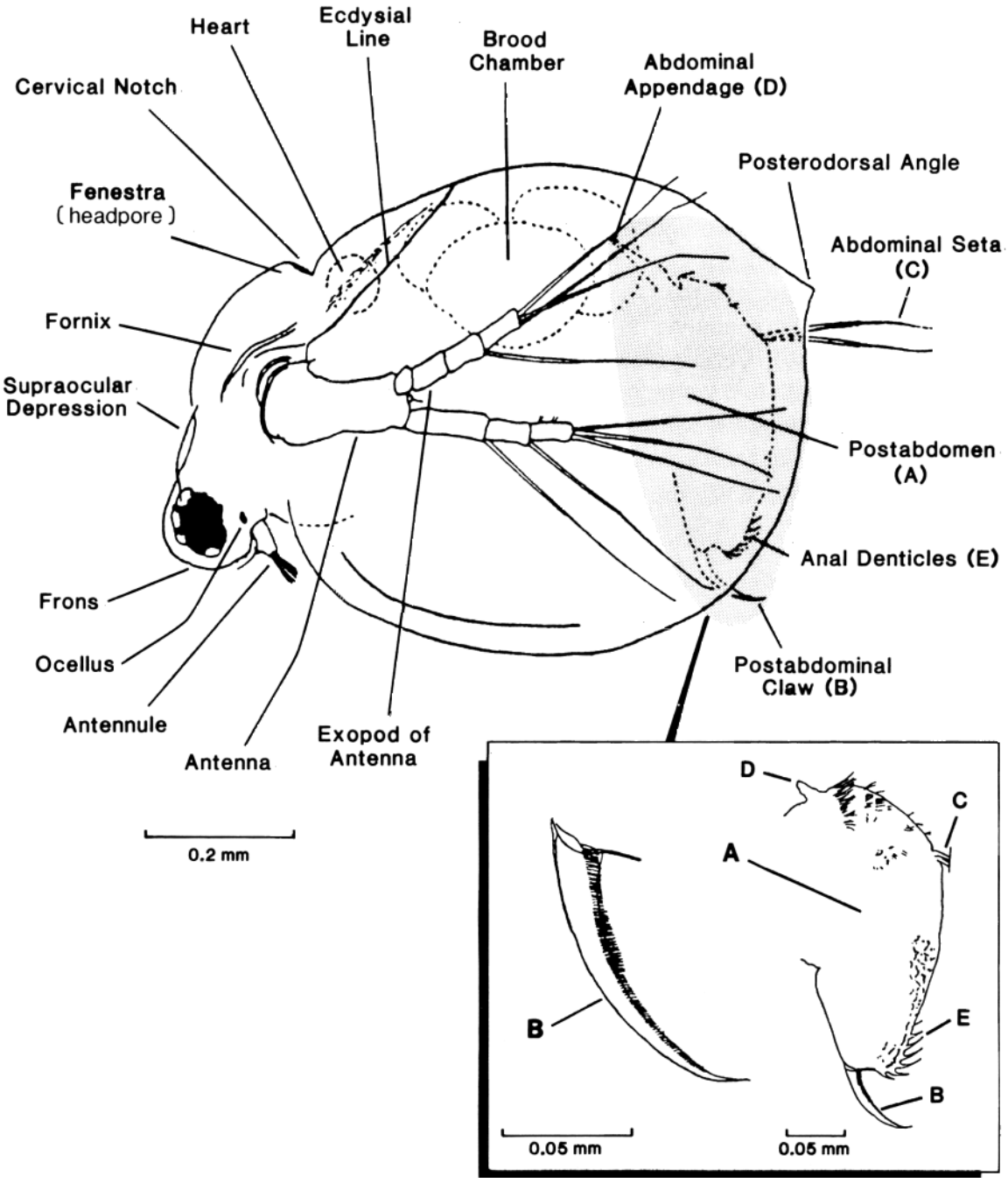

Figure 2 Anatomy of Female *Ceriodaphnia dubia* (from Berner, 1986)

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% DO saturation; 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
Lighting	–	"cool white" fluorescent, 100–600 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 within the first three broods, during the week before the test; with ≥8 young produced by each brood organism in its third or fourth brood; no ephippia produced in culture

A training video and supplemental report was prepared 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).

The parentage of all organisms used to start a test must originate from the same *mass 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[2]. 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[3]. 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 (Section 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; USEPA, 1989, 1994, 2002). To provide cultures of overlapping ages, new cultures are started weekly using adults which produce at least eight 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 substances or materials. Cultures should also be isolated from regions of the laboratory where stock or test solutions are prepared, effluent or other test material or substance 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, 2006). Materials or substances 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.

Items made of materials or substances other that 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, 2005; ASTM, 1989,

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[2] 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.

[3] 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.

2006) and rinsed with culture water between uses. New glass beakers used as cultures 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* (Section 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) 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, drilled to hold up to ten rows of 10 cups or beakers, are suitable for holding culture/test cups or beakers; 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[4]. Cool-white fluorescent or alternate light skewed towards the blue end of the spectrum (colour-rendering index ≥90) is normally suitable (Buikema, 1973); other light sources and wavelengths might also be used for specialized tests (ASTM, 1996). Light intensity at the water surface should be 100 to 600 *lux*.

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*; USEPA, 1989, 1994, 2002), or *reconstituted water* adjusted to the desired *hardness* and *pH* (see Section 2.4.8)[5]. 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 (Section 2.4.1) should be uniform. The culture water should consistently support good survival, growth, and reproduction of daphnids (see Section 2.4.11). A given batch of culture water (or control/dilution water) should not be stored for than 14 days[6]. 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

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[4] 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).

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

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

reconstituted water have been identified[7] (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, 2006; Cowgill, 1989). Guidance for preparing reconstituted water with a desired hardness is given in Section 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, 2006).

Culture water should 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 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 (Section 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

[7] 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 reconstitued water prepared according to USEPA (1989, 1994, 2002) using either the formula given in Table 2 or an alternative (USEPA, 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.

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 Section 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 Section 2.4.11) in soft or hard water (ASTM, 1989, 2006). 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[8]. 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; other suitable formulae 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 (USEPA, 1989, 1994, 2002). 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*[9]. The food used

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[8] 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.

[9] Organisms could be stressed by less frequent feedings, resulting in low numbers of young, large numbers of males, and/or ephippial females (USEPA, 1989, 1994, 2002).

Table 2 Preparation of Reconstituted Water of a Desired Hardness (USEPA, 1985a)[a]

	Reagent Added [b, c] (mg/L)				Final Water Quality	
Water Type	**$NaHCO_3$**	**$CaSO_4$[d]**	**$MgSO_4$**	**KCl**	**Hardness [e]**	**pH [f]**
very soft	12	7.5	7.5	0.5	10 to 13	6.4 to 6.8
soft	48	30	30	2	40 to 48	7.2 to 7.6
moderately hard	96	60	60	4	80 to 100	7.4 to 7.8
hard	192	120	120	8	160 to 180	7.6 to 8.0
very hard	384	240	240	16	280 to 320	8.0 to 8.4

[a] 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 (USEPA, 1989).

[b] 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.

[c] 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, USEPA, Environmental Monitoring Systems Laboratory, Quality Assurance Research Division, 3411 Church Street, Cinicinnati, Ohio, 45244.

[d] $CaSO_4 \cdot 2H_2O$

[e] Expressed in mg/L, as $CaCO_3$.

[f] Approximate pH after aerating for 24 h.

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 Section 2.4.11. Various combinations of yeast, Cerophyll™ and trout chow[10] (YCT), if provided along with unicellular algae (most frequently

Pseudokirchneriella subcapitata, formerly *Selenastrum capricornutum*[11], will provide suitable nutrition if fed daily (Cooney *et al*., 1988; ASTM, 1989, 2006; Cowgill, 1989; USEPA, 1989, 1994, 2002). A mixed algal diet, usually a green alga (*Ankistrodesmus convolutus* or *P. subcapitata*) and a freshwater diatom (*Nitzchia frustulum*) appears to sustain healthier animals that unialgal diets (Cowgill, 1989). Other food sources have also been used with success (Anon., 1989).

The USEPA (1989, 1994, 2002) recommends that *C. dubia* routinely be fed YCT and algae

[10] Researchers at USEPA'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).

[11] Other algae used as a food for *Ceriodaphnia* include *Ankistrodesmus convolutus*, *A. falactus*, *Chlamydomonas reinhardtii* and *Scenedesmus* sp. (Cooney *et al*., 1988; NWRI, 1988; ASTM, 1989, 2006; 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).

