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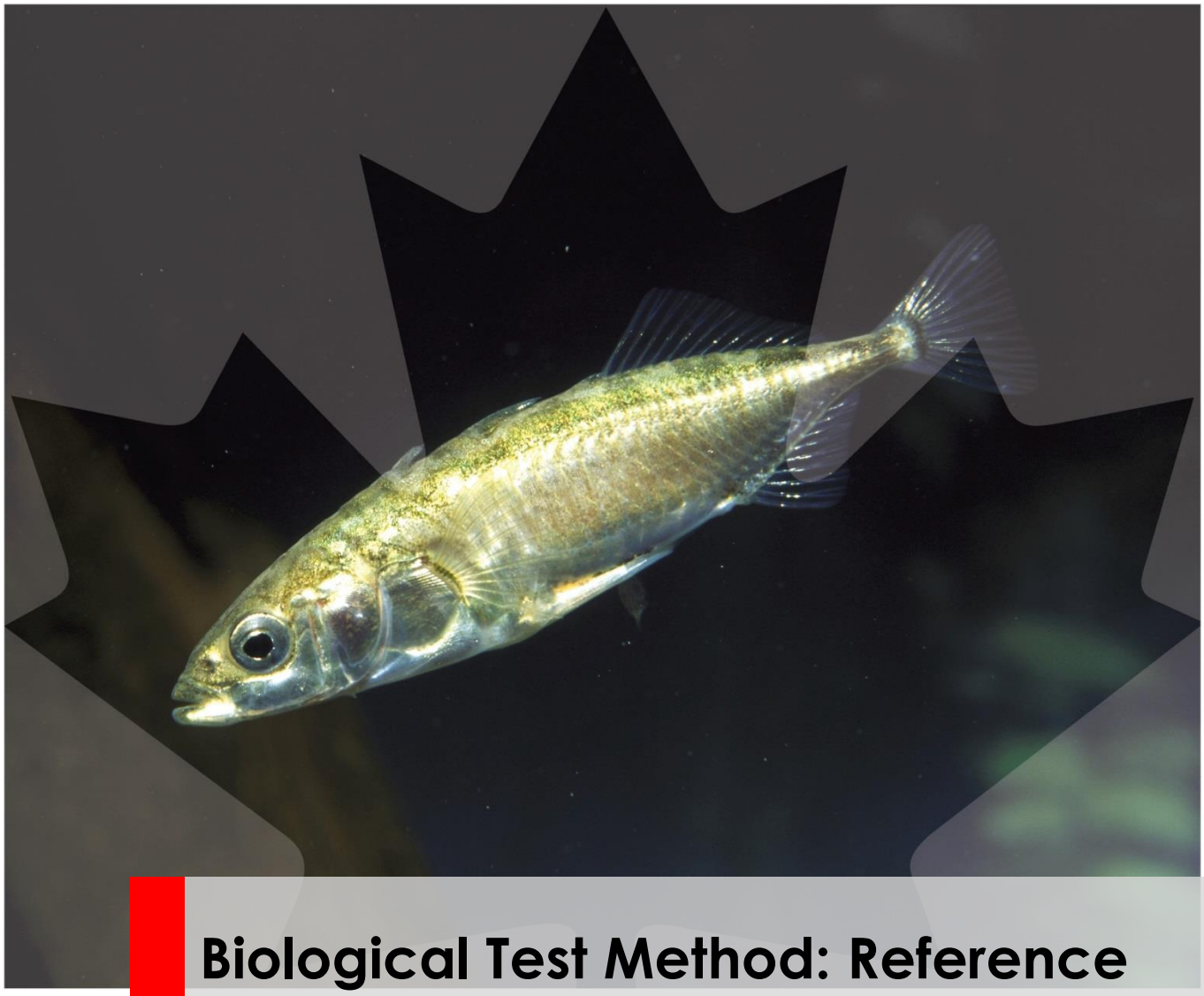

Biological Test Method: Reference Method for Determining Acute Lethality Using Threespine Stickleback

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Environment and Climate Change Canada
Public Inquiries Centre
12th Floor, Fontaine Building
200 Sacré-Coeur Boulevard
Gatineau QC K1A 0H3
Telephone: 819-938-3860
Toll Free: 1-800-668-6767 (in Canada only)
Email: ec.enviroinfo.ec@canada.ca

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Biological Test Method: Reference Method for Determining Acute Lethality Using Threespine Stickleback

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

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EPS 1/RM/10
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Readers' Comments

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

Richard Scroggins, Chief
Biological Assessment and Standardization Section
Science and Technology Branch
Environment and Climate Change Canada
335 River Road
Ottawa, Ontario
K1A 0H3

General inquiries regarding this method can be addressed to:
ec.methodes-methods.ec@canada.ca

Review Notice

This report has been reviewed by the staff from the Science and Technology Branch, Environment and Climate Change Canada, and approved for publication. Mention of trade names or commercial products does not constitute endorsement by Environment and Climate Change Canada for use. Other products of similar value are available.

Abstract

Explicit standard or reference methods for measuring the acute lethal toxicity of effluents to marine or estuarine threespine stickleback (*Gasterosteus aculeatus*) are described in this report. Specific instructions are provided for performing acute lethality tests with effluent samples having a salinity of > 10 g/kg discharging directly to estuarine or marine receiving waters.

This second edition report replaces the first edition of Environment Canada's Biological Test Method EPS 1/RM/10 (EC, 1990a), which was published in July 1990 and amended in March 2000. It supersedes that earlier version, and is to be applied as Environment and Climate Change Canada's (previously Environment Canada) current reference method for determining the acute lethality of effluents to threespine stickleback. This revised version of Report EPS 1/RM/10 includes numerous updates such as: the conversion from "generic" to "reference" method; the revision of methods for salinity adjustments and preparation of artificial seawater; the requirement that test organisms be obtained from estuarine or marine waters or cultures; a narrowed size range of fish recommended for use as test organisms; the revision of holding and acclimation guidance to reflect current and varied laboratory practices; and the review and revision of the recommendations and requirements of the test method.

Methods are given for:

i) a single-concentration test, with full-strength effluent unless otherwise specified;

ii) a multi-concentration test to determine the median lethal concentration (LC50); and

iii) a test with a reference toxicant.

Instructions are included on holding sticklebacks in the laboratory, facilities and water supply, handling and storage of samples, preparation of solutions, test conditions, observations to be made, endpoints with methods of calculations, and the use of reference toxicants. Specific procedures for testing chemicals, formulated products, or chemical mixtures are also provided.

Foreword

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

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

Reference methods are those that have been developed and published by Environment and Climate Change Canada, and are favoured:

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

Appendix A lists those **reference methods** prepared for publication by Environment and Climate Change Canada's Method Development and Applications Unit in Ottawa, Ontario, along with other generic (more widely applicable) biological test methods and supporting guidance documents.

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

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List of Abbreviations and Chemical Formulae

°C	degree(s) Celsius
cm	centimetre(s)
CV	coefficient of variation
DO	dissolved oxygen (concentration)
g	gram(s)
g/kg	gram(s) per kilogram
h	hour(s)
L	litre(s)
LC50	median lethal concentration
m	metre(s)
mg	milligram(s)
min	minute(s)
mL	millilitre(s)
mS	millisiemen(s)
nm	nanometer(s)
®	Registered Trade Mark
s	second(s)
SD	standard deviation
SI	International System of Units
™	Trade Mark
µg	microgram(s)
µmhos	micromhos
µmol	micromol(s)
×	times
÷	divided by
>	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 or percent
‰	parts per thousand (salinity)

Terminology

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

Grammatical Terms

Must is used to express an absolute requirement.

Should is used to state that the specified condition or procedure is recommended and ought to be met if possible.

May is used to mean "is (are) allowed to".

Can is used to mean "is (are) able to".

Might is used to express the possibility that something could exist or happen.

Technical Terms

Acclimation is the physiological adjustment to a particular level of one or more environmental factors such as temperature or salinity. The term usually refers to the adjustment to controlled laboratory conditions.

Accuracy is the closeness of the measured (or estimated) value to the "true" value. Determination of accuracy of a measurement usually requires *calibration* of the analytical method with a known standard.

Anadromous fish are those that are born in fresh water, spend most of their life in the sea, and return to fresh water to spawn.

Batch means a single group of threespine sticklebacks received from a supplier 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*). It *might* also refer to a volume of seawater (artificial or natural) intended for use for holding/*acclimation* or in a discrete toxicity test (including any associated reference toxicity test).

Calibration is the comparison of measurement values delivered by a device under test with those of a calibration standard of known *accuracy*. Such a standard could be another measurement device of known accuracy; a device generating the quantity to be measured such as a voltage; or a physical artefact, such as a metre ruler.

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, on their valence and mobility, and on the solution's temperature. Conductivity readings in water is typically temperature-adjusted to the standard temperature of 25°C, and is normally reported in the SI unit of millisiemens/metre, or as micromhos/centimetre (1 mS/m = 10 µmhos/cm). Conductivity is an indirect method for measuring *salinity*, with the result converted to g/kg or "parts per thousand" (‰).

Euryhaline is the ability of an organism to tolerate a wide variation in salinity without stress.

Fork Length is the length of a fish, measured from the tip of the nose to the tip of the medial (i.e., middle) caudal-fin ray.

Lux is a unit of illumination based on units per square metre. One lux = 0.0929 foot candles and one foot-candle = 10.76 lux. 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 is properly described in terms of quantal flux (photon fluence rate) in the photosynthetically effective wavelength range of approximately 400−700 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, 2014a). Conversions between quantal flux and lux, for full-spectrum fluorescent light (e.g., Vita-Lux® by Duro-Test®) is as follows: 1 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) observation and measurement of certain biological or water quality variables, or the collection and testing of samples (e.g., *effluent*) for *toxicity*.

Percentage (%) is a concentration expressed in parts per hundred. With respect to effluents or *chemicals*, 10 percent (10%) represents 10 units of effluent (or a chemical) diluted with water to a total of 100 parts. Concentrations *can* be prepared on a weight-to-weight, weight-to-volume, or volume-to-volume basis, and are expressed as the percentage of effluent or chemical sample in the final solution.

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

Precision is the closeness of repeated measurements to each other (i.e., the degree to which data generated from replicate measurements differ), and is often assessed by the variance or standard deviation. It measures random contributions to uncertainty.

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

Refractometry is a technique that measures the extent to which light is bent (i.e., refracted) when it moves from air into a sample and is typically used to determine the index of refraction (i.e., refractive index) of a liquid sample. The refractive index, which is highly dependent on temperature, is then used to determine the *salinity* of a sample. A refractometer is an instrument used for measuring the refractive index.

Salinity is the total mass of dissolved salts in a given mass of solution. For the purposes of this method, salinity must be measured using *conductivity* or *refractometry* (see Section 4.2). Salinity is reported here as g/kg. The term “parts per thousand” (‰) is synonymous with g/kg.

Verification is a procedure used for checking that an instrument or analytical system meets a set of requirements or specifications and that the performance of the instrument has not changed significantly from the initial *calibration*.

Terms for Effluents or Chemicals

Artificial seawater is fresh water to which commercially available dry ocean salts have been added in a quantity that provides the salinity (and pH) desired for holding/acclimating organisms and for testing purposes (*control/dilution water*). See *seawater (natural)*.

Chemical is, in this report, any element, compound, formulation, or mixture of a substance that might be mixed with, deposited in or found in association with water; or enter the aquatic environment through spillage, application, or discharge.

