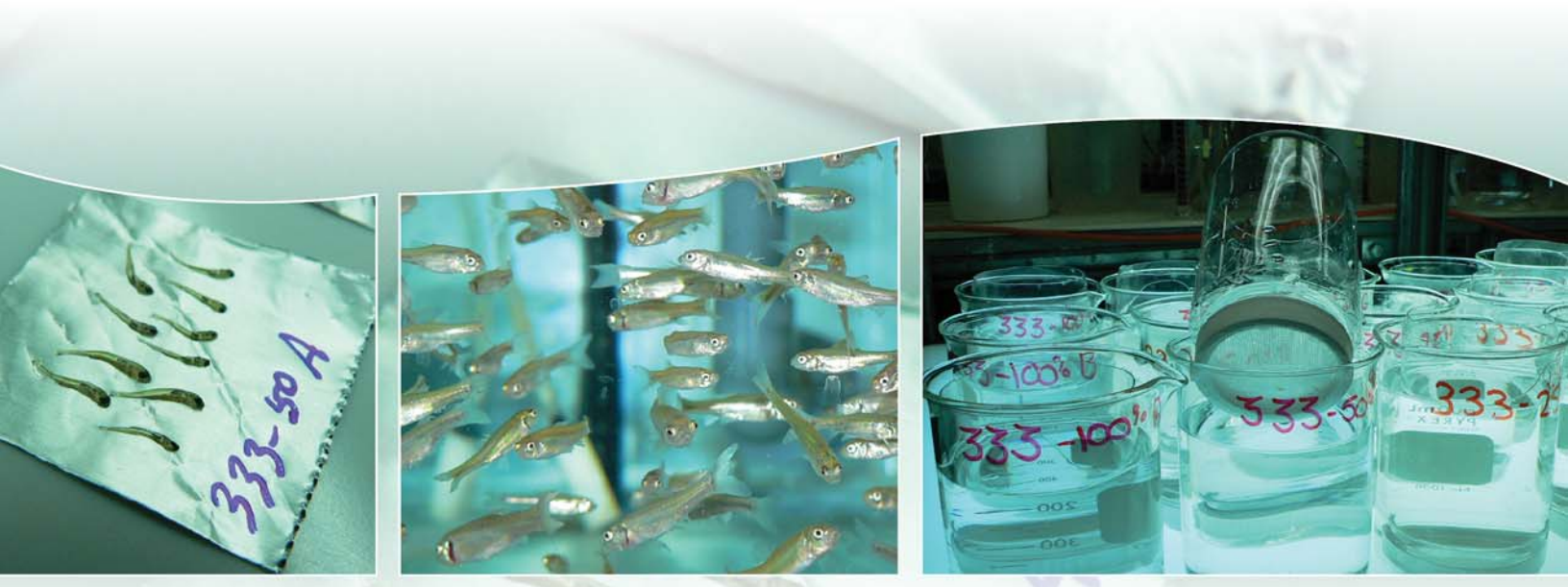
Environment Canada Environnement Canada

Biological Test Method: Test of Larval Growth and Survival Using Fathead Minnows

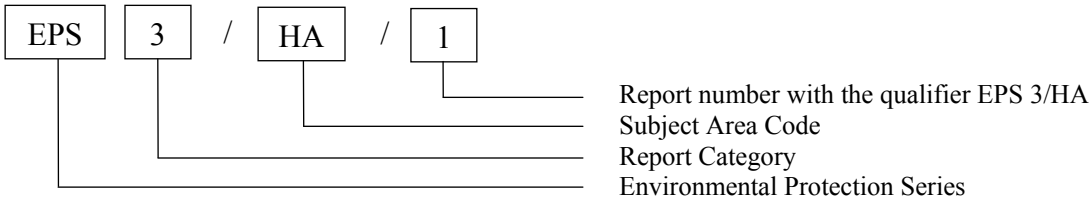
EPS 1/RM/22 Second Edition – February 2011
Science and Technology Branch
Environment Canada



Canada

Environmental Protection Series

Sample Number:



Categories

1 Regulations/Guidelines/Codes of Practice
2 Problem Assessments and Control Options
3 Research and Technology Development
4 Literature Reviews
5 Surveys
6 Social, Economic and Environmental Impact Assessments
7 Surveillance
8 Policy Proposals and Statements
9 Manuals

Subject Areas

AG Agriculture
AN Anaerobic Technology
AP Airborne Pollutants
AT Aquatic Toxicity
CC Commercial Chemicals
CE Consumers and the Environment
CI Chemical Industries
FA Federal Activities
FP Food Processing
HA Hazardous Wastes
IC Inorganic Chemicals
MA Marine Pollutants
MM Mining and Ore Processing
NR Northern and Rural Regions
PF Paper and Fibres
PG Power Generation
PN Petroleum and Natural Gas
RA Refrigeration and Air Conditioning
RM Reference Methods
SF Surface Finishing
SP Oil and Chemical Spills
SRM Standard Reference Methods
TS Transportation Systems
TX Textiles
UP Urban Pollution
WP Wood Protection/Preservation

New subject areas and codes are introduced as they become necessary. A list of EPS reports may be obtained from Communications Services, Environment Canada, Ottawa, Ontario, K1A 0H3, Canada.

Biological Test Method:
Test of Larval Growth and Survival Using Fathead Minnows

Method Development and Applications Unit
Science and Technology Branch
Environment Canada
Ottawa, Ontario

Report EPS 1/RM/22
2nd Edition
February 2011

Print version
Cat. No.: En49-7/1-22E
ISBN 978-1-100-17595-9

PDF version
Cat. No.: En49-7/1-22E-PDF
ISBN 978-1-100-17596-6

Aussi disponible en français

Readers' Comments

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

Richard Scroggins, Chief
Biological Assessment and Standardization Section
Environment Canada
335 River Road
Ottawa, ON
K1A OH3

Lisa Taylor, Manager
Method Development & Applications Unit
Environment Canada
335 River Road
Ottawa, ON
K1A 0H3

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

Services des communications
Environnement Canada
Ottawa (Ontario) K1A 0H3

Review Notice

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

Abstract

A revised method now recommended by Environment Canada for performing toxicity tests of seven days' duration, that measures growth and survival of very young (larval) fathead minnows (Pimephales promelas), *is described in this report. This revised version of Report EPS 1/RM/22 includes numerous updates such as the use of regression analyses for quantitative endpoint data, as well as the "biomass" endpoint as a combined measure of effects on survival and growth that is currently applied by USEPA (2002) in its 7-day test for toxic effects on the survival and growth of larval fathead minnows. When published by Environment Canada's Method Development and Applications Unit (Ottawa, ON), this revised method will supersede Environment Canada's 7-day test for larval growth and survival of fathead minnows, that was published as Report EPS 1/RM/22 in February 1992 and amended thereafter on two occasions (i.e., in November 1997 and September 2008).*

Procedures are given for culturing fathead minnows in the laboratory, obtaining eggs, and hatching the young for use in the tests. General or universal conditions and procedures are outlined for testing a variety of materials or substances for their effects on larval growth and mortality. Additional specific conditions and procedures are stipulated for testing samples of chemicals, effluents, elutriates, leachates, or receiving waters. Instructions and requirements are included on test facilities, handling and storing samples, preparing test solutions and initiating tests, specific test conditions, appropriate observations and measurements, endpoints and methods of calculation, and the use of reference toxicants.

Résumé

*Le présent document expose une méthode révisée, maintenant recommandée par Environnement Canada, pour l'exécution d'essais de toxicité d'une durée de sept jours qui mesurent les effets sur la croissance et la survie de têtes-de-boule (*Pimephales promelas*) au stade larvaire. Il s'agit d'une version révisée du rapport SPE 1/RM/22 qui comprend plusieurs éléments nouveaux, comme l'utilisation d'analyses de régression pour les résultats quantitatifs, ainsi que l'emploi du paramètre « biomasse » pour obtenir une mesure combinée des effets sur la survie et la croissance, comme le fait l'EPA des États-Unis (2002) dans son essai de détermination des effets toxiques sur la survie et la croissance des larves de tête-de-boule d'une durée de sept jours. Après sa publication par l'Unité de l'élaboration et de l'application des méthodes d'Environnement Canada [Ottawa (Ontario)], cette méthode révisée remplacera la méthode décrite dans le rapport SPE 1/RM/22 d'Environnement Canada qui a été publié en février 1992 et modifié à deux reprises (soit en novembre 1997 et septembre 2008).*

Ce document présente des modes opératoires pour l'élevage de têtes-de-boule en laboratoire, l'obtention d'œufs et l'éclosion de larves devant servir aux essais. Il présente les conditions et modes opératoires généraux ou universels permettant de réaliser des essais sur un large éventail de matières ou de substances pour déterminer leur effet sur la croissance et sur la mortalité des larves. On y précise aussi d'autres conditions et modes opératoires propres à l'évaluation d'échantillons de produits chimiques, d'effluents, d'élutriats, de lixiviats ou de milieux récepteurs. Le lecteur y trouvera des instructions et des exigences concernant les installations d'essai, la manipulation et le stockage des échantillons, la préparation des solutions d'essai et la mise en route des essais, les conditions prescrites pour les essais, les observations et mesures appropriées, les résultats des essais, les méthodes de calcul et l'utilisation de produits toxiques de référence.

Foreword

*This is one of a series of **recommended methods** for measuring and assessing the 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 program or standard reference method.*

The different types of tests included in this series were selected because of their acceptability for the needs of environmental protection and management programs 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 F 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.

Table of Contents

List of Tables

List of Figures

List of Abbreviations and Chemical Formulae

ANOVA	analysis of variance
°C	degree(s) Celsius
$CaCO_3$	calcium carbonate
cm	centimetre(s)
CV	coefficient of variation
d	day(s)
DO	dissolved oxygen (concentration)
g	gram(s)
g/kg	grams per kilogram
h	hour(s)
HCl	hydrochloric acid
H_2O	water
ICp	inhibiting concentration for a (specific) percent effect
L	litre(s)
LC	lethal concentration
LC50	median lethal concentration
LOEC	lowest-observed-effect concentration
LT50	time to 50% mortality (lethality)
m	metre(s)
mg	milligram(s)
min	minute(s)
ml	millilitre(s)
mm	millimetre(s)
mS	millisiemen(s)
N	Normal
NaOH	sodium hydroxide
nm	nanometre(s)
NOEC	no-observed-effect concentration
P	probability
SD	standard deviation
SI	Système international d'unités
sp	species
$TM^{(TM)}$	Trade Mark
µg	microgram(s)
µm	micrometre(s)
µmhos	micromhos
µmol	micromole(s)
>	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.

General 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.

Batch means a single group of embryos or young (≤24-h post-hatch) larvae taken from a *culture* at a discrete time, in order to provide all of the test organisms intended for use in a discrete toxicity test (including any associated reference toxicity test). The embryos or larvae in a batch are normally derived from three or more spawnings (i.e., different parentage).

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/centimetre (1 mS/m = 10 μmhos/cm).

Culture, as a noun, means the stock of animals or plants that is raised under defined and controlled conditions 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.

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

Embryo means the undeveloped young fish, before it hatches from the egg. In literature on fathead minnows, the term 'embryo' is usually used instead of 'egg'.

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

Growth means increase in size or weight as the result of proliferation of new tissues. In this test it is limited to increase in dry weight.

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.

Larva (plural, larvae) means a recently hatched fish which has physical characteristics other than those seen in the adult fish. The larval period begins with hatching of the *embryo* and lasts until the disappearance of the last vestige of the median fin fold and the appearance of a full complement of fin rays and spines. Fathead minnows are considered larvae for the first few days after hatching.

Lux is a unit of illumination based on units per square metre. One lux = 0.929 foot-candles and one foot-candle = 10.76 lux. For conversion of lux to quantal flux [μmol/(m^2 · s)], the spectral quality of the light source must be known. Light conditions or irradiance are properly described in terms of quantal flux (photon fluence rate) in the photosynthetically effective wavelength range of approximately 400 to 700 nm. The relationship between quantal flux and lux or foot-candles is highly variable and depends on the light source, the light meter used, the geometrical arrangement, and the possibilities of reflections (see ASTM, 1999). An approximate conversion between quantal flux and lux, for full-spectrum fluorescent light (e.g., Vita-Lite® by Duro-Test®), is as follows: one lux is approximately equal to 0.016 μmol/(m^2 · s) (Deitzer, 1994; Sager and McFarlane, 1997).

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.

Nauplius (plural, nauplii) is the earliest larval stage characteristic of many marine crustaceans and some other inverterbrates. It is microscopic, free-swimming, has only three pairs of appendages, and one median eye in the front of the head.

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, 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 equilvalents 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 a solid (i.e., precipitate) from some or all of the dissolved components of a solution.

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

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 1kg 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 chemical substance that might enter the aquatic environment through spillage, application, or discharge. Examples of chemicals that 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 measurable 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 means the water used for diluting the test material, or for the control test, or both.

Dechlorinated water means 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 fish 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 obtained by the laboratory for that chemical.

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 that includes effluents, leachates, and elutriates.

Statistical and Toxicological Terms

Acute means within a short period of exposure in relation to the life span of the organism, usually ≤4 days for fish. An acute toxic effect would be induced and observable within the short period.

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 96 h for fish.

Biomass means, in this test method, the total (dry) weight of living fish in a *replicate* or *treatment* at the end of the test, divided by the number of larvae that started in the replicate or treatment. The biomass *endpoint* represents a combination of sublethal effect and mortality.

Chronic means occurring during a relatively long period of exposure, usually a significant portion of the life span of the organism such as 10% or more.

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

Coefficient of Variation (CV) is the standard deviation (SD) of a set of data divided by the mean of the data set, expressed as a percentage. It is calculated according to the following formula: CV (%) = 100 (SD ÷ mean).

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 *biomass* of live organisms).

Flow-through describes tests in which solutions in test vessels are renewed continuously by the constant inflow of a fresh solution, or by a frequent intermittent inflow.

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 quantitiative biological function such as growth of fish. For example, an IC25 could be the concentration estimated to cause a 25% reduction in growth (including that measured as *biomass)* of larval fish, relative to the control. This term should be used for any toxicological test which measures a change in rate, such as reproduction, growth (including that measured and expressed

as biomass), 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 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., 7-d LC50).

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

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, effects which are detected by the observer and are statistically signicant. For example, the LOEC might be the lowest concentration at which growth of fish differed significantly from that in the control.

LT50 is the time (period of exposure) estimated to cause 50% mortality in a group of fish 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 E).

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 growth did not differ significantly from growth 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.

Renewed static 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 "batch replacement", "static renewal", "renewal", "static replacement" and "semi-static".

Replicate (*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* in a treatment must be an independent test unit; therefore, any transfer of organisms or test substance or 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.

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 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 either (a) the proportions of organisms affected (*quantal*), and/or (b) the degree of effect shown (*graded or quantitative* or graded), after exposure to specific concentrations of chemical, effluent, elutriate, leachate, or receiving water.

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

In February 1992, Environment Canada published Report EPS 1/RM/22 "Biological Test Method: Test of Larval Growth and Survival Using Fathead Minnows". This report (which was amended in November 1997 and again in September 2008 in keeping with the current revision of it) was co- authored by J.B. Sprague (Sprague Associates Ltd., Guelph, Ontario) and D.J. McLeay (McLeay Associates Ltd., West Vancouver, BC). It was based on pre-existing reports describing a 7-day test for measuring the survival and growth of larval fathead minnows, that was applied in the USA (Norberg and Mount, 1985; Denny, 1987; USEPA, 1989) and in Ontario by C.M. Neville (1989) of the Ontario Ministry of the Environment. G.A. Sergy and R.P. Scroggins (Environmental Protection, C & P, Environment Canada) acted as Scientific Authorities and provided technical input and guidance throughout the preparation of Report EPS 1/RM/22. The 1991 members of the Inter-Governmental Aquatic Toxicity Group (now referred to as the Inter-Governmental Ecotoxicological Testing Group; see Appendix A for the 2009 membership list) participated actively in the development and review of the first edition of this document. The laboratory testing support of the Ontario Ministry of the Environment at Rexdale, Ont., and of Environment Canada (Appendix B) also contributed to its preparation. Richard Chong-Kit of the Ontario Ministry of the Environment is thanked for providing the cover photos.

The current report represents a revised and updated version of Report EPS 1/RM/22 (including its November 1997 and March 2008 amendments). It was prepared by D. McLeay (McLeay Environmental Ltd., Victoria, B.C.), with assistance and guidance from L. Taylor (Manager, Method Development and Applications Unit) and R. Scroggins (Chief, Biological Methods Section) of Environment Canada, Ottawa, Ontario.

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 aquatic life. Recognizing two decades ago that no single test method or test organism can be expected to satisfy a comprehensive approach to environmental conservation and protection, the Inter-Governmental Ecotoxicological Testing Group (see Appendix A) 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). The test based on *growth* and mortality of *larval* fathead minnows was on several "core" aquatic toxicity tests which was then selected to be standardized sufficiently to help meet Environment Canada's testing requirements.

In February 1992, Environment Canada published a 7-day test for measuring the toxic effects of environmental contaminants on the growth and survival of larval fathead minnows (EC, 1992). This biological test method (Report EPS 1/RM/22) was amended in November 1997 and September 2008. The current report represents a revised and updated version of Report EPS 1/RM/22 (including, with appropriate modifications, its two sets of amendments). When published, the biological test method described in the current report is intended to supersede and replace Environment Canada's earlier guidance in the amended (twice) version of Report EPS 1/RM/22 (1992) for performing a 7-day test for growth and survival of larval fathead minnows.

Universal procedures for a test with larval fathead minnows in the laboratory are described in this revised and updated version of Environment Canada's Report EPS 1/RM/22 (2010; including its November 1992 and September 2008 amendments). Also presented herein are specific sets of test conditions and procedures, required or recommended when using the test for evaluating different types of substances or materials (namely samples of *chemicals*, *effluents*, *elutriates*, *leachates*, or *receiving water*; see Figure 1). Those procedures and conditions relevant to the conduct of the test are delineated and, as appropriate, discussed in explanatory footnotes. In formulating these test conditions and procedures, an attempt was made to balance scientific, practical, and financial considerations, and to ensure that the results will be accurate and precise enough for the majority of situations in which they will be applied. Explicit instructions that might be required in a regulatory *protocol* or *reference method* are not provided, although the report is intended to serve as a guidance document useful for that and other applications.

This revised and updated version of Report EPS 1/RM/22 defines and applies the "*biomass*" *endpoint* (see Section 4.5) to measure combined toxic effects on larval fish survival and growth. The use of the "biomass" endpoint provides a more sensitive and ecologically relevant test endpoint than the more strict *sublethal* endpoint for growth (based on mean dry weight attained by surviving fish) that was measured and applied

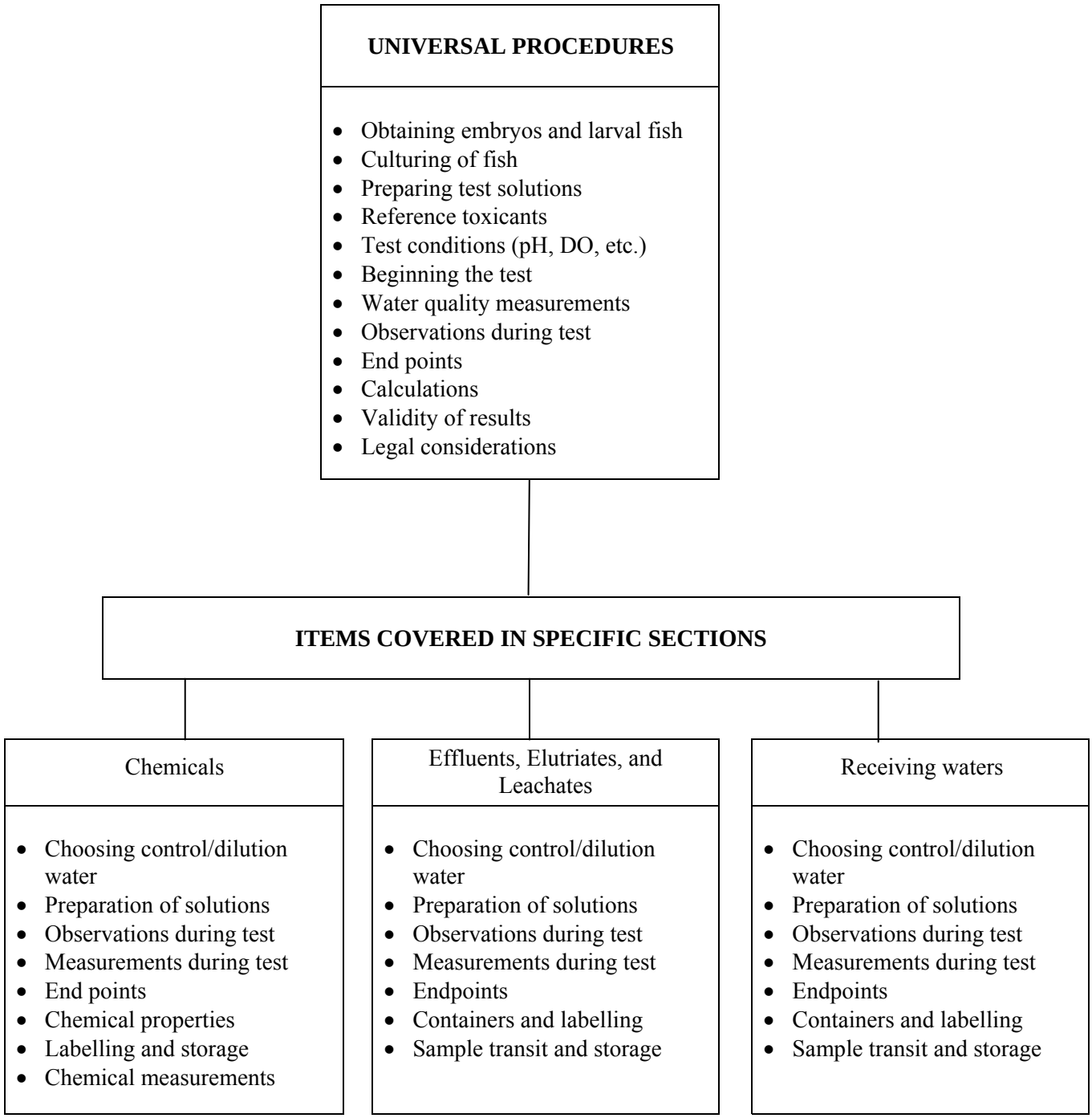


Figure 1 Diagram of Approach Taken in Delineating Test Conditions and Procedures Appropriate for Various Types of Materials or Substances

according to Report EPS 1/RM/22 (EC, 1992, up to and including its November 1997 amendments). The "biomass" endpoint is also consistent with the approach used by the United States Environmental Protection Agency in its short-term method for estimating the *chronic toxicity* of effluents or receiving waters on fathead minnows in a 7-day test of larval survival and growth (Section 11 in USEPA, 2002). Consistent with the September 2008 amendments to EC (1992), the current report also includes instructions for the use of revised statistics (i.e., regression analyses) when calculating the statistical endpoint for a multi-concentration test (i.e., *ICp*; see Section 4.5.1) using the "biomass" calculations.