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 (Section 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 (USEPA, 1989, 1994, 2002). 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 (USEPA, 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 must meet the following health criteria (ASTM, 1989, 2006; USEPA, 1989, 1994, 2002):

- During the 7-day period prior to test initiation, the average mortality rate for brood organisms in the individual cultures must not exceed 20%.

- Neonates used to start a test must be taken only from individual brood cultures containing at least eight young that were produced during the third or subsequent brood.

- Within the seven-day period before testing, brood organisms in individual cultures must produce an average of at least 15 young per adult during their first three broods.

- 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 substance or material (see Section 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 Section 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. Glass containers should be used for tests involving chemicals (Section 5). Supporting boards or racks suitable for holding large numbers of small test vessels (e.g., ten rows of ten test vessels per board) are recommended for use (see Section 2.4.2). Sheets of glass should be used to cover test vessels[12].

3.4 Control/Dilution Water

The choice of control/dilution water depends on a number of variables including the test substance or 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 (Section 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[13]; reconstituted water of a desired pH and hardness (see Section 2.4.8); or sample of receiving water collected upstream of the influence of the contaminant source, or adjacent to the source, but removed from it.

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 (USEPA, 1989, 1994, 2002). If receiving water is to be used, conditions for its collection, transport,

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

[13] 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.

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 (Section 2.4.8).

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 or substances.

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 USEPA (1989, 1994, 2002). Control/dilution water should be used as the final rinse water.

Reconstituted water with the desired hardness (Section 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 tests results[14]. Freshly prepared reconstituted water should be aerated vigorously in a nontoxic vessel for at least 24 h before use (USEPA, 1989, 1994, 2002).

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 7 to 10 test concentrations plus a control) and for the required chemical analyses.

The control/dilution water must be adjusted to the test temperature (25 ± 1 °C) before use. This water should not be supersaturated with excess gases (Section 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 required volume of control/dilution water should be aerated vigorously (oil-free compressed air passed through air stones) immediately before use, and its dissolved

[14] USEPA (1989, 1994, 2002) 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 the dilute mineral water (e.g., 20% Perrier ™ water, USEPA, 1989, 1994, 2002) 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	–	must end as soon as 60% (or more) of control organisms have three broods; maximum duration of test is 8 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
Number of concentrations	–	minimum of 7, plus control(s); recommend more (i.e.,≥10), plus control(s)
Number of replicates	–	10 replicates/treatment
Organisms	–	neonates (<24 h old and within 12 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 6.5 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, 100–600 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 throughout test, for the death of the first-generation daphnid in each replicate test solution ; daily during first three broods, for numbers of live neonates produced in each replicate test solution; discard all offspring produced during fourth or subsequent broods upon their observation, without recording these numbers

Endpoints	–	mortality and reproduction; if multi-concentration tests, LC50, plus ICp for decreased reproduction (see Section 4.6)
Reference toxicant	–	one or more of sodium chloride, phenol, or zinc sulphate; standard test of ≤8d for LC50 and ICp (for decreased reproduction), within 14 days of the start of the definitive test and following the same method and procedures used for that test
Test validity	–	invalid if mean mortality of first-generation controls is >20%; invalid if at least 60% of controls have not produced three broods within 8 days; invalid if an average of <15 live young produced per surviving female in the control solutions during the first three broods ; invalid if ephippia observed in any control solutions at any time

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, either three subsamples from a single sampling or ≥3 separate samples are collected (or prepared, if elutriate) and handled 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 ≥2L (multiple-concentration test)
Transport and storage	–	if warm (>7 °C), must cool to 1 to 7 °C with regular ice (not dry ice) or frozen gel packs upon collection; transport in the dark at 1 to 7 °C (preferably 4 ± 2 °C) using regular ice or frozen gel packs as necessary; sample must not freeze during transit or storage; store in the dark at 4 ± 2 °C; use in testing should begin within 1 day and must start within 3 days of sample collection or elutriate 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

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 any test that is intended to estimate an *LC50* (mortality endpoint) as well as *ICp* (reproduction endpoint), at least seven test concentrations plus a control solution (100% control/dilution water) must be prepared[15], and more ($\geq$10 plus a control) are recommended. 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 (e.g., 100, 75, 56, 42, 32, 24, 18, 13, 10, 7.5; see column 7 in Appendix E). If a high rate of mortality is observed within the initial 2 h of the test, additional dilutions should 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 uncertainity about the range of concentrations likely to be toxic.

When water other than that in which the organisms have been cultured (e.g, receiving water from upstream of the area of concern) is used as dilution and control water, a second control solution must be prepared using the culture water[16]. Upstream receiving water is normally considered unsuitable as control/dilution water if it cannot meet the criteria for a valid test (see Section 4.4). In such cases, the culture water would normally be used as the control water and for all dilutions (see footnote 16). The investigator might choose to attempt to acclimate the culture to *upstream water* beforehand, in which instance at least two generations of brood organisms preceding the neonates should be reared in this water.

For each definitive test, control solution(s) must be prepared at the same time as the experimental *treatments*, using an identical number of *replicates*. Any dilution water used to prepare test concentrations must also be used for preparing one set of controls. Each test solution must be mixed well using a glass rod, Teflon™ stir bar, or other device made of nontoxic material. The temperature, dissolved oxygen, and pH of each test solution should be checked upon mixing. Sample/solution temperature must be

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[15] The use of ten or more test concentrations plus the control solution(s) is recommended to enable calculation of both the LC50 (for adult daphnids) and the ICp for reproduction using regression analyses (EC, 2005). 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 24 h or less.

[16] If the intent of the test is to measure the extent to which a particular receiving water might modify the toxicity of the test material or substance due to its physicochemical characteristics (e.g., hardness, *pH*, *turbidity*, humic or fulvic acid content) and/or the presence of other contaminants, the investigator might choose to use the *upstream water* to prepare the test concentrations and as one of the control solutions. A comparison of results for this water with those for the controls held in laboratory water will identify toxic responses that might be contributed by the upstream water. A clearer understanding of the differing influence of each type of control/dilution water on the toxicity of the test material or substance can be achieved by undertaking a side-by-side comparison for toxic effects using each control/dilution water to prepare a series of test concentrations.

adjusted as required to attain an acceptable value for each solution (25 ± 1 °C). Samples or test solutions must not be heated by immersion heaters, since this could alter chemical constituents and toxicity. 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, each prepared test solution should be pre-aerated before it is divided between the individual *replicate test vessels*. To achieve this, oil-free compressed air should be dispensed through airline tubing and a disposable plastic or glass tube (e.g., capillary tubing or a pipette with an Eppendorf tip) with a small aperture (e.g., 0.5 mm ID). Rate of aeration should not exceed 100 bubbles/min. Duration of pre-aeration must 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 must be discontinued at ≤20 minutes, at which time each test solution should be divided between the replicate test chambers and the test initiated or the solutions used for renewals, regardless of whether 40 to 100% saturation was achieved in all test solutions. Any pre-aeration must be reported, including the duration and rate (Section 8).

Adjustment of sample/solution pH might be necessary (see Section 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[17] 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 (Section 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

[17] If the test material or substance 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 or substance. 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.

exception of special investigations[18], no attempt should be made to adjust the hardness of samples or test solutions.

Each treatment, consisting of ten replicates of a particular test concentration or the control(s), must be randomly assigned to a position on a test board. If a template is used, it should be one of several available (to prevent the same ordering for each set). Once a numbered position for each treatment has been 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 12 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 Section 2.4.1 and health criteria given in Section 2.4.11. For multi-concentration tests, up to 20 brood cups/beakers[19], each with eight or more young, are identified on one or more brood boards[20] for use in setting up the test. To begin the test, one neonate from the first one or two brood cups is transferred to each of the ten test vessels in the first row on the test board (each board normally holds ten rows and up to ten columns of test vessels). A second one or, if required, two brood cups is (are) chosen at random, and one neonate from these one or two cups is transferred to each of the 8 to 10 (or more) test vessels in the second row. This procedure is repeated until each of the 80 to 100 (or more) test vessels[21] contains one neonate.

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 (Section 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.

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[18] 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.

[19] Small glass test tubes, held in racks, may be substituted for cups or beakers held on brood boards (see Section 2.4.2).

[20] One brood board would suffice if there were eight (or more) neonates in each brood cup and the test involved seven test concentrations plus a control. Two brood boards with eight (or more) neonates per cup might be required if the test involved more than 8 treatments. The design of the brood board is somewhat arbitrary. The number of rows reflects the number of replicates per test concentration (i.e., 10), whereas the number of columns reflects the number of treatments (i.e., ≥ 7 test concentrations plus the control).