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 effluent or *chemical* sample. The *control* is used as a check for the absence of measurable toxicity due to basic test conditions (e.g., quality of *dilution water*, health of test organisms, or effects due to their handling). In this method, the term "dilution-water control" is synonymous with *control*, and consists of *control water*.

Control/dilution water is the water used for diluting the sample of effluent (or chemical), and for the *control* of a test. Control/dilution water is frequently identical to the holding/ acclimation water.

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

Deionized water is water that has been purified by passing it through resin columns or a reverse osmosis system.

Dilution water is water used to dilute an effluent or chemical sample in order to prepare different concentrations for the various toxicity test treatments.

Dispersant is 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 throughout the water as an emulsion.

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.

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

Estuarine is of brackish seawater, residing in or obtained from a coastal body of ocean water that is measurably diluted with fresh water derived from land drainage.

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

Marine is of salt water, residing in or obtained from the open ocean and without appreciable dilution by natural fresh water derived from land drainage.

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

Receiving water is surface water (e.g., *marine* or *estuarine* water body, stream, river, or lake) that has received a discharged waste, or else is about to receive such a waste. Further description must be provided to indicate which meaning is intended.

Reconstituted seawater – see *artificial seawater*.

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

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

Salinity control for the purpose of this method is a sample of control/dilution water with the *salinity* adjusted to within 1‰ of the effluent sample or, for chemical testing, the highest concentration of the test sample. In addition to the dilution-water control, a salinity control must be included in a test if the salinity of the sample is > 5 g/kg higher or lower than the salinity to which fish are acclimated. The salinity control is used to check for the absence of effects due solely to the sudden change in salinity (i.e., salinity shock). The salinity control must be > 10 g/kg and ≤ 35 g/kg, and salinity adjustment is carried out using commercially available dry ocean salts (see Section 2.3).

Seawater (natural) is salt water residing in or obtained from the open ocean and without appreciable dilution by natural fresh water derived from land drainage. See *artificial seawater*.

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

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.

Statistical and Toxicological Terms

Acute means occurring within a short period of exposure in relation to the life span of the test organism, usually taken as ≤ 96 hours for fish. An acute toxic effect would be induced and observable within the short period.

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 derived value(s) that characterize the results of the test (e.g., LC50, percent mortality). It also means the response of the test organisms that is measured (e.g., death).

Flow-through describes test or holding conditions in which solutions 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, or as the antilogarithm of the mean of the logarithms of the "n" values.

LC50 is the median lethal concentration, i.e., the concentration of effluent or chemical in water (% or mg/L) that is estimated to be *lethal* to 50% of the test organisms. The LC50 and its 95% confidence limits are usually derived by statistical analysis of percent mortalities in several test concentrations, after a fixed period of exposure. The duration of exposure must be specified (e.g., 96-h LC50).

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

Overt means obviously discernible under the test/holding conditions employed.

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

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

Static-replacement describes test or holding conditions in which solutions are renewed (replaced) periodically, usually every 24 hours. Synonymous terms are "static renewal", "renewal", "batch replacement", and "semi-static".

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

Toxicant is a toxic effluent or chemical.

Toxicity is the inherent potential or capacity of an effluent or chemical to cause adverse effect(s) on fish or other living organisms. These effect(s) could be *lethal* or *sublethal*.

Toxicity Identification Evaluation describes a systematic sample *pre-treatment* (e.g., pH adjustment, filtration, aeration) followed by tests for *acute* toxicity. This evaluation is used to identify the causative agent(s) that are primarily responsible for acute lethality in a complex mixture.

Toxicity test is a determination of the effect of an effluent or chemical on a group of selected organisms, under defined conditions. An aquatic toxicity test usually measures the proportions of organisms affected by their exposure to specific concentration(s) of a test effluent or chemical.

Treatment is, in general, an intervention or procedure whose effect is to be measured. More specifically, in testing for toxicity, it is a condition or procedure applied to the test organisms by an investigator,

with the intention of measuring the effect(s) on those organisms. The treatment could be a full-strength sample of effluent, a specific concentration of an effluent or chemical, or control water.

Warning chart is a graph used to follow changes in the *endpoints* for a reference toxicant over time. The date of the test is on the horizontal axis and the concentration causing an effect 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.

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This (second) edition, prepared as a reference method by Jennifer Miller (Miller Environmental Sciences), supersedes the first edition of this biological test method. Leana Van der Vliet (Biological Assessment and Standardization Section, Environment and Climate Change Canada) was the Scientific Authority for the project, providing guidance, technical assistance, and detailed review throughout the work. Leana Van der Vliet, Rick Scroggins, and Lisa Taylor (Biological Assessment and Standardization Section, Environment and Climate Change Canada) provided significant contributions to various sections of the document. Improvements to this second edition method were based on research conducted by Environment and Climate Change Canada's Atlantic Laboratory for Environmental Testing (ALET) and Harris Industrial Testing Service Ltd. (HITS). The tremendous efforts of Paula Jackman (ALET), Gary Harris (HITS), and Karen Marks (HITS) towards development of the method and review of this test method document are gratefully acknowledged.

The inter-laboratory studies undertaken to validate the test method described herein were coordinated by Paula Antunes (AquaTox Testing & Consulting Inc.) and performed by the following participating laboratories: Environment and Climate Change Canada's Atlantic Laboratory for Environmental Testing (ALET) and Pacific & Yukon Laboratory for Environmental Testing (PYLET); AquaTox Testing & Consulting Inc. (AquaTox); Harris Industrial Testing Service Ltd. (HITS); Integrated Resource Consultants Inc. (IRC); Maxxam Analytics (Maxxam); Nautilus Environmental Company Inc. (Nautilus); CanmetMINING at Natural Resources Canada (CanmetMINING); and Petroforma Laboratories (Petroforma). We gratefully acknowledge the contributions of all of the inter-laboratory participants: Megan Bauer and Paula Jackman from ALET; Craig Buday, Christie Laing, and Grant Schroeder from PYLET; Paula Antunes, Kim Dube, Shawna Kirkpatrick, Derek Kozakiewicz, Nancy Kreager, Jasmine Lauinger, Tim Lauinger, Lesley Novak, Martina Rendas, Frank Saliani, and Adam Wartman from AquaTox; Gary Harris, Amanda Huybers, and Karen Marks from HITS; Wade Britz, Richard Chea, Melissa Hebert, Ditty Kakkassery, and Catherine Wong from IRC; Michael Brassil, Marriah Grey, Pam Howes, and Donald Lai from Maxxam; Eric Cheung, Yvonne Lam, and Armando Tang from Nautilus; Morgan King, Carrie Rickwood, and Emily Suominen from CanmetMINING; and Jennifer Mews, Stephen Rossiter, Suzette Winter, and Amanda Woodrow from Petroforma. The ongoing support of members of the Inter-Governmental Ecotoxicological Group (Appendix B) is also acknowledged.

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Section 1

Introduction

This reference method specifies the procedures and conditions for an *acute* lethality test with threespine stickleback (*Gasterosteus aculeatus*), as specified by Canadian governments involved in pollution *monitoring* and control of industrial or municipal effluents. The present test method is intended for use with *effluent* samples having a *salinity* of > 10‰ discharging directly to *estuarine* or *marine receiving waters*. This report replaces the first edition of Environment Canada's Biological Test Method EPS 1/RM/10 (EC, 1990a), which was published in July 1990 and amended in March 2000. This reference method represents one of the biological test methods to be used as part of effluent assessments for monitoring and *compliance* under the *Metal Mining Effluent Regulations* promulgated under the Federal *Fisheries Act*. Two other reference methods, published by Environment Canada (2000a, 2000b and Appendix A) are used for assessing effluents containing fresh water or having a salinity of ≤ 10‰, and those effluents that are > 10‰ discharging into fresh water.

Procedures are also provided herein to evaluate different types of substances such as *chemicals*, formulated products, or chemical mixtures (see Section 8), and could be used to provide data for pesticide management and regulation as well as chemicals of concern at contaminated sites. Additionally, results from chemical-specific tests can be incorporated into national or provincial guidelines for environmental quality.

This reference method is based on the generic method published by Environment Canada in 1990 with contributions from method development research conducted by Environment and Climate Change Canada's Atlantic Laboratory for Environmental Testing (unpublished data) and Harris Industrial Testing Services Ltd (HITS, 2017). Procedures and conditions stipulated in this reference method must be taken as the definitive ones for regulatory purposes.

Before finalizing this reference method, two inter-laboratory studies were performed to assess inter-laboratory *precision* and to validate the test method (AquaTox, 2017). Phenol (a common *reference toxicant*) and cadmium were the two *toxicants* evaluated. Results from the first reference toxicant round using phenol yielded a *Coefficient of Variation* (CV) of 19.6%, which is within an acceptable range of variability for inter-laboratory tests. The second round, which tested cadmium as the reference toxicant, produced results that were more variable (AquaTox, 2017). Environment Canada (2005) has suggested that a CV of ≤ 30% would be within a reasonable range of variability expected in repeated toxicity tests with a reference toxicant. As follow-up to the inter-laboratory study, an effluent sample was divided and tested concurrently in two laboratories, and the results were in good agreement (unpublished data).

The threespine stickleback (*Gasterosteus aculeatus*), a common *anadromous* and freshwater fish, is *euryhaline*, occupying mainly the shallow-water areas of marine, estuarine, and freshwater environments. Almost circumpolar in coastal habitats, it prefers the temperate and subarctic zones of the northern hemisphere. It is widely distributed in the northern hemisphere on all coasts, with the exception of the most northern coastlines of cold Arctic seas.

Threespine stickleback occur on the Pacific coast from California to northwestern Alaska and on the Atlantic coast from Nova Scotia to northern Labrador (Hart, 1973; Wootton, 1976; Scott and Scott, 1988). *G. aculeatus* has been used for many years by both government and private sector laboratories in Canada by investigators concerned with evaluating the *acute* toxic effects of effluents discharged to the estuarine or marine environment. This fish species has also been recommended as an appropriate marine/estuarine toxicity test organism by both the U.S. Environmental Protection Agency (USEPA, 2002) and the American Society for Testing and Materials (ASTM, 2014b). It has also gained international

popularity as a model species in ecology, evolution, genomics, ecotoxicology, and endocrine disruption studies (Katsiadaki, 2007; Katsiadaki *et al*., 2007; Barber and Nettleship, 2010).

Three procedures are described in this test method document. One uses a single concentration of effluent (full strength unless otherwise specified) or a chemical and a *control*(s), as would be suitable for a pass/fail test. A second procedure is a multi-concentration test that estimates the median *lethal* concentration (*LC50*) (i.e., it determines the degree of *toxicity* using several concentrations of effluent including full-strength, or a chemical). A third procedure is a multi-concentration test with a *reference toxicant*, to assess the sensitivity of the test fish to a standard *toxicant* and the *precision* of the data produced by the laboratory for that chemical. Additional guidance for testing chemical samples is included (see Section 8).

This reference method is to be used with saline (> 10‰) effluents discharging directly to estuarine or marine *receiving waters*. Effluent *salinity* must be measured by *conductivity* or *refractometry* using an acceptable method and calibrated instrument with a tolerance limit for *accuracy* within ± 1‰, as described in Section 4.2.

Section 2

Test Organisms

2.1 Species and Source

The threespine stickleback (*Gasterosteus aculeatus*) is to be used as the test species in this reference method. The average wet weight of test fish must be between 0.20 and 1.2 g, and the *fork length* of the largest fish should not be more than 1.6 times that of the smallest in the same test. Juvenile or adult life stages may be used as test fish, however males displaying breeding colours (typically, blue eyes and/or red throat/fore-belly) and gravid females (swollen abdomen) must not be used for testing.