1.2 Species Distribution and Historical Use in Tests

Fathead minnows belong to the family Cyprinidae, the carps and minnows, the dominant freshwater family in terms of number of species. Forty-four species of the family are found in Canada, most of them small minnows similar in appearance to the fathead minnow. Maximum lengths of fathead minnows in Canada are 8.3 to 9.4 cm, with mature females being smaller, normally 4 to 7 cm (Andrews and Flickinger, 1973; Scott and rossman, 1973). A male fish of 7 cm would weigh about 3.5 to 5 g depending on nutritional status, and a female fish of 5 to 6 cm would weigh about 1.5 to 2 g (Benoit and Carlson, 1977; Korver and Sprague, 1989).

Fathead minnows *(Pimephales promelas)* are native to much of Canada. Their range touches the Northwest Territories (southern drainage of Great Slave Lake), covers most of Alberta, the southern two-thirds of Saskatchewan and Manitoba, most of Ontario (reaching Hudson Bay), southwestern Quebec, and the northwestern corner of New Brunswick. Moving southward through the United States, the range narrows to the central part of that country, and touches northern Mexico (Scott and Crossman, 1973). This fish is not native west of the Rocky Mountains, nor to the three most easterly Atlantic provinces, and a permit should be obtained before bringing the species to a laboratory in those places (see Section 2.2). The fathead minnow thrives in ponds, lakes, ditches, and slow muddy streams, and in alkaline or saline lakes such as those in Saskatchewan. It is an omnivore, feeding opportunistically on anything from living invertebrates to detritus, but is well-suited to diet high in vegetable matter.

Fathead minnows will commence spawning in May or June in the northern part of their range when average water temperatures are as low as 13 to 17 °C. Both temperature and *photoperiod* appear to play a role in initiating spawning, which could continue through the summer, ending by August or September (Andrews and Flickinger, 1973).

During the spawning season, the male fathead minnow selects an overhanging object (log or rock), cleans it, and defends it and the territory around it. Females are allowed to enter for spawning on the underside of the object, then the male continues to guard and clean the eggs. Cleaning is important in preventing fungus on the eggs. A female can produce 1000 to 10 000 eggs in a season depending on conditions, and could deposit 300 to 500 at one time. These characteristics make the species ideal for laboratory studies, because the male will adopt an inverted section of semicircular tile as a spawning territory, and the investigator can conveniently find and recover the *embryos* for counting or obtaining

young fish. Fathead minnows are, in fact, good laboratory or aquarium fish, taking readily to that life and adapting well to dry commercial fish food, brine shrimp, etc. The species has been commonly reared in ponds in the USA for use as a bait fish (Brauhn *et al.*, 1975).

Fathead minnows have been used for life-cycle toxicity tests in the U.S. since the 1960s (Mount and Stephan, 1967), and it is now a standard species for tests of both *acute lethality* and *sublethal* or *chronic* effect (USEPA, 1989, 1994, 2002). A toxicological data bank of appreciable magnitude has been assembled for this species.

The seven-day test with larval fish is a sensitive sublethal test, but is not of long duration relative to the life span of the fish, and is therefore not a chronic test. It might not estimate exactly, the results of longer exposures (Suter, 1990; Norberg, 1990). The seven-day test is sensitive, however, because larval fish are usually among the most vulnerable stages of the entire life cycle (reviewed in Woltering, 1984; McKim, 1985; Norberg and Mount, 1985; Suter *et al.,* 1987; Norberg-King, 1989). In general (from NOECs reported by Norberg-King, 1989[1]), the seven-day test could be expected to estimate the toxicity in a 30-day exposure of early life- stages of fathead minnows closely in some cases, and within a factor of 2 in other cases, but it might sometimes under-predict by an order of magnitude. The seven-day test could underestimate the *sublethal toxicity* in a life-cycle exposure of fathead minnows by factors of 2 to 3 in many cases, but sometimes by factors of 25 or more. The larval test described in this report does not necessarily replace chronic toxicity tests, but comes much closer to results of such chronic tests than would a conventional lethality test with juvenile fish (e.g., Environment Canada, 1990a).

The seven-day larval test has shown excellent correlation with ecological evaluations of polluted waters. In a Kentucky river, the degree of mortality of larval fathead minnows had correlation coefficients of 0.92 to 0.96 with the number of fish species resident in sections of the river, and with the number of invertebrate species and their diversity (Birge *et al.,* 1989). McKim (1985) presents a rationale for use of tests with early life stages, and descriptions of these stages.

Precision of the seven-day test with larval fathead minnows has been satisfactory in the existing comparisons, for example there was good agreement in an intensive comparison among ten U.S. laboratories (API, 1988), with inter-laboratory coefficients of variation of 31% for survival of *larvae* and 52% for final weight. A *coefficient of variation* of 31% was

[1] Norberg-King (1989) carried out many five- to seven-day larval tests with fathead minnows and also some 30- to 32-day exposures of early life-stages (starting with eggs). Those results were compared with each other and with the findings of five tests taken from literature, for life-cycle exposures of fathead minnows with some of the same chemicals. The results are also discussed in Suter (1990) and Norberg-King (1990). Comparing the five- to seven- day larval exposures (24 tests using four chemicals) with approximately 30-day exposures, the latter tests showed somewhat greater toxicity. Average ratios of five- to seven-day NOEC to 30-day NOEC were: Carbaryl, 0.88; zinc, 1.3; cadmium, 2.3; and Diazinon, 8.6. For Dursban, a seven-day NOEC was compared to three, 30-day values from the literature, and the longer tests were more toxic by an average ratio of 1.8.

Comparing five- to seven-day larval NOECs with life-cycle NOECs, the latter showed appreciably greater toxicity. The ratios (seven-day NOEC divided by life-cycle NOEC) averaged 2.2 for zinc, 3.0 for chromium and Carbaryl, >25 for Dursban, and >45 for Diazinon. Suter *et al.* (1987) point out that fecundity of adults is usually the most sensitive effect in a life-cycle test, with larval growth and survival less sensitive and about equal in sensitivity to mortality of adults.

shown for results from nine laboratories in the San Francisco area (Anderson and Norberg-King, 1991). That precision is somewhat better than in chemical analyses of priority pollutants, for which a comparable average inter-laboratory coefficient of variation was ≥60% (Rue *et al.*, 1988).

Fathead minnows are used in several Canadian aquatic toxicity laboratories, both governmental and industrial, for *lethal* and *sublethal* testing. A standard test method has been described in Ontario (Neville, 1989), but no standard method for the species has previously been published by a Canadian federal government agency.

In the United States, written descriptions of standard methods for sublethal toxicity tests using fathead minnows, in a seven-day test for effects on survival and growth, have been provided by several groups. The most authoritative is from the Environmental Protection Agency (USEPA, 1989, 1994, 2002), while other descriptions are essentially adaptations or abbreviated versions of the basic USEPA procedure (e.g., Battelle, 1987; NJ, 1989).

Provided herein is a standardized Canadian methodology for undertaking 7-day tests of sublethal toxicity of various substances or materials, using larval fathead minnows. The test procedures detailed in the U.S. documents vary in their coverage of *endpoints*, issues such as *pH* adjustment, differing methodology for various objectives, criteria for *control/dilution water*, and how to deal with samples that contain appreciable solids or floating material. This method is intended for evaluating the sublethal toxicity of samples of chemical, effluent, leachate, elutriate, or receiving water, and the rationale for selecting certain approaches is given.

The method is meant for use with freshwater- acclimated fish, with fresh water as the dilution and *control* water, and with effluents, leachates, or elutriates that are essentially fresh water (i.e., *salinity* ≤10 g/kg) or saline but destined for discharge to fresh water. Its application may be varied but includes instances where the impact or potential impact of materials or substances on the freshwater environment is under investigation. Other tests, using other species acclimated to seawater, may be used to assess the impact or potential impact of materials or substances 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 and Life Stage

The test species is the fathead minnow (*Pimephales promelas*). Larvae that have been hatched for 24 hours or less must be used in tests[2].

[2] Larvae that have been hatched for 24 h or less are used in tests because the very young fish are considered to be particularly sensitive, although there does not seem to be published evidence on this topic. Unpublished trials by the Duluth laboratory of USEPA have apparently indicated that there can be decreased sensitivity among older larvae. Although fish of 1, 4, and 7 days age showed similar sensitivity to metals, the one-day-old fish were more sensitive to carbamate insecticides and other organic *toxicants* (personal communication, T.J. Norberg-King, USEPA).

There would be one advantage in using older larvae. Some of them do not start feeding until 24 h after hatching, and perhaps about 6% of the larvae are that category (API, 1988). Some of those larvae might never start to feed, in which case they would die within seven days and influence the results of the test or at least increase the variation in data obtained. By 48 h, larvae that are feeding can be distinguished by an orange colour of brine shrimp in the gut, and non-feeding larvae can be rejected, increasing the precision of the test. At present there does not appear to be quantitative information available, to permit an objective comparison of the relative importance of eliminating non-feeders, and the greater sensitivity of younger larvae.

The present method requires larvae of age ≤ 24 h in order to use more sensitive animals, and in order to increase the relevance and usefulness of data from tests conducted elsewhere, which will apparently be standardized on the 24-h age limit. The USEPA method will continue to use fish of ≤1day post-hatching (personal communication, T.J. Norberg-King), and in practice that means that all agencies and organizations in the USA will follow the procedure. The only exception in the USEPA procedure is that larvae as old as 48 h may be used if they must be shipped to a remote site for the test (USEPA, 1989, 1994, 2002), but that seems an unlikely prospect. It is clear that large amounts of test-data will be generated in the U.S. using larvae of age ≤24 h, and that data will be immediately useful for predictive purposes in Canada if the Canadian method is comparable. The method used by the Ontario Ministry of Environment uses larvae ≤24 h post-hatching (Neville, 1989).

2.2 Source

Breeding stock are best acquired from another laboratory that is known to have disease-free fish (Section 2.3.11). The least risk of carrying disease is by transfer of embryos, a procedure that also provides the greatest ease of shipment. Less desirably, fish may be acquired by collection in the field, but careful identification is required to separate this species from similar ones (Scott and Crossman, 1973). Parasites and disease are likely in wild fish, which should be carefully examined, reared in small isolated groups, and bred through a full generation before obtaining the next generation of progeny for use in tests (Brauhn *et al.*, 1975; Denny, 1987).

Confirmation of the species of test organisms received from a supplier must be made by a qualified taxonomist, at least once for any shipments of fathead minnows provided by that supplier. Thereafter, periodic confirmation of the species can be made by the testing laboratory by comparing an organism from a given *batch* to a representative specimen previously confirmed as to species by a taxonomist and maintained as a preserved specimen at that laboratory (EC, 1999).

Procurement, shipment, and transfer of fish must be approved, if required by provincial or

regional authorities. Provincial governments might require a permit to import fish or their eggs whether or not the species is native to the area, or movements of fish stocks might be controlled by a Federal-Provincial Introductions and Transplant Committee. Advice on contacting the committee or provincial authorities and on sources of fish, can be obtained from the regional Environmental Protection office (Appendix B). In areas where fathead minnows are not native (B.C., P.E.I., N.S., Newfoundland, and parts of other provinces and territories (see Section 1.2), application for a permit must be made to the above-mentioned committee, to the appropriate provincial agency, or to the Regional Director-General of the Department of Fisheries and Oceans (DFO), depending on procedures in place locally.

It is strongly recommended that the test organisms (i.e., larvae that have been hatched for 24 h or less) be obtained from an in-house *culture* (see Section 2.3). If, however, it is necessary to import embryos or young (i.e., hatched for ≤24 h) larvae for use in a test, Environment Canada's recommended procedures for the importation of test organisms for sublethal toxicity testing (EC, 1999; see website http://www.ec.gc.ca/eem/ for document) should be consulted and the guidance therein followed.

If it is necessary to import test organisms, it is recommended that they be transported as newly-eyed embryos rather than young (i.e., hatched for 24 h) larvae. Since embryos of fathead minnows typically hatch within 4 to 5 days when held at 22 to 25°C, their transportation as eyed eggs within 2 days of egg deposition on tiles is a preferred approach. Given no more than 2 days (and, ideally, ≤1 day) for shipment, this approach should enable sufficient time for *acclimation* of the embryos to laboratory control/dilution water at the test temperature (i.e., 25 ± 1°C; Section 4.3), before the embryos hatch. It would also prevent any transportation stress on young larvae before the test is started, and would allow the testing laboratory to determine with confidence the age of the larvae (must have been hatched for 24 h or less) at the start of the test. Each shipment of imported test organisms must include a written statement that identifies the age of the embryos or larvae shipped, as well as the date and time of that shipment.

If test organisms are imported to a testing laboratory, the mortality rate for larval fish must not exceed 10% (EC, 1999). Confirmation that this mortality rate is not exceeded requires a count of the number of larvae hatched from the *batch* of eyed eggs shipped as well as a count of the number of surviving larvae in this batch just prior to their transfer to test vessels, in instances where eyed eggs are delivered to the testing laboratory. If young (i.e., <24 h since hatching) larvae are imported, the testing laboratory must determine and compare the number of live larvae in the batch just prior to their transfer to test vessels, to the total number of larvae (live and dead) received from the supplier.

Section 6.0 "*Acclimation and Holding*" in EC (1999) provides useful guidance on the *acclimation* of imported test organisms to laboratory conditions before the test is started. Included there are useful procedures for their gradual adaptation to the control/dilution water to be used in the test as well as to the test temperature. This guidance should be reviewed and followed in instances where the importation of test organisms is necessary.

In each instance where test organisms (as eyed eggs or <24-h old larvae) are imported to the testing laboratory, the temperature and

dissolved oxygen concentration in the water within the shipping container(s) must be measured and recorded upon departure from the supplier's facility, as well as upon arrival at the testing laboratory (EC, 1999). During transportation, the temperature of this water must be maintained at or near the required test conditions, and must not change by more than 3°C during any 24-h period in transit. Additionally, its dissolved oxygen content must be ≥80% saturation (EC, 1999). Water used for transporting test organisms must be well oxygenated (e.g., 90 to 100% saturation) before shipment. A record of the temperature and dissolved oxygen of the water in which the test organisms are transported should accompany the shipment, together with information on the number of organisms in the shipment and their age and life stage.

2.3 Culturing

2.3.1 General

The recommended and required conditions for holding and culturing fathead minnows, summarized in Table 1, are intended to allow some degree of flexibility within a laboratory, while at the same time standardizing those elements which, if uncontrolled, might affect the health of fish or viability of their offspring. Much of Section 2.3 that specifically concerns fathead minnows is derived from Denny (1987) and Norberg-King and Denny (1989), reports that should be consulted if further details are required.

A training video prepared by the U.S. Environmental Protection Agency (1988), shows procedures used by the Environmental Research Laboratory at Duluth, Minnesota for culturing fathead minnows. This video is available within Canada, courtesy of T. Norberg-King (USEPA, Duluth), and can be obtained for viewing by contacting a regional office of Environment Canada (see Appendix B).

Small groups of male and female fathead minnows are held in aquaria provided with spawning substrates. The substrates are inspected daily, those with embryos are moved to hatching tanks, and new substrates are provided. Collections from the hatching tanks yield larvae, hatched for 24 hours or less, for use in tests. Some fish are reared as a source of new generations of adults. Two dozen pairs of spawning adults should provide at least 200 embryos per day on average, on a continuing basis if non-performing fish are periodically replaced with maturing fish, and 500 or more embryos per day under good conditions. All larval fish used in a test must be from the same *batch*, and must be of known age. The larval fish should represent at least three spawnings (i.e., different parentage), although that is not an absolute requirement (USEPA, 1989, 1994, 2002). It is strongly recommended that the culture of fathead minnows used to provide a *batch* of test organisms be maintained in the laboratory that carries out the toxicity tests. If necessary, however, the test organisms can be imported (as embryos or larvae that are <24-h old since hatching) to the testing laboratory, provided that Environment Canada's required conditions and procedures for importing toxicity test organisms (see Table 1 in EC, 1999) are adhered to unless specified otherwise in this methodology document. In this case, each shipment or group imported would represent a discrete batch of test organisms.

2.3.2 Facilities

Embryos and larvae may be hatched and reared in containers made of nontoxic materials such as glass, stainless steel, porcelain, fibreglass- reinforced polyester, perfluorocarbon plastics (Teflon™), acrylic, polyethylene, or polypropylene.

Table 1 Checklist of Recommended and Required Conditions and Procedures for Culturing Fathead Minnows

Source of fish	–	disease-free stock from another laboratory; captured in the wild if special care taken in identifying species and eliminating disease
Water	–	uncontaminated ground, surface, or, if necessary, dechlorinated municipal water; flow to culture aquaria 1.4 L/g fish per day
Temperature	–	holding temperature within the range 4 to 26°C; culturing at 25°C (range 22 to 26°C) achieved at rate ≤3°C/d and held at 22 to 26°C for ≥2 weeks
Oxygen/aeration	–	dissolved oxygen 80 to 100% saturation; maintained by aeration with filtered, oil-free air if necessary
pH	–	within the range 6.8 to 8.5, preferably 7.0 to 8.5
Water quality	–	temperature, dissolved oxygen, pH, and flow to each holding or culturing aquarium to be monitored, preferably daily
Lighting	–	broad spectrum (fluorescent or equivalent), 100–500 lux at surface, 16 ± 1h light : 8 ±1 h dark, preferably gradual transition between light and dark
Feeding	–	at least once a day with frozen brine shrimp supplemented by commercial pelleted or flaked food; feeding rate judged by amount consumed in 10 minutes; food stored as recommended by manufacturer
Cleaning	–	siphoning of debris, daily or as required
Mortalities and disease	–	mortalities monitored ≥5 days/week (preferably daily), and moribund fish removed; mortality <5% during seven days preceding collection of eggs; discard breeding stock if combined incidence of mortalities and disease >10%/ week at any time; if treated for disease prevention or control, allow at least two weeks before collecting eggs for use in toxicity tests

Juvenile and adult fathead minnows may be reared in aquaria, troughs, or tanks that receive flowing water. These must also be made of nontoxic materials such as listed previously. Aquaria containing about 40 L of water, and provided with a standpipe drain, are most commonly used. The fish culture operation should be located away from any physical disturbances and preferably in a location separate from the test containers. Aquaria for rearing are usually indoors but may be outdoors; aquaria for obtaining embryos and young should be in the laboratory, exposed to the standard lighting, temperature, water, and other test conditions.