[21] 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 range from 80 to ≥100 in multiples of ten (i.e.,7 to≥10 test concentrations plus the control solutions.

4.3 Renewing Test Solutions

Each test solution must be renewed at least once daily[22]. When doing so, any brood organism observed to be in the process of releasing its young must be given sufficient time to do so before changing that test solution. Also, care and attention must be given to prevent the carryover of any neonates from an aged test solution to a fresh one.

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 (USEPA, 1989, 1994, 2002), 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 incorporates a static-renewal procedure. Each test solution must be replaced at intervals of $\leq$24 h, throughout the test.

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 Section 2.4.9.

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

Test solutions are normally not aerated.

For each *replicate* and treatment (including the control), any neonates produced as a fourth or subsequent brood must not be included in the total number of neonates produced for those treatments during the test (USEPA, 2002).

The test must end when 60% or more of the first-generation daphnids in the control solutions have produced three broods, or at the end of 8 days, whichever occurs first[23].

The presence of two or more neonates in any test chamber, during any given day of the test, constitutes a brood. To determine when to end a test (i.e., as soon as it is observed that $\geq$60% of the first-generation daphnids in the control solution have produced three broods), the following scoring for daily number of broods is to be applied. When only one neonate was observed and counted for a particular test chamber containing control water, and one or more neonates were found in the same test chamber on the following or previous day, that single neonate must be scored as part of the count

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

[23] Under normal conditions, three broods can be achieved by the first-generation adults in the control solutions as early as five days, although in some instances this does not occur until eight days.

for the following or previous day.[24]

If ≥60% of the first-generation adults in the control solutions have not produced three broods by the end of the eighth day of the test, the test is to be declared invalid and must be terminated at that time. The test is also not valid if the mean mortality rate for the first-generation test organisms in the control water exceeds 20% at any time. Additionally, the test is not valid if reproduction in the controls averages less than 15 live young per surviving adult upon ≥60% of the adults achieving their third brood[25]. A test must be terminated early (and declared invalid) if, at any time during the test, the mean mortality rate for the first-generation control daphnids is ≥20% or if ephippia are evident in one or more control solutions.

[24] The presence of a single neonate in a test chamber can occur if the release of a brood is inadvertently interrupted during the daily transfer of the adult brood organism to a fresh test solution, resulting in a split in the brood count between two consecutive days. This can also occur if the brood organism has just started to release its young when it is transferred to a fresh test solution.

[25] 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 (Section 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).

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 organism 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, 2006). In those instances where the test material or substance 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[26].

In certain cases (usually experimental), the investigators might wish to aerate oxygen-deficient test solutions during the test. Alternatively, they might choose to 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, 2006). Any aeration of solutions prior to ("pre-aeration"; see Section 4.1) or during the test must be at a minimal and controlled rate. For this purpose, oil-free compressed air should be dispensed through airline tubing and a disposable plastic or glass tube of small

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

aperture (e.g., capillary tubing or a pipette with an Eppendorf tip, with an opening of about 0.5 mm). The rate of any aeration provided during the test should be as slow as possible, and should not exceed 100 bubbles/min. Any aeration during testing must be reported, including the rate.

4.4.2 pH

Toxicity tests should normally be carried out without adjustment of pH. However, if the sample or subsample of test material or substance causes the pH of any test solution to be outside the range 6.5 to 8.5, and it is desired to assess toxic chemicals rather than the deleterious or modifying effects of pH, then the pH of the test solutions or sample/subsample should be adjusted before use, or a second (pH-adjusted) test conducted concurrently using a portion of the same sample or subsample. For this second test, the initial pH of the sample, or of each test solution (see footnote 17, 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) upwards to pH 6.5 to 7.0 (if the sample/subsample has/causes pH <6.5), or downwards to pH 8.0 to 8.5 (if sample/subsample has/causes pH >8.5). Solutions of hydrochloric acid (HCl) or sodium hydroxide (NaOH) at strengths $\leq 1N$ should normally be used for all pH adjustments. Some situations (e.g. effluent samples with highly buffered pH) might require higher strengths of acid or base.

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.

Test solutions that are opaque due to colour or suspended solids should be transferred temporarily to a shallow dish (eg., 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).

For each replicate test solution (including each of the control replicates), the death of any first-generation daphnid must 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[27]. Each live first-generation daphnid is to be transferred to its respective new test solution (see Section 2.4.10 and Section 4.3) immediately thereafter.

For each replicate test solution (including each of the control replicates), the number of live neonates produced by each first-generation daphnid during each of its first three broods must be counted and recorded at the time of each daily observation. These young are discarded after counting. Any dead young observed should be discarded; counting of dead neonates is normally not required[28].

For each replicate and treatment (including the control), any neonates produced as a fourth or subsequent brood must not be included in the total number of neonates produced for those replicates or treatments during the test (USEPA, 2002). Rather, all offspring produced during the fourth or subsequent brood are to be discarded without recording their number(s).

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[29], 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

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[27] 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.

[28] 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, 2006; USEPA, 1989, 1994, 2002). Such information could, however, be useful for Toxicity Identification Evaluations (Mount and Anderson-Carnahan, 1988) or other research investigations.

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

beginning and end of their use might be desirable for certain test materials or substances[30].

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 or substances on daphnid survival and reproduction. There are two biological endpoints for 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 its first three broods.

4.6.1 Multi-Concentration Tests

In a multi-concentration test, the required statistical endpoints are: (i) an LC50 and its 95% confidence limits for the mortality of first-generation daphnids, and (ii) an ICp[31,32] and its 95% confidence limits for reproduction (i.e., number of live neonates produced by first-generation daphnids). Environment Canada (2005) provides direction and advice for calculating the LC50 and the ICp, including decision flowcharts to guide the selection of appropriate statistical tests. All statistical tests used to derive endpoints require that concentrations be entered as logarithms.

An initial plot of the raw data (number of live neonates) against the logarithm of concentration is highly recommended, both for a visual representation of the data, and to check for reasonable results by comparison with later statistical computations[33]. Any major disparity between the approximate graphic ICp and the subsequent computer-derived ICp must be resolved. The graph would also show whether a logical relationship was obtained between log concentration (or, in certain instances, concentration) and effect, a desirable feature of a valid test (EC, 2005).

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

[31] The *ICp* is the *inhibiting concentration* for a specified *percent* effect. The "p" represents a fixed *percentage* of reduction, and is chosen by the investigator. Typically, its value is chosen as 25% or 20%.

[32] Historically, investigators have frequently analyzed *quantitative sublethal* endpoints from multi-concentration tests by calculating the *no-observed-effect concentration* (*NOEC*) and the *lowest-observed-effect-concentration* (*LOEC*). Disadvantages of these statistical endpoints include their dependence on the test concentrations chosen and the inability to provide any indication of *precision* (i.e., no 95% or other confidence limits can be derived) (Section 7.1 in EC, 2005). Given these disadvantages, ICp is the required statistical endpoint for reproduction data derived from a multi-concentration test using *Ceriodaphnia dubia*.

[33] As an alternative to plotting the raw data, investigators might choose to calculate and plot the percent inhibition for each test concentration; this calculation is the difference between the average control response and the treatment response (average control response minus average treatment response in the numerator), divided by the average control response (denominator), expressed as a percentage (multiplied by 100%). The value for each treatment is graphed against the concentration; see ASTM (1991) for more details. The x-axis represents log concentration or, in some instances, concentration, depending on the preferences and purpose of the investigator. For example, using a log scale will match the regression data scales, but concentration might be clearer in the final report. To improve the use of a graph as a visual representation of the data, the investigator might choose to include the regression line as well as the raw data.

Regression analysis is the principal statistical technique and must be used for the calculation of the ICp, provided that the assumptions below are met. A number of models are available to assess reproduction data (using a *quantitative* statistical test) via regression analysis. Use of regression techniques requires that the data meet assumptions of *normality* and *homoscedasticity*. Weighting techniques may be applied to achieve the assumption of *homoscedasticity*. The data are also assessed for outliers using one of the recommended techniques (see Section 10.2 in EC, 2005). An attempt must be made to fit more than one model to the data. Finally, the model with the best fit[34] must be chosen as the one that is most appropriate for generation of the ICp and associated 95% confidence limits. The lowest residual mean square error is recommended to determine best fit; it is available in the ANOVA table for any of the models. Endpoints generated by regression analysis must be bracketed by test concentrations; extrapolation of endpoints beyond the highest test concentration is not an acceptable practice.

In the analyses of reproductive performance, a value of zero is assigned for number of neonates in a replicate, if the adult female died before producing young. If a female died during the test, after producing young, the number of neonates produced is still to be used in the analyses.