All fish used in a test must be derived from the same population and source, and must be free of *overt* signs of disease or parasites (see footnote 1). Fish must be cultured or captured from coastal *marine* or *estuarine* waters, and acclimated subsequently to laboratory conditions. Beach seines, cast nets, or minnow traps are suitable for capturing these fish. *G. aculeatus* originating from populations inhabiting fresh water must not be used in this test.

A commercial source for marine or estuarine threespine stickleback includes:

Seacology
3025 Sunnyhurst Road,
North Vancouver, BC V7K 2G4
Tel.: 604-987-4675
Fax: 604-987-4675
Website: http://www3.telus.net/seacology/
Email: seacology@telus.net
Contact: Douglas Swanston

For current information on suppliers for *G. aculeatus* contact:

Method Development and Applications Unit
Science and Technology Branch
Environment and Climate Change Canada
335 River Road
Ottawa ON K1A 0H3
Email: ec.methodes-methods.ec@canada.ca

Regional, provincial, or federal authorities (e.g., Federal-Provincial Introductions and Transfers Committee) might require approval for the procurement, shipment, or transfer of threespine sticklebacks. For further information on federal or provincial permit requirements, contact Environment and Climate Change Canada's regional environmental testing laboratories (see Appendix C).

Each *batch* of sticklebacks captured from Atlantic waters must be examined carefully to remove any blackspotted stickleback (*Gasterosteus wheatlandi*) that might have been captured along with *G. aculeatus* (see Appendix D), and to ensure that only *G. aculeatus* are used in this test. Each batch of sticklebacks collected for testing should also be examined to ensure that only morphs with a caudal keel (i.e., caudal keel is present in complete-plated and partial-plated morphs) are used in this test, and that those lacking a caudal keel (i.e., caudal keel is lacking in low-plated morphs) are removed from the batch. Taxonomic descriptions and illustrations of the distinguishing features of threespine stickleback and the various morphs are given in Appendix D.

Taxonomic identification and documentation of the species of test organisms must be made by a qualified taxonomist, at least once for each collection site or supplier of threespine stickleback, using distinguishing taxonomic features described in taxonomic keys, or using DNA-based taxonomic identification (i.e., barcoding). Organisms that are purchased from a commercial supplier may be supplied with certification of the organisms' species

identification, and the taxonomic reference or name(s) of the taxonomic expert(s) consulted. After the initial taxonomic identification (i.e., provided by a given supplier, taxonomist, or through barcoding), confirmation of the species of test organisms in each shipment must be made, and can be conducted by the supplier or by the testing laboratory using the distinguishing taxonomic and morphological features described and illustrated in Appendix D. Records accompanying each batch of test organisms must include, at a minimum: the quantity and source of test organisms in each shipment; supplier's and/or collector's name; date of shipment; date of arrival at the testing laboratory; and arrival condition (i.e., mortality, temperature, DO, *pH*, and salinity).

2.2 Holding and Acclimation

Threespine sticklebacks employed in this reference method have been successfully maintained in the laboratory for extended periods of time (i.e., 3 months to 1 year) and laboratories report very little mortality (i.e., cumulative weekly mortality of < 2%) with these fish during holding and *acclimation*.

Fish should be held and acclimated in tanks or troughs. These must be made of nontoxic materials (e.g., glass, stainless steel, porcelain, fibreglass-reinforced polyester, polyethylene, acrylic, or polypropylene). Troughs or tanks used for holding and acclimating test fish should be located away from any physical disturbances and preferably in a location separate from the test tanks.

Holding (rearing) tanks or troughs may be outdoors or indoors as long as they provide the conditions required and recommended herein; however, tanks for acclimating fish to laboratory test conditions should be indoors or, if outdoors, covered with lids fitted with *photoperiod*-controlled lighting.

Following the transport of fish to the rearing/acclimation facilities of the testing laboratory, they must be held for a minimum period of one week under conditions specified in Section 2.4. This *acclimation* period must immediately precede their use in a test.

Tanks should be kept clean, with siphoning of excess food and faeces as frequently as necessary. Tanks with central, double standpipes are partially self-cleaning and have been successfully used at Canadian laboratories. Tanks should be disinfected and rinsed thoroughly with water used for holding/acclimating fish before introducing a new batch of fish. Disinfectants such as those containing chlorinated or iodophore compounds, n-alkyldimethyl-benzyl ammonium chloride, or a biodegradable detergent should be used.

Feeding may be withheld for 24 hours immediately after receipt of fish. Fish might not feed while recovering from transport stress, and the addition of uneaten food could lead to deterioration of water quality. Feeding should be once or more per day with bloodworms and/or brine shrimp (fresh or frozen), at a daily ration (dry food basis) approximating 1% to 5% of wet body weight, depending on temperature, fish size, and consumption rate. Alternatively, freeze-dried bloodworms and/or brine shrimp or a recognized (standard) commercial fish food or tropical fish food may be used at a ration equivalent to that described above. Pellet size and type should be chosen in consideration of fish size and age, water temperature, and the manufacturer's recommendations. The maximum duration and method of food storage should also follow the manufacturer's recommendation.

Fish should be inspected daily for signs of disease or parasites.[1] Dead or moribund fish

[1] Symptoms of unhealthy fish include loss of appetite, abnormal distribution in the tank, lethargy, erratic or atypical swimming behavior, darkened colouration, pale gills, eroded or frayed fins, and external lesions. Parasites and disease are not uncommon among populations of sticklebacks collected from the wild. One of the most commonly observed and obvious infections is that caused by a microsporidian parasite of the genus *Glugea*. Microsporidian parasites are acquired by the host fish when they either ingest infected aquatic invertebrates or free spores in the water column. Once ingested, *Glugea* invades the host's cells producing tumours that are externally

should be removed immediately after daily inspection. Fish with any overt signs of disease or parasites (e.g., tumours), as well as males displaying breeding colours (typically, blue eyes and/or red throat/fore-belly), and gravid females (swollen abdomen) should also be removed from holding and acclimation tanks if observed. Mortalities in the holding and acclimation tank(s) from which test fish are to be taken should be monitored and recorded daily and, as a minimum, must be monitored and recorded 5 days per week. The cumulative fish mortality during the 7-day period preceding the day that the *toxicity test* is started must be < 2%. If the cumulative fish mortality during this period is 2% to 10%, acclimation must be extended for at least an additional 7 days and until a cumulative 7-day mortality of < 2% is achieved for the 7-day period preceding the day that the toxicity test is started. A cumulative mortality of > 10% per week, during any 7-day period makes the batch of fish unacceptable for future use if deaths are caused by disease or aquatic contaminants. If deaths result from other factors (e.g., high initial mortalities following fish transfer), the fish may be used for future toxicity tests provided that mortalities in the acclimation tank(s) from which fish are to be taken decline to < 2% during the 7 days preceding the day that the test is started.

Chemical treatment of diseased fish should be avoided. If the use of chemically treated fish cannot be avoided, a minimum two-week period must follow their treatment before they are used in tests. The test with a *reference toxicant* (see Section 7) gives some indication of the suitability of the fish for use in toxicity tests.

2.3 Water

Water for holding and acclimating fish may be either an uncontaminated supply of natural *seawater* or "*reconstituted*" *seawater* (also known as *artificial seawater*) made up to a desired salinity using commercially available dry ocean salts. If natural seawater is to be used for holding and/or acclimation, it may be filtered (to remove particulates and indigenous organisms) and aerated, if necessary. If artificial seawater is to be used for holding and/or acclimating fish, it must be made up to the desired salinity by adding commercially available dry ocean salts to the appropriate quantity of suitable fresh water and mixing thoroughly during salt addition. Artificial seawater prepared by the direct addition of dry salts must be aerated continuously and vigorously for a minimum of 12 hours before being used, however longer periods are recommended (≥ 3 days). Artificial seawater may be filtered after the 12-hour aeration period and/or prior to use to remove any undissolved ocean salts. Any commercially available sea salts used to prepare the artificial seawater should have previously been shown to consistently and reliably support good survival and health of threespine sticklebacks (e.g., Instant Ocean®, H2Ocean Pro+, OmegaSea® Premium Marine Salt). A given *batch* of natural seawater may be stored for up to 4 months, and artificial seawater up to 2 weeks in covered containers protected from light. The chemical quality of the laboratory's artificial or natural seawater supply should be monitored and assessed as frequently as required to document quality and variation. This should include at least salinity, pH, dissolved oxygen (DO), and total residual chlorine (if municipal drinking water is used as a source for artificial seawater). Salinity measurements must be carried out using either conductivity or refractometry, as described in Section 4.2.

In addition, and as appropriate, suspended solids, total organic carbon, ammonia, metals, and pesticides should be monitored. Alkalinity and total dissolved gases can also be monitored. Any supersaturation with gases should be remedied (see Section 2.4.3 in EC, 1990b).

Sources of water used for preparing artificial seawater may be *deionized water*, *distilled water*, an uncontaminated supply of groundwater or surface water, or dechlorinated municipal drinking water. If *dechlorinated water* is used, it

visible as white cysts on the skin, mouth, and opercula. Spores are released following the rupture of these tumours. There is a metabolic cost to the host associated with *Glugea* parasitism (Ward *et al*., 2005).

must be free of any harmful concentration of chlorine or chlorinated compounds upon fish exposure (see Section 2.4.3 in EC, 1990b). A readily measurable total residual chlorine value of 20 µg/L has been shown not to affect stickleback survival (unpublished data).[2]

The water in containers holding fish should be renewed continuously (i.e., *flow-through* system), renewed periodically (i.e., *static-replacement* system), or recirculated (i.e., with biological filtration) to prevent a build-up of metabolic wastes. Holding densities for static-replacement, and recirculated or low flow systems with filtration should be ≤ 0.5 g fish/L of water and must be ≤ 0.9 g fish/L of water. Higher densities of fish can be held under flow-through conditions. For holding/acclimating systems in which water is recirculated, a biological filter suitable for removing metabolic wastes should be used. Recycled water should be filtered to remove solid waste. Biobeads and charcoal (or equivalent) can be added to the filter to control ammonia concentrations and to remove other possible contaminants from the water. Water in the holding/acclimation tanks should be siphoned out and replaced with clean water as required to remove faeces and debris and/or to maintain water quality (e.g., to maintain pH and manage ammonia levels). In such cases of water reuse, ammonia should be measured frequently to check that it does not reach harmful levels. More frequent *monitoring* and water changes might be necessary when aquaria are first established (i.e., the first month), prior to stabilization of the biological filtration system. A target value for holding/culturing threespine sticklebacks recommended herein is ≤ 2 mg/L of total ammonia. For static-replacement (i.e., no filter and water quality maintained simply through water changes) more frequent water changes might be necessary (e.g., three times per week) to avoid water quality problems. For flow-through systems without recirculation, flow of fresh (new) water through tanks used for holding and acclimating fish must be adequate to maintain fish health (e.g., ≥ 2 L/min).

Ten or more fish should be weighed at regular intervals to determine or adjust feeding rates and to assure that the holding density requirements (i.e., ≤ 0.9 g fish/L of water for static-replacement, recirculated, and low flow systems) are met. This can be accomplished by randomly removing fish from each holding/acclimation tank or by using the weight measurements of the control fish determined at the end of each test. The mean wet weight of individual fish should be determined and recorded, for each of these samples. These measurements can also be used as a guide when determining the volume of effluent required for a test, and to ensure that the required fish size (0.20 to 1.2 g) and maximum loading density (0.5 g fish/L solution in each test vessel) during the toxicity test (see Section 4.2) will not be exceeded.