Breeding aquaria are divided or partially divided for spawning purposes with stainless steel screens or rigid plastic sheets, opaque or transparent. A spawning substrate, intended as territory for one male minnow, is placed in each of the areas created. There are variations

in the exact arrangements which are not crucial. A 40-L aquarium might have two areas, with a spawning substrate in each, and a partial divider with an open "doorway" in it for fish (essentially the females) to move back and forth. Alternatively, the aquarium might be divided into four areas, intended for four substrates with a male and female in each area (Denny, 1987).

The spawning substrate is one half of a cylinder of tile or pipe. The material is not critical and could be PVC plastic, or a porous material such as clay or concrete (Benoit and Carlson, 1977). Tile of about 10 cm in diameter is cut into lengths of 7 to 10 cm, then cut in half lengthwise. One half is inverted in the area intended for each male.

White plastic dishpans are convenient "hatchery trays" if the embryos are hatched on the tiles. Up to six such trays can stand partly immersed in a large water bath. If that bath is under the breeding aquaria, wastewater from those aquaria can flow to the bath, providing the basic heating of the water bath. Alternatively, the eggs may be removed from the tiles and hatched in a separatory funnel (Section 2.3.8).

2.3.3 Lighting

Depending on test requirements and intent, lighting during rearing and breeding should be natural or as provided by overhead full-spectrum[3] fixtures. If photoperiod control is required, the photoperiod should normally be a constant sequence of 16 ± 1 hours of light and 8 ± 1 hours of darkness. Light intensity at the tank-water surface should be 100 to 500 *lux*. A 15- to 30-minute transition period between light and dark is recommended if artificial lighting is provided[4].

2.3.4 Water

Sources of water for holding and culturing fish can be "uncontaminated" groundwater, surface water, or if necessary, dechlorinated municipal drinking water (see the following paragraph). The water supply should previously have been demonstrated to consistently and reliably support good survival, health, and growth of fathead minnows. *Monitoring* and assessment of variables such as residual chlorine (if municipal water is used), pH, *hardness*, alkalinity, total organic carbon, *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.

---

[3] For most tests, fluorescent or other tubes with a "full-specturm" wavelength lamp (e.g., Vitalite™ or Benelux 55™, supplemented if desired with natural outdoor illumination, should be used to simulate the visible range of natural light (Denny, 1987). Other artificial illumination may be used, and various laboratories report success in rearing fathead minnows using the less expensive cool white or warm white fluorescent lighting. It should be noted that "full-spectrum" or other fluorescent lights do not emit the intensity of ultraviolet (UV-B) radiation approaching that of natural illumination, and that the toxicity of certain effluents and chemicals can be altered markedly by photolysis reactions caused by UV-B radiation. For certain tests (e.g., photoactivation or photodegradation of toxic materials due to ultraviolet radiation), special lights (e.g., high-pressure mercury arc lamps) with differing spectral qualities should be used. ASTM (1996) provides useful guidance in this regard. Studies designed to determine the influence of lighting conditions on toxicity should conduct concurrent side-by-side comparisons with *replicate* solutions held under differing lighting conditions (e.g., full-spectrum versus mercury arc).

[4] A "dawn/dusk" transition period is recommended because abrupt changes in intensity startle and stress fish. Automated control systems are available for dimming and brightening the intensity of fluorescent lights, although they are costly. Alternatively, a secondary incandescent light source, regulated by time clock and automated rheostat, may be used to provide the transition period.

Dechlorinated water is not recommended for culturing of fish and, in particular, not for hatching of embryos or rearing of larvae. It is difficult to remove the last traces of residual chlorine and chlorinated organic substances, and they could be toxic to the larval fathead minnows. If municipal drinking water is to be used for culturing fish and as control/dilution water, effective dechlorination must rid the water of any harmful concentration of chlorine. Vigorous aeration of the water can be applied to strip out part of the volatile chlorine gas. That could be followed by use of activated carbon (bone charcoal) filters and subsequent ultraviolet radiation (Armstrong and Scott, 1974) for removing most of the residual chloramine and other chlorinated organic compounds. Aging the water in aerated holding tanks might be of further benefit. A target value for total residual chlorine, recommended for the protection of freshwater aquatic life, is ≤0.002 mg/L (CCREM, 1987). Anything greater than 0.002 mg/L might risk interaction of chlorine toxicity with whatever was being tested[5]. In addition to measurements of chlorine, monitoring of egg production and fish survival can provide evidence of satisfactory water.

If surface water is used for culturing fish, it should be filtered to remove potential predators and competitors of fathead minnows. A conventional sand filter or commercial in-line filter would be suitable. Small quantities might be filtered through a fine-mesh net (≤60 µm). Ultraviolet sterilization is recommended to reduce the possibility of introducing pathogens into the colony of fish.

If *reconstituted water* is to be used as dilution and control water (see Section 5.3), adult fish must be acclimated to that reconstituted water or to a similar water, for at least the five days immediately before embryos are obtained for the test[6]. The similar water could be: (a) a natural water with hardness within 20% of the reconstituted water; (b) a harder natural water adjusted downwards to the desired hardness with *deionized water*; or (c) a softer natural water adjusted upwards with the appropriate quantities and ratio of reagent-grade salts (e.g., ASTM, 1980; Environment Canada, 1990b, Table 2).

The water in aquaria containing adult fish should be renewed to prevent a buildup of metabolic wastes. At least 1 mL/min of fresh (new) water should flow into the tank for every gram of fish being held (equals 1.4 L/g fish · d, or 0.69 g fish · d/L)[7]. For an aquarium with 50

---

[5] Chlorine is quite toxic to aquatic organisms, especially to crustaceans and the early life-stages of fish. For example, 0.011 mg/L of total residual chlorine causes chronic sublethal effects in fathead minnows, and somewhat higher concentrations of 0.040 to 0.045 mg/L might cause acute lethality of rainbow trout and a species of minnow (Arthur and Eaton, 1971; Wolf *et al*., 1975; Ward and DeGraeve, 1978; CCREM, 1987). An exposure to 0.08 mg/L of total residual chlorine for only 2 h/d might prove lethal to juvenile fathead minnows (Wilde *et al.*, 1983), suggesting that sublethal effects could be expected at concentrations at least an order of magnitude lower.

The recommended limit of 0.002 mg/L taken from Canadian guidelines (CCREM, 1987) is in line with recommendations in the United States. A maximum of 0.002 mg/L was recommended for protection of most aquatic life in receiving waters by Brungs (1973), and a maximum of 0.003 mg/L at any time or place was recommended for freshwater life by NAS/NAE (1974).

[6] Without such acclimation, the benefit of a standardized *dilution water* might be lost. For example, it takes several days for fish to readjust their tolerance to heavy metals when moved to a water of different mineral content (Lloyd, 1965).

[7] If necessary (e.g., if fish are being acclimated to reconstituted water, receiving water, or some other water source that is restricted in amount), water-

g of fish, that would be an inflow of 70 L/d or 50 mL/min. Unusual circumstances such as acclimation of fish to reconstituted water might require the filtration and recirculation of water, or its periodic renewal in *static* systems. A recirculating culture system is described by Rottmann and Campton (1989). In such cases of water reuse, ammonia and nitrite should be measured frequently to check that they do not reach harmful levels. Target values, recommended for the protection of freshwater aquatic life, are ≤0.02 mg/L of un-ionized ammonia (OME, 1984), and ≤0.06 mg/L of nitrite (CCREM, 1987).

Water entering the aquaria should not be supersaturated with gases. In situations where gas supersaturation within the water supply is a valid concern, total gas pressure within water supplies should be frequently checked (Bouck, 1982). Remedial measures must be taken (e.g., use of aeration columns or vigorous aeration in an open reservoir) 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, pH, and flow should be monitored for each aquarium or tank, preferably daily. Weekly or more frequent monitoring of levels of ammonia, nitrite, and total residual chlorine (if municipal water source) is recommended.

2.3.5 Temperature

Groups of fish may be held for later use at temperatures as low as 4°C. High temperatures should be avoided, the optimum for the species being 23.5°C, and 32°C marking a limit for failure of reproduction and the onset of effect on growth (Brungs, 1971b). When preparing a group of fish for breeding, water temperature may be changed at a rate not exceeding 3°C per day, until a value near 25°C is achieved. Fish should be maintained within the range 22 to 26°C for a minimum of two weeks and preferably ≥3 weeks, before using their embryos to obtain larvae for toxicity tests. Temperatures outside the 22 to 26°C range are known to decrease egg production (Brungs, 1971b).

2.3.6 Dissolved Oxygen

The dissolved oxygen (DO) content of the water within holding and culture aquaria should be 80 to 100% air saturation. Mild aeration of the tanks should be carried out using filtered, oil-free compressed air. Such aeration through a commercial aquarium airstone is customary and assists in mixing the water and maintaining uniform physicochemical conditions. Avoid vigorous aeration, especially if larval or young fish are present.

2.3.7 pH

The *pH* of water used for holding and culturing fish should be within the range 6.8 to 8.5, and preferably[8] 7.0 to 8.5.

volume requirements for fish acclimation may be decreased substantially by recirculating the flow to the fish tank through a filter suitable for removing metabolic wastes (e.g., Rottmann and Campton, 1989). If a recirculation system is used, ammonia and nitrite concentrations in the water should be monitored and kept below levels harmful to fish health.

[8] The objective in holding and culturing is to provide conditions that are quite favourable for the fish. Fathead minnows are not well adapted to acidic pH values. There is evidence of marginal effects of acidic conditions, up to pH 6.6, hence the recommended minimum of pH 6.8 for culture of fish. Maintaining pH 7.0 or higher is considered a more realistic margin of safety to avoid any sublethal effects during culture. That pH is therefore preferred, although a scarcity of data in the region between pH 6 and 7 do not provide much direct support for the limit.

A pH of 6.0 is clearly unsatisfactory for reproduction of fathead minnows and appears unsatisfactory for rearing the species. In soft water in the laboratory, McCormick *et al.* (1989) demonstrated an appreciable reduction in survival from spawned egg to 4 days' posthatch, at pH 6

2.3.8 Growing and Breeding the Fish

Post-larval fish, juveniles, and maturing fish are normally reared in aquaria. The number per aquarium should be gradually reduced as they grow, by moving fish to other aquaria. As the fish approach adult size, males and females can be obtained for stocking the breeding aquarium. The sexes can be distinguished only as the fish approach breeding condition, usually at about 16 to 24 weeks of age (Figure 2). Females retain the appearance of a silvery minnow but develop an ovipositor ahead of the anal fin. Males are bigger, become blackened on the sides with two light-coloured vertical bars near the front of the body, and develop a pad with turbercles on the back part of the head and tubercules on the "forehead" or snout. Two or three spawning substrates should be placed in the culturing aquaria as maturation becomes evident, and some males will claim them. They stay under the shelter (i.e., the tile or other spawning substrate) most of the time and keep other fish away, except for the purpose of spawning.

Upon signs of maturation, individual males and females may be selected from the culture tank and used to stock the breeding aquaria. Other males will eventually take over the substrates and other females will develop, and more breeding aquaria can be stocked. If a breeding aquarium is divided into two areas, it might be stocked with two males and four to six females. Alternatively, one male and one female might be placed in each of four screened-off areas in an aquarium; Denny (1987) indicates that the paired method increases total egg reproduction and points out that it allows good records of egg production, so that non-productive fish can be replaced. Fish should be replaced with others if there is a three-week period without egg production. Automatic replacement of fish could be practiced, after a fixed period of three or six months.

Eggs will be laid inside the tile substrate on the "ceiling". Daily inspection should be made at mid-morning, because spawning often takes place in early morning. Inspection may be done by feeling inside the tile with a finger, or less desirably, by removing and inspecting the tile. Daily estimates of the

---

compared to pH 7.5. At pH 7.5, 6, and 5.5, survivals were respectively 92%, 60%, and 0%, with a trace level of aluminum present that should not have affected results. In a long experiment in an outdoor artificial stream (one of the most meaningful types of toxicity test), Zischke *et al.* (1983) found that at pH 8 (control), pH 6, and pH 5, the estimated numbers of eggs produced by parallel populations of fathead minnows were respectively 41 000, 33 100, and 110, while the numbers of young surviving at the end of the experiment were respectively 2924, 14, and 1. Zischke and his co-authors concluded that "*Continued acidification would likely have caused extinction of the fathead minnow populations at both pH 5 and pH 6. Our results... give support to the proposed protection pH recommendations (6.5 or higher) set by the National Academy of Sciences (1973) and the U.S. Environmental Protection Agency (1976)*" [NAS/NAE, 1974; and USEPA, 1976].

One Canadian laboratory had severe difficulties in testing with larval fathead minnows at pH 6.5, and suffered control mortalities severe enough that some tests had to be repeated six or more times (pers. com., T. Kovacs, Pulp and Paper Research Institute of Canada, Pointe Claire, Que.). Mount (1973) did a life- cycle test with fathead minnows in hard water which would be favourable to the fish (hardness = 200 mg/L), with results that indicated some deleterious effects on reproduction at pH 6.6. For exposures at four values of pH, Mount (1973) recorded the following numbers of eggs produced per female in two replicates: pH 7.5 = 480 and 968; pH 6.6 = 210 and 394; pH 5.9 = 66 and 101; and pH 5.2 = 0 and 0. The number of spawnings per female in the two replicates were respectively, for the same four values of pH: 4.2 and 5.4; 3.0 and 4.0; 1.0 and 1.2; 0 and 0. Statistical analysis of Mount's results cannot be done without access to the raw data, but the reduction of average number of eggs at pH 6.6, to about 42% of the number at pH 7.5, has the appearance of a deleterious effect.

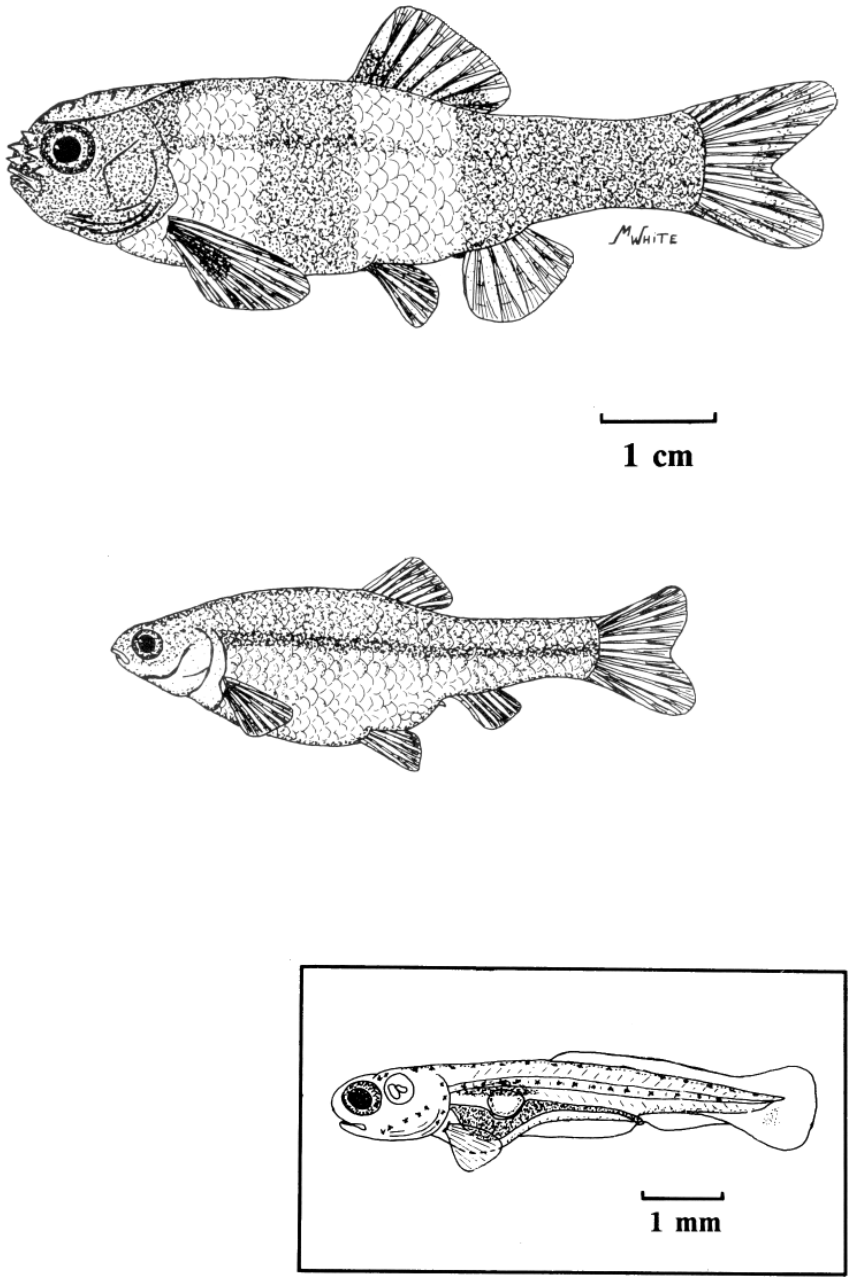


Figure 2 General Appearance of Male and Female Fathead Minnows in Breeding Condition, and of a Larva About Four Days After Hatching
(original drawings from specimens, by M.A. White)

Non-breeding males would not have the tubercules on the snout, the rugose pad on the top of the head, or the vertical bands of colour. Non-breeding females would not show the ovipositor ahead of the anal fin, nor the ventral distension from carrying eggs.

number of eggs laid should be made and recorded; values for each 7-day period should be combined to provide weekly fecundity rates.

If there are embryos, the tile is removed and replaced with a clean one. The tile with embryos is moved to a hatching tray. Two such tiles might be placed on end in a circle, with an airstone inside to keep the water moving. To help prevent the spread of fungal infections, it is desirable to aerate tiles individually in beakers immersed in a hatching tray (Section 2.3.2).

Incubating embryos must be inspected daily. Dead embryos or those with fungal growth must be removed and discarded, the dead ones appearing opaque or having a white spot inside. Tiles with severe fungal growth should be removed. Disturbance should be minimal on Days 3 to 5, because it might cause early hatching. Embryos will hatch in 4 to 5 days depending on temperature, which should be maintained in the 22 to 25°C range. Larvae are then removed with a large-bore pipette with rubber bulb, and used for tests. The used spawning tiles are disinfected (Section 2.3.10) and thoroughly soaked and rinsed in water before reuse.

To culture fish towards adulthood for a new stock of breeders, groups of 200 to 300 larvae are placed in aquaria with a water depth of 20 cm. For these groups of fish being cultured, there should be an approximate estimate of hatching success, as well as an estimate of mortality among the ensuing larvae during the first 30 days of life.

An alternative technique for culturing is to gently roll the eggs off a smooth spawning tile (PVC) using a wet finger. Eggs are placed in culture water within a separatory funnel that is aerated to keep them suspended in the water. Dead eggs or those with fungal growth are removed and discarded at 24 and 48 hours. At 48 h, viable eggs are moved to culture water within small pans or aquaria that is vigorously aerated until hatching.

If there is long-continued culture of minnows in a laboratory, steps should be taken to avoid selecting a homogenous strain. Larvae for future spawning stock should be selected from different parents at intervals, rather than keeping many larvae from a few spawnings or a single spawning. Every two years, the gene pool might be supplemented by exchanging with another laboratory; however, bringing in some wild fish would be preferred. The new fish should be carefully examined by a taxonomic expert. Any diseased fish should be rejected and the rest treated for disease (Sections 2.3.10 and 2.3.11; Denny, 1987), kept segregated in small groups, and held through breeding so that it is actually their progeny that are added to the laboratory stock. It is encouraging that when ten laboratories engaged in round-robin studies, source of fish did not appear to affect results (API, 1988).

2.3.9 Feeding

It is recommended that juvenile and adult fathead minnows be fed with frozen brine shrimp, supplemented with other commercial fish food. Commercial “flake” food may be used for part of the diet, but only as supplement to the frozen brine shrimp. Commercial pelleted fish food of suitably small size may also be used, again as a supplement. Depending on water temperature and fish size, feeding should be one or more times daily, normally with a daily ration approximating 1 to 5% of wet body weight. In practice, the amount of food required is best judged by the amount the fish consume in

about 10 minutes, the amount left over on the bottom of the tank, and by the appearance and condition of the fish. The method and maximum duration for storing fish food should be as recommended by the manufacturer.

Newly hatched fish to be raised as future breeding pairs should be fed with *nauplii* of brine shrimp *(Artemia salina)* that have been hatched from embryos within the previous 24 hours (Appendix C). Fathead minnow larvae begin feeding toward the end of their first day of life or shortly thereafter, so feeding could start when they are about 12 hours old. Brine shrimp should be supplied at least twice a day, since they might live in fresh water for only about 8 hours. The first daily feeding of the minnow larvae should be early in the day, so that live nauplii are always available during the daylight hours. Fish in the early larval stages can ingest only small, newly hatched nauplii of brine shrimp (maximum size 0.24 to 0.28 mm).

As the larvae grow they can be fed larger brine-shrimp nauplii, and, after about 30 days, can be gradually weaned from living to frozen brine shrimp and supplements of other food. The amount to be fed (i.e., the volume of fluid containing an estimated concentration of nauplii to be added on each feeding occasion) depends to some extent on the nature of the rearing system (Appendix C).