With some highly toxic test materials or substances, it is possible to record zero neonates in all ten replicates at one or more exposure concentration(s). In these cases, the results from the high test concentration(s) provide no further information on the response of the organism, and the repetitive zeroes interfere with regression assumptions of *normality* and *homoscedasticity*. Accordingly, data from any high test concentration(s) resulting in zero neonates in all test replicates must be removed before the regression analyses.

The ability to mathematically describe *hormesis* (i.e., a stimulatory or "better than the control" response occurring only at low exposure concentrations) in the dose-response curve has been incorporated into recent regression models for quantitative data (see Section 10.3 in EC, 2005). Data exhibiting *hormesis* can be entered directly, as the model can accommodate and incorporate all data points; there is no trimming of data points which show a hormetic response.

In the event that the data do not lend themselves to regression analysis (i.e., assumptions of *normality* and *homoscedasticity* cannot be met), linear interpolation (e.g., ICPIN; see Section 6.4.3 in EC, 2005) can be used to derive an ICp.

For each test concentration, including the control treatment(s), the following calculations must be performed and reported: (i) the (cumulative) mean percent mortality for the ten first-generation daphnids, at the end of the test; and (ii) the (cumulative) mean number (including its SD) of live neonates produced per first-generation daphnid, during its first three broods only.

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[34] As described in Section 6.5.8 of EC (2005), Environment Canada's current guidance on statistical methods for environmental toxicity tests specifies the use of the following five models for regression analysis, when estimating the ICp: linear, logistic, Gompertz, exponential and *hormesis* (logistic adapted for hormetic effect at low doses). Specific mathematical expressions of the model, including worked examples for a common statistics package, are also provided in that guidance document (Section 6.5.8 and Appendix O in EC, 2005).

4.6.2 Single-Concentration Tests

In single-concentration tests, the response in the test concentration is compared with the control response[35]. If mortality (a *quantal* endpoint) is assessed at only one site and a control site, the choice of statistical tests depends on whether replicates exist. If several locations are being assessed, the investigator is advised to contact a statistician. If reproduction (a *quantitative* endpoint) is assessed at a single test site and control site, a t-test[36] is normally the appropriate method of comparing the data from the test concentration with that for the control. In situations where more than one test site is under study, and the investigator wishes to compare multiple sites with the control, or compare sites with each other, a variety of ANOVA (or non-parametric equivalent) tests exist (Section 3.3 in EC 2005). Choice of the test to use depends on:

(i) the type of comparison that is sought (e.g. complete a series of pairwise comparisons between all sites or compare the data for each location with that for the control only);
(ii) if a chemical and/or biological response gradient is expected, and
(iii) if the assumptions of *normality* and *homoscedasticity* are met.

As with multi-concentration tests, other calculations which must be performed and reported when performing a single-concentration test include: (i) the (cumulative) mean percent mortality for the ten first-generation daphnids, at the end of the test; and (ii) the (cumulative) mean number (including its SD) of live neonates produced per first-generation daphnid, during its first three broods only.

4.7 Reference Toxicant

The routine use of a reference toxicant or toxicants is required to assess, under standardized 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) must be evaluated within 14 days before or after the date that the toxicity test is started, or during it. The same stock of brood animals should be used for tests on both the reference toxicant and sample. The reference toxicant test must be performed under the same experimental conditions as those used with the test sample(s).

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*;

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[35] See Sections 4.1, 5.3, 6.3, and 7.3 for a description of the type(s) of control/dilution water that could be used in a single-concentration test.

[36] Strictly speaking, the t-test assumes a t-distribution and equal variances in the two groups. Tests for distribution and equal variances have been outlined, and alternatives in the case of unequal variances are recommended (see Section 3.2 in EC, 2005).

- 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) should be used as reference toxicant(s) for this test: sodium chloride; zinc sulphate; phenol. Daphnid sensitivity must be evaluated by standard tests following the methods given in this document, to determine the LC50 (for survival) as well as the ICp (for reproduction), for at least one 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 desired[37].

Once sufficient data are available (EC, 1990b), a *warning chart* which plots values for LC50 and/or ICp must be prepared and updated for each reference toxicant used. Successive LC50s for survival and/or ICps for reproduction are plotted separately on this chart, and examined to determine whether the results are within ± 2 SD of respective values obtained in previous tests. The *geometric mean* LC50 and/or ICp , together with its respective upper and lower *warning limits* (± 2 SD calculated on a logarithmic basis) are recalculated with each successive test until the statistics stabilize (USEPA, 1989, 1994, 2002).

The logarithm of concentration (i.e., LC50 and/or ICp as a logarithm) must be used in all calculations of mean and standard deviation, and in all plotting procedures. This simply represents continued adherence to the assumption by which each endpoint value was estimated on the basis of logarithms of concentrations. The warning chart may be constructed by plotting the logarithms of the mean and ± 2 SD on arithmetic paper, or by plotting arithmetic values on the logarithmic scale of semi-log paper. If it were definitely shown that the LC50s or ICps failed to fit a log-normal distribution, an arithmetic mean and SD might prove more suitable.

If a particular ICp or LC50 fell outside the warning limits, the sensitivity of the test organisms and the performance and precision of the test would be suspect. Since this might occur 5% of the time due to chance alone, an outlying ICp or LC50 would not necessarily indicate abnormal sensitivity of the test organisms or unsatisfactory precision of toxicity data. Rather, it would provide a warning that there might be a problem.

A thorough check of the health of the culture (Section 2.4.11) together with all culturing and test conditions should be carried out. Depending on the findings, it might be necessary to repeat the *reference toxicity test*, to obtain new breeding stock, and/or to establish new cultures, before undertaking further toxicity tests with *C. dubia*.

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

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[37] 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 (USEPA, 1989, 1994, 2002). 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.

limits but still represent undesirable variation in results obtained in tests. For guidance, in terms of reasonable variation associated with warning limits for a warning chart, see Section 2.8.1 and Appendix F in EC, 2005.

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, 2005). 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 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, 2005). 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 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 or substance being sampled; uncontaminated by foreign substances or materials; identifiable as to date, time and location of origin; clearly documented as to the chain of custody; and analyzed as soon as possible after collection. Persons responsible for conducting the test and reporting findings must maintain continuity of evidence for court proceedings (McCaffrey, 1979), and ensure the integrity of the test results.

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 test substance(s) 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[38]. 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 substance or substances 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* LC50 (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[39], or by adding measured volumes of a stock solution. If the latter is used, the concentration and stability of the test substance(s) in the stock solution should be determined before beginning the

[38] 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.

[39] 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.

test. Stock solutions subject to photolysis 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[40]. 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 must 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 (USEPA, 1985b; ASTM, 1989, 2006): 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[41, 42]. Examples of such situations would include appraisals of the toxic effect of

[40] 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.

[41] Contaminants already in the receiving water could add toxicity to that of the chemical 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 substance(s), 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 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.

If the intent of the test is to measure the extent to which a particular receiving water might modify the toxicity of the test material or substance due to its physicochemical characteristics (e.g., hardness, pH, turbidity, humic or fulvic acid content) and/or the presence of other contaminants, the investigator might choose to use the upstream water to prepare the test concentrations and as one of the control solutions. A comparison of results for this water with those for the controls held in laboratory water will identify toxic responses that might be contributed by the upstream water. A clearer understanding of the differing influence of each type of control/dilution water on the toxicity of the test material or substance can be achieved by undertaking a side-by-side comparison for toxic effects using each control/dilution water to prepare a series of test concentrations.

[42] 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.

chemical spills or intentional applications of chemical (e.g., spraying of a pesticide) on a water body. 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., 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 must be prepared using the culture water (see Section 2.4.4 and Section 4.1). The survival and reproduction rates for *C. dubia* held in ten replicate solutions of culture water must be compared to those for test organisms held in the ten replicate solutions of receiving water[43]. The sample of upstream water would normally be considered to be 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 their first three broods under test conditions (see Section 4.4) using this water. 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[44]. The use of moderately hard (80 to 100 mg/L) water is recommended for such purposes (see Section 4.1, including footnote 14). 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*

[43] 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.

[44] Since the hardness, pH, and other characteristics of the control/dilution water can markedly influence the toxicity of the test substance(s), the use of 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.

are exposed[45]. 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, 2006). Samples from the old (used) test 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 endpoints for tests performed with chemicals will usually be the LC50 at the end of the test, and the ICp for reproductive performance (see 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 during their first three broods (Section 4.4).