2.4 Physicochemical Conditions

2.4.1 Temperature

During the holding period preceding acclimation to test conditions, fish may be held within the temperature range shown previously to be suitable for the species (i.e., 8 to 17°C). Fish must be acclimated for ≥ 1 week at 15 ± 2°C before use in a test. The recommended rate of temperature change is ≤ 5°C/day.

[2] The guideline value for total residual chlorine for the protection of marine life is ≤ 0.5 µg/L (CCME, 1999). Values > 0.5 µg/L might risk interaction of chlorine/chloramines toxicity with the contaminant(s) being tested. The limit of detection for the analytical technique used to measure residual chlorine or chloramines in the treated supply of dechlorinated water should ideally be low enough to assure that residual chlorine is ≤ 0.5 µg/L; however, this might be unrealistic for methods used in the laboratory for routine measurements. Using equipment that can, in a particular laboratory, measure down to 20 µg/L, is acceptable.

2.4.2 Salinity

Fish must be acclimated for ≥ 1 week to a salinity within 5 g/kg of that used for the *control/dilution water* to be used in the test. The recommended rate of salinity change is ≤ 5 g/kg per day. A second control (*salinity control*) must be included in the test if the salinity of the effluent sample (or the highest test concentration for chemical testing; see Section 8) is more than 5 g/kg greater than or less than the salinity to which the fish have been acclimated (see Section 4.2).

2.4.3 Dissolved Oxygen and pH

The dissolved oxygen (DO) content of the water within holding containers should be maintained at 80% to 100% saturation. Supplementary aeration to the tanks should be provided if necessary, using filtered, oil-free compressed air.

The *pH* of water used for holding threespine sticklebacks should be in the range of 7.0 to 8.5.

2.4.4 Lighting

Lighting should be full spectrum, with 100 to 500 *lux* intensity at the water surface. For at least one week before a test, *photoperiod* must be a constant at 16 ± 1 hours of light and 8 ± 1 hours of darkness, preferably with a 15- to 30-minute transition period (see footnote c in Section 2.4.2 of EC, 1990b).

2.4.5 Monitoring

Water temperature, DO, salinity, pH, water flow (if applicable), ammonia, and fish mortalities must be monitored for each holding and acclimation tank at regular intervals.

Section 3

Facilities

The need for any special facilities would be governed by the degree of hazard associated with the samples that are to be tested, and by the risk of sample and apparatus contamination. Test must be performed in a facility that is isolated from general laboratory disturbances; either a separate room or a section walled or curtained off. The area should be well ventilated, and free from physical disturbances or airborne contaminants that might affect the test organisms. Dust and fumes should be minimized. The testing facilities should also be isolated from areas in which test solutions are prepared, and removed from areas in which equipment is cleaned. Control of test temperature (15 ± 1°C) can be achieved by thermostatically controlled air conditioning or by immersing test vessels in regulated water baths.

Test vessels, equipment, and supplies that might contact test or *stock solutions* or control/dilution water must not contain substances that can be leached or dissolved in amounts that adversely affect the test organisms. Equipment and supplies should be chosen carefully to minimize sorption of materials from water.

Test vessels must be glass, Plexiglas®, acrylic, polypropylene, polyethylene, or have high-quality (nontoxic) polyethylene liners. If liners are used, they must be discarded at the end of the test. It is recommended that test vessels be loosely covered with clean, nontoxic screens, polyethylene bag liners, or glass if necessary to prevent fish from escaping. All containers (i.e., type, size, and shape) used for a test vessel must be identical, and the minimum water depth must be 15 cm and identical for each test solution. Equipment must be thoroughly cleaned and rinsed in accordance with good laboratory practice.

The laboratory must have the instruments to measure the basic variables of water quality (temperature, salinity, dissolved oxygen, and pH), and must be prepared to undertake prompt and accurate analysis of other variables such as ammonia.

The *control/dilution water* should be the type described in Sections 2.3 and 4.3, and it should preferably be identical to that used for holding and/or acclimating the fish.

Section 4

General Procedure for Determining Acute Lethality of Effluent

4.1 Sample Labelling, Transport, and Storage

Sample-volume requirements depend on fish size and numbers per test solution, loading density requirements, test concentrations, and the use of *replicates*. For single-concentration tests, sample volumes of 20 to 40 L or more are normally required. For tests to determine an LC50, sample volumes of 40 to 80 L or more are normally required.

Containers for transportation and storage of samples must be made of nontoxic material (e.g., polyethylene or polypropylene carboys or pails). The containers must be new or thoroughly cleaned and rinsed with clean water and should then be rinsed with the sample to be collected. Each sample container should be filled completely, to exclude air. Immediately after filling, each sample container must be sealed (e.g., using a snap-on lid if the sample container is a pail), and labelled or coded. Labelling and accompanying records made at this time must include at least a code that can be used to identify the sample or subsample. Labelling or a cross-referenced record, which might or might not accompany the sample(s), must include at least sample type, source, sampling method, date and time of collection, and name of sampler(s).

Samples must be kept from freezing during transport or storage. During transport, samples should be kept in the dark, and at a temperature of 1 to 8°C if more than 2 days are spent in transit or when ambient temperatures are extreme (i.e., > 30°C or < 1°C). Upon receipt of sample(s) at the laboratory, the date and time of receipt and the temperature of the effluent in each sample container must be measured and recorded. Each sample to be used in the *toxicity test* must be adjusted to 15 ± 1°C before the toxicity test can be started.

To enable the toxicity test to be started on the day the sample is received in the laboratory, temperature adjustment of the effluent sample(s) can be done quickly (see Section 4.3). Alternatively, the laboratory might choose to store the sample(s) in the dark at 4 ± 2°C for a brief period (e.g., over the weekend, if the sample(s) arrived on a Friday afternoon), provided that the test commences within the time period specified below. Using this option, the sample(s) must be stored in full, sealed containers, which are held in the dark within a refrigerated facility. A third option is to hold the sample(s) overnight within a facility adjusted to the test temperature (i.e., 15 ± 1°C), in which instance the test must be started the next day. If a sample is warmed or cooled at 15 ± 1°C overnight, it must be kept in one or more full, sealed containers during that time.

Testing of samples should commence as soon as possible after collection. The test should begin within 3 days and must commence no later than 5 days after termination of sampling.

The contents of each sample container must be agitated thoroughly (i.e., to re-suspend settleable solids) just before pouring aliquots to prepare solutions. Samples of effluent must not be filtered prior to testing or agitated (other than that provided by the required aeration described herein) during the test. If concern exists about the contribution of elevated concentrations of suspended or settleable solids in test samples to the sample toxicity, additional tests (i.e., with filtration, or maintaining solids in suspension during the exposure) are described elsewhere (see Section 6.4 in EC, 1990b). Subsamples (i.e., aliquots of a sample divided between two or more containers) must be combined prior to solution preparation.

4.2 Test Conditions

This is a 96-hour *static* test (i.e., there is no replacement of solutions during the test). Loading of fish into each test vessel must not exceed a density of 0.5 g fish/L; adherence to this requirement is based on mean wet weight of control fish at the end of the test (Section 4.5). Fish must

not be fed during the test, nor during the 16-hour period immediately preceding it. The test is not valid if > 10% control fish die and/or exhibit atypical/stressed behaviour (Section 4.6).

The test must be conducted at 15 ± 1°C (as measured in test solutions). All solutions, including the control(s) must be aerated throughout the test at a controlled rate of 6.5 ± 1 mL/min · L. Lighting must be the same as that defined for acclimation (see Section 2.4.4). Photoperiod (a light:dark cycle of 16 ± 1 hours:8 ± 1 hours) must coincide with the timing which prevailed during acclimation.

The test must be conducted without adjustment of sample or test solution pH. If, however, it is desired to understand the extent to which extremes in solution or sample pH (e.g., outside the range of 6.5 to 8.5)[3] might contribute to acute lethality, a parallel (pH-adjusted) test may be used. If both pH-adjusted and non-adjusted tests are run, definitive results must be those derived from the non-adjusted test. Rationale and procedural details regarding pH adjustment are provided elsewhere (see Section 4.3.2 in EC, 1990b). Adjustment of pH is also one of a number of "*Toxicity Identification Evaluation*" techniques for characterizing the cause of sample toxicity (USEPA, 1991, 1996).

This reference method is suitable for effluents with salinity values of greater than 10 parts per thousand salinity (‰). The salinity of the effluent must be measured before testing commences. There are two acceptable methods to measure salinity: *conductivity* and *refractometry*. A performance-based approach is used to confirm the suitability/acceptability of the method and instruments.

If using conductivity, an acceptable method and instrument (e.g., Fisher Accumet™ AR50 meter, Fisher Accumet™ 13-620-162 Conductivity cell 10.0 cm^{-1} or more recent equivalents) must:

i) be calibrated daily when in use with a certified conductivity standard, and

ii) be verified to accurately measure seawater salinity using a certified seawater standard (e.g., those offered by Ocean Scientific International Ltd); the tolerance limit for accuracy is within 1 part per thousand.

The *verification* for *accuracy* should be carried out after *calibration*. A conductivity standard close to the conductivity of the effluent sample is recommended. A conductivity cell with a cell constant appropriate for use in high ionic strength solutions is recommended. Conductivity measurements are sensitive to temperature, and reported conductivity must account for temperature. This can be achieved via automatic temperature compensation offered on some instruments. Some instruments automatically convert conductivity to salinity; others provide only conductivity readings, which necessitates the use of a conversion table to determine salinity. Conversion methods that use the formulas described in Standard Methods for the Examination of Water and Wastewater (APHA *et al*., 2012) are recommended[4]. Both automatic conversion using instruments and the use of on-line conversion tables are acceptable provided the performance criteria are met.

If using refractometry, an acceptable method and instrument (e.g., Reichert® Goldberg Salinity Refractometer) must:

i) be calibrated daily when in use with purified water at 0‰, and

ii) be verified to accurately measure seawater salinity using a certified seawater standard (e.g., those offered by Ocean Scientific International Ltd); the tolerance limit for accuracy is within 1 part per thousand.

[3] The pH of natural, uncontaminated seawater is normally within the range of 7.5 to 8.5. Seawater solutions with pH values beyond the 6.5 to 8.5 range are atypical of the estuarine or marine environment. In this context, such pH values are considered as (environmentally) atypical.

[4] Several on-line conversion calculators are available, including: www.chemiasoft.com/chemd/salinity_calculator.

The verification for accuracy should be carried out after calibration. Deionized water or reverse osmosis water are examples of appropriate purified water.

Instruments for measuring salinity, either via conductivity or refractometry, must be properly operated (e.g., temperature compensation with conductivity is needed) and maintained, as required by accreditation programs. Instruments must be calibrated and verified routinely.

The acceptable methods for measuring salinity rely on physical properties (electrical conductance and ability to refract light) that are closely associated with salinity. These methods do not identify the ions contributing to the conductance or refraction. As a result, these methods cannot distinguish between an effluent dominated by sodium and chloride ions and an effluent dominated by high total dissolved solids, which might have a different ionic composition. Further analytical investigation of effluent ion composition is recommended if it is suspected that the effluent sample is high in total dissolved solids.