It is desirable to assess toxic contaminants in all fish food, but particularly dry flake food and brine-shrimp eggs (Appendix C). *Toxicants* of concern are bioaccumulative metals and pesticides. Guidance can often be obtained from the experience of other laboratories, and the measurements that they have done. It is desirable to report the origin of the brine shrimp eggs, so that any association between the source and success in rearing and testing can be detected over time.

2.3.10 Cleaning of Tanks

Tanks used to hold and culture fish should be kept reasonably clean. Excess food and faeces should be siphoned out with minimum disturbance of fish, once a day or as frequently as necessary to eliminate a buildup. Excessive growths of fungi or blue-green algae should be scraped and removed, and an effort made to eliminate whatever conditions are favouring their growth. However, a light growth of other algae and invertebrates on the walls of the aquaria should be tolerated because that might provide supplementary food and activity for the fish.

To minimize the occurrence of disease, tanks should be disinfected before introducing a new batch of fish. Suitable disinfectants include those containing chlorinated or idophore compounds or n-alkyldimethyl-benzylammonium chloride (e.g., Comet™, Ovidine™, Argentyne™, Roccal™). As disinfectants are toxic to fish, tanks should be rinsed thoroughly with the water used for culturing fish, following disinfection.

2.3.11 Fish Morbidity, Mortality, Disease, and Treatment

Adult and pre-adult fish being cultured should be inspected daily for signs of disease (Amlacher, 1970; Brown and Gratzek, 1980; Roberts and Shephard, 1986)[9]. Mortality rates and any evidence of disease must be monitored and recorded at least five days per

[9] Symptoms of unhealthy fish include loss of appetite, abnormal distribution in the tank, lethargy, erratic or atypical swimming behaviour, darkened coloration, pale gills, eroded or frayed fins, and external lesions. Books by Amlacher (1970) and Brown and Gratzek (1980) are useful guides for preliminary identification and diagnosis of fish diseases. *The Handbook of Trout and Salmon Diseases, 2nd ed.* (Roberts and Shephard, 1986) is useful for the same purposes for a variety of fishes, as well as salmonids.

week (e.g., Monday to Friday), as a minimum. Dead and moribund individuals should be removed immediately. During the seven-day period preceding the collection of eggs, mortalities must be less than 5% of the general population being reared; and less than 5% of the fish in individual tanks or aquaria, or limited to one fish in the case of breeding aquaria containing small numbers of fish. If the 7-day mortality rate for either of these groups is between 5 and 10%, holding of fish must be extended for at least another seven days before collection of eggs, until less than 5% mortality in seven days is realized. If the combined incidence of mortalities and noticeably diseased fish in the adult breeding stock exceeds 10% per week at any time[10], that stock of fish must not be used to produce test fish. An intensive search for the cause of this unacceptably high rate of mortality and/or disease should be carried out at this time, and cultures should be started anew from apparently healthy stock.

Treatment of fish with chemicals for disease prevention or control should be avoided if possible. It is strongly recommended that groups of fish showing signs of disease be discarded rather than treated. This might be a feasible approach if groups are held separately in a number of aquaria or tanks.

If the use of chemically treated fish cannot be avoided, a minimum 2-week period should follow their treatment before their eggs are collected for tests. Records (including each date of treatment, chemical and quantity applied, and reason for administration) must be kept of any treatment of breeding stock for disease prevention or control.

[10] A maximum acceptable mortality rate of 10% per week is specified by the Organization for Economic Cooperation and Development (OECD, 1984). For this biological test method, the incidence of disease is included with that for mortality in requiring (as a single rate of adverse effect) that no more than 10% of the adult breeding stock are apparently unhealthy and/or die, since diseased fish are unacceptable for use as breeding stock.

Test System

3.1 Facilities and Apparatus

The test is to be conducted in a facility isolated from general laboratory disturbances. If a separate room is unavailable, the test area should be surrounded with an opaque curtain (e.g., black plastic) to minimize stress to fish during testing. Dust and fumes should be minimized within the test and culturing facilities.

A test facility is required that will maintain the temperature of all test solutions at a mean temperature of 25 ± 1°C with extreme fluctuations within the range 23 to 27°C. This may be achieved using various types of equipment such as thermostat-controlled air conditioning unit or a temperature-controlled water bath in which test vessels are immersed.

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 2.3.2). 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 should be the same as those defined in Section 2.3.3. The photoperiod is to be timed to coincide with that at which the parent fish were held.

3.3 Test Vessels

Vessels may be beakers or rectangular containers of borosilicate glass[11] (such as Pyrex™), perfluorocarbon plastics (Teflon™), or disposable polystyrene. Nontoxic containers of other plastic such as polypropylene or polyethylene may be used, but should not generally be reused in a second test, in case the plastic sorbs toxicants that could be released during a subsequent test. For chemical testing (see Section 5), glass test vessels must be used to limit sorption.

The test vessel must contain at least 250 mL of solution during the test, and 500 mL is recommended. Volumes of up to 1 L are suitable and would give additional protection against depletion of toxicant or dissolved oxygen. The minimum water depth in any test vessel should be 3 cm. The vessel should not unduly restrict the surface area of the test solution, because diffusion of oxygen through the surface could be important when testing effluents or other test materials with an oxygen demand. As a guideline, the diameter of the vessel should approximate the depth of the test solution. Using that guideline, 500 mL of liquid should fill a container of 8.6-cm diameter to a depth of 8.6 cm, yielding a surface area of about 58 cm^2.

[11] Glass containers are inert and easily cleaned, and permit the unimpeded observation of test fish. Adsorption to non-glass containers (e.g., polyethylene, polypropylene, stainless steel, etc.) is markedly different for certain chemicals. Accordingly, for tests with chemicals (Section 5), glass beakers must be used.

Considerable latitude is allowed in the design and shape of test vessels. They may be specially constructed for easy renewal of test solutions without damaging the fish. For example, a screened sump at one end of a glass container can be used to remove the old test solution by siphoning, with larvae held safely on the other side of the screen (Norberg and Mount, 1985). (That method still requires dislodging and siphoning debris directly from the area containing larvae.) Alternatively, plastic containers with a screened bottom or other types of mesh "cages" can be submerged in a larger vessel of test solution and lifted easily to transfer fish to another vessel with fresh solution. Nytex™ netting of 500-µm size has been found advantageous, since dead brine shrimp can pass through it but the fish larvae will be retained. For a given test, water depth, and container type, size and shape should be identical for each test solution and each *replicate* used in that test. Vessels should be covered with glass during the test, to avoid potential contamination from the air and loss of volatile components.

3.4 Control/Dilution Water

Depending on the test material and intent (Sections 5 to 7), the control/dilution water may be: "uncontaminated" groundwater or surface water from a stream, river, or lake; reconstituted water of a desired pH and hardness (e.g., simulating that of the receiving water); a sample of receiving water collected upstream of the source of contamination, or adjacent to the source but removed from it; or dechlorinated municipal water[12] (see Section 2.3.4). Conditions for the collection, transport, and storage of samples of receiving water should be as described in Section 6.1. If surface water is used, it should be filtered through a fine-mesh net (≤60 µm) to remove potential predators and competitors of fathead minnows.

The control/dilution water must be adjusted to 25 ± 1°C before use. This water should not be supersaturated with excess gases (see Section 2.3.4). Before it is used, control/dilution water should have a dissolved oxygen content that is 90 to 100% of the air-saturation value. If necessary, aerate it vigorously (oil-free compressed air passed through air stones) immediately before use, and confirm that dissolved oxygen levels representing 90 to 100% saturation have been achieved.

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

Universal Test Procedures

Procedures described in this section apply to all the tests of particular chemicals, wastewaters, or receiving-water samples described in Sections 5, 6, and 7. All aspects of the test system described in Section 3 must be incorporated into these universal test procedures. The summary checklist of recommended and required conditions and procedures in Table 2 includes not only universal procedures but also those for specific types of test materials or substances.

4.1 Preparing Test Solutions

All test vessels, measurement devices, stirring equipment, and fish-transfer pails must be thoroughly cleaned and rinsed in accordance with standard operational procedures. Control/dilution water should be used as the final rinse water.

For any test that is intended to estimate the *LC50* (Section 4.5.1) as well as the *ICp* for growth (i.e., "*biomass*"; see Section 4.5.1), at least seven test concentrations plus a control solution (100% dilution water) must be prepared[13], and more (≥8 plus a control) are recommended. A geometric dilution series might be used in which each successive concentration is about 50% of the previous one (e.g., 100, 50, 25, 12.5, 6.3, 3.1, 1.6, etc.). Test concentrations may also 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 D). 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 uncertainty about the range of concentrations likely to be toxic.

In cases of appreciable uncertainty about sample toxicity, it is beneficial to run a range-finding or screening test for the sole purpose of choosing concentrations for the definitive test. Conditions and procedures for running this test can be relaxed. A 24-h exposure to determine mortality of larvae at a wide range of concentrations spanning ≥2 orders of magnitude (e.g., 100, 32, 10, 3.2, and 1.0 for 2 orders of magnitude; or 100, 10, 1, 0.1, and 0.01 for 4 orders of magnitude) should be of assistance in selecting concentrations for the full test. The highest concentration for the definitive test should be one that did not cause more than 20 to 30% mortality in the range-finding test. If there are severe time-limitations on starting the definitive test, a range-finding test of shorter duration such as 8 h would still provide useful guidance.

Single-concentration tests could be used for regulatory purposes (e.g., pass/fail). They would normally use full-strength effluent, elutriate, leachate, or receiving water, or an

[13] The use of eight or more test concentrations plus the control solution(s) is recommended to enable calculation of both the LC50 and the ICp for growth (based on biomass) using regression analyses (see Option 3 in Section 8.2.1 of EC, 2005). A preliminary or 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.

Table 2 Checklist of Recommended and Required Test Conditions and Procedures

Universal		
Test Type	–	*renewed static*, 7-d duration*
Control/dilution water	–	ground, surface, or if necessary, dechlorinated municipal water; "upstream" water to assess toxic impact at a specific location**; reconstituted water if requiring a high degree of standardization; dissolved oxygen (DO) 90 to 100% saturation at time of use in a test
Fish	–	larval fathead minnows, hatched for <24 h and inflated swim bladder evident; all from the same batch; at least ten larvae in each replicate
Replicates	–	minimum of three replicate test vessels required at each concentration, and four replicates recommended
Number of concentrations	–	minimum of 7, plus control(s); recommend more (i.e., >8), plus control(s)
Vessel and solution	–	depth >3 cm, and ≈ diameter; volume >250 mL, preferably 500 mL
Temperature	–	daily means 25 ± 1 ºC with extreme fluctuations within the range 23 to 27 ºC
Oxygen/aeration	–	no pre-aeration unless a test solution has DO <40% or >100% saturation upon preparation, in which case aerate all test solutions for ≤20 minutes at minimal rate before starting test or renewing solution; DO 40 to 100% saturation throughout the test, with more frequent renewal if required to maintain DO; if necessary to meet objectives of test, gentle aeration of all vessels
pH	–	no adjustment if pH of test solutions is in range 6.5 to 8.5***; a second (pH-adjusted) test might be required or appropriate, for pH beyond that range, or at any pH below 7.0
Lighting	–	normally full spectrum, 100 to 500 lux at surface of test solution; normally 16 ± 1 h light : 8 ± 1 h dark; preferably gradual transition
Feeding	–	two or three times/day with newly hatched brine shrimp nauplii; begin this feeding schedule at the start of the test but do not feed during the final 12 h
Observations	–	mortality, every 24 h; mean dry weight at 7.0 d; for controls only, combined incidence of fish observed to be dead, dying, showing loss of equilibrium, or displaying overt signs of atypical swimming behaviour; optionally (e.g., for research purposes), daily observations for number of surviving fish in each replicate and treatment that are moribund, or show loss of equilibrium and/or atypical swimming behaviour
Measurements	–	temperature, pH, and DO at start and end of 24-h periods, representative concentrations; conductivity at least at start of 24-h periods; hardness of control/dilution water and highest concentration at start of test
Endpoints	–	mortality and biomass (as a measure of growth); if multi-concentration test, 7-day LC50, plus 7-day ICp for decreased biomass (see Section 4.5)
Reference toxicant	–	sodium chloride, phenol and/or zinc; 7-day test for LC50 and ICp (for decreased biomass); perform within 14 days of the start of the definitive test, following the same method and procedures used for that test; if test organisms are imported,

		test fish from this batch for tolerance to the reference toxicant using the same procedure as the definitive test
Test validity	–	test to be declared invalid if, for the controls, the combined (i.e., for all replicates) and cumulative (over time) incidence of any mortalities, moribund fish, or fish showing loss of equilibrium or other signs of clearly atypical swimming behaviour, is >20% at any period of observation including that at test end; results also invalid if the average dry weight of surviving control fish is not >250 μg at test end
Chemicals		
Solvents	–	to be used only in special circumstances; maximum concentration, 0.1 mL/L
Concentration	–	recommended measurements are at beginning and end of 24-h renewal periods, in high, medium, and low strengths and control(s); if concentrations decline 20%, re- test with more frequent renewal or flow-through methods
Control/dilution water	–	as specified and/or depending on intent; reconstituted water if high degree of standardization required; receiving water if concerned with local toxic impact; otherwise, uncontaminated laboratory water
Effluents, Leachates, and Elutriates		
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; daily volumes of 8-10 L are normally adequate
Transport and storage	–	if warm (>7 ºC), must cool to 1 to 7 ºC with regular ice (not dry ice) or frozen gel packs upon collection; transport in the dark at 1 to 7 ºC (preferably 4 ± 2 ºC) using regular ice or frozen gel packs as necessary; sample must not freeze during transit or storage; store in the dark at 4 ± 2 ºC; 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 or "upstream" receiving water for monitoring and compliance
High solids	–	second test with filtered sample is an option, to assess 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

---

* Special situations (e.g., volatile or unstable chemicals in solution) might require the use of flow-through tests.
** For this option, there must be an additional control using a separate water supply (natural or reconstituted) that has been shown by the testing laboratory to routinely achieve valid test results in previous 7-day tests for growth and survival of larval fathead minnows.
*** If pH is outside this range or below pH 7.0, results might reflect toxicity due to biologically adverse pH.

arbitrary or prescribed concentration of chemical. Use of controls would follow the same rationale as multi-concentration tests. Single-concentration tests are not specifically described here, but procedures are evident, and all items apply except for testing a single concentration and a control.

Each *treatment* including the control(s) must include a minimum of three *replicate test vessels* if point-estimates are intended (i.e., LC50 and ICp; see Section 4.5), and four replicates per treatment are recommended[14]. The test must start with an equal number of replicates for each concentration including the controls. If there is accidental loss of a replicate during the test, unbalanced sets of results can be analyzed with less power.

When receiving water from upstream of the discharge is used as control/dilution water (see Sections 5.3, 6.3, and 7.3), a second control solution must be prepared using a supply (source) of laboratory water shown previously by the testing laboratory to routinely enable valid test results in a 7-day test for survival and growth of larval fathead minnows[15]. Unless the testing laboratory is importing test organisms rather than maintaining cultures of fathead minnows at their facility, the laboratory water in which fish were kept for production of embryos, and in which the embryos hatched into larvae, must be used for this purpose. In instances where the testing laboratory imports the test organisms, an alternate source of uncontaminated laboratory water shown previously by that laboratory to enable valid test results may be used as the second control solution.

Upstream receiving water is considered unsuitable as control/dilution water if it cannot meet the criteria for a valid test (see Section 4.3). In such cases, the laboratory water used for breeding should normally be used as the control/dilution water. The investigator might choose to attempt to acclimate the breeding stock to *upstream water* beforehand, in which instance any larvae generated would be held in this water until used as test organisms.

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. Temperatures must be adjusted as required to 25 ± 1°C. It might be necessary to adjust the pH of the sample of

[14] It has been estimated that increasing the number of replicates from two to three in this test increased the amount of work by only 15%, but resulted in a major improvement in variation and sensitivity of the test. The addition of the third replicate was considered "worth the investment" (API, 1988). An increase from three to four replicates resulted in only a small additional decrease in variability; increased effort would not be warranted on that account, but use of four replicates is recommended here because of the infrequent but possible need in statistical analysis.

[15] 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 effects 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 of toxic effects using each control/dilution water to prepare a series of test concentrations.

test material or the test solutions (see Section 4.3.2), or to provide preliminary aeration of the test solutions (Section 4.3.1).

4.2 Beginning the Test

At least ten fish per test vessel (replicate) must be used, with an equal number in each vessel. A minimum of three replicates per treatment (concentration), including the control treatment(s), must be included in each test, and four replicates per treatment are recommended. Additionally, for a multi-concentration test intended to determine an ICp for growth (determined as *biomass*) inhibition by regression analyses (see Section 4.5.1), a minimum of seven concentrations plus the control(s) must be included in the test, and more treatments (i.e., at least eight concentrations plus the control) are recommended.

A test with eight concentrations plus a control, and with three replicates per treatment (concentration), requires at least 270 fish. The larvae should, if possible, represent three or more different spawnings (Section 2.3.1), and must be all from the same *batch*. Each concentration including the control must start the test with the same number of replicates (at least three; see Section 4.1).

Larvae must be ≤24 h old at the start of the test, and must also have an inflated swim bladder (see Figure 3). The batch of larvae to be used in a test should not be fed until after they have been transferred to the test vessels (see Section 4.3.3).

There is some indication that variation in results might be caused by age differences within the 24-h collection period, perhaps because very young larvae with undeveloped swim bladders might be less tolerant of handling. Accordingly, this possible source of variation is reduced by selecting larvae with swim bladders already developed (Figure 3), and using them for the test. This would normally mean that the larvae would have been hatched for between ~7 and 24 h (Figure 3). Alternatively, frequent inspection of the tiles and associated breeding aquaria would allow selection of larvae that had been hatched for ≥7 and ≤24 h.

Since it is possible that larvae from a given spawning might be particularly sensitive or particularly tolerant, an attempt must be made to achieve "homogeneity of the experimental units", i.e., to avoid any differences among vessels that are related to the spawning. There are two ways to achieve that. They are both valid and are suitable for the same statistical analyses of results (personal communication, Prof. J.J. Hubert, Dept. of Mathematics and Statistics, Univ. of Guelph, Guelph, Ontario). In the first method, larvae from different parents or spawnings which have been held separately may be combined (pooled) before assigning larvae to vessels. In the second method, larvae from a given spawning may be divided evenly among all replicates of all concentrations, then larvae from other spawnings are similiarly allotted evenly to all vessels, to make up the full number of 10. The second method requires more care and effort in culturing and handling. It should, however, reduce the "noise" of the variation between replicates at the same concentration and avoid the chance that exists in the first method, of getting high proportions of weak larvae or strong larvae in a particular vessel, assuming that such spawning-related variation exists. This latter method is recommended by Neville (1989).