[45] Such analyses need not be undertaken in all instances, due to analytical limitations, cost, or previous technical data including chemical stability in solution under conditions similar to those in the test. Chemical analyses are particularly advisable if (USEPA, 1985b): the test substance(s) 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 measurements of chemical concentrations in test solutions.

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 or subsamples of effluent, elutriate, or leachate must be made of nontoxic material. Collapsible polyethylene or polypropylene containers manufactured for transporting drinking water (e.g., Reliance™ plastic containers) are recommended. Their volume can be reduced to fit into a cooler for transport, and the air space within kept to a minimum when portions are removed in the laboratory for the toxicity test or for chemical analyses. 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, leachate, or elutriate will be performed "off-site" in a controlled laboratory facility. Each off-site test must be conducted using one of the following two procedures and approaches:

1. A single sample may be used throughout the test. However, it must be divided into three separate containers (i.e., three subsamples) upon collection or (in the case of elutriate) preparation[46].

2. In instances where the toxicity of the wastewater is known or anticipated to change significantly if stored for up to 7–10 days before use, fresh samples must be collected (or, in the case of elutriate, prepared) on at least three separate occasions using sampling intervals of 2–3 days or less[47].

In those instances where the test 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 (USEPA, 1989, 1994, 2002).

A 2-L sample is adequate for an off-site multiple-concentration test and the associated routine sample analysis. Lesser amounts are required for single-concentration tests (Section 4.6). Upon collection, each sample container

[46] Using this approach, the first subsample must be used for test initiation (Day 0) plus renewals on Days 1 and 2, the second subsample for renewals on Days 3 and 4, and the third subsample for renewals on Days 5, 6, and 7.

[47] If three samples are collected at 2- to 3-day intervals (e.g., on Monday, Wednesday, and Friday), the first must be used for test initiation (Day 0) plus renewals on Days 1 and 2, the second for renewals on Days 3 and 4, and the third for renewals on Days 5, 6, and 7. Wastewaters known or anticipated to be particularly unstable could, if tested off-site, be sampled at daily intervals for seven consecutive days, and each sample used for only one day of the test in order of sampling.

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.

Testing of effluents and leachates should start as soon as possible after collection. Use of any sample in a test should begin within 1 day whenever possible, and must begin no later than 3 days after sampling. Samples of sediment, soil, or other solid material 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 must begin within 3 days of preparation or as specified in a regulation or protocol.

An effort must be made to keep samples of effluent or leachate cool (1 to 7 °C, preferably 4 ± 2 °C) throughout their period of transport. Upon collection, warm (>7 °C) samples must be cooled to 1 to 7 °C with regular ice (not dry ice) or frozen gel packs. As necessary, ample quantities of regular ice, gel packs, or other means of refrigeration must be included in the transport container in an attempt to maintain sample temperature within 1 to 7 °C during transit. Samples must not freeze during transport or storage.

Upon arrival at the laboratory, the temperature of the sample or, if collected, one of the subsamples (with the remaining subsamples left unopened and sealed), must be measured and recorded. 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 or subsamples required for subsequent solution renewals must be stored in darkness in sealed containers, without air headspace, 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

Each sample or subsample in a collection container must be agitated thoroughly just before pouring, to ensure the re-suspension of settleable solids. The dissolved oxygen content and pH of each sample or subsample must be measured just before its use. As necessary, each test solution should be pre-aerated (see Section 4.1) before aliquots are distributed to replicate test chambers.

Filtration of samples or subsamples is normally not required nor recommended. However, if they contain organisms which might be confused with the test organisms, attack them, or compete with them for food, the samples or subsamples must be filtered through a sieve with 60 µm mesh openings before use (USEPA, 1989, 1994, 2002). In instances where concern exists regarding the effect of this filtration on sample toxicity[48], a second (concurrent) test should be conducted using portions of the unfiltered sample.

[48] 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.

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[49, 50]. 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[49, 50]. An important example of such a situation would be testing for *sublethal* 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 must 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[51]. Situations where such use is appropriate include investigative studies intended to

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[49] Contaminants already in the receiving water could add toxicity to that of the 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 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.

If the intent of the test is to measure the extent to which a particular receiving water might modify the toxicity of the test material due to its physicochemical characteristics (e.g., hardness, pH, turbidity, humic or fulvic acid content) and/or the presence of other contaminants, the investigator might choose to use the upstream water to prepare the test concentrations and as one of the control solutions. A comparison of results for this water with those for the controls held in laboratory water will identify toxic responses that might be contributed by the upstream water. A clearer understanding of the differing influence of each type of control/dilution water on the toxicity of the test material can be achieved by undertaking a side-by-side comparison for toxic effects using each control/dilution water to prepare a series of test concentrations.

[50] 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.

[51] Since the hardness, pH, and other characteristics of the control/dilution water can markedly influence the toxicity of the test material, the use of 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.

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 (USEPA, 1989, 1994, 2002). 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, 2005) 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 samples/subsamples (or a specified dilution thereof), and ten replicate control solutions. Depending on the specified regulatory requirements, tests for compliance might be restricted to a single concentration (100% wastewater unless otherwise specified). Multi-concentration tests might also be required for regulatory compliance or monitoring purposes, in which instance the LC50 at the end of the test must be determined together with the ICp for the reproductive data

(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, interlaboratory investigations) could, depending on the study objectives, be single-concentration tests (100% or an appropriate dilution, plus a control), or multi-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/subsamples 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 percent mortality and number of live neonates (first three broods only) produced in the wastewater and control solutions at the end of the test (Section 4.6). Multi-concentration tests of effluent, leachate, or elutriate samples/subsamples should be performed and the appropriate endpoints (i.e., LC50 at test end, plus ICp for reproduction) calculated. A multi-concentration test should be performed 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.

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 or subsamples of receiving water should be as described in Section 6.1. Testing of samples/subsamples should commence as soon as possible after collection, preferably within 1 day and no later than 3 days after sampling.

7.2 Preparing Test Solutions

Samples in the collection container(s) should be agitated before pouring to ensure their homogeneity. Subsamples should be collected as described in Section 6.1.

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[52].

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[53, 54]. This control/dilution water

[52] 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.

[53] Contaminants already in the "upstream" water could add toxicity to that of the downstream sample 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 downstream sample 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.

If the intent of the test is to measure the extent to which a particular receiving water might modify the toxicity of the test material due to its physicochemical characteristics (e.g., hardness, pH, turbidity, humic or fulvic acid content) and/or the presence of other contaminants, the investigator might choose to use the upstream water to prepare the test concentrations and as one of the control solutions. A comparison of results for this water with those for the controls held in laboratory water will identify toxic responses that might be contributed by the upstream water. A clearer understanding of the differing influence of each type of control/dilution water on the toxicity of the test material can be achieved by undertaking a side-by-side comparison for toxic effects using each control/dilution water to prepare a series of test concentrations.

[54] 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.

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 must 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 (Section 2.4.4), control water, and for all dilutions (see footnote 54). 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 daphnid survival and reproduction, obtained for *C. dubia* exposed to full-strength receiving water (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 multi-concentration test to determine the LC50 at the end of the test as well as the ICp for reproduction should be conducted as outlined in Section 4. Any multi-concentration test should include the undiluted (100%) receiving water 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.

Reporting Requirements

Each test-specific report must indicate if there has been any deviation from any of the "must" requirements delineated in Sections 2 to 7 of this biological test method, and, if so, provide details as to the deviation. The reader must be able to establish from the test-specific report whether the conditions and procedures preceding and during the test rendered the results valid and acceptable for the use intended.

Section 8.1 provides a list of the items which must be included in each test-specific report. Section 8.2 gives a list of those items which must either be included in the test-specific report, provided separately in a general report, or held on file for a minimum of five years. Specific monitoring programs or related test protocols might require selected test-specific items listed in Section 8.2 to be included in the test-specific report, or might relegate certain test-specific information (e.g., details regarding the test substance or material and/or explicit procedures and conditions during sample collection, handling, transport, and storage) as "data to be held on file".

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 document, may be referred to by citation or by attachment of a general report which outlines standard laboratory practice.

Details pertinent to the conduct and findings of the test, which are not conveyed by the test-specific report or general report, must be kept on file by the laboratory for a minimum of five years, so that the appropriate information can be provided if an audit of the test is required. Filed information might include:

- a record of the chain-of-continuity for samples tested for regulatory or monitoring purposes;
- a copy of the record of acquisition for the sample(s);
- certain chemical analytical data on the sample(s);
- bench sheets for the observations and measurements recorded during the test;
- bench sheets and warning chart(s) for the reference toxicity tests;
- detailed records of the source and health of the breeding stock; and
- information on the calibration of equipment and instruments.

Original data sheets must be signed and dated by the laboratory personnel conducting the tests.