Toxicity tests must be carried out without the adjustment of the test sample salinity. If the salinity of the sample is > 5‰ higher or lower than the salinity to which to fish are acclimated, a second control (*salinity control*) with the salinity adjusted to that (i.e., within 1 g/kg) of the sample must be included in the test. This salinity control must be prepared as described for control/dilution water (see Sections 2.3 and 4.3). The salinity control must be > 10‰ (below which this test method is not applicable) and ≤ 35‰ (the upper limit of salinity in natural *seawater*), even if the sample salinity is outside that range (i.e., > 35‰). When performing a multi-concentration test, the water used as the *dilution water* (typically holding and acclimation water) must also be used as the dilution-water control. In instances where a further understanding of the contribution of salinity to sample *toxicity* is desired, the water used as the salinity control (i.e., adjusted to the salinity of the test sample) can be used as the dilution water in a second, parallel, multi-concentration test. The results for each set of controls used in a toxicity test (i.e., dilution water and salinity controls) must be examined to determine if they independently meet the test-specific criteria for test validity (Section 4.6). In instances where two sets of controls are used, the results for the toxicity test are considered valid and acceptable only if each set of control solutions independently met the respective validity requirement(s).

4.3 Preparing Test Solutions

Measurements of pH, temperature, dissolved oxygen, and salinity must be made in the unadjusted, undiluted effluent just before preparing test solutions. Adjustment of the effluent sample and control/dilution water to 15 ± 1°C must be done if the temperature is outside that range. This can be accomplished using different ambient temperatures as needed for cooling or warming. The sample can also be cooled using a cold-water bath or immersion cooler made of nontoxic material (e.g., stainless steel), or warmed using a hot-water bath. Samples or test solutions must not be heated by immersion heaters or microwaves.

For a given test, the same water is to be used for preparing the control(s) and all test concentrations less than 100%. This is almost always the same water as that used for acclimation. If the temperature of this water is adjusted upwards, supersaturation with gases must be avoided. The water must have an oxygen content within the range of 90% to 100% air saturation, achieved if necessary by vigorous aeration with oil-free compressed air passed through clean air stones. Air stones acceptable for use are:

i) Marina® air stone, 2.5 cm length × 1.5 cm diameter, cylindrical (one use only);

ii) AS1 silica glass, 3.8 cm length × 1.3 cm width, rectangular (re-usable after proper cleaning; as described in Section 4.3.1 of EC, 1990b); or

iii) alternate air stone that has been shown to perform equivalently to the Marina® or AS1 air stone.[5]

[5] The Marina® (Hagen®) air stones are available from numerous local suppliers and from Rolf C. Hagen Inc. (1-800-554-2436). For a complete description, go to

If *artificial seawater* is to be used as the dilution and control water, it must be prepared as described in Section 2.3. Test vessels should be rinsed with control/dilution water just before use, although that might not be necessary if polyethylene liners are used. Each test solution must be made up to an identical volume, and well mixed with a glass rod, Teflon™ stir bar, or other device made of nontoxic material, just before its use. All test vessels, measurement devices, stirring equipment, and fish-transfer pails must be thoroughly cleaned and rinsed in accordance with standard operating procedures (SOPs).

The depth of solution in each test vessel must be at least 15 cm. Upon preparation of the test solutions including the control(s), each must be aerated for a period of 30 minutes at 6.5 ± 1 mL/min · L. Thereafter, the concentration of dissolved oxygen must be measured in at least the highest test concentration (normally 100% effluent). If (and only if) DO in the highest test concentration is < 70% or > 100% of air saturation, then pre-aeration (i.e., before exposure of the fish) of all solutions including the control(s) must be continued at 6.5 ± 1 mL/min · L. This period of pre-aeration must be restricted to the lesser of 90 additional minutes and attaining 70% saturation in the highest test concentration (or 100% saturation if supersaturation is evident). Immediately thereafter, fish must be placed in each test solution and the test initiated, regardless of whether 70% to 100% saturation was achieved in all test solutions. Aeration of test solutions must be provided by bubbling compressed air through clean air stones, described previously. Aeration of each test solution at a rate of 6.5 ± 1 mL/min · L must be continued throughout the test. Rationale for the use of pre-aeration and aeration in fish tests is provided elsewhere (see Section 4.3.1 and footnote "n" in EC, 1990b).

www.hagen.com and search for product A960, A961, or A962. The silica glass air stones model AS1 (Sweetwater® Air Diffusers) are available directly from Pentair Aquatic Eco-Systems®, Nanaimo BC (1-866-714-0141 or www.pentairaes.com), Dynamic Aqua Supply, Surrey BC (1-604-543-7504 or www.dynamicaqua.com), Valox Ltd, Fredericton NB (1-800-825-6997 or www.valoxltd.com), and Fish Farm Supply, Elmira ON (1-877-669-1096 or www.fishfarmsupply.ca). Alternate brands are acceptable, provided they are approximately the same size as the Marina® and AS1 air stones, produce an equivalent quality of aeration, and have been verified by the laboratory as a suitable replacement for the Marina® and AS1 air stones.

4.4 Beginning the Test

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

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

Healthy fish, which have been acclimated for a minimum of one week to the temperature, salinity, and lighting conditions used in the test (see Section 2), must be taken randomly from the acclimation tank(s) for use in the test. Handling and transfer procedures should minimize stress. Any fish dropped or injured during transfer must be discarded. Dip nets should be rinsed (dilution water) between transfers if contact is made with a test solution. Seawater within fish-transfer pails should be aerated if necessary to maintain dissolved oxygen levels at 80% to 100% of air saturation during the period required for introduction of fish to test vessels. At least ten fish must be introduced into each test concentration including each control solution. They may be divided between two or more vessels at the same concentration to meet the required limit on loading (see Section 4.2).

Besides positioning the test concentrations at random within the testing facility, the order of adding fish to each test solution must also be randomized. This can be accomplished by introducing one or two fish sequentially to each test solution, including the control solution(s), until 10 fish have been placed in each. Individual fish must be used only once as test or control organisms.

If one or more test solutions are highly coloured, opaque, or foamy, baskets made of nontoxic,

nonabrasive material (e.g., nylon, polyethylene, polypropylene) can be used to permit inspections of fish during the test. If used, a basket must be placed in each test vessel including the control(s). Baskets must be big enough to allow fish to move throughout the test vessel. Each basket must be thoroughly cleaned and rinsed with control/dilution water before being used.

4.5 Observations and Measurements

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

Measurements of dissolved oxygen, pH, and temperature must be made in each test solution including the control(s), at the start and end of the test as a minimum. Temperature should be measured and aeration should be checked in each test solution including the control(s) every 24 hours. DO and pH can also be measured during the test (e.g., every 24 hours or when test organisms die or appear stressed). Initial measurements on each test solution should be carried out after the pre-aeration period (see Section 4.3). Final measurements should be done after biological observations are complete. The salinity of each test solution must be measured at the start of the test as a minimum.

The frequent and routine observation of fish in each test vessel is required to obtain information regarding their time to death as well as any *overt sublethal* responses evident, and to remove dead fish that could otherwise foul the test solution. Fish in each test vessel must be inspected at least at 24, 48, 72, and 96 hours; more frequent observations (e.g., 0.5, 1, 2, 4, 8 hours) are recommended during the initial day of the test. During each observation period, all dead fish must be recorded and removed. Fish are considered to be dead when they fail to show evidence of opercular or other activity, and do not respond to subsequent gentle prodding. Overt sublethal toxic effects should also be recorded (e.g., any abnormal appearance or behaviour, increased respiratory "coughing" rates, erratic swimming behaviour, surfacing, discolouration, loss of equilibrium; see Appendix E in EC, 1990b). Any differences from control fish should be noted. For highly coloured, opaque, or foamy test solutions, fish can be inspected using a dip net (cleaned and rinsed before use), or by raising them to the surface within a suitable basket (Section 4.4).

The mean *fork length* of the fish in the dilution-water control (measured from the tip of the nose to the tip of the middle caudal-fin ray) must be determined and recorded at the end of the test. The measurement of fork length for each control fish should be used to assess if the size of the test fish met with the recommendation (i.e., the largest fish should not be more than 1.6 times the length of the smallest in the same test; see Section 2.1).

The mean wet weight of individual fish in the dilution-water control (after being blotted dry and weighed within 30 minutes of removal from the tank) must be determined and recorded at the end of the test. This measurement must be used to confirm that the required range of weights for test fish (i.e., average wet weight must be between 0.20 and 1.2 g; see Section 2.1), and loading density (i.e., loading density of fish in each test vessel must be $\leq$ 0.5 g fish/L; see Section 4.2) were met.

Each individual fish in each *treatment* should be examined to determine its morphological form (see Section 2.1 and Appendix D). This can be accomplished through examination for the presence of a caudal keel, staining techniques for plate counts, or by submitting fish for taxonomic analysis.

All surviving fish (including controls) used in the test must be disposed of in a humane manner at the end of the test. Euthanasia must be carried out according to CCAC guidelines on the euthanasia of animals used in science (CCAC, 2010). Test organisms can be euthanized at the end of the test following final observations of mortality and any abnormal appearance or behaviour to facilitate final fork length and wet weight measurements. Overdosing the fish with an anaesthetic such as tricaine methane sulphonate (TMS) is recommended. Fish can be transferred to a bucket containing TMS at 2 g/L, buffered by the addition of sodium bicarbonate to pH 6.5 (Paula Jackman, Atlantic Laboratory for Environmental Testing, Environment and Climate Change Canada, personal communication, 2017).

4.6 Validity Criteria

The test is not valid if > 10% control fish die and/or exhibit atypical/stressed behaviour (atypical swimming, twitching, skittering at the surface, loss of equilibrium, etc.; see Appendix E in EC, 1990b). For the results of any toxicity test which includes two sets of controls (i.e., a dilution-water control and a salinity control) to be considered as valid and acceptable, both controls must independently meet the criteria for test validity.

Section 5

Procedure for a Single-concentration Test to Determine Percent Mortality at 96 Hours

All conditions, procedures, and facilities specified in Sections 1, 2, 3, 4, 7, and 9 apply to the procedure for testing a single concentration of *effluent*.

This procedure uses one concentration of effluent, 100%, unless otherwise specified, plus a control (*control water* only), which is normally the same as the holding/acclimation water. If the salinity of the effluent and the salinity to which the fish are acclimated differ by more than 5 g/kg, a second control (i.e., salinity control) adjusted to the salinity of the effluent must also be used (see Section 4.2). The use of *replicate* solutions (e.g., three replicates of the 100% concentration and three replicates for each control solution, using 10 fish in each replicate solution) is recommended for this test, to provide greater confidence in the test results and their interpretation. At least 10 fish must be exposed to each effluent and each of any controls.

The test is invalidated if > 10% of the control fish (combined data, if replicates are used) exhibit atypical/stressed behaviour and/or mortality (see Section 4.6). For the results of any toxicity test which includes two sets of controls (i.e., a dilution-water control and a salinity control) to be considered as valid and acceptable, both controls must independently meet the criteria for test validity.

The *endpoint* for this test is *percentage* mortality at 96 hours. Mortality of > 50% is commonly used to define whether or not a sample would receive a "pass" or "fail" rating. For example, the *Metal Mining Effluent Regulations* define an effluent as failing this test if the effluent at 100% concentration kills more than 50% of the fish.

Section 6

Procedure for a Multi-concentration Test to Determine the 96-h LC50

All conditions, procedures, and facilities specified in Sections 1, 2, 3, 4, 7, and 9 apply to this procedure.