With either of these methods, an attempt to achieve homogeneity must be made by assigning fish to vessels in the following manner. Larvae should be counted into a series of small beakers or plastic cups, introducing one, two, or three larvae at a

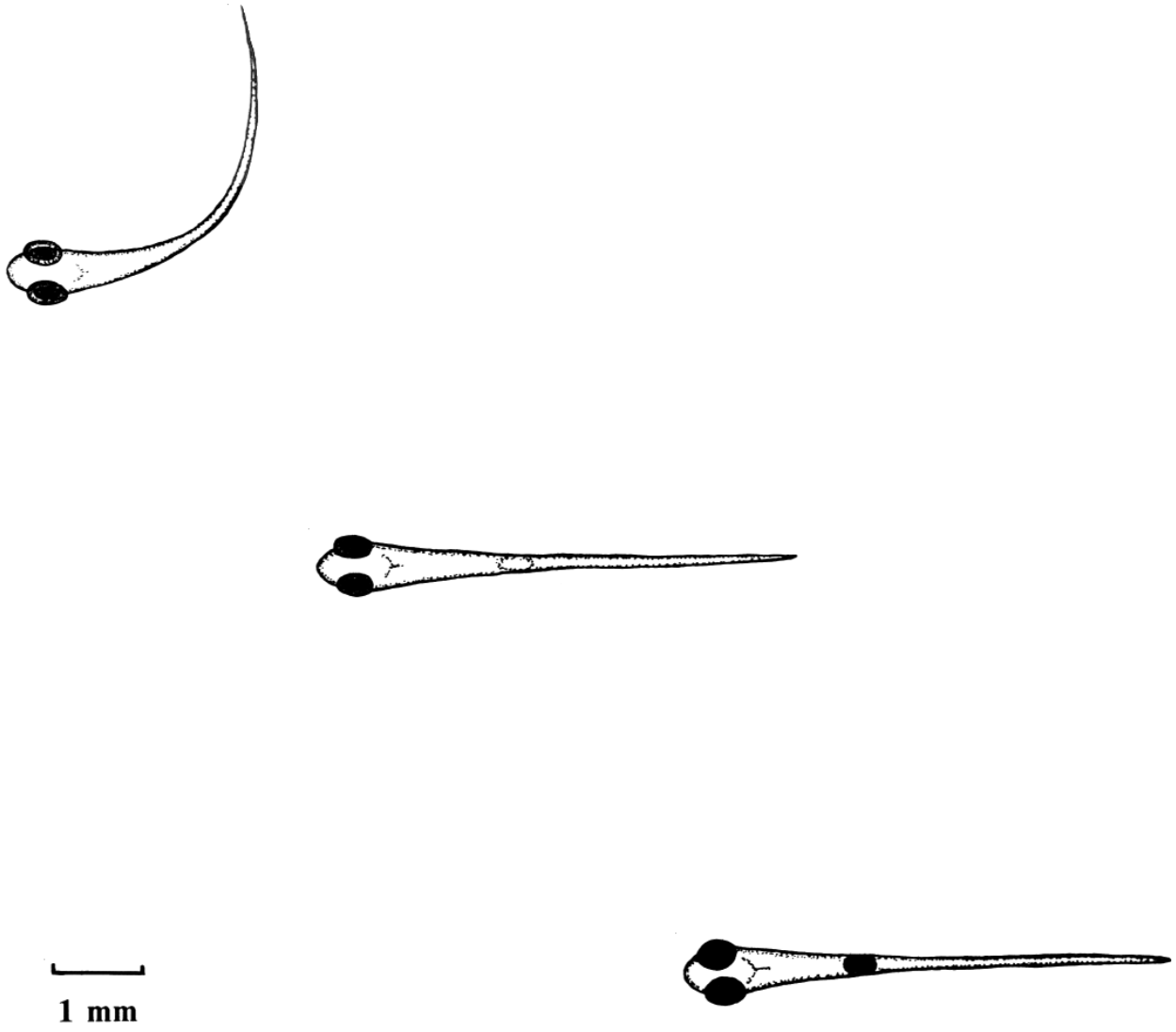


Figure 3 **Larvae of Fathead Minnows as They Appear if Viewed Dorsally**
(original drawings from specimens, by C.M. Neville and M.A. White)

On the upper left is a larva that has just hatched. The eyes are the most conspicuous feature. In the centre is a larva which hatched three or four hours earlier, and has not yet inflated its swim bladder. It might swim rapidly on the bottom of the container. On the lower right is a larvae with an inflated swim bladder, which might be ~7- to 24-h old. It can swim at any depth in the water.

time into each beaker in rotation, until the desired total numbers are attained in all. Fish appearing abnormal in any way must not be selected for the test. Fish should be moved by means of a large-bore pipette with rubber bulb, and any fish injured or possibly injured during transfer must be discarded. The amount of culture water carried over to the test vessels, with the fish in the pipette, must be minimal.

In addition to these procedures, there must be formal random assignment of each group of ten or more larvae (i.e., those in the transfer vessels) to each test vessel. The group of replicate vessels representing a particular treatment (e.g., a specific test concentration) must also be in randomized positions in the water bath or other temperature-control facility. Each test vessel must be clearly coded or labelled to identify the test material or substance and concentration being tested, and the date and time of starting. Temperature, dissolved oxygen, and pH levels in the vessels should be checked and adjusted, if required/permitted, to acceptable levels (Section 4.3, including 4.3.1 and 4.3.2) before introducing fish. As a check on test concentrations, it is recommended that conductivity be measured in each new preparation of test solution, before dispensing it to the test vessels.

4.3 Test Conditions and Validity Criteria

This is a 7-day test with replacement of solutions at 24-h intervals[16]. Fish are fed brine shrimp.

Sample/solution temperature must be 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. Each day of the test, the mean temperature determined for all fresh and aged test solutions for which temperature is measured must be 25 ± 1°C, with extreme fluctuations within the range 23 to 27°C. Temperature must be determined by measurements in representative vessels (i.e., in at least the high, medium, and low concentrations plus control solutions if a multi-concentration test). Measurements must be made at the beginning and end of each 24-h period of exposure, in both the fresh test solution and the used solution just before it is changed, or just after it has been changed[17].

The test must be declared invalid and its results unacceptable if, for the (laboratory) control solutions, the combined (i.e., for all replicates) and cumulative (over time) incidence of any mortalities, moribund fish, or fish showing loss of equilibrium or other signs of clearly atypical swimming behaviour, is >20%[18]. Should this occur at any time during a test, the test must be terminated at that time. The test is also not valid and its results unacceptable if the average dry weight per surviving control larvae does not equal or exceed 250 µg at the end of the test[19].

[16] Special situations (e.g., volatile or unstable chemicals in solution) require more frequent renewal of solutions, the use of *flow-through* tests, or modified duration of the test.

[17] Although measurements in the old solution, after organisms have been moved to the new solution, are theoretically less relevant, there is a major advantage in using this approach, since no damage can be done to the fish larvae by the measuring device. The likelihood of damage to the organisms might not be great for a thermometer, but is more likely for oxygen or pH probes which are moved around in the water.

[18] In a ten-laboratory round-robin comparison, average mortality in controls was 6%, and mortality was 20% or greater in only 16 of 270 individual control vessels (API, 1988).

[19] Larval fathead minnows can be expected to average about 90 µg at the start of a test (API, 1988). Tests with

4.3.1 Dissolved Oxygen and Aeration

If (and only if) the measured dissolved oxygen is <40% or >100% of air saturation in one or more test solutions when they have been freshly prepared, each test solution should be pre-aerated before the fish larvae are exposed to it. To achieve this, oil-free compressed air should be dispensed through airline tubing and a disposable plastic or glass tube of small aperture (e.g., capillary tubing or a pipette with an Eppendorf tip, with an opening of about 0.5 mm). The 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)[20]. 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).

Dissolved oxygen must be recorded at the beginning of each 24-h period in representative concentrations of the freshly prepared test solutions including the highest, which must again meet the requirements in the preceding paragraph. Measurements must also be made in representative concentrations at the end of each 24-h period, to establish the extent of oxygen depletion before the solutions are changed[21] (see Section 4.4).

Oxygen in the vessels should not fall below 40% of saturation (3.3 mg/L at 25°C). If it does, the investigator should be aware that the test is not measuring the toxic quality, *per se*, of the material or substance being tested. Rather, such a test would measure the total effect of the material (e.g., effluent) or substance (chemical) including its deoxygenating influence[22]. Potential problems with dissolved oxygen will be foretold by the initial measurements, and in such a case a running check on oxygen concentrations is required. The required use of

---

good procedure should obtain a final average dry weight of 350 µg for control fish in soft water (hardness ≤50 mg/L), an average of 500 µg in water that is hard or moderately hard (hardness ≥130 mg/L), and proportional weights for the middle range of hardness. Measuring a statistically significant difference becomes more difficult with lower growth, and thus the test becomes less sensitive. On the basis of experience of Canadian and U.S. workers, an average dry weight of 250 µg or greater for control fish is a suitable requirement for considering that the test is valid.

[20] Aeration can strip volatile chemicals from solution or can increase the rate of chemical oxidation and degradation to other substances. However, aeration of test solutions before fish exposure might be necessary due to the oxygen demand of the test material (e.g., oxygen depleted in the sample during storage). Aeration also assists in re-mixing the test solution. If it is necessary to aerate any test solution, *all* solutions are to be aerated in the manner stipulated in Section 4.3.1.

[21] DO measurements may be made on a test solution after it has been removed from the test vessels by siphoning that solution into a sample bottle, or other means that does not aerate it. This is allowed in order to avoid damage to the larvae (see footnote for temperature measurement in Section 4.3).

[22] It should be realized that the lower limit of 40% saturation (3.3 mg/L) for dissolved oxygen in test solutions is an arbitrary one, and that oxygen levels above that value are also stressful to the fish. Growth of larval fathead minnows is reduced at 5 mg/L compared to growth at 7.2 mg/L (Brungs, 1971a). Any reduction below saturation, in fact, results in some metabolic loading of fish and decreases their performance (Doudoroff and Shumway, 1970). Thus at oxygen values above the limit of 3.3 mg/L, stress from low oxygen might be expected to interact with any stress from toxicants, and this will be measured as part of the effect of the sample, be it effluent or other test material or substance. Such interaction has been accepted in this test procedure, as part of the impact being measured.

oxygen-saturated control/dilution water and daily renewal of test solutions will, in most instances, keep dissolved oxygen above the levels that severely stress the larvae and have a major influence on test results. If the test material or substance has a strong oxygen demand, more frequent renewal of test solutions might be required to maintain DO at ≥40% of saturation. If frequent renewal is not successful, and the objectives of the test require DO ≥40% saturation in order to measure toxicity *per se*, then each test vessel should be aerated. For this purpose, air should be delivered through a disposable glass or plastic pipette with a narrow-bore tip (e.g., 0.5 mm ID) at a rate which does not exceed 100 bubbles/min. Any aeration of solutions prior to ("pre-aeration"; see Section 4.1) or during the test must be at a minimal and controlled rate. Any aeration during testing must be reported, including the rate (Section 8).

Alternatively, the objective of the test might require that oxygen demand be included as part of the measurement of total effects of the sample, in which case the daily renewal frequency would be retained, and no aeration would be used.

4.3.2 pH

The pH must be measured in the control, high, medium, and low concentrations at the beginning of the test, before fish are added. The pH must also be measured in representative vessels at the beginning and end of each 24-h period, i.e., in the fresh test solution and the used solution just before it is changed, or just after it has been changed (see earlier footnote 17, as well as the paragraph on physicochemical measurements in Section 4.4).

Toxicity tests should normally be carried out without adjustments of pH. However, if the sample 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 chemical(s) rather than the deleterious or modifying effect of pH, then the pH of the solutions or sample should be adjusted before adding fish, or a second, pH-adjusted test should be conducted concurrently using a portion of the sample[23]. For this second test,

---

[23] The usual justification for not adjusting sample/solution pH is that pH might have a strong influence on the toxicity of a chemical, or substances in a wastewater. Thus, for the (generally) low concentrations of waste found in receiving water after dilution, any change from the natural pH, with concomitant modification of toxicity, should be accepted as part of the pollution "package". That leads to the rationale that the pH should not be adjusted in tests, and that is the requirement for the procedure to be followed in most instances, if test solutions are in the pH range 6.5 to 8.5.

Some chemicals and wastewaters, however, will create levels of pH that have appreciable direct sublethal or lethal effects at the high concentrations used in tests. That is especially true in monitoring or *compliance* tests with full-strength effluent. It seems unlikely that an investigator would be primarily interested in ascertaining whether extreme pH in full-strength effluent had a toxic effect on fish, because such a pH would be unrepresentative of what would prevail after even moderate dilution in receiving water. If pH *per se* were of primary interest, a toxicity test would not seem necessary, because the toxicity of extreme pH is well-documented, and any danger could be much more economically assessed by a simple physicochemical measurement. The investigator would usually wish to know if toxic substances were present in a wastewater, and determining that would require that any masking by toxic action of pH should be eliminated. The rationale leads to the use of pH-adjusted samples or test solutions, where appropriate. The rationale is exactly parallel to standardizing the temperature and dissolved oxygen in the toxicity tests, even if the wastewater itself were 90°C or had low (e.g., <2mg/L) dissolved oxygen, either of which would have a rapid toxic effect in itself. Adjusting the pH before testing, or running a second pH-adjusted test, are options in the procedure described herein, and the exact method for adjustment depends on the objectives of the test.

Investigators using the present test with fathead minnows should be aware that major effects on

the initial pH of the sample, or of each test solution[24] could, depending on objectives, be neutralized (adjusted to pH 7.0) or adjusted to within ± 0.5 pH units of that of the control/dilution water, before fish 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 test sample has/causes pH <6.5), or downwards to pH 8.0 to 8.5 (if sample has/causes pH >8.5). Solutions of hydrochloric acid (HCl) or sodium hydroxide (NaOH) at strengths ≤*N* 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.

In some circumstances it could be desired to carry out the most sensitive test possible for detecting toxic chemicals, rather than including pH as part of the total effect of a chemical, effluent, elutriate, or leachate. In such a case, depressing effects of low pH on growth and survival of larvae should be eliminated by raising pH of test solutions as necessary, to ≥6.8 or preferably ≥7.0 (see preceding footnote 8 in Section 2.3.7).

Abernethy and Westlake (1989) provide useful guidelines for adjusting pH. Aliquots of samples or test solutions receiving pH-adjustment[24] 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 the test is initiated, the pH of each test solution is monitored. (Section 4.4) but not adjusted.

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 as one of a number of techniques (e.g., oxidation, filtration, air stripping, addition of a chelating agent, etc.) for characterizing sample toxicity. Mount and Anderson-Carnahan (1988) list pH adjustment as one of nine "Toxicty Identification Evaluation" (TIE) techniques which, when performed with an acutely toxic aqueous sample, provide the investigator with a useful method assessing the physical/chemical nature of the toxicant(s) and its (their) susceptibility to detoxification.

4.3.3 Feeding

Larval fish should not be fed until they are placed in the test vessels and the test is started. Immediately after starting the test, the fish in each test vessel are fed live brine shrimp nauplii (Section 2.3.9 and Appendix C). The objective is to keep live brine shrimp available for the fish during the daylight hours, but not to have a large excess of these crustaceans in the test vessels. This is because brine shrimp will sorb some of the toxic material, and because they die after some time in fresh water (and the test solutions), which could depress the dissolved oxygen level in each test vessel.

Groups of ten fish should be fed at least 1500 nauplii per day, and preferably 2250 nauplii/d. This daily quantity of food should be apportioned over at least two feedings each day, starting in the early

---

reproduction and larval survival have been found at about pH 6, with marginal effects apparent at pH 6.6. As discussed elsewhere (see earlier footnote 8 in Section 2.3.7), pH≥6.8 and preferably ≥ 7.0 are recommended as a lower limit for eliminating effects of pH *per se*.

[24] 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 sample(s) of receiving water normally adjust an aliquot of the undiluted sample, before preparing the test concentrations.

morning. Levels above that do not improve growth (Appendix C; Silberhorn, 1989). For more fish larvae per container (i.e., due to the accidental addition of more than ten fish per test vessel), or fewer fish (due to mortalities or inadvertent losses), numbers of nauplii should be appropriately (i.e., proportionately) adjusted.

There is no feeding during the final 12 hours of the test, in order to avoid weighing food in the gut of the fish.

4.3.4 Renewal of Test Solutions

This a static-replacement test and the solutions are to be almost completely (80%) renewed at 24-h intervals after the start of the test. Siphoning or use of a pipette is the usual procedure. It is desirable to replace solutions in random order across the replicates within a concentration, particularly if the material or substance being tested is difficult to keep mixed because some of the contents settle.

During renewal, dead brine shrimp and other detritus on the bottom of each chamber should first be removed. Then the remaining solution is drawn down to a height of 7 to 10 mm (sufficient to allow the fish to continue swimming). Depending on the shape of vessel, tilting might be required to achieve the 80% removal and the minimum height. New test solution is slowly added to make up the original total volume of test solution in each vessel. The entire procedure must be done cautiously to avoid any injury to the fish; working on a light-table assists in making the larval fish visible. In any case, the solution that is siphoned out or otherwise removed should go into a white tray, so that an inspection can be made for larvae that have been accidentally removed. Such fish are likely to be injured and should be discarded and noted on the benchsheet as accidentally removed at that time; the results of the test should be analyzed as if the discarded fish had not been present.

The siphoning procedure is much easier to accomplish without accidentally removing a fish, if the vessel is of the style with a screened sump. If containers with screened bottoms are used, siphoning of debris is done, then the container is quickly but gently removed from the vessel of old test solution to a vessel that is ready with new solution. The momentary removal from liquid does no apparent harm to the larvae, if done delicately. Other techniques are allowed if the apparatus is made of nontoxic materials mentioned in Section 3.3, the required replacement of solution is done, and the control organisms show acceptable growth (see the minimum growth requirement specified in Section 4.3, along with footnote 19). For example, a technique of "submerged pouring" of the larvae from a used screen-bottomed container to a new one was developed by Parrott (1988), allowing the fish to be held in containers that were clean at the start of each 24-h period.

4.4 Test Observations and Measurements

The mortality in each test vessel must be determined and recorded, at intervals of 24 hours from the start of exposure until the end of the test at 7 days of exposure. Fish are considered dead when they fail to show any swimming activity, even when stimulated by a gentle jet of test solution from a wide-mouthed pipette. Any dead fish that are seen should be discarded. Often, dead larval fish will simply not be found upon inspection, because they decompose quickly or cannot be distinguished from debris on the bottom.

For the control treatment(s) only, daily observations must be made and recorded

which show the combined and cumulative (over time) number of larval fish in each replicate that are found to be dead, dying, exhibiting loss of equilibrium, or displaying overt signs of atypical swimming behaviour. Optionally (e.g., when using this biological test method for research purposes), the investigator(s) might wish to make daily observations and records of the combined number of surviving fish in each replicate and treatment that are moribund, show a loss of equilibrium, and/or display clearly abnormal swimming behaviour. For a multi-concentration test, such observations could be used for determining an ECp (e.g., EC20 or EC50) for impaired swimming ability (see Section 4 in EC, 2005 for guidance with respect to this statistical determination).

At the end of the 7-d exposure, living fish are counted, dried, and weighed. For *each vessel* of test solution (i.e., each replicate in a treatment, including one or more control treatments), dry weight is determined for the fish as a group. Larvae may be transferred individually from the test chamber to a rinse of clean dilution water, to avoid including any debris in the weight. The rinse should be brief. After rinsing, the fish can be netted together and transferred to the weighing trays by means of fine forceps, making sure that no parts of the fish are broken off. Alternative techniques may be used with advantage[25].

Fish should be dried immediately in small, tared and numbered weighing-boats, at either 100°C for 6 h, or at 60°C for 24 h[26]. Upon removal from the oven, the boats must be moved immediately to a desiccator. Thereafter, the boats should be individually and randomly removed from the desiccator, and weighed on a balance that measures consistently to 10 µg. The fish take up water vapour readily, so rapid weighing and standard timing among boats is necessary. At the same time, care must be taken because rapid movement can blow any unattached larvae out of the weighing dish. Trays should be removed in random order for weighing, and the first one weighed should be replaced in the desiccator and weighed again at the end, as a check on gain of water by the last trays weighed (see preceding footnote 25). The change should not be >5%; if it is, redrying of the trays for <2 h and re-weighing might be carried out. A few weighing boats should be tared, dried, and weighed without fish, and results should conform to the laboratory's quality control standards.

Physicochemical measurements during the test must include temperature, dissolved oxygen, and pH in representative test vessels at the beginning and end of each 24-h period of exposure (Section 4.3). Measurements must be made in both the fresh test solution and the used solution just before or after it is changed (see preceding footnote 17 in Section 4.3). For a multi-concentration test, these measurements must include at least the high, medium, and low concentrations plus control solutions. Conductivity should also be measured in the test solutions, at least at the start of the 24-h periods. Hardness of the control/dilution water and, as a minimum, the

[25] If they are demonstrated to be satisfactory, equivalent methods may be used for collecting the fish at the end of the test. One method successfully employed in a Canadian laboratory is to pour the contents of a test vessel onto a mesh of 1 mm pore size. Very hot water is immediately poured onto the mesh to kill and "fix" the fish, and they are then easy to handle with forceps. They must still be rinsed.

[26] Alternatively, fish may be preserved in 70% ethanol at the end of the test, and within two weeks rinsed in *distilled water* before drying and weighing in the same way as described for non-preserved fish.

highest test concentration, should be measured and reported for the start of the test.

4.5 Test Endpoints and Calculations

The endpoints for this 7-day test are based on the adverse effects of test materials or substances on the survival and growth of larval fathead minnows. There are two biological endpoints for the test, the first being based on increased mortality of the contaminant-exposed test organisms. The other endpoint is based on a reduction in the *biomass* of the larvae due to their contaminant exposure.

At the end of the exposure, the number of fish alive and number dead are recorded for each replicate of the control and the various concentrations of the test material or substance. Fish in any replicate that were accidentally killed or accidentally removed during the daily renewal of test solutions (Section 4.3.4) should be deducted from the initial number of larvae for that replicate at the start of the test, as if they had not been in the test.

The average dry weight per fish is calculated for the surviving fish in each vessel (i.e., in each replicate of each concentration and the control). In keeping with USEPA (2002; see Sections 11.10.9 and 11.13.3.1), the average dry weight per fish must be calculated as a measurement of *biomass*. This is achieved by dividing the total (dry) weight of fish surviving to test end in each vessel, by the number of larvae that were placed in that vessel at the start of the test (presumably, ten fish). When making this calculation for biomass, fish in any replicate that were accidentally killed or accidentally removed during the daily renewal of test solutions (Section 4.3.4) should be deducted from the initial number of larvae for that replicate at the start of the test, as if they had not been in the test.