8.1 Minimum Requirements for Test-Specific Report

Following is a list of items that must be included in each test-specific report.

8.1.1 Test Substance or Material

- Brief subscription of sample type (e.g., chemical or chemical substance, effluent, elutriate, leachate, or receiving water), if and as provided to the laboratory personnel;

- information on labelling or coding for each sample or subsample;

- date of sample/subsample collection; date and time sample(s)/subsample(s) were received at test facility;

- dates or days during test when individual samples or subsamples used;

- for effluent or leachate, measurement of temperature of sample or, if multiple subsamples, one only of these subsamples, upon receipt at test facility;

- measurements of dissolved oxygen and pH of sample or subsample of wastewater or receiving water, just before its preparation and use in toxicity test; and

- date of elutriate generation and description of procedure for preparation; dates or days during an elutriate test when individual samples or subsamples are used.

8.1.2 Test Organisms

- species and source of brood organisms;

- confirmation that the parentage of all organisms that were taken from a series of individual cultures to initiate the test, originated from the same mass culture (see Section 2.4.1);

- range of age at start of test:

- any unusual appearance, behaviour, or treatment of test organisms, before their use in the test; and

- data showing health of individual brood cultures, including: mean % mortality of brood organisms during 7-day period preceding test; number of young produced by each brood organism in its third or subsequent brood; mean number of surviving young produced within the first three broods of each adult during the 7-day period preceding the test; and any observations of ephippia (see Section 2.4.11)

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8.1.3 Test Facilities and Apparatus

- name address of test laboratory;

- name of person(s) performing the test; and

- brief description of test vessels (size, shape, type of material).

8.1.4 Control/Dilution Water

- type(s) and source(s) of water used as control and dilution water; and

- type and quantity of any chemical(s) added to control or dilution water.

8.1.5 Test Method

- citation of biological test method used (i.e., as per this document);

- brief description of procedure(s) in those instances in which a sample, subsample, or test solution has been filtered or adjusted for hardness or pH;

- design and description if specialized procedure (e.g., renewal of test solutions at intervals other than daily; preparation and use of elutriate; preparation and use of solvent and, if so, solvent control);

- brief description of frequency and type of all observations and all measurements made during test; and

- name and citation of program(s) and methods used for calculating statistical endpoints.

8.1.6 Test Conditions and Procedures

- design and description of any deviation from or exclusion of any of the procedures and conditions specified in this document;
- number, concentration, volume, and depth of solutions in test vessels, including controls;
- number of individuals per test vessel, and number of replicates per treatment;
- brief statement (including procedure, rate, and duration) of any pre-aeration of test solutions;
- brief statement concerning aeration (if any, give rate, duration) of test solutions during exposure of test organisms;
- confirmation that all neonates produced in each test solution (including the control solutions) by a brood organism during its fourth or subsequent broods were discarded without including them in counts of number of young produced per replicate or treatment;
- dates when test was started and ended;
- all required (see Section 4.5) measurements of temperature, pH, and dissolved oxygen (mg/L and percent saturation) in test solutions (including controls), made during the test; and
- brief statement indicating whether the reference toxicity test was performed under the same experimental conditions as those used with the test sample(s); and description of any deviation(s) from or exclusion(s) of any of the procedures and conditions specified for the reference toxicity test in this document.

8.1.7 Test Results

- for each replicate test solution (including each of the control replicates): the presence or absence of mortality of the first-generation adult in that test solution at each period of observation including at the end of the test;
- for each treatment, including the control treatment(s): the (cumulative) mean % mortality for the first-generation daphnids at the end of the test;
- for each replicate test solution (including each of the control replicates): number of live neonates produced by each first-generation daphnid during its first three broods only, as noted during each observation period and at test end;
- for each treatment, including the control treatment(s): the (cumulative) mean number (including its SD) of live neonates produced by each first-generation daphnid during its first three broods only;
- LC50 and 95% confidence limits and indication of quantal method used; ICp and 95% confidence limits for the reproduction data; details regarding any weighting techniques applied to the data; and indication of quantitative method used;
- any outliers and the justification for their removal
- the results and duration of any toxicity tests with the reference toxicant(s) performed within 14 days of the start 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; and

- anything unusual about the test, any problems encountered, any remedial measures taken.

8.2 Additional Reporting Requirements

Following is a list of items that must be either included in the test-specific report or the general report, or held on file for a minimum of five years.

8.2.1 Test Substance or Material

- identification of person(s) who collected and/or provided the sample or subsamples;
- records of sample/subsample chain-of-continuity and log-entry sheets; and
- conditions (e.g., temperature, in darkness, in sealed container) of samples/subsamples upon receipt and during storage.

8.2.2 Test Organisms

- description of culture conditions and procedures, including: temperature and lighting conditions; water source and quality; water *pre-treatment*; breeding method including frequency of water exchange and procedure for replacement; and age of culture; and
- type, source, and method of preparation of food for cultures and test; records of nutritive value and known contaminants in foods; procedures used to store food; feeding procedures, frequency, and ration.

8.2.3 Test Facilities and Apparatus

- description of systems for regulating light and temperature within the facility;
- description of procedures used to clean or rinse test apparatus; and
- for any test involving aeration of test solutions, description of apparatus and rate.

8.2.4 Control/Dilution Water

- sampling and storage details if the control/dilution water was "upstream" receiving water;
- details regarding any water pre-treatment (e.g., filtration; sterilization; chlorination/dechlorination; adjustment for pH, temperature, and/or hardness; de-gassing; aeration); and
- any ancillary water-quality variables (e.g., dissolved metals, ammonia, pesticides, suspended solids, residual chlorine, humic and fulvic acids) measured before and/or during the toxicity test.

8.2.5 Test Method

- description of laboratory's previous experience with this biological test method for measuring toxicity using *C. dubia*;
- procedure used in preparing and storing stock and/or test solutions of chemicals; description and concentration(s) of any solvent used;
- methods used (with citations) for chemical analyses of sample or test solutions; details concerning sampling, sample/solution preparation and storage, before chemical analyses; and

- use and description of preliminary or range-finding test.

8.2.6 Test Conditions and Procedures

- photoperiod, light source, and intensity adjacent to the surface of test solutions;

- conditions, procedures, and frequency for toxicity tests with reference toxicant(s) and neonate daphnids;

- water quality measurements for culture/control/dilution water (see Section 2.4.4);

- total hardness of control/dilution water and at least the highest test concentration at the start of the test; conductivity of each newly prepared test solution;

- any other chemical measurements on sample, stock solutions, or test solutions (e.g., chemical concentration, suspended solids content, conductivity, hardness, alkalinity), before and/or during test; and

- appearance of sample or test solutions; changes in appearance noted during test.

8.2.7 Test Results

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

- results for any statistical analyses conducted both with outliers and with outliers removed; for regression analyses, hold on file information indicating sample size (e.g, number of replicates per treatment), parameter estimates with variance or standard error, any ANOVA table(s) generated, plots of fitted and observed values of any models used, results of outlier tests, and results of tests for normality and homoscedasticity;

- warning chart showing the most recent and historic results for toxicity tests with the reference toxicant(s);

- graphical presentation of dose-response data; and

- original bench sheets and other data sheets, signed and dated by the laboratory personnel performing the test and related analyses.

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Appendix A

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[a] Previously named the "Inter-Governmental Aquatic Toxicity Group" (IGATG).

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Appendix B

Environment Canada Regional and Headquarters' Office Addresses

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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
USEPA 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]
USEPA 1989	effl., RW	St-R	3 broods[g, h]

[a] Full names of agencies are given in reference list.

[b] effl. = effluent; chem. = chemical(s); leach. = leachate; RW = receiving water; sed. = sediment

[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	Life Stage
USEPA 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]
USEPA 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[a]	Temperature (°C)	Hardness (mg/L)	Aeration
USEPA 1985a	Rc (NW, DW)	25 ± 2[b]	80 to 100	if needed, DO ≥5
Anon. 1986	"as in USEPA 1985a"			
Battelle 1986	NW	25 ± 2	100	none
Battelle 1987	Rc	25 ± 2	NI[c]	NI
Battelle 1988	Rc[d]	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 USEPA 1989"			
USEPA 1989	Rc	25 ± 1	80 to 100	NI

[a] Rc = Reconstituted water NW = Natural surface or groundwater, uncontaminated source.
DW = Dechlorinated municipal water

[b] Range

[c] NI = not indicated.

[d] With added bacterial inoculum.

4. Lighting Conditions During Culturing

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

[a] L = light, D = dark

[b] Fluorescent with colour rendering index ≥90

[c] Desirable to minimize stress due to abrubt change.