At least five concentrations of *effluent* plus a control (*dilution water* only), which is normally the same as the holding/acclimation water, must be used in tests to estimate an *LC50*. If the salinity of the effluent and the salinity to which the fish are acclimated differ by more than 5 g/kg, a second control (i.e., salinity control) adjusted to the salinity of the effluent must also be used (see Section 4.2). At least ten fish must be exposed to each test concentration, including the undiluted (100%) concentration and the control(s). The highest concentration must be full-strength (100%) effluent, and each successive concentration must have at least 50% of the strength of the next higher one. A geometric (logarithmic) series is beneficial (e.g., percent concentrations such as 100, 50, 25, 12.5, 6.3). Concentrations may be based on other proportions or on standard dilution-series (see Appendix D in EC, 1990b).

Since this LC50 test must include full strength (100%) effluent as the highest concentration, the single-concentration *endpoint* of percent mortality in 100% effluent at 96 hours (see Section 5) can also be determined from the results of this test.

Replicates of each concentration may be used. The use of *replicate* solutions (i.e., exposing a greater number of fish) could provide a more accurate representation of the concentration-response curve (USEPA, 2016a), and therefore greater confidence in the test results and their interpretation. The 96-h LC50 and its 95% confidence limits must be calculated if the data are amenable to this calculation, and the method of calculation must be reported. Environment Canada's guidance document on statistical methods for environmental toxicity tests, EPS 1/RM/46 (EC, 2005) provides further direction and advice for calculating the LC50. Computer programs for calculating LC50 and confidence limits are available (EC, 2005) and should be used.

The test is invalidated if > 10% of the control fish (combined data if replicates are used) exhibit atypical/stressed behaviour and/or mortality (Section 4.6). For the results of any toxicity test which includes two sets of controls (i.e., a dilution-water control and a salinity control) to be considered as valid and acceptable, both controls must independently meet the criteria for test validity. Only the dilution-water control is used in the calculation of the LC50, or for calculating any other statistical endpoints involving comparisons of the findings for each set of test concentrations versus those for control solutions.

Section 7

Procedure for Testing a Reference Toxicant

A *reference toxicant* must be used to assess the relative sensitivity of the *batch* of fish used in the toxicity test, and the *precision* and reliability of data produced by the laboratory for that reference toxicant under standardized test conditions, as well as the technical proficiency of the laboratory staff conducting the test (EC, 1990c).

The selected reference chemical(s) must be tested in a multi-concentration test started within 14 days before or after the date that the toxicity test is initiated, and upon acclimation of a new batch of fish. Fish used in the *reference toxicity test* conducted in conjunction with a test for determining the acute lethality of effluent must be from the same batch held at the laboratory and used in the effluent test. The procedures and conditions to be followed are identical to those in Sections 4 and 6 and as described in Environment Canada (1990c), except that aliquots of a reference chemical are added to dilution water and tested instead of an effluent. The control/dilution water used routinely in effluent tests must also be used for the reference toxicity test.

Reagent-grade phenol is recommended for use as a reference toxicant with *G. aculeatus*. The 96-h LC50 should be calculated for the reference toxicant used and expressed as mg/L based on phenol. Based on results generated during inter-laboratory testing, the mean 96-h LC50 for threespine sticklebacks was 14.6 mg phenol/L (CV = 19.6%; n = 9) (AquaTox, 2017). *Stock solutions* of phenol must be made up on the day of use or shown to remain stable during holding if stored prior to use. Some metals have been shown to be problematic for use as reference toxicants using sticklebacks with solubility problems for zinc and copper in saline water, and higher inter-laboratory variability with cadmium relative to phenol in inter-laboratory testing (AquaTox, 2017). Metals are therefore not recommended for use as a reference toxicant with sticklebacks.

Concentrations of reference toxicant in all stock solutions should be measured chemically using appropriate methods (APHA *et al*., 2012). 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 LC50 be atypical (i.e., outside *warning limits*). If stored, sample aliquots must be held in the dark at 4 ± 2°C. Phenol solutions should be preserved before storage (APHA *et al*., 2012). Stored aliquots requiring chemical measurement should be analyzed promptly upon completion of the toxicity test. It is desirable, but not required, to measure concentrations in the same solutions at the end of the test after completing biological observations. Calculations of LC50 should be based on measured concentrations if they are appreciably (i.e., ≥ 20%) different from nominal ones and if the accuracy of the chemical analyses is satisfactory.

Once sufficient data (e.g., minimum of five data points) are available (EC, 1990c, 2005), a *warning chart* which plots values for LC50 must be prepared, and continually updated, with each new reference toxicity test. The warning chart should plot logarithm of concentration on the vertical axis against date of the test or test number on the horizontal axis. Each new LC50 for the reference toxicant should be compared with the previously established warning limits; the LC50 is acceptable if it falls within the warning limits (± 2 SD). All calculations of mean and standard deviation must be made on the basis of log(LC50). This represents continued adherence to the assumption by which each LC50 was estimated on the basis of logarithm of concentrations. The *mean* of log(LC50), together with its upper and lower warning limits (± 2 SD) as calculated by using the available values of log(LC50), are recalculated with each successive LC50 (EC, 1990c, 2005). If the test is run frequently, the most recent 20 reference toxicant points may be used to calculate means and warning limits.

The warning chart can be constructed by simply plotting mean and ± 2 SD as the logarithms, or if desired, by converting them to arithmetic values and plotting LC50 and ± 2 SD on a logarithmic scale of concentration. Different approaches to creating a warning chart (e.g., Levey-Jennings, moving average) are acceptable. Warning charts can be used to detect trends over time. Examples of trends that might be observed include an increasing or decreasing trend, several successive points on one side of the mean, changes that are observed at different times of the year, and successive LC50 values outside the ± 2 SD warning limits.

If a particular LC50 falls outside the warning limits, the sensitivity of the test fish and the performance and precision of the test are suspect. Since this might occur 5% of the time due to chance alone, an outlying value does not necessarily mean that the sensitivity of the batch of fish or the precision of the toxicity data produced by the laboratory are in question. Rather, it provides a warning that this might be the case. A thorough check of all acclimation, holding, and test conditions, as well as technical proficiency is required at this time.

Depending on the findings, further acclimation and re-evaluation of the fish with one or more reference toxicant(s) should be undertaken, or a new batch of fish should be procured and acclimated for use in subsequent toxicity tests with effluent(s) and reference toxicant(s).

Test results that usually fall within warning limits do 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 the test. For guidance on reasonable variation among reference toxicant data (i.e., warning limits for a warning chart), please refer to Section 2.8.1 and Appendix F in Environment Canada, 2005.

If an LC50 fell outside the control limits (mean ± 3 SD), it would be highly probable that the test was unacceptable and should be repeated, with all aspects of the test being carefully scrutinized. If endpoints fell between the control and warning limits more than 5% of the time, a deterioration in precision would be indicated, and again the most recent test should be repeated with careful scrutiny of procedures, conditions, and calculations.

Section 8

Procedure for Testing Chemicals

This section gives specific instructions for testing individual *chemicals*, chemical substances (e.g., formulated products), or chemical mixtures (i.e., water samples amended with a test substance), in addition to the procedures listed in Sections 1 to 7.

8.1 Properties, Labelling, and Storage of Sample

Information should be obtained about the properties of the chemical, formulated product, or chemical mixture to be tested, including the concentration of major ingredients, solubility in seawater (natural or artificial), vapour pressure, chemical stability, dissociation constants, toxicity to humans and aquatic organisms, and biodegradability. Data-sheets on safety aspects of the test substance(s) (e.g., Safety Data Sheets) 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 and/or chemical solubility in control/dilution water should be determined experimentally. Other available information such as structural formulae, degree of purity, nature and percentage of significant impurities, presence and amounts of additives, and n-octanol:water partition coefficient should be obtained and recorded.[6] An acceptable analytical method for measuring the chemical in seawater at concentrations intended for the test should also be known, together with data indicating the *precision* and *accuracy* of the analysis.

Chemical containers must be sealed and coded or labelled upon receipt. Required information (i.e., chemical name, supplier, date received) must be indicated on the label and/or recorded on a separate datasheet dedicated to the sample, as appropriate. 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.

8.2 Preparing Test Solutions

For testing chemicals, a multi-concentration test is usually performed, to determine the LC50 (see Section 6). It might be desirable to have *replicates* (e.g., two or three) of each test concentration for purposes of evaluating the *toxicity* of chemicals or chemical mixtures for federal registration or other regulatory purposes. Replicates could be required under regulations for registering a chemical, pesticide, or similar category of chemical. Since the objective for a multi-concentration test is to determine the 96-h LC50 (based on mortality data), a test using a minimum of five concentrations plus control(s) is recommended. The number of replicates and *treatments* could be reduced or eliminated for range-finding tests and depending on the expected variance among test vessels within a treatment, could also be reduced or eliminated for non-regulatory screening assays or research studies.

Test solutions of the chemical to be tested are usually prepared by adding aliquots of a *stock solution* made up in control/dilution water. Alternatively, for strong solutions or large volumes, weighed (using an appropriate balance) quantities of chemical can be added to control/dilution water to give the nominal strengths for testing. For aqueous samples (e.g., chemical formulations in water), test solutions can also be prepared by adding appropriate quantities of commercially available dry ocean salts (see Section 2.3) directly to the sample or each of the test solutions to adjust the salinity to the within the desired range. Nominal test concentrations must be prepared and reported in consideration of any salinity adjustment. If the salinity of the highest test concentration is > 5 g/kg higher or lower than the salinity to which to fish are

[6] 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). Information regarding chemical solubility and stability in seawater will also be of use in interpreting test results.

acclimated, a second control with the salinity adjusted to that (i.e., within 1 g/kg) of the highest test concentration (salinity control) must be included in the test. For guidance on the use of a salinity control, see Section 4.2. 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 and unstable stock solutions must be newly prepared as necessary. If deionized water, distilled water, or fresh water is used to make the stock solution, commercially available dry ocean salts should be added, as necessary, to adjust the salinity of each test solution to within the desired range.

For chemicals that do not dissolve readily in water, guidance provided in OECD's document on the aquatic toxicity testing of difficult substances and mixtures (OECD, 2000) should be followed. *Emulsifiers* or *dispersants* should not be used to increase chemical solubility except in instances where these substances might be formulated with the test chemical for its normal commercial purposes. The use of a solvent other than water should be avoided if possible. An organic solvent may be used for the dissolution of the test substance in dilution water where no other acceptable method of test solution preparation is available. If used, an additional control solution must be prepared containing the control/dilution water and the same concentration of solubilizing agent as that present in the most concentrated solution of the test chemical (i.e., solvent control). Such agents should be used sparingly (i.e., using the minimum volume necessary to dissolve or suspend the test substance in dilution water) and should not exceed the concentration that affects the survival of threespine sticklebacks or a maximum of 0.1 mL/L in any test solution (OECD, 2000; Hutchison *et al*., 2006; Green and Wheeler, 2013). If this information is unknown, a preliminary solvent only test, using various concentrations of the solvent should be conducted to determine the threshold-effect concentration of the particular solvent being considered for use in the definitive test. If solvents are used, the following are preferred (OECD, 2000; USEPA, 2016b): dimethyl formamide, triethylene glycol, methanol, acetone, and ethanol.

Upon preparation of each test solution including the control(s), the dissolved oxygen content should be measured. Thereafter, either fish should be introduced and the test initiated (see Section 4.4), or each test solution should be pre-aerated (see Section 4.3) and then fish added. In most instances, the pre-aeration of test solutions is not necessary nor warranted.[7] For those situations in which pre-aeration is appropriate (i.e., if, upon preparation, the DO content of one or more test solutions is < 70% or > 100% of air saturation), the guidance for pre-aeration of solutions given in Section 4.3 should be followed.