The "biomass" endpoint represents a combination of sublethal effect (i.e., reduced total dry weight of surviving fish in each replicate at test end) and mortality. Since it integrates effects on survival with those on growth (dry weight of surviving fish), the "biomass" endpoint has potential to show a greater sensitivity to toxic samples than the growth endpoint based on sublethal effect alone (i.e., as determined according to the first edition of Environment Canada's 7-day test of larval growth and survival using fathead minnows; EC, 1992)[27]. Section 8.2 in Environment Canada's guidance document on statistical methods for environmental toxicity tests (EC, 2005) describes the use of this endpoint as one of three options for measuring growth as a quantitative sublethal effect.

Using final biomass as the statistical endpoint, a value of zero would be assigned if all fish died in a replicate (EC, 2005).

As is stated in Section 4.3, the test is invalid if, for the (laboratory) control solutions, the combined (i.e., for all replicates) and cumulative (over time) incidence of any mortalities, moribund fish, or fish showing loss of equilibrium or other signs of clearly atypical swimming behaviour, is >20%. The test is also invalid if the combined (for all replicates) average final dry weight of the surviving control fish does not attain 250 µg when the fish are dried and weighed (USEPA, 2002). With reasonable procedures, it should not be difficult to attain average final dry

[27] An analysis of data derived for 7-day toxicity tests with larval fathead minnows exposed to more than 100 samples of Canadian mining effluent from various sources showed appreciably greater test sensitivity overall, when growth was calculated using the biomass endpoint rather than the endpoint based on total dry weight of surviving fish alone (as per EC, 1992; Holtze, 2007).

weights of 350 µg in soft water and 500 µg in hard water[28].

4.5.1 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 larval fathead minnows, and (ii) an ICp[29],[30] and its 95% confidence limits for *biomass* (i.e., total dry weight of surviving larvae at test end, divided by the initial number of larvae placed in that replicate at the start of the test). 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 for biomass 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.[31] 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).

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

[28] Control weights averaging greater than the required value of 250 µg, but less than the desirable values of 350 to 500 µg, might indicate that feeding or some other condition was less than favourable, although results of the test should still provide useful information (see preceding Section 4.3 including its footnote 19).

[29] 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%.

[30] 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 biomass data derived from a multi-concentration test using larval fathead minnows.

[31] 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.

data. Finally, the model with the best fit[32] 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.

If all fish in a particular replicate died during the test, a value of zero weight (and zero biomass) would be assigned to that replicate. If any larvae had been accidentally lost or damaged during the exposure, they would be deducted from the initial number of larvae in that replicate when calculating its biomass (as per "Option 3" described in Section 8.2 of EC, 2005).

With some highly toxic test materials or substances, it is possible to record zero surviving larvae in all of the 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 surviving larvae in all test replicates must be removed before performing 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 (± SD) percent mortality for the larvae, at the end of the test; and (ii) the (cumulative) mean (± SD) biomass of live larvae at the end of the test. Section 8.1 lists these and other minimum requirements for a test-specific report.

4.5.2 Single-Concentration Tests

In single-concentration tests, the response in the test concentration is compared with the control response.[33] If mortality (a *quantal* endpoint) is assessed at only one site and a

[32] 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).

[33] 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.

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 biomass (a *quantitative* endpoint) is assessed at a single test site and control site, a t-test[34] 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 (± SD) percent mortality for the larval fathead minnows for each treatment, at the end of the test; and (ii) the (cumulative) mean (± SD) biomass, for each treatment, at the end of the test. Section 8.1 provides these and other minimum requirements for a test-specific report.

[34] 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).

4.6 Reference Toxicant

The routine use of a *reference toxicant* or toxicants is required to assess, under standardized conditions, the relative sensitivity of the group of fish that are used, and the precision and reliablility of data produced by the laboratory for that/those reference toxicants (Environment Canada, 1990c). Sensitivity of young (≤24-h post-hatch) larval fathead minnows to the reference toxicant(s) must be determined by starting a *reference toxicity test* with this life stage within 14 days before or after the date that the toxicity test is initiated, or by performing this test concurrently with the definitive one. The same stock of brood animals should be used to generate the larvae required for tests with both the reference toxicant and sample. When a reference toxicity test is performed at the same time as the definitive toxicity test, the same *batch* of test organisms must be used for each of these two tests. The reference toxicant test must be performed under the same experimental conditions as those used with the test sample(s).

If test organisms are imported to the testing laboratory, rather than selecting them from an in-house culture which is the recommended approach (see Section 2.2), a portion of the larvae from each batch of imported organisms must be tested for its tolerance to the reference toxicant(s). The reference toxicant test must be performed under the same experimental conditions as those used with the test sample(s). Testing must be performed at the same time as the definitive test.

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-response curve for fathead minnows;
- known influence of pH on toxicity of chemical to test organism; and
- known influence of water hardness on toxicity of chemical to fathead minnows.

One or more of the following three chemicals (reagent grade) should be used as the reference toxicant(s) for this test: sodium chloride, phenol, and/or zinc (prepared using zinc sulphate). Fish 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 biomass), for at least one of these chemicals. The tests should use the control/dilution water that is customary at the laboratory, or reconstituted water if a greater degree of standardization is desired.[35]

[35] Because the pH, hardness, and other characteristics of the dilution water can markedly influence the toxicity of the test substance, use of a standard reconstituted water provides results that can be compared in a meaningful way with results from other laboratories. Soft reconstituted water is recommended for this purpose. This water is prepared by adding the following quantities of reagent-grade salts to carbon- filtered, deionized water, or glass-distilled water (ASTM, 1980):

	salt	mg
Sodium bicarbonate	$NaHCO_3$	48
Calcium sulphate	$CaSO_4 \cdot 2H_2O$	30
Magnesium sulphate	$MgSO_4$	30
Potassium chloride	KCl	2

The reconstituted water should be aged several days (USEPA, 1985) and intensely aerated before use. It can be expected to have a total hardness of 40 to 48 mg/L and a pH of 7.4 ± 0.2.

Test conditions and procedures for tests with reference toxicants are to be consistent and as described in this document.

Once sufficient data are available (EC, 1990c), 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 biomass 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; EC, 1990c).

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 2.3.11) 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 larval fathead minnows.

Use of warning limits does not necessarily indicate that a laboratory is generating consistent results. A laboratory that produced extremely variable data for a reference toxicant would have wide warning limits; a new datum point could be within the warning limits but still represent undesirable variation in results obtained in tests. A coefficient of variation of 20% or 30% is tentatively suggested as a suitable limit by Environment Canada (1990c). That seems a reasonable range since round-robin tests in the San Francisco area showed a coefficient of variation between laboratories of 22% when calculated on a logarithmic basis (CV = 31% when calculated on an arithmetic basis; Anderson and Norberg-King, 1991). However, establishing a limit for allowable variation of results for testing reference toxicants would require more data on the reproducibility that can be achieved in Canadian laboratories for the seven-day test with fathead minnows.

Stock solutions of phenol should be made up on the day of use. Zinc sulphate (usually $ZnSO4 \cdot 7H2O$, molecular weight 4.398 times that of 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. Concentrations of zinc should be expressed as mg Zn++/L. Concentrations of sodium chloride should be expressed as the weight of the total salt (NaC1) in the water (g/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 (i.e., outside warning limits). If stored, sample aliquots must be held in the dark at 4 ± 2 °C. Both zinc and phenol 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 mean measured concentrations if they are appreciably (i.e., ≥20%) different from nominal ones and if the accuracy of the chemical analyses is reliable.

4.7 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 continuity; and analyzed as soon as possible after collection. Persons responsible for conducting the test and reporting the findings must maintain continuity of evidence for court proceedings (McCaffrey, 1979), and ensure the integrity of the test results.

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. Where aqueous solubility 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.[36] 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 larval fathead minnows is useful in predicting chemical concentrations appropriate for the chronic (7-day) toxicity test. The results of a 48-h *static* LC50 (see Section 4.5 and Appendix E), conducted at 25 ± 1 °C using the control/dilution water intended for the chronic test, will provide this information. Larval fish, cultured under conditions similar or identical to those used for organisms to be employed in the 7-day 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 are usually prepared by adding aliquots of a stock solution made up in control/dilution water. Alternatively, for strong solutions or large volumes, weighed (analytical balance) quantities of chemical may be added to control/dilution water to give the nominal strengths for testing. If stock solutions are used, the concentration and stability of the test chemical in the solution should be determined before the test. Stock solutions subject to photolysis should be shielded from light. Unstable stock solutions must be prepared

[36] 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 well-ventilated facility, need for solvent, etc.). Information regarding chemical solubility and stability in fresh water will also be useful in interpreting test results.

daily or as frequently as is necessary to maintain consistent 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.[37] Organic solvents, *emulsifiers*, or *dispersants* should not be used to increase chemical solubility except in instances where they 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, 1985): dimethyl formamide, triethylene glycol, methanol, ethanol, and acetone.

5.3 Control/Dilution Water

Control/dilution water may be one of the following: reconstituted water; the freshwater source in which the adults were cultured and the larvae hatched (natural groundwater, surface water, or dechlorinated municipal water as a last choice); an alternate source of uncontaminated natural water shown previously by the testing laboratory to be suitable for 7-day tests of larval growth and survival using fathead minnows; or a particular sample of receiving water if there is special interest in a local situation. The choice of control/dilution water depends upon the intent of the test.

If a high degree of standardization is required (e.g., the measured toxicity of a chemical is to be assessed relative to values derived elsewhere, for this and/or other chemicals), soft reconstituted water (hardness 40 to 48 mg/L as $CaCO_3$, pH 7.2 to 7.5) should be prepared and used for all dilutions and as the control water (USEPA, 1985). Guidance on preparing this water is provided in Section 4.6 (see footnote 35).

If 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[38,39,40]. Examples of such situations

[37] 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 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.

[38] Contaminants already in the receiving water might add toxicity to that of the chemical (or wastewater; see Section 6.3) being tested. In such cases, uncontaminated dilution water (reconstituted, natural, or dechlorinated municipal) would give a more accurate estimate of the individual toxicity of the spill or spray, but not necessarily of the total effect on the site of interest.

If the intent of the test is to determine the effect of a specific chemical (or wastewater; see Section 6.3) on a specific receiving water, it does not matter if that receiving water modifies sample toxicity by the presence of additional toxicants, or conversely by the presence of substances that reduce toxic effects, such as humic acids. However, due to the possibility of toxic effects attributable to the "upstream" receiving water, the following must be included in any test that uses "upstream" water as the control/dilution water: (1) as a minimum, a second control using the laboratory's uncontaminated water supply that is normally used for 7-day tests of larval growth and survival using fathead minnows; and (2) as a maximum, another series of concentrations using this same water source as the diluent.

[39] While it would be desirable to acclimate the breeding fish, and hold the embryos in the receiving water

include appraisals of the toxic effect of chemical spills (real or potential) or intentional chemical applications (e.g., spraying of a pesticide) on a particular waterbody. If "upstream" water is used as control/dilution water, a separate control solution must be prepared using the laboratory water supply that is normally used for 7-day toxicity tests with fathead minnows and able to achieve valid test results on a routine basis[38].

The laboratory supply of uncontaminated natural water may also be used to appraise the toxic effect of a chemical on a particular receiving water, especially where the collection and use of receiving water is impractical. The laboratory's normal water supply might also be appropriate for use in other instances (e.g., preliminary or intra-laboratory assessment of chemical toxicity).

5.4 Test Observations and Measurements

In addition to the observations on toxicity described in Section 4.4, 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 periods 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 fish are exposed.[41] 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 periods on the first and last days of the test. These should be preserved, stored, and analyzed according to best proven methodologies available for determining the concentration of the particular chemical in aqueous solution.

If chemical measurements indicate that concentrations declined by more than 20% during the test, the toxicity of the chemical should be re-evaluated by a test in which solutions are renewed more frequently than

---

before using the larvae in a test with that water used for dilution and control, that is seldom feasible because of the need to transport large volumes of water. If tests were carried out near the site of interest, it might be feasible to use receiving water in the breeding aquaria for at least five days before embryos were selected, and to hold the embryos in receiving water until the larvae had hatched.

[40] 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 might be to seasonal means, or to values measured in the receiving water at a particular time. Adjustments may be made by methods mentioned in Section 2.3.4, including the addition of appropriate quantities and ratio of reagent-grade salts (ASTM, 1980; also given in Table 2 of Environment Canada, 1990b).

[41] Such analyses need not be undertaken in all instances, due to analytical limitations, cost, or previous technical data indicating chemical stability in solution under conditions similar to those in the test. Chemical analyses are particularly advisable if (USEPA, 1985): the test solutions are aerated; the test substance is volatile, insoluble, or precipitates out of solution; the test chemical is known to sorb to the material(s) from which the test vessels are constructed; or a *flow-through* system is used. Some situations (e.g., testing of pesticides for purposes of registration) might require the measurement of chemical concentrations in test solutions.

once a day. If necessary, a *flow-through* test could be considered although it requires special design to accommodate the small larvae (McKim, 1985).

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 test 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 the calculations, each test solution should be characterized by the geometric average measured concentration to which fish were 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 biomass (growth) (see Section 4.5).

If a solvent control is used, the test is rendered invalid if, for either the solvent control solutions or those comprised solely of untreated control water, the combined (i.e., for all replicates in the same treatment) and cumulative (over time) incidence of any mortalities, moribund fish, or fish showing loss of equilibrium or other signs of clearly atypical swimming behaviour, is >20%. The test is also invalid if, for either the solvent control or the untreated laboratory control, the combined (for all replicates of the same treatment) average final dry weight of the surviving control fish does not attain 250 µg when the fish are dried and weighed. Sections 4.3 and 4.5 provide the (identical) test validity criteria for the solutions of untreated control/dilution water included in any test involving solvent and a solvent control, which apply here as well.

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 at least three separate containers (i.e., three or more subsamples) upon collection or (in the case of elutriate) preparation. 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 and 6.

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. 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 and 6. 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.

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 sample volume of 60 to 80 L is adequate for an off-site multiple-concentration test and the associated routine sample analyses, using the

preceding approach #1. If approach #2 is followed, a per-sample volume (for each of the three samples required to perform the test) of 20 to 25 L should prove adequate in most instances. Greater volumes of effluent would of course be required if the same samples were to be used for other toxicity tests (e.g., a 7-day test with *Ceriodaphnia dubia* performed according to EC 2007). Lesser amounts are required for single-concentration tests (Section 4.5). Upon collection, each sample container must be filled, sealed, and labelled or coded. Labelling should include at least sample type, source, date and time of collection, and name of sampler(s). Unlabelled or uncoded containers arriving at the laboratory should not be tested. Nor should samples arriving in partially filled or unsealed containers be routinely tested, since volatile toxicants escape into the air space. However, if it is known that volatility is not a factor, such samples might be tested at the discretion of the investigator.

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 for samples of effluent or leachate.

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 4.3.1) 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). Such filtration could remove suspended solids that

are characteristic of the sample or subsample, and might otherwise contribute to part of the toxicity or modify the toxicity. In instances where concern exists regarding the effect of this filtration on sample toxicity, a second test should be conducted concurrently using an unfiltered portion of the sample or subsample.

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 a supply (source) of laboratory water shown previously by the testing laboratory to routinely enable valid test results in a 7-day test for survival and growth of larval fathead minnows, or a sample of the receiving water. Because results could be different for the two sources of water, the objectives of the test must be decided before a choice is made. Difficulties and costs associated with the collection and shipment of receiving-water samples for use as control/dilution water should also be considered.

The use of receiving water as the control/dilution water might be desirable in certain instances where site-specific information is required regarding the potential toxic impact of an effluent, leachate, or elutriate on a particular receiving water (see Section 5.3 including its associated footnotes 38-40 that apply equally here). 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. Surface water should be filtered to remove organisms, as described in Section 6.2.

If a sample of upstream receiving water is to be used as control/dilution water, a separate control solution must be prepared using the laboratory water supply that is normally used for performing 7-day toxicity tests with larval fathead minnows (i.e., culture water or other suitable laboratory water; see Section 4.1). The survival and growth (i.e., biomass) of fish (Section 4.5) in the laboratory control water must be compared to that in the sample of upstream receiving water.

Tests requiring a high degree of standardization may be undertaken using reconstituted water of a specified hardness (see the preceding footnote 35 in Section 4.6) as the control/dilution water. Situations where such use is appropriate include investigative studies intended to interrelate toxicity data for various effluent, leachate, or elutriate types and sources, derived from a number of test facilities or from a single facility where water quality is variable. In such instances, it is desirable to minimize any modifying influence due to differing dilution-water chemistry.

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 could be particularly stressful to larval fish, and can be *acutely lethal*, even to juvenile fish if present in sufficiently high strengths (e.g., ≥2000 mg/L, Noggle 1978; McLeay *et al*., 1987; Servizi *et al*., 1987; Hall and Hall, 1989). High concentrations of biological solids in certain types of treated effluent might also contribute to sample toxicity because of ammonia and/or nitrite production (Servizi and Gordon, 1986). An additional test should be conducted concurrently if there is concern 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

Mortality at 24-h intervals and dry weight at the end of the 7-day test must be determined, as described in Section 4.4.

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

For tests with highly coloured or opaque solutions, or for samples producing foam in the test vessel, tests should use the screen-bottomed vessels mentioned in Sections 3.3 and 4.3.4. Fish should be inspected by raising the vessel in its container of test solution until they can be seen. If necessary, the vessel could be moved briefly to a container of clear dilution water while observations were made on mortality. Experience indicates that the brief period of transfer between liquids and of immersion in a "clean" liquid does not damage the fish to any degree or noticeably affect the results of the toxicity test (Parrott, 1988).

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 must include, as a minimum, three replicates of the undiluted sample/subsamples (or a specified dilution thereof), and three 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 biomass (see Section 4.5).

Toxicity tests conducted for other purposes (e.g., determination of in-plant sources of toxicity, treatment effectiveness, or effects of process changes on toxicity) might, depending on the study objectives, be single-concentration tests (100% or an appropriate dilution, plus a control), or multiple-concentration tests. Single-concentration tests are often cost-effective for determining the presence or absence of measurable toxicity or as a method for screening a large number of samples for relative toxicity. Endpoints for these tests would again depend on the objectives of the undertaking, but could include arbitrary "pass" or "fail" ratings, or percentage mortality of fish at a suitable time period such as seven days. Items in Section 4.5 provide instructions that are relevant here, on statistical analysis and reporting of results from a set of tests on different samples, each tested at only one concentration. 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 (i.e., for 7 days using this biological test method).

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 containers should be agitated before pouring to ensure their homogeneity. Samples that might contain predators or competitors of larval fathead minnows should be filtered through a 60-µm plankton net before use. A second unfiltered test could be conducted concurrently if there is concern about changes in toxicity due to filtration. For instance, 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.

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 samples (see Section 5.3 including its associated footnotes 38-40 that apply equally here). This control/dilution water should be collected as close as possible to the contaminant source(s) of concern, but upstream of the zone of influence or outside it. Such surface water should be filtered to remove organisms, as described in Section 6.2.

If "upstream" water is used as control/dilution water, a separate control solution must be prepared using a supply (source) of laboratory water shown previously by the testing laboratory to routinely enable valid test results in a 7-day test for survival and growth of larval fathead minnows. Test conditions and procedures for preparing and evaluating each control solution should be identical, and as described in Sections 4.1, 5.3, and 6.3.

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, a suitable laboratory water supply should be used as control water and for all dilutions. For laboratories rearing their own test organisms, culture water is recommended for this purpose. If, however, the test organisms are imported from an outside supplier, an alternate source of laboratory water known to routinely achieve valid test results using this biological test method should be used. The pH and hardness of this laboratory water source could be adjusted to partially simulate those characteristics of the upstream water; footnote

40 in Section 5.3 provides useful guidance in this respect.

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 and measurements on fish that are described in Section 4.4.

7.5 Test Endpoints and Calculations

Endpoints for tests with samples of receiving water are consistent with the options and approaches identified in Sections 4.5 and 6.6.

Testing of each receiving-water sample must include a minimum of three replicate solutions of the undiluted test water and three replicate control solutions. Endpoints for tests with receiving-water samples might often be restricted to data on fish survival and biomass, obtained for larval fathead minnows exposed to samples of full-strength receiving water in single-concentration tests (see Section 4.5).

If toxicity of receiving-water samples is likely, a multi-concentration test to determine the LC50 at the end of the test as well as the ICp for biomass weight should be conducted as outlined in Section 4. The undiluted (100%) sample should be included in the test as the highest concentration of the series.

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 4.5.

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 description of sample type (e.g., chemical or chemical substance, effluent, elutriate, leachate, or receiving water) and volume or weight (if a dry chemical), 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) 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 breeding stock and test larvae;
- age of larvae (i.e., hours since hatched), at start of test; brief statement confirming that their swim bladders are inflated
- any unusual appearance, behaviour, or treatment of larvae, before their use in the test;
- data for breeding stock (including that if test organisms are imported; see Section 2.2), showing combined incidence (expressed as a percentage) of mortalities and disease on a weekly basis, up to and including the 7-day period preceding test; and.
- larval mortality rate (must be <10%; Section 2.2), for any batch of embryos or larvae imported to a testing laboratory.