5. Feeding Conditions During Culture and Testing

Document	Food	Feeding of Culture	Feeding During Test (days after start)
USEPA 1985a	YCT[a]	daily	daily
Anon. 1986	"as in USEPA 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,d]	regularly	daily desirable
NWRI 1988	alga[e] + yeast	daily	0, 2, 4
Anon. 1989	brine shrimp[f]	NI	twice daily
USEPA 1989	alga[b] + YCT	daily	daily

[a] YCT = Yeast + Cerophyll™ (dried plant material) + trout chow.

[b] *Pseudokirchneriella subcapitata*.

[c] As suitably documented, mixtures of various algae/Y/T/C.

[d] NI = not indicated.

[e] *Scenedesmus* sp.

[f] Newly hatched nauplii.

6. Test Conditions

Document	Container	Test Volume (mL)	No. of Daphnids /vessel	No. of replicates
USEPA 1985a	30-mL BSGB[a] or plastic cup	15	1	10
Anon. 1986	"as in USEPA 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
USEPA 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	Renewal Times (days)
USEPA 1985a	Rc, NW, RW (DW[b])	80 to 100[c]	NI[d]	aerate if low	daily
Anon. 1986	"as in USEPA 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[b])	NI[e]	NI	90 to 100%	daily
NWRI 1988	DW	NI	NI	NI	2, 4
Anon. 1989	NW, Rc, ≤20%DMW[f]	NI	NI	aerate ≥24h	daily
USEPA 1989	Rc[g], NW, RW, DW	NI[g]	NI	aerate if low	daily

[a] Rc = Reconstitutued water
NW = surface or groundwater from uncontaminated source
RW = receiving water
DW = dechlorinated municipal water
DMW = diluted mineral water.

[b] "To be used as a last resort".

[c] If reconstituted water. Similar to the receiving water if used for dilution.

[d] NI = not indicated.

[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
USEPA 1985a	25 ± 1[a]	none[b]	NI[c] ("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% [d]	NI
NWRI 1988	25 ± 1	none	NI	NI
Anon. 1989	25 ± 1	none	NI	NI
USEPA 1989	25 ± 1	none	NI	NI

[a] Range
[b] Aerate sample before testing, if required
[c] NI = not indicated.
[d] 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
USEPA 1985a	16L / 8D	ambient (10 to 20 $\mu E/m^2 \cdot s$)	NI[a]	NI
Anon. 1986	16L / 8D	"as in USEPA 1985a"·····		
Battelle 1986	16L / 8D	540 to 1607 lux	Fl[b]	NI
Battelle 1987	16L / 8D	ambient (323 to 1076 lux)	Fl	NI
Battelle 1988	16L / 8D	540 to 1607 lux	Fl[b]	NI
ASTM 1988	16L / 8D	NI	NI	15 to 30 min[c]
NWRI 1988	NI	NI	NI	NI
Anon. 1989	16L / 8D	"as in USEPA 1989"·····		
USEPA 1989	16L / 8D	ambient (10 to 20 $\mu E/m^2 \cdot s$)	NI	NI

[a] NI = not indicated.
[b] Fluorescent with colour rendering index ≥90.
[c] Desirable to minimize stress due to abrupt change.

10. Monitoring Water Quality During Tests

Document	Variable[a]	At Exposure Time (days)
USEPA 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 USEPA 1989".....	
USEPA 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 concentrations, at least at start, middle and end of test. Alkalinity and pH at least once in new and used testwater of high concentrations.

[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)
USEPA 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
USEPA 1989	mortality, no. of live young	daily

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

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

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

Document	Endpoint(s)	Criterion
USEPA 1985a	NOEC, LOEC	sig. diff. from control in mortality, no. live young [a]
Anon. 1986	pass/fail, mortality	≥20% in any concentration
	pass/fail, no. of young	sig. diff. from control
	NOEC for reduced young	NI[b]
Battelle 1986	NI	mortality, no. of 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 for reduced young	no. in conc'n/no. in control
Anon. 1989	"as in USEPA 1989"	·····
USEPA 1989	NOEC, LOEC	sig. diff. from control in mortality, no. live young[a]
	LC5, LC10, LC50	mortality, female adults[d]
	IC25, IC50	mortaltiy, no. live young[d]

[a] 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.
[b] NI = not indicated.
[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
USEPA 1985a	NI[a]	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
USEPA 1989	20%	≥15/surviving female

[a] NI = not indicated.

Procedure for Preparing YCT and Algal Food for *C. dubia* [a]

Preparing Digested Trout Chow [b]

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[c] 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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[a] From USEPA (1989).

[b] Researchers at USEPA's Environmental Monitoring Systems Laboratory in Cincinnati, Ohio recommended 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).

[c] Available as "Cereal grass media" -Cerophyll™ from Ward's Natural Science Ltd. (1-800-387-7822).

3. Mix in a blender at high speed for 5 minutes 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.

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 (*P. subcapitata*) Food

A. Algal culture medium

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

2. Add 1 mL of each stock solution, in the order listed in Table D.1, to approximately 900 mL of deionized water. Mix well after each solution is added. Dilute to l L, mix well, and adjust the pH to 7.5 ± 0.1, using 0.1 *N* NaOH or HC1, as appropriate. The final concentration of macronutrients and micronutrients in the culture medium
is given in Table D.2.

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.

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 36.3 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 1000 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.

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.

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_2HPO_4	1.04	P	0.186
$NaHCO_3$	15.0	Na	11.0
		K	0.469
		C	2.14

Macronutrient	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	—	—

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 micro-organisms.

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 Section B), containing 1.5×10^6 cells/mL, are added to each litre of fresh algal culture medium (i.e., 3L of medium in a 4-L bottle, or 18 L in a 20-L bottle). The 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].

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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[d] 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[a]

Column (Number of concentrations between 100 and 10, or between 10 and 1)[b]						
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

[a] Modified from Rocchini *et al*. (1982).

[b] A series of seven (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 divided by any power of 10. Column 1 might be used if there was considerable uncertainty about the degree of toxicity. More widely spaced concentrations 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.

Analysis of Mortality to Estimate the Median Lethal Concentration

Data permitting, the LC50 (and its 95% confidence limits) for the first-generation daphnids must be calculated based on the mean percent mortality in each test concentration at test end (Section 4.6). The investigator might also wish to estimate the LC50 for earlier periods of exposure (e.g., 24-h LC50, 48-h LC50, 96-h LC50). General procedures for estimating an LC50 are summarized briefly here; the reader should consult Section 4 in EC (2005) for detailed guidance.

To estimate an LC50, data are combined for all replicates at each concentration. If mortality is not ≥ 50% in at least one concentration, the LC50 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 LC50, 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 LC50.

Various computer programs for calculating LC50 and confidence limits are suitable for use. Stephan (1977) developed a program for estimating the LC50 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 (USEPA, 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, 2005; USEPA, 1989, 1994, 2002) may be used. Programs using the Trimmed Spearman-Kärber method (Hamilton *et al*., 1977) are available for personal computers but should be applied cautiously (EC, 2005) 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 LC50 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 LC50 with conservative (wide) confidence limits.

Any computer-derived LC50 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, 2005, see example in Figure F.1). Any major disparity between the estimated LC50 derived form this plot and the computer-derived LC50 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 LC50 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 LC50s (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-Kärber method: (Hamilton *et al*., 1977)	0% trim:	5.64 (4.38 to 7.26)
	10% trim:	5.73 (4.34 to 7.58)
	20% trim:	5.95 (4.34 to 9.80)

The bionomial 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"(*LT50*) 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 percent 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 LT50s could be estimated from successive records of mortality at 24-h intervals. Observed mortality must be greater than 50% in order to estimate at LT50.

Neither an LT50 nor the percent 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 substance or 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 LT50s instead of an LC50 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).