8.3 Control/Dilution Water

Control/dilution water may be *artificial seawater*, the laboratory's supply of natural "uncontaminated" *seawater* (see Section 2.3), or a particular sample of estuarine or marine *receiving water* if there is special interest in a local situation. The choice of control/dilution water to be used depends on the intent of the test.

If a high degree of standardization is required (e.g., the measured toxicity of a chemical is to be compared and assessed relative to values derived elsewhere for this and/or other chemicals), artificial seawater adjusted to one or more salinities common to all tests should be prepared and used as the control/dilution water. Additionally, the salinity of all test concentrations should be within 1 g/kg of the controls.

If the toxic effect of a chemical on a particular marine or estuarine receiving water is to be assessed, sample(s) of the receiving water, could be taken from an area that was isolated from influences of the chemical, and used as the dilution and control

[7] 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 substance. If it is necessary to aerate any test solution, all solutions are to be aerated as described in Section 4.3.

water.[8, 9, 10] Examples of such situations would include appraisals of the toxic effects of chemical spills (real or potential) or intentional applications of a chemical (e.g., spraying of a pesticide) on a particular estuarine or marine water body. If a sample of receiving water is to be used as control/dilution water, a separate control solution must be prepared using the control/dilution water that is normally used for the stickleback acute lethality test and is able to achieve valid test results on a routine basis (see Section 4.3).

The laboratory supply of uncontaminated natural seawater or artificial seawater may also be used to appraise the toxic effect of a chemical on a particular receiving environment, especially where logistical constraints make the collection and use of receiving water impractical or if there is already an interfering toxicity in the receiving water. This supply of natural or artificial seawater is also appropriate for use as control/dilution water in other instances (e.g., preliminary or intra-laboratory assessment of chemical toxicity).

If information is desired regarding the influence of salinity on the toxicity of the chemical under investigation, separate tests should be conducted concurrently at three or more salinities. Control/dilution water for such comparative tests should be from a single source. This source may be artificial seawater (see Section 2.3) or natural, full-strength seawater adjusted for salinity as necessary using dry salts, deionized water, distilled water, or an "uncontaminated" fresh water.

8.4 Test Observations and Measurements

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

During solution preparation and at each of the prescribed observation periods during the test, each test 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 aliquots of test solutions be analyzed to determine the concentrations of chemicals to which fish are exposed.[11] In instances where chemicals are to be

[8] Contaminants already in the receiving water might add toxicity to that of the chemical being tested. In such cases, uncontaminated dilution water (artificial seawater, or the laboratory's supply of natural seawater) would give a more accurate estimate of the individual toxicity of the chemical 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 on a specific receiving environment, 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: as a minimum, a second control using the laboratory's uncontaminated water supply that is normally used in stickleback lethality tests; and as a maximum, another series of concentrations using this same water source as the diluent.

[9] While it would be desirable to acclimate a batch of fish to the receiving water before being used in a test with that water used for dilution and control, it is seldom feasible because of the need to transport large volumes of water to the laboratory. If possible and appropriate, tests using receiving water could be carried out near the site of interest, in which case acclimation should last for at least 5 days.

[10] An alternative (compromise) to using receiving water as dilution and control water is to use artificial seawater or the laboratory's natural seawater supply, adjusted to the salinity and pH 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 to salinity can be made by methods mentioned in Section 2.3, including the addition of appropriate quantities and ratio of commercially available sea salts, and to pH as described in Section 4.3.2 in EC, 1990b.

[11] Such analyses need not be undertaken in all instances, due to cost, analytical limitations, or previous technical data indicating chemical stability in solution under conditions similar to those in the test. Chemical analyses

measured, samples should be taken from the high, medium, and low test-concentrations and the control solution(s) at the beginning and end of the test as a minimum. These samples should be preserved, stored, and analyzed according to best proven, validated methods with acceptable detection limits, available for determining the concentration of the particular chemical in an aqueous (seawater) solution.

If chemical measurements indicate that the concentrations declined by more than 20% during the test period, the acute lethal toxicity of the chemical should be re-evaluated by a test in which solutions are renewed periodically (*static-replacement* test) or continuously (*flow-through* test) (OECD, 1992).

Toxicity results for any tests in which concentrations are measured should be calculated and expressed in terms of those measured concentrations, unless there is good reason to believe that the chemical measurements are not accurate. In making these calculations, each test solution should be characterized by the *geometric mean* measured concentration to which fish were exposed.

8.5 Test Endpoints and Calculations

The *endpoint* for tests performed with chemicals will usually be a 96-h LC50 for fish mortality (see Section 4.5). Accepted procedures for calculating the LC50 and its 95% confidence interval are given in Section 6. Section 5 provides guidance for calculating and comparing endpoints for single-concentration tests. For further information on the appropriate statistics to apply to the endpoint data, the investigator should consult Environment Canada's guidance document on statistical methods for environmental toxicity tests, EPS 1/RM/46 (EC, 2005).

If additional controls (e.g., solvent, salinity, and/or other) are used, the results must be examined to determine if they independently meet the test validity criteria (Section 4.6). The test is rendered invalid if fish mortality and/or atypical/stressed behaviour in any additional control or in the untreated dilution-water control is > 10%. If solvents are used to prepare test solutions, only the data from the solvent control should be used to calculate the LC50, or for calculating any other statistical endpoints involving comparisons of the findings for each set of test concentrations versus those for control solutions.

For each test concentration, including the control treatment(s) the mean percent mortality for the fish at the end of the test must be calculated and reported, if the test is performed using replicate solutions.

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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.

Section 9

Reporting Requirements

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

Unless otherwise specified by Environment and Climate Change Canada, all items listed in Section 9.1 must be reported to Environment and Climate Change Canada for each toxicity test that is initiated. The information is to be provided in accordance with pertinent regulations, and in a manner and format specified by Environment and Climate Change Canada (i.e., manual or electronic, transmission mode, form, and content).

Information additional to that in Section 9.1 such as that required by or distinctive to a regulation, or information that is necessary to clarify reporting and data assessment, might also be specified by Environment and Climate Change Canada.

Unless otherwise specified by Environment and Climate Change Canada, those items listed under Section 9.2 must be recorded and held on file for a period of five years. This information is to be provided as and when requested by Environment and Climate Change Canada. It will be required on a less frequent basis, such as during an audit or investigation.

Each test report must indicate if there has been any deviation from any of the "must" requirements delineated in Sections 2 to 7 of this reference method, for effluent testing and Sections 2 to 8 of this reference method for chemical testing, and, if so, provide details of the deviation. The reader must be able to establish from the test report whether the conditions and procedures preceding and during the test rendered the results valid and acceptable for the use intended.

9.1 Data to be Reported

This section provides a list of items that must be included in each test report.

9.1.1 Effluent or Chemical

- name and location of operation generating the effluent;
- date and time of sampling;
- type of sample (e.g., "whole effluent from plant", "final mill effluent", "discharge from emergency spill lagoon", "leachate", name of chemical or substance) or coding, as provided to the laboratory personnel;
- information on labelling or coding for each sample;
- brief description of sampling point;
- sampling method (e.g., "grab", "batch", 24-h composite with sub-samples at 1-h intervals");
- name of person(s) collecting sample; and
- date and time sample received at test facility and temperature of sample upon receipt.

9.1.2 Test Facilities and Conditions

- test type and method; e.g., "single-concentration test method as specified in the second edition of EPS 1/RM/10";
- name and city of testing laboratory;
- percent mortality of fish in holding/acclimation tank(s) from which test fish are taken, as recorded daily (or, as a minimum, for 5 of the 7 days spanning a weekly period) for the 7-day period immediately preceding the test;
- species of test organism;

- date and time for start of toxicity test;
- person(s) performing the test and verifying the results;
- the pH, temperature, DO, and salinity of unadjusted, undiluted effluent, just before preparing test solutions;
- method used (with citation) for measuring salinity of effluent (or chemical sample), control/dilution water, and test solutions;
- confirmation that sample or solution was not filtered; indication if any additional tests with filtration or maintaining solids in suspension were performed (see Section 4.1)
- confirmation that no adjustment of sample or solution pH occurred; indication of procedure used for any pH adjustment if both pH-adjusted and non-adjusted tests were run (see Section 4.2);
- confirmation that no adjustment of sample or solution salinity occurred; indication if any parallel test run using salinity-control water as dilution water (see Section 4.2);
- indication of aeration of test solutions (rate and time) before introduction of fish; rate of aeration throughout the test;
- concentrations and volumes tested, including control(s), and indication of any replication;
- measurements of DO, pH, and temperature determined for each test solution including control(s) at the beginning and end of the test, as a minimum; as well as salinity of each test solution at the beginning of the test;
- number of fish added to each test vessel;
- mean fork length of fish in the dilution-water control at the end of the test, together with the range of the values measured;
- mean wet weight of individual fish in the dilution-water control at the end of the test; and
- calculated loading density (g/L) of fish in dilution-water control solution(s).

9.1.3 Results

- number of mortalities of fish in each test solution including the control(s), at 24, 48, 72, and 96 hours; number of control fish showing atypical/stressed behaviour;
- mean percent mortality of fish in test solutions and control(s), at 96 hours, if a single-concentration or multi-concentration test is performed using replicate solutions; mean percent of control fish showing atypical/stressed behaviour if replicate control solutions;
- estimate of 96-h LC50 and 95% confidence limits in multi-concentration tests, if statistically achievable; methods used for calculating statistical endpoints;
- most recent 96-h LC50 (with 95% confidence limits) for reference toxicity test(s) performed with fish from the same batch of fish used in the effluent (or chemical) test; reference chemical(s); date test initiated; historic *geometric mean* LC50 and warning limits (± 2 SD); and
- anything unusual about the test, any problems encountered, and any remedial measures taken.

9.2 Data to be Held on File

This section provides a list of items that must be either included in the test report, or held on file for a minimum of five years. Filed information must also include the following, if available:

- a record of the chain-of-continuity for samples tested for regulatory or monitoring purposes;
- a copy of the record of receipt 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 fish used for this test; and
- information on the calibration of equipment and instruments.

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

9.2.1 Effluent or Chemical

- all information (e.g., code, sample description, date/time of sampling) affixed to label(s) on sample container(s); description of sample container (size and material);
- volume of sample;
- transport and storage conditions (e.g., times, in sealed container, in darkness; temperature during storage at the laboratory; indication if sample frozen on arrival);
- appearance and other properties (observations on colour, turbidity, odour, floating or settleable material);
- colour change, precipitation, flocculation, release of volatiles or other changes when making up test solution(s) and during the test; and
- procedures and results for any chemical analyses performed on the sample, if available (e.g., suspended solids content, total dissolved solids).