8.1.3 Test Facilities and Apparatus

- name and 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 and 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, settled and decanted, 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 if any deviation from or exclusion of any of the procedures and conditions specified in this document;
- number, concentration, volume, and depth of test solutions, including controls;
- number of individuals per test vessel, and number of replicates per treatment;
- brief statement (including procedure, rate, and duration) if any pre-aeration of test solutions;
- brief statement concerning aeration (if any, give rate, duration) of test solutions during exposure of test organisms;
- dates when test was started and ended;
- all required (see Section 4.4) 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 number and percentage of mortalities in each test vessel, as recorded during each 24-h observation period over the 7 days;
- for each treatment (i.e., each concentration, including the control treatment): mean (± SD) percent mortality, at the end of the test;
- for each control treatment: the combined and cumulative (over time) mean (± SD) percentage of fish that either died, appeared moribund, displayed loss of equilibrium, or showed clearly atypical swimming behaviour, at each period of observation including at the end of the test; average dry weight per surviving larva at the end of the test, as used for the dry-weight criterion for test validity;
- for each treatment, including the control treatment(s): mean (± SD) biomass (expressed on a dry-weight basis) at the end of the test, as used for the ICp calculation;
- LC50 and 95% confidences limits and indication of quantal method used; ICp and 95% confidence limits for the data on biomass; details regarding any transformation of data that was required, 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 at the same time or 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 procedures, including: temperature and lighting conditions; water source and quality; water *pre-treatment*; water exchange rate and method; ages and densities of fish in cultures; type and quantity of substrate;
- type, source, and method of preparation of food for cultures and test; records of nutritive value and known contaminants in food; procedures used to prepare and store food; feeding procedures, frequency, and ration;
- history of breeding and stock, including weekly fecundity rates up to and including the 7-day period preceding test;
- records of any treatment of breeding stock for disease prevention and control, including nature of disease, approximate percentage of stock affected, symptoms of infection, and specifics (including type, dose, frequency, and dates) of any treatment; and
- if test organisms are imported (see Section 2.2): records of confirmation of species, by a qualified taxonomist; records of species confirmation made by laboratory personnel; all supplier's records provided with each shipment, including life stage (i.e., eyed eggs or larvae), age, and number of test organisms shipped, as well as date and time of shipment; temperature and dissolved oxygen concentration of water in shipment container(s) when shipped and upon arrival.

8.2.3 Test Facilities and Apparatus

- description of systems for regulating light and temperature within the test facility;
- description of any system for providing air and regulating air flow to test vessels; and
- description of procedures used to clean or rinse test apparatus.

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 the laboratory's previous experience with this biological test method for measuring toxicity using fathead minnows;
- 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(s).

8.2.6 Test Conditions and Procedures

- photoperiod, light source, and intensity adjacent to the surface of test solutions;
- description of food source, type, and ration (quantity and frequency of feeding);
- conditions, procedures, and frequency for toxicity tests with reference toxicant(s) and larval (<24 h-old) fish;
- water quality measurements for water supply used as culture/control/dilution water, and for water in aquaria or tanks containing breeding stock (see Section 2.3.4);
- total hardness of the 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 the sample, stock solutions, or test solutions (e.g., chemical concentration, suspended solids content, conductivity, hardness, alkalinity), before and/or during the test; and
- appearance of sample or test solutions; changes in appearance noted during the test.

8.2.7 Test Results

- results for any range-finding test(s) conducted in conjunction with the definitive test;
- results for any statistical analyses conducted both with outliers and with outliers removed; for regression analyses, 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 historical 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.

References

Abernethy, S.G. and G.F. Westlake, "Guidelines for pH Adjustment of Effluent Samples for Toxicity Testing," Ontario Ministry of Environment, Rexdale, Ontario [ISBN No. 0-7729-5947-1], 11p. (1989).

Amlacher, E., *Textbook of Fish Diseases*, T.F.H. Publications, Neptune City, NJ [ISBN 0-87666- 037-5], 302 p. (1970).

Anderson, S.L. and T.J. Norberg-King, Letter to the Editor, "Precision of Short-term Chronic Toxicity Tests in the Real World", *Environ. Toxicol. Chem., 10*:143–145 (1991).

Andrews, A.K. and S.A. Flickinger, Spawning Requirements and Characteristics of the Fathead Minnow, *Proc. 27th Annual Conf. SE Assoc. Game and Fish Commissioners*, p. 759–766 (1973).

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

APHA, AWWA, and WEF, "Toxicity", Part 8000, p.8-1–8-173, in: *Standard Methods for the Examination of Water and Wastewater*, 21st ed. American Public Health Association, American Water Works Association, and Water Environment Federation, Washington, D.C. (2005).

API, "Fathead Minnow 7-day Test: Round Robin Study. Intra- and Interlaboratory Study to Determine the Reproducibility of the Seven-day Fathead Minnow Larval Survival and Growth Test". American Petroleum Inst., Health and Environ. Sci. Dept., Washington, D.C. API Pub. No. 4468 (1988).

Armstrong, F.A.J. and D.P. Scott, "Photochemical Dechlorination of Water Supply for Fish Tanks with Commercial Water Sterilizers", *J. Fish. Res. Board Can., 31:*1881–1885 (1974).

Arthur, J.W. and J.G. Eaton, "Chloramine Toxicity to the Amphipod *Gammarus pseudolimnaeus* and the Fathead Minnow (*Pimephales promelas*)", *J. Fish. Res. Board Can., 28*:1841–1845 (1971).

ASTM, "Standard Practice for Conducting Acute Toxicity Tests with Fishes, Macroinverterbrates, and Amphibians", Amer. Soc. for Testing and Materials, Philadelphia, PA, Report E729-80, 25 p. (1980).

ASTM, "Standard Practice for Using Brine Shrimp Nauplii as Food for Test Animals in Aquatic Toxicology", Sect. 11 (Water and Environmental Technology), E-1203, p. 796–800, in: *Annual Book of ASTM Standards*, Amer. Soc. For Testing and Materials, Philadelphia, PA (1989).

ASTM, "Standard Guide for the Use of Lighting in Laboratory Testing", E1733-95, p. 1279–1289, in: *1996 Annual Book of ASTM Standards*, Vol. 11.05, American Society for Testing and Materials, Philadelphia, PA (1996).

ASTM (American Society for Testing and Materials), "Standard Guide for Conducting Laboratory Soil Toxicity or Bioaccumulation Tests With the Lumbricid Earthworm *Eisenia fetida*", E1676-97, p. 1062-1079 in: *1999 Annual Book of ASTM Standards — Biological Effects and Environmental Fate; Biotechnology; Pesticides, Vol. 11.05*, ASTM, Philadelphia, PA (1999b).

Battelle, "Standard Operating Procedure. Static-renewal Short-term Chronic Toxicity Tests with Fathead Minnows (*Pimephales*

promelas)", Battelle, Columbus Division, Columbus, Ohio, Manuscript EEF-E-19-01, 10 p. (1987).

Benoit, D.A. and R.W. Carlson, "Spawning Success of Fathead Minnows on Selected Artificial Substrates", *Prog. Fish-Culturist, 39* (2):67–69 (1977).

Billington, J.W., G.-L. Huang, F. Szeto, W.Y Shiu, and D. MacKay, "Preparation of Aqueous Solutions of Sparingly Soluble Organic Substances: I. Single Component Systems", *Environ. Toxicol. Chem., 7:*117–124 (1988).

Birge, W.J., J.A. Black, T.M. Short, and A.G. Westerman, "A Comparative Ecological and Toxicological Investigation of a Secondary Wastewater Treatment Plant Effluent and its Receiving Stream". *Environ. Toxicol. and Chem., 8*:437–450 (1989).

Bouck, G.R., "Gasometer: An Inexpensive Device for Continuous Monitoring of Dissolved Gases and Supersaturation", *Trans. Amer. Fish. Soc., III*: 505–516 (1982).

Brauhn, J.L., R. A. Schoettger, and L.H. Mueller, "Acquisition and Culture of Research Fish: Rainbow Trout, Fathead Minnows, Channel Catfish, and Bluegills", U. S. Environmental Protection Agency, Corvallis, Oregon, Ecolog. Res. Ser., EPA-660/3-75-011, 45 p. (1975).

Brown, E.E. and R.B. Gratzek (eds.), *Fish Farming Handbook: Food, Bait, Tropicals and Goldfish*, AVI Publishing Co. Inc., Westport, Connecticut [ISBN 0-87055-341-0], 31 p. (1980).

Brungs, W.A., "Chronic Effects of Low Dissolved Oxygen Concentrations on the Fathead Minnow (*Pimephales promelas*)", *J. Fish. Res. Board of Can., 28:*1119–1123 (1971a).

Brungs, W.A., "Chronic Effects of Constant Elevated Temperature on the Fathead Minnow (*Pimephales promelas*), *Trans. Amer. Fish Soc., 100*:659–664 (1971b).

Brungs, W.A., "Effects of Residual Chlorine on Aquatic Life", *J. Water Pollut. Control Fed., 45*:2180–2193 (1973).

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

Deitzer G. "Spectral comparisons of sunlight and different lamps" *c74*, p. 197 - 199 in: *Proceedings of International Lighting in Controlled Environments Workshop* (Tibbits, T.W., ed), 1 Mar. 1994, Madison, WI (1994).

Denny, J.S., "Guidelines for the Culture of Fathead Minnows *Pimephales promelas* for use in Toxicity Tests", U.S. Environmental Protection Agency, Duluth, Minn. Report EPA/600/3-87/001, 42 p. (1987).

Doudoroff, P. and D.L. Shumway, "Dissolved Oxygen Requirements of Freshwater Fishes", Food and Agriculture Organization of the United Nations, Rome, Fisheries Tech. Paper 86, 291 p. (1970).

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

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

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

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

EC (Environment Canada), "Recommended Procedure for the Importation of Test Organisms for Sublethal Toxicity Testing", Method Development and Applications Section, Environmental Technology Centre, Ottawa, ON, unpublished report, September 1999, 22 p. (1999) [see website http://www.ec.gc.ca/eem/ for document].

EC (Environment Canada), "Guidance Document on Statistical Methods for Environmental Toxicity Tests", Environment Canada, Environmental Protection Service, Ottawa, Ontario, Report EPS 1/RM/46, 241 p. (2005).

EC (Environment Canada), "Biological Test Method: Test of Reproduction and Survival Using the Cladoceran *Ceriodaphnia dubia*", Environment Canada, Environmental Protection Service, Ottawa, Ontario, Report EPS 1/RM/21, second edition, 74 p. (2007).

Hall, W.S. and L.W. Hall, "Toxicity of Alum Sludge to *Ceriodaphnia dubia* and *Pimephales promelas*", *Bull. Environ. Contam. Toxicol., 42(5):*791–798 (1989).

Hamilton, M.A., R.C. Russo, and R.V. Thurston , "Trimmed Spearman-Kärber Method for Estimating Median Lethal Concentrations in Toxicity Bioassays", *Environ. Sci. Technol., 11*:714–719 (1977).

Holtze, K., "Unpublished Data", derived by Stantec Consultants Ltd. (Guelph, ON) for the Method Development and Applications Section, Environment Canada, Ottawa, ON (2007).

Hubert, J.J., "PROBIT2: A Microcomputer Program for Probit Analysis", Dept. of Mathematics and Statistics, Univ. of Guelph, Guelph, Ontario, Canada N1G 2W1 (1987).

Korver, R. M. and J.B. Sprague, "Zinc Avoidance by Fathead Minnows (*Pimephales promelas*): Computerized Tracking and Greater Ecological Relevance", *Can. J. Fish. Aquat. Sci., 46*: 494–502 (1989).

Litchfield, J.T., "A Method for Rapid Graphic Solution of Time-percent Effect Curves", *Pharmacol. Exp. Ther., 97*:399–408 (1949).

Lloyd, R., "Factors that Affect the Tolerance of Fish to Heavy Metal Poisoning", p. 181–187, in: *Biological Problems in Water Pollution*, Third Seminar, 1962, U.S. Public Health Service, Washington, D.C. Publ. 999-WP-25 (1965).

McCaffrey, L., "The Role of Toxicity Testing in Prosecutions Under Section 14 (1)(a) of the Environmental Protection Act, 1971 and Section 32(1) of the Ontario Water Resources Act", p. 15–22, in: *Proc. Fifth Annual Aquatic Toxicity Workshop*, Hamilton, Ontario, Nov. 7–9, 1978, Canada Fisheries and Marine Service, Ottawa, Ontario. Fish. Mar. Serv. Tech. Rep. 862 (1979).

McCormick, J.H., K.H. Jensen, and L.E. Anderson, "Chronic Effects of Low pH and Elevated Aluminum on Survival, Maturation, Spawning and Embryo-larval Development of the Fathead Minnow in Soft Water", *Water Air Soil Pollut., 43*: 293–307 (1989).

McKim, J. M., "Early Life Stage Toxicity Tests", p. 58–95, in: *Fundamentals of Aquatic Toxicology. Methods and Applications*, G.M. Rand and S.R. Petrocelli (eds.), Hemisphere Pub. Corp., Washington, D.C. (1985).

McLeay, D.J., I.K. Birtwell, G.F. Hartman, and G.L. Ennis, "Responses of Arctic Grayling *(Thymallus arcticus)* to Acute and Prolonged Exposure to Yukon Place Mining Effluent", *Canad. J. Fish Aquat. Sci., 44*:658–673 (1987).

Mount D.I., "Chronic Effect by Low pH on Fathead Minnow Survival, Growth, and Reproduction", *Water Res., 7:*987–993 (1973).

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

Mount, D.I. and C.E. Stephan, "A Method for Establishing Acceptable Toxicant Limits for Fish—Malathion and Butoxyethanol Ester of 2, 4-D", *Trans. Amer. Fish Soc., 96*:185–193 (1967).

NAS/NAE, "Water Quality Criteria 1972," [U.S. National Acad. Sciences/National Acad. Engineering.] U.S. Environmental Protection Agency, Ecolog. Res. Serv., EPA, R3.033, 594 p. Supt. of Documents, Washington, D.C. (1974).

Neville, C.M., "Fathead Minnow (*Pimephales promelas*) Larval Growth Test", Ontario Ministry of Environment, Aquatic Toxicity Unit, Rexdale, Ontario, Unpub. Report, 26 p. (1989).

NJ, "Fathead Minnow (*Pimephales promelas*) Larval Survival and Growth Test", in: *Interim Chronic Toxicity Testing Methodologies for Use in the NPDES Permit Program*, Dept. of Environ. Protection, State of New Jersey, Trenton, NJ (February, 1989).

Noggle, C., "The Behavorial and Physiological Effects of Suspended Sediment of Juvenile Salmonids", p. 54–63, in: *Proc. Fourth Annual Aquatic Toxicity Workshop*, Vancouver, B.C., Nov. 8–10, 1977, Canada, Fisheries and Marine Service, Ottawa, Ontario, Fish. Mar. Serv. Tech. Rep. 818 (1978).

Norberg, T. J. and D.I. Mount, "A New Fathead Minnow (*Pimephales promelas*) Subchronic Toxicity Test", *Environ. Toxicol. Chem., 4*:711–718 (1985).

Norberg-King, T.J. "An Evaluation of the Fathead Minnow Seven-day Subchronic Test for Estimating Chronic Toxicity", *Environ. Toxicol. Chem., 8*:1075–1089 (1989).

Norberg-King, T.J., "Seven-day Tests and Chronic Tests", Author's reply. *Environ. Toxicol. Chem., 9*:1436 (1990).

Norberg-King, T. J. and J. Denny, "Culturing of Fathead Minnows *(Pimephales promelas)*: Supplemental Report for Video Training Tape", U.S. Environmental Protection Agency, Office of Water Environment and Permits, Washington, D.C. Rept EPA/505/8-89/002b, 22p. (1989).

OECD, "Guideline for Testing of Chemicals - Fish, Acute Toxicity Test", Organization for Economic Cooperation and Development, Paris, France, Document No. 203, 12 p. (1984).

OME, "Water Management. Goals, Policies, Objectives and Implementation Procedures of the Ministry of the Environment", Revised, Ontario Ministry of the Environment, Toronto, Ontario, 70 p. (1984).

Parrott, J. L., "Patterns of Sublethal Joint Toxicity Determined by Three Rapid Assays", University of Guelph, Guelph, Ontario, M.Sc. thesis (1988).

Roberts, R.J. and C.J. Shephard, *Handbook of Trout and Salmon Diseases*, Second edition, Fishing News Books Ltd., Surrey, England, 222 p. [ISBN 0-85238-066-6](1986).

Rocchini, R.J., M.J.R. Clark, A.J. Jordan, S. Horvath, D.J. McLeay, J.A. Servizi A. Sholund, H.J. Singleton, R G. Watts and R.H. Young, "Provincial Guidelines and Laboratory Procedures for Measuring Acute Lethal Toxicity of Liquid Effluents to Fish", B.C. Ministry of Environment, Victoria, B.C. 18p. (1982).

Rottmann, R.W. and D.E. Campton, "Multiple-tank Aquarium System with Recirculating Water for Laboratory Studies of Freshwater

Fishes", *Progr. Fish-Cult., 51*:238–243 (1989).

Rue, W.J., J.A. Fava, and D.R. Grothe, "A Review of Inter- and Intralaboratory Effluent Toxicity Test Method Variability", p. 190–203, in: *Aquatic Toxicology and Hazard Evaluation: 10th Volume*, W.J. Adams, G.A. Chapman, and W.G. Landis (eds.), Amer. Soc. for Testing and Materials, Philadelphia, PA, ASTM STP 971 (1988).

Sager J.C. and C. McFarlane, "Radiation", p. 1-30 in: *Plant Growth Chamber Handbook,* R.W. Langhans and T.W. Tibbits (eds.), North Central Regional Research Publication No. 340, Iowa Agriculture and Home Economics Experiment Station Special Report No. 99, Iowa State University of Science and Technology, Ames Iowa (1997).

Scott, W.B. and E.F. Crossman, *Freshwater Fishes of Canada*, Fisheries Research Board of Canada, Ottawa, Ontario, Bulletin No. 184, 966 p. (1973).

Sergy, G., "Recommendations on Aquatic Biological Tests and Procedures for Environment Protection", C&P, DOE. Environment Canada, Environ. Prot., Conservation and Prot., Edmonton, Alberta, Unnumbered Report (July, 1987).

Servizi, J.A. and R.W. Gordon, "Detoxification of TMP and CTMP Effluents Alternating in a Pilot Scale Aerated Lagoon", *Pulp Paper Can., 87(11):*T404–409(1986).

Servizi, J.A. and D.W. Martens, "Some Effects of Suspended Fraser River Sediments on Sockeye Salmon (*Oncorhynchus nerka*)", p. 254–264, in: *Sockeye salmon* (Oncorhynchus nerka)*Population Biology and Future Management*, H.D. Smith, L. Margolis and C.C. Wood (eds.). Department of Fisheries and Oceans, Ottawa, Ontario, Canad. Spec. Pub. Fish. Aquat. Sci., 96 (1987).

Shiu, W.Y., A. Maijanen, A.L.Y. Ng, and D. Mackay, "Preparation of Aqueous Solutions of Sparingly Soluble Organic Substances: II. Multicomponent Systems - Hydrocarbon Mixtures and Petroleum Products", *Environ. Toxicol. Chem., 7*:125–137 (1988).

Silberhorn, E.M., Personal communication, Jan. 20, 1989. Graduate Center for Toxicology, University of Kentucky, Lexington, Kentucky (1989).

Sprague, J. B., "Measurement of Pollutant Toxicity to Fish, I. Bioassay Methods for Acute Toxicity", *Water Res., 3:*793–821(1969).

Stephan, C.E., "Methods for Calculating an LC50", p. 65–84, in: *Aquatic Toxicology and Hazard Evaluation*, Amer. Soc. for Testing and Materials, F.L. Mayer and J. L. Hamelink (eds.), Philadelphia, PA, ASTM STP 634 (1977).

Suter, G.W. II, "Letter to the Editor. Seven-day Tests and Chronic Tests", *Environ. Toxicol. Chem., 9:*1435 (1990).

Suter, G.W. II, A.E. Rosen, E. Linder, and D.F. Parkhurst, "Endpoints for Responses of Fish to Chronic Toxic Exposures", *Environ. Toxicol. Chem., 6*:793–809 (1987).

USEPA, "Quality Criteria for Water", U. S. Environmental Protection Agency, Office of Water and Hazardous Materials, Washington, D.C., 256 p. (1976).

USEPA, "Acute Toxicity Test for Freshwater Fish. Standard Evaluation Procedure", U.S. Environmental Protection Agency, Hazard Evaluation Div., Washington, D.C., Report EPA- 540/9-85-006, 12p. (1985).