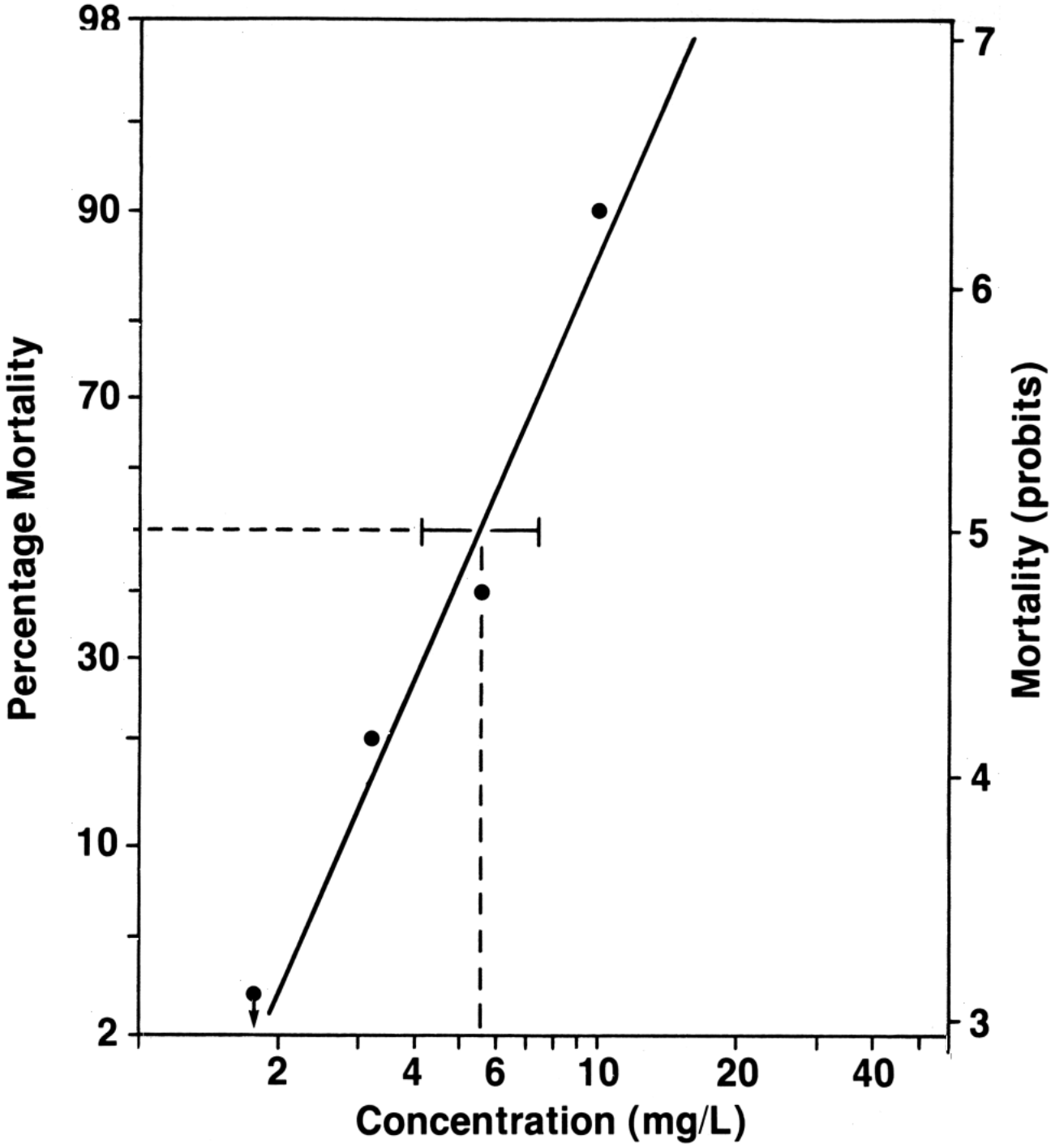

Figure F.1 **Estimating a Median Lethal Concentration by Plotting Mortalities on Logarithmic-Probability Paper**

Appendix G

Biological Test Methods and Supporting Guidance Documents Published by Environment Canada's Method Development & Applications Section [a]

Title of Biological Test Method or Guidance Document	Report Number	Publication Date	Applicable Amendments
A. Generic (Universal) Biological Test Methods			
Acute Lethality Test Using Rainbow Trout	EPS 1/RM/9	July 1990	May 1996
Acute Lethality Test Using Threespine Stickleback (*Gasterosteus aculeatus)*	EPS 1/RM/10	July 1990	March 2000
Acute Lethality Test Using *Daphnia* spp.	EPS 1/RM/11	July 1990	May 1996
Test of Reproduction and Survival Using the Cladoceran *Ceriodaphnia dubia*	EPS 1/RM/21 2nd Edition	February 2007	—
Test of Larval Growth and Survival Using Fathead Minnows	EPS 1/RM/22	February 1992	November 1997
Toxicity Test Using Luminescent Bacteria (*Photobacterium phosphoreum*)	EPS 1/RM/24	November 1992	—
Growth Inhibition Test Using a Freshwater Alga	EPS 1/RM/25 2nd Edition	March 2007	—
Acute Test for Sediment Toxicity Using Marine or Estuarine Amphipods	EPS 1/RM/26	December 1992	October 1998
Fertilization Assay Using Echinoids (Sea Urchins and Sand Dollars)	EPS 1/RM/27	December 1992	November 1997
Toxicity Tests Using Early Life Stages of Salmonid Fish (Rainbow Trout, Coho Salmon, or Atlantic Salmon)	EPS 1/RM/28 1st Edition	December 1992	January 1995
Toxicity Tests Using Early Life Stages of Salmonid Fish (Rainbow Trout)	EPS 1/RM/28 2nd Edition	July 1998	—
Test for Survival and Growth in Sediment Using the Larvae of Freshwater Midges (*Chironomus tentans* or *Chironomus riparius*)	EPS 1/RM/32	December 1997	—

[a] These documents are available for purchase from Communications Services, Environment Canada, Ottawa, Ontario, K1A 0H3, Canada. Printed copies can also be requested by e-mail at: epspubs@ec.gc.ca. These documents are freely available in PDF at the following website: http://www.etc-cte.ec.gc.ca/organization/bmd/bmd_publist_e.html.
For further information or comments, contact the Chief, Biological Methods Division, Environmental Science and Technology Centre, Environment Canada, Ottawa, Ontario K1A 0H3.

Title of Biological Test Method or Guidance Document	Report Number	Publication Date	Applicable Amendments
A. Generic (Universal) Biological Test Methods (cont'd.)			
Test for Survival and Growth in Sediment Using the Freshwater Amphipod *Hyalella azteca*	EPS 1/RM/33	December 1997	—
Test for Measuring the Inhibition of Growth Using the Freshwater Macrophyte, *Lemna minor*	EPS 1/RM/37 2nd Edition	January 2007	—
Test for Survival and Growth in Sediment Using Spionid Polychaete Worms (*Polydora cornuta*)	EPS 1/RM/41	December 2001	—
Tests for Toxicity of Contaminated Soil to Earthworms (*Eisenia andrei, Eisenia fetida*, or *Lumbricus terrestris*)	EPS 1/RM/43	June 2004	—
Tests for Measuring Emergence and Growth of Terrestrial Plants Exposed to Contaminants in Soil	EPS 1/RM/45	February 2005	—
Test for Measuring Survival and Reproduction of Springtails Exposed to Contaminants in Soil	EPS 1/RM/47	December 2006	—
B. Reference Methods[b]			
Reference Method for Determining Acute Lethality of Effluents to Rainbow Trout	EPS 1/RM/13 1st Edition	July 1990	May 1996, December 2000
Reference Method for Determining Acute Lethality of Effluents to Rainbow Trout	EPS 1/RM/13 2nd Edition	December 2000	—
Reference Method for Determining Acute Lethality of Effluents to *Daphnia magna*	EPS 1/RM/14 1st Edition	July 1990	May 1996, December 2000
Reference Method for Determining Acute Lethality of Effluents to *Daphnia magna*	EPS 1/RM/14 2nd Edition	December 2000	—
Reference Method for Determining Acute Lethality of Sediment to Marine or Estuarine Amphipods	EPS 1/RM/35	December 1998	—
Reference Method for Determining the Toxicity of Sediment Using Luminescent Bacteria in a Solid-Phase Test	EPS 1/RM/42	April 2002	—

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

Title of Biological Test Method or Guidance Document	Report Number	Publication Date	Applicable Amendments
C. Supporting Guidance Documents			
Guidance Document on Control of Toxicity Test Precision Using Reference Toxicants	EPS 1/RM/12	August 1990	—
Guidance Document on Collection and Preparation of Sediment for Physicochemical Characterization and Biological Testing	EPS 1/RM/29	December 1994	—
Guidance Document on Measurement of Toxicity Test Precision Using Control Sediments Spiked with a Reference Toxicant	EPS 1/RM/30	September 1995	—
Guidance Document on Application and Interpretation of Single-Species Tests in Environmental Toxicology	EPS 1/RM/34	December 1999	—
Guidance Document for Testing the Pathogenicity and Toxicity of New Microbial Substances to Aquatic and Terrestrial Organisms	EPS 1/RM/44	March 2004	—
Guidance Document on Statistical Methods for Environmental Toxicity Tests	EPS 1/RM/46	March 2005	—