9.2.2 Test Facilities and Conditions

- address of testing laboratory;
- description of rearing/acclimation and test facilities including general layout of each and means of isolation;
- normal holding and acclimating conditions (containers; location; lighting; temperature, including maximum rate of change; salinity, including maximum rate of change; aeration; volumes and flows of water; procedure for water renewal/filtration; numbers and densities of fish; handling procedures; food type, ration, and frequency of feeding; disease incidence and treatment if any; and weekly cumulative percent mortality);
- duration of acclimation immediately preceding the test;
- source of test fish, including name of supplier and/or collector, and location of collection; date of collection; records of taxonomic confirmation of species; all supplier's records provided with each shipment, including number of test organisms shipped, as well as date of shipment; date of arrival at the testing laboratory; temperature, DO, salinity, and pH of water in shipment container(s) and mortality upon arrival at the laboratory;
- brief history of test-specific conditions and procedures for holding and acclimating fish [e.g., times; water source; loading density; characteristics such as temperature, salinity, pH, and DO; food type and ration; display of breeding characteristics (i.e., male breeding colours or swollen female abdomen), if any; fish lacking a caudal keel (i.e., low-plated morph or blackspotted stickleback), if any; disease incidence and treatment; and weekly cumulative percent mortality] if different from usual practice;
- description of source(s) of water used for rearing and acclimating fish and as control/dilution water;
- brief description of procedure(s), products used, and duration of aeration and holding for preparation and/or salinity adjustment of control/dilution water and salinity-control water, if used; and/or test solutions for chemical testing;

- *pre-treatment* of acclimation and control/dilution water, if any (e.g., adjustment of temperature and salinity, aeration rate and duration, type and quantity of any chemical added, storage details);

- quality (mean and range values) of acclimation and control/dilution water as measured for source water and within holding tank(s); to include pH, salinity, DO, and total residual chlorine (if dechlorinated municipal drinking water used to prepare control/dilution water), and total ammonia; preferably also, solids, organic carbon, colour, mineral ions, metals, un-ionized ammonia, and pesticides; and total dissolved gases and alkalinity, if measured;

- systems to regulate light and temperature;

- light source, photoperiod, and past measures of intensity at rearing/acclimation tanks and at surface of test vessels;

- description of test vessels (size, shape, and material), covers, and baskets (if used for inspecting fish); routine cleaning procedures for each;

- procedures used to randomize the introduction of fish to test vessels and to randomize the positioning of the test concentrations within the testing facility;

- procedure and apparatus for aeration of test solutions

- procedures 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;

- any other chemical measurements on sample, stock solutions, or test solutions (e.g., concentrations of one or more specific chemicals, suspended solids content), before and/or at the time of the test;

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

- all measurements of fork length and wet weight of individual fish used in test (together with the mean value and range of values for each sample);

- depth of test solutions; appearance of solutions, including any changes evident during test;

- test concentrations of reference toxicant(s), both nominal and measured; indication of data set used to estimate LC50; and description of any deviation from or exclusion(s) of any of the procedures and conditions specified for the reference toxicity test; and

- any measurements of water quality in test solutions not included in data reported (Section 9.1.2).

9.2.3 Results

- any observations of mortalities of fish not included in data reported (e.g., more frequent observations on the initial day of the test) (see Sections 4.5 and 9.1.3);

- observations of fish behaviour and appearance recorded for each test solution during the test; and

- any manual plot(s) of data used to verify a computer-derived LC50.

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Appendix A

Biological Test Methods and Supporting Guidance Documents Published by Environment and Climate Change Canada's Method Development and 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 and May 2007
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 2nd Edition	February 2011	–
Toxicity Test Using Luminescent Bacteria *(Photobacterium phosphoreum)*	EPS 1/RM/24	November 1992	–
Growth Inhibition Test Using a Freshwater Algae	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 2nd Edition	February 2011	–
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 Publication Catalogue, Environment and Climate Change Canada, Ottawa ON K1A 0H3, Canada. Printed copies can also be requested by email at: enviroinfo@ec.gc.ca. These documents are freely available in PDF at the following website: 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 and Climate Change Canada, Ottawa ON K1A 0H3.

Title of Biological Test Method or Guidance Document	Report Number	Publication Date	Applicable Amendments
A. Generic (Universal) Biological Test Methods (continued)			
Test for Survival and Growth in Sediment and Water Using the Freshwater Amphipod *Hyalella azteca*	EPS 1/RM/33 2nd Edition	January 2013	–
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	June 2007
Tests for Measuring Emergence and Growth of Terrestrial Plants Exposed to Contaminants in Soil	EPS 1/RM/45	February 2005	June 2007
Test for Measuring Survival and Reproduction of Springtails Exposed to Contaminants in Soil	EPS 1/RM/47 2nd Edition	February 2014	–
Test for Growth in Contaminated Soil Using Terrestrial Plants Native to the Boreal Region	EPS 1/RM/56	August 2013	–
B. Reference Methods[b]			
Reference Method for Determining Acute Lethality of Effluents to Rainbow Trout	EPS 1/RM/13 2nd Edition	December 2000	May 2007 and February 2016
Reference Method for Determining Acute Lethality of Effluents to *Daphnia magna*	EPS 1/RM/14 2nd Edition	December 2000	February 2016
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	–
Reference Method for Measuring the Toxicity of Contaminated Sediment to Embryos and Larvae of Echinoids (Sea Urchins or Sand Dollars)	EPS 1/RM/58	July 2014	–

[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 and Climate Change 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 2nd Edition	December 2016	–
Guidance Document on Statistical Methods for Environmental Toxicity Tests	EPS 1/RM/46	March 2005	June 2007
Procedure for pH Stabilization During the Testing of Acute Lethality of Wastewater Effluent to Rainbow Trout	EPS 1/RM/50	March 2008	–
Supplementary Background and Guidance for Investigating Acute Lethality of Wastewater Effluent to Rainbow Trout	–	March 2008	–
Guidance Document on the Sampling and Preparation of Contaminated Soil for Use in Biological Testing	EPS 1/RM/53	February 2012	–

Appendix B

Members of the Inter-Governmental Ecotoxicological Testing Group (as of June 2017)

Federal, Environment and Climate Change Canada

Suzanne Agius
Marine Protection Programs Section
Gatineau, Québec

Deborah Austin
Marine Protection Programs Section
Gatineau, Québec

Adrienne Bartlett
Aquatic Ecosystem Protection Research Division
Burlington, Ontario

Lee Beaudette
Science & Technology Laboratories
Ottawa, Ontario

Rene Beaulieu
Prairie & Northern Laboratory for Environmental Testing
Edmonton, Alberta

Christian Blaise (Emeritus)
Centre St. Laurent,
Montréal, Québec

Lorraine Brown
Pacific & Yukon Laboratory for Environmental Testing
North Vancouver, British Columbia

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

Julia Brydon
Marine Protection Programs Section
Gatineau, Québec

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

Melanie Camplin
Prairie & Northern Laboratory for Environmental Testing
Edmonton, Alberta

Heather Dillon
Prairie & Northern Laboratory for Environmental Testing
Edmonton, Alberta

Ken Doe (Emeritus)
Atlantic Laboratory for Environmental Testing
Moncton, New Brunswick

Tamzin El-Fityani
National Guidelines and Standards Office
Ottawa, Ontario

Chris Fraser
Science & Technology Laboratories
Ottawa, Ontario

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

Christina Heise
Prairie & Northern Laboratory for Environmental Testing
Edmonton, Alberta

Ryan Hennessy
Science & Technology Laboratories
Ottawa, Ontario

Natasha Hostal
Prairie & Northern Laboratory for Environmental Testing
Edmonton, Alberta

Dale Hughes
Atlantic Laboratory for Environmental Testing
Moncton, New Brunswick

Paula Jackman
Atlantic Laboratory for Environmental Testing
Moncton, New Brunswick

Stephanie Kvas
Ecotoxicology and Wildlife Section
Ottawa, Ontario

Heather Lemieux
Science & Technology Laboratories
Ottawa, Ontario

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

Danielle Milani
Aquatic Ecosystem Impacts Research Division
Burlington, Ontario

Rachel Miliano
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
Ellyn Ritchie
Science & Technology Laboratories
Ottawa, Ontario

Ajith Dias Samarajeewa
Science & Technology Laboratories
Ottawa, Ontario

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

Rick Scroggins
Science & Technology Laboratories
Ottawa, Ontario

David Taillefer
Marine Environmental Protection
Gatineau, Québec

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, Natural Resources Canada

Philippa Huntsman-Mapila
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

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

Provincial

Richard Chong-Kit
Ontario Ministry of Environment and Climate Change
Etobicoke, Ontario

Olesya Hursky
Saskatchewan Research Council
Saskatoon, Saskatchewan

Lisa Kennedy (co-Chair)
Ontario Ministry of Environment and Climate Change
Etobicoke, Ontario

David Poirier
Ontario Ministry of Environment and Climate Change
Etobicoke, Ontario

Trudy Watson-Leung (co-Chair)
Ontario Ministry of Environment and Climate Change
Etobicoke, Ontario

Private Research Facilities/Others

Eloise Veilleux
Centre d'expertise en analyse environnementale du Québec
Ste. Foy, Québec

Appendix C

Environment and Climate Change Canada, Regional Environmental Testing Laboratories

Atlantic Laboratory for Environmental Testing
Environmental Science Building
443 Université Avenue, Université de Moncton
Moncton, New Brunswick
E1A 3E9

Pacific and Yukon Laboratory for Environmental Testing
Pacific Environmental Science Centre
2645 Dollarton Hwy
North Vancouver, British Columbia
V7H 1B1

Québec Laboratory for Environmental Testing
105, rue McGill
Montréal, Québec
H2Y 2E7

Prairie and Northern Laboratory for Environmental Testing
Northern Forestry Building
5320 122 St NW
Edmonton, Alberta
T6H 3S5

For current regional laboratory contact information please contact:

Method Development and Applications Unit
Science and Technology Branch
Environment and Climate Change Canada
335 River Road
Ottawa, Ontario
K1A 0H3
Email: ec.methodes-methods.ec@canada.ca

Appendix D

Distinguishing Features of Threespine Stickleback

The threespine stickleback (*Gasterosteus aculeatus* Linnaeus 1758) is a well-studied small, ubiquitous fish from the northern hemisphere, found in freshwater, estuarine, and marine environments. Laterally compressed, torpedo shaped, and characterized by prominent dorsal and pelvic spines, these fish inhabit shallow areas of coastal marine and estuarine environments as well as small streams, rivers, and lakes. *G. aculeatus* is typically *anadromous*, spending most of its life in coastal marine waters, returning to estuarine and freshwater environments for spawning, however populations are varied with some employing entirely freshwater existences. In addition to its ecological and scientific importance, the species has been used widely in toxicity testing (see Section 1.0).

Lacking the typical scales of most teleost fish, sticklebacks are characterized by numerous bony lateral plates that form a distinctive row running down each flank of the body (Wootton, 1984). There are three morphological forms (or morphs), namely complete-, partial-, and low-plated forms (Mattern, 2007). These morphs are also known as trachurus, semiarmatus, and leiurus respectively (Wootton, 1976). In the complete-plated form, each fish typically has an uninterrupted row of 30 to 35 lateral plates along its length (pectoral fin to tail) and a distinct caudal keel. The partial-plated form can have a range of 8 to 30 plates which are located in two regions (anterior and posterior), separated by a gap in the row of lateral plates. The caudal keel is present, but is not as prominent when compared with the complete-plated morph. The low-plated form has 1 to 9 lateral plates along the anterior part of the body and lacks a caudal keel (Wooton, 1976; Mattern, 2007). Figures D-1 and D-2 illustrate the lateral plating and caudal keel variations between the low-plated and the complete-plated morphs. The complete-plated morphs are prevalent in marine, anadromous fish populations, while the low-plated morphs are prevalent in fresh-water populations (Wootton, 1976, 1984; Mattern, 2007). In Canadian waters, the distribution of the partial-plated morph cannot be easily described in terms of association with marine water or fresh water habitats (Hagen and Moodie, 1982). Although phenotypes (complete-, partial-, and low-plated morphs) are fairly consistent in a given population, the number of plates can vary among individuals and populations consisting of more than one morph are known to occur in both marine and freshwater environments (Wootton, 1984, 2009).

The blackspotted stickleback, *Gasterosteus wheatlandi*, is found in coastal waters of the North American Atlantic, and is similar in appearance to the threespine stickleback (Scott and Scott, 1988). Blackspotted sticklebacks have few lateral plates (5–11) in the anterior region, and lack a