USEPA, "Culturing of Fathead Minnows *Pimephales promelas*. Fathead Minnow Larval Survival and Growth Toxicity Test", Unnumbered Video Tapes, U.S. Environmental Protection Agency, Office of

Water Protection and Permits, Washington, D. C. (1988).

USEPA, "Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms", second edition [prepared by C.I. Weber, W.H. Peltier, R.J. Norberg-King, W.B. Horning, F.A. Kessler, J.R. Menkedick, T.W. Nieheisel, P. A. Lewis, D.J. Klemm, W. H. Pickering, E.L. Robinson, J. Lazorchak, L.J. Wymer, and R.W. Freyberg], U. S. Environmental Protection Agency, Cincinnati, Ohio, Report EPA/600/4-89/001, 248 p. (1989).

USEPA, "Short-term Methods for Estimating the Chronic Toxicity of Effluents and Organisms", 3rd ed., U.S. Environmental Protection Agency, Report EPA/600/4-91-002, Cincinnati, Ohio (1994).

USEPA, "Short-term Methods for Estimating the Chronic Toxicity of Effluents and Organisms", 4th ed., U.S. Environmental Protection Agency, Report EPA-821-R-02-013, Washington, DC (2002).

Ward, R.W. and G.M. DeGraeve, "Acute Residual Toxicity of Several Wastewater Disinfectants to Aquatic Life",*Water Resources Bull., 14:*696–709 (1978).

Wilde, E.W., R.J. Soracco, L.A. Mayack, R.R. Shealy, T.L. Broadwell, and R.F. Steffen, "Comparison of Chlorine and Chlorine Dioxide Toxicity to Fathead Minnows and Bluegill", *Water Res., 17*: 1327–1331 (1983).

Wolf, E.G., M.J. Schneider, K.O. Schwarzmiller, and T.O. Thatcher, "Toxicity Tests on the Combined Effects of Chlorine and Temperature on Rainbow (*Salmo gairdneri*) and Brook (*Salvelinus fontinalis*) Trout", Battelle Pacific Northwest Labs, Richland, Washington, BNWL- SA-5349, 20 p. (1975).

Woltering, D.M., "The Growth Response in Fish Chronic and Early Life Stage Toxicity Tests: A Critical Review", *Aquatic Toxicol., 5*:1–21 (1984).

Zischke, J. A., J.W. Arthur, K. J. Nordlie, R. O. Hermanutz, D.A. Standen, and T.P. Henry, "Acidification Effects on Macroinvertebrates and Fathead Minnows *(Pimephales promelas)* in Outdoor Experimental Channels", *Water Res., 17:*47–63 (1983).

Appendix A

Members of the Inter-Governmental Ecotoxicological Testing Group[a] (as of October 2009)

Federal, Environment Canada

Suzanne Agius
Marine Protection Programs Section
Gatineau, Québec

Adrienne Bartlett
National Water Research Institute
Burlington, Ontario

Christian Blaise
Centre St. Laurent
Montréal, Québec

Joy Bruno
Pacific & Yukon Laboratory for Environmental Testing
North Vancouver, British Columbia

Craig Buday
Pacific & Yukon Laboratory for Environmental Testing
North Vancouver, British Columbia

Ken Doe
Atlantic Laboratory for Environmental Testing
Moncton, New Brunswick

Garth Elliott
Prairie & Northern Laboratory for Environmental Testing
Edmonton, Alberta

François Gagné
Fluvial Ecosystem Research
Montréal, Québec

Patricia Gillis
Aquatic Ecosystem Protection Research Division
Burlington, Ontario

Manon Harwood
Québec Laboratory for Environmental Testing
Montréal, Québec

Dale Hughes
Atlantic Laboratory for Environmental Testing
Moncton, New Brunswick

Paula Jackman
Atlantic Laboratory for Environmental Testing
Moncton, New Brunswick

Nancy Kruper
Prairie & Northern Laboratory for Environmental Testing
Edmonton, Alberta

Michelle Linssen-Sauvé
Pacific & Yukon Laboratory for Environmental Testing
Norrth Vancouver, British Columbia

Danielle Milani
Aquatic Ecosystem Impacts Research Division
Burlington, Ontario

Warren Norwood
Aquatic Ecosystem Protection Research Division
Burlington, Ontario

Heather Osachoff
Pacific & Yukon Laboratory for Environmental Testing
North Vancouver, British Columbia

Joanne Parrott
Aquatic Ecosystem Protection Research Division
Burlington, Ontario

Linda Porebski
Marine Protection Programs Section
Gatineau, Québec

Juliska Princz
Science & Technology Laboratories
Ottawa, Ontario

Jessica Rahn
Science & Technology Laboratories
Ottawa, Ontario

Grant Schroeder
Pacific & Yukon Laboratory for Environmental Testing
North Vancouver, British Columbia

Rick Scroggins
Science & Technology Laboratories
Ottawa, Ontario

Rachel Skirrow
Pacific & Yukon Laboratory for Environmental Testing
North Vancouver, British Columbia

Troy Steeves
Atlantic Laboratory for Environmental Testing
Moncton, New Brunswick

David Taillefer
Marine Environmental Protection
Gatineau, Québec

Lisa Taylor (Chairperson)
Science & Technology Laboratories
Ottawa, Ontario

Sylvain Trottier
Québec Laboratory for Environmental Testing
Montréal, Québec

Graham van Aggelen
Pacific & Yukon Laboratory for Environmental Testing
North Vancouver, British Columbia

Leana Van der Vliet
Science & Technology Laboratories
Ottawa, Ontario

Brian Walker
Québec Laboratory for Environmental Testing
Montréal, Québec

Peter Wells (Emeritus)
Environmental Conservation Service
Dartmouth, Nova Scotia

Federal, Fisheries & Oceans Canada

Robert Roy
Institut Maurice Lamontagne
Mont-Joli, Québec

Federal, Natural Resources Canada

Melissa Desforges
Ecosystem Risk Management Program
Mining & Mineral Sciences Laboratory, CANMET
NRCan
Ottawa, Ontario

Morgan King
Ecosystem Risk Management Program
Mining & Mineral Sciences Laboratory, CANMET
NRCan
Ottawa, Ontario

Philippa Huntsman-Mapila
Ecosystem Risk Management Program
Mining & Mineral Sciences Laboratory, CANMET
NRCan
Ottawa, Ontario

Carrie Rickwood
Ecosystem Risk Management Program
Mining & Mineral Sciences Laboratory, CANMET
NRCan
Ottawa, Ontario

Bernard Vigneault
Ecosystem Risk Management Program
Mining & Mineral Sciences Laboratory, CANMET
NRCan
Ottawa, Ontario

Provincial

Richard Chong-Kit
Ontario Ministry of Environment
Etobicoke, Ontario

Kim Hunter
Ontario Ministry of Environment
Etobicoke, Ontario

David Poirier
Ontario Ministry of Environment
Etobicoke, Ontario

Julie Schroeder
Ontario Ministry of Environment
Toronto, Ontario
Trudy Watson-Leung
Ontario Ministry of Environment
Etobicoke, Ontario

Private Research Facilities/ Others

Christian Bastien
Centre d'expertise en analyse environnementale du Québec
Ste. Foy, Québec

Barbara Bayer
Manitoba Technology Centre, ALS Laboratory
Winnipeg, Manitoba

Mary Moody
Saskatchewan Research Council
Saskatoon, Saskatchewan

Jim Somers
Standards Council of Canada
Ottawa, Ontario

Appendix B

Environment Canada Regional and Headquarters' Office Addresses

Headquarters
351 St. Joseph Boulevard
Place Vincent Massey
Gatineau, Quebec
K1A 0H3

Ontario Region
4905 Dufferin Street, 2nd Floor
Downsview, Ontario
M3H 5T4

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

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

Quebec Region
1141 Route de l'église
Montreal, Quebec
G1V 3W5

Pacific and Yukon Region
401 Burrard Street
Vancouver, British Columbia
V6C 3S5

Use of Brine Shrimp for Feeding Fathead Minnows

Brine shrimp eggs can be purchased at aquarium supply stores and most pet stores. Also commercially available and recommended, are systems for hatching brine shrimp eggs, usually an elongated plastic bag and salts to provide water of 15 mg/kg salinity for these crustaceans. Instructions for using the apparatus and hatching the eggs are included with the particular device. Brine shrimp eggs can, however, be hatched in almost any conical container with air entering the narrow part at the bottom to keep the eggs in continual motion (Denny, 1987). The detailed advice and discussion on using brine shrimp provided by ASTM (1989) is recommended.

Depending on the apparatus, the person making the preparation usually concentrates the hatched brine shrimp *nauplii* densely in their culture fluid before drawing them off, by briefly (temporarily) removing the air source. For feeding during the toxicity test, the shrimp should be rinsed in fresh water in order to avoid adding salt to the test solutions. The concentrate of brine shrimp nauplii in their culture medium should be rinsed in fresh water at 25°C in a separatory funnel, and allowed to settle for 2 min, during which time some of the unhatched eggs might conveniently float to the top. The settled concentrate of nauplii is then drawn into a small beaker or container with 20 μm screened bottom, and re-suspended for feeding to the fish with a repeater pipette or dropper (Neville, 1989). Usually, about 0.05 to 0.1 mL of the concentrate, i.e., 1 or 2 drops, will be sufficient for one feeding of a test vessel containing 10 larval fish. That feeding should contain about 700 to 1000 brine shrimp nauplii (Neville, 1989). A check should be made at the beginning of the procedural setup, to determine how many drops of shrimp concentrate are required to deliver those 700 to 1000 nauplii with the particular technique used in a laboratory (check by estimating numbers in an aliquot under a microscope, using a haemocytometer or other appropriate device).

It is essential that all test vessels get the same amount of food, and standardized techniques must be set up to accomplish that, such as mixing the suspension in the small beaker, and standard timing and positioning for refilling the dropper and delivering the nauplii. In addition, occasional inspections should be made, particularly during early stages of testing in a laboratory, to make sure that there is a small excess of nauplii in the chambers throughout the daylight hours.

Two such feedings of the test vessels during each day should ensure near-maximum growth, if the first feeding is done early in the morning. Two feedings have been shown to achieve better growth of larvae than one feeding, although the difference is less noticeable if the single feeding is a heavy one (Silberhorn, 1989). Three feedings do not result in appreciably better growth than two feedings. Growth increases with more nauplii per day, up to a plateau in the region of about 2000 nauplii per day.

For aquaria containing large numbers of fathead minnow larvae, proportionally more concentrate of brine shrimp nauplii would be required. For delivering shrimp to aquaria with a continuous flow of new water, it is not necessary to rinse the shrimp in fresh water before delivery.

Because the larval minnows are very sensitive, and because the brine shrimp are their only food during the test, any contaminants in the food could be a distinct problem since they might cause combined action with toxicants being tested, and bias the results. Therefore, an effort should be made to use brine shrimp eggs which are known (by measurement) to contain low amounts of contaminants, especially persistent organochlorine compounds. USEPA (1989, 2002) recommends chemical analysis of each new batch of brine shrimp eggs, with a maximum limit for total organic chlorine of 0.15 µg/g wet weight and a maximum limit for organochlorine pesticides plus PCBS of 0.30 µg/g wet weight. Additionally, USEPA (1989, 2002) recommends brine shrimp eggs originating in Brazil or Columbia because of their record of low levels of contaminants, but also gives a U.S. source. Because sources and suppliers will change for the brine shrimp commercially available in Canada, they are not specified here. The best indications of quality will come from measurement of contaminants in different supplies of eggs, success in hatching the shrimp and growing fathead minnows, and an exchange of information on those subjects among laboratories.

The nutritive quality of brine shrimp might also vary with their origin. This factor is as difficult to assess on a continuing basis as the contamination question, but should be dealt with by keeping track of sources and success of rearing, and by sharing information on measured nuturitive values with other laboratories.

For adult or juvenile fathead minnows, foods other than frozen brine shrimp could be used. Other kinds of collected or cultured invertebrates or chopped meat, fresh or frozen, can be satisfactory or superior, but the frozen brine shrimp are convenient and of proven performance.

Logarithmic Series of Concentrations Suitable for Toxicity Tests[1]

Column (Number of concentrations between 100 and 10, or between 10 and 1)[2]						
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

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

[2] A series of successive concentrations (minimum of seven; recommend 8 or more) may be chosen from a column. Mid-points 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 multilplied or divided by any power of 10. Column 2, which spans two orders of magnitude in concentration, 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 columns 3 or 4; the finer gradations of columns 4 to 7 might occasionally be useful for testing chemicals that have an abrupt threshold of effect (i.e., a steep dose-response curve).

Appendix E

Analysis of Mortality to Estimate the Median Lethal Concentration

The 7-day growth and survival test with fathead minnow larvae is intended to be a sensitive and meaningful sublethal test, because early life stages are usually among the most sensitive in a life- cycle test. Therefore, this assay usually focuses on determining the ICp based on mean *biomass*. However, mortality of larval fathead minnows is a relatively sensitive effect in the life cycle, and is sometimes the most sensitive effect that is documented during the 7-day larval exposure (Woltering, 1984; McKim, 1985; Suter *et al.,* 1987). A point-estimate of the concentration causing lethality is also useful, and must be calculated (data permitting) as one of the statistical endpoints for this test (see Section 4.5). Section 4 in Environment Canada (2005) should be consulted for further guidance when calculating the LC50.

A larval LC50 obtained in the course of the present test would be much lower than the usual acute LC50s for fathead minnows that are recorded in the literature, since those published values are typically obtained from tests with juvenile fish that are more tolerant of contaminants (for which, accordingly, higher LC50s are derived).

To estimate an LC50, data are combined for all replicates at each concentration, for a given exposure-time that would normally be seven days, the length of this test. 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 highest concentration of the series, i.e., the zero-effect 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 100% 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. 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 and available for use. Section 4 in Environment Canada's guidance document on statistical methods for environmental toxicity tests (EC, 2005) provides detailed guidance on commercial programs that are readily available (e.g., within the statistical packages identified as TOXCALC, CETIS, TOXSTAT, SAS), which should be followed when estimating an LC50 for larval fathead minnows. Choice of methods including the two preferred methods using *probit* or *logit regression by maximum likelihood*, are described in Section 4.3 of EC (2005). The *Spearman-Kärber* method (Hamilton *et al*., 1977) without trimming (or with "minimal" trim) is recommended only if results cannot be analyzed using one or both of these two (preferred) methods, and the "trimmed" *Spearman-Kärber* method is not recommended (EC, 2005). In situations with no partial effect, but

0% and 100% effects, the *binomial* method is recommended (see Section 4.3 "*Choice of Methods*" in EC, 2005).

Any computer-derived LC50 should be checked by examining a plot on logarithmic-probability scales of percent mortalities at a fixed observation time (e.g,. seven days) for the various test concentrations (APHA *et al*., 1989) (see example in Figure E.1). Any major disparity between the estimated LC50 derived from this plot and the computer-derived LC50 must be resolved.

In the hypothetical example shown in Figure E.1, ten fish were tested at each of five concentrations (1.8, 3.2, 5.6, 10, and 18 mg/L), and a control (with no control mortality). Mortalities in the test concentrations, of 0, 2, 4, 9, and 10 fish, were plotted and a line fitted by eye. The concentration expected to be lethal to half the fish 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 E.1, relatively more emphasis should be assigned to points that are near 50% mortality. Logarithmic-probability paper ("log-probit", as in Figure E.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 set of data in Figure E.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:		
0% trim:	5.64	(4.38 to 7.26)
(Hamilton *et al.*,1977)		
10% trim:	5.73	(4.34 to 7.58)
20% trim:	5.95	(4.34 to 9.80)

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

It is also possible, if desired, to estimate the "time to 50% mortality" (*LT50*) in a given concentration. A graph similar to Figure E.1 can be plotted using logarithm of time as the horizontal axis. Individual times to death of organisms would not be available for use, since tests would not be inspected continuously. The cumulative percent mortality at successive inspections (normally, daily) is quite satisfactory for plotting, and an eye-fitted line leads to estimates of confidence limits following the steps listed in Litchfield (1949).

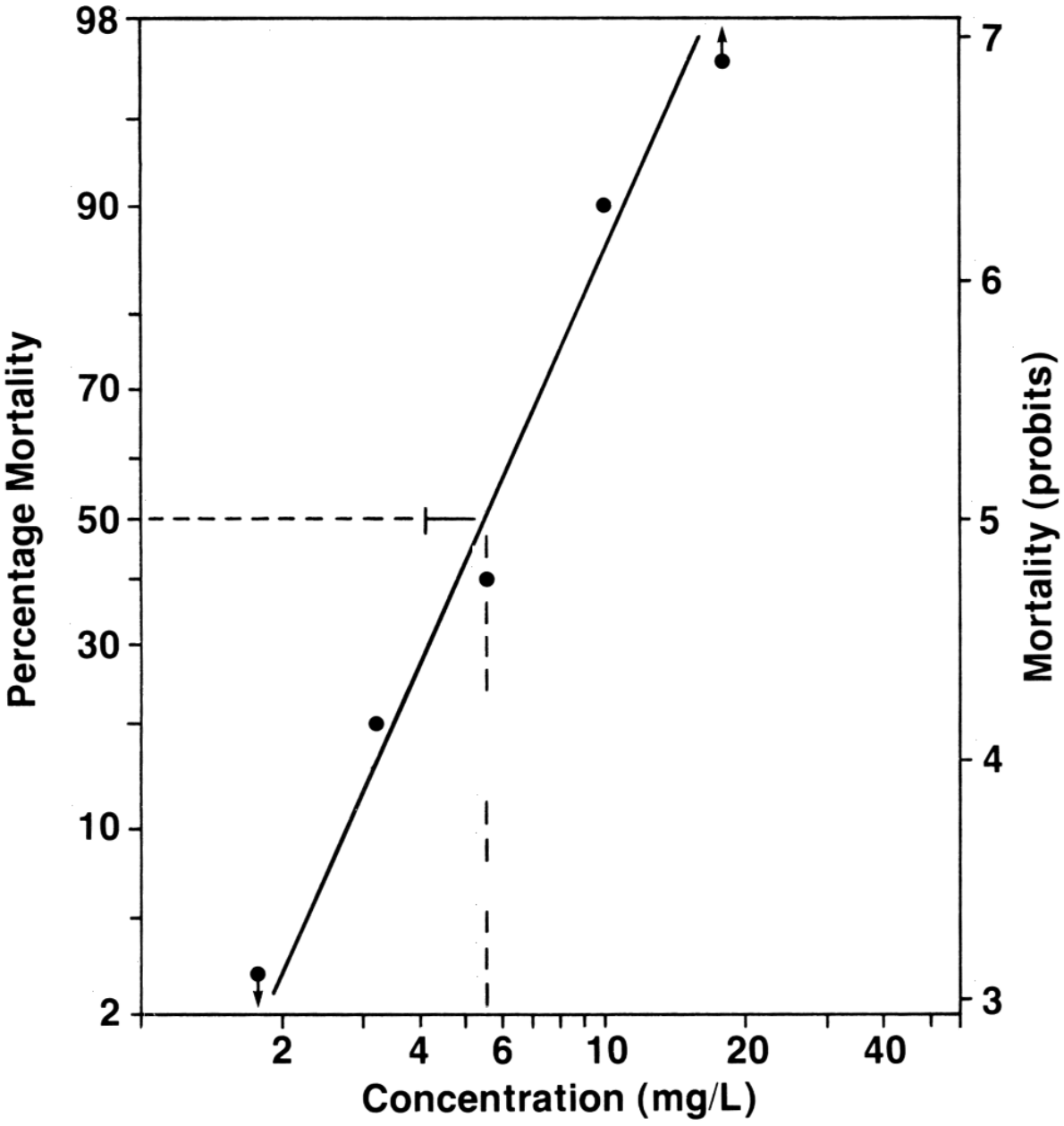


Figure E.1 Estimating Median Lethal Concentration by Plotting Mortalities on Logarithmic-probablity Paper

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 an LT50.

Neither an LT50 nor the percent mortality at short exposure-times is a dependable method of judging ultimate toxicity, thus comparisons based on those endpoints give only semi-quantitative guidance. However, it might sometimes be useful 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).

Appendix F

Biological Test Methods and Supporting Guidance Documents Published by Environment Canada's Method Development & Applications Unit[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	February 2010
Toxicity Test Using Luminescent Bacteria	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.ec.gc.ca/faunescience-wildlifescience/default.asp?lang=En&n=0BB80E7B-1. For further information or comments, contact the Chief, Biological Assessment and Standardization Section, 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	September 2007	—
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	June 2007

www.ec.gc.ca

Additional information can be obtained at:

Environment Canada
Inquiry Centre
351 St. Joseph Boulevard
Place Vincent Massey, 8th Floor
Gatineau QC K1A 0H3
Telephone: 1-800-668-6767 (in Canada only) or 819-997-2800
Fax: 819-994-1412
TTY: 819-994-0736
Email: enviroinfo@ec.gc.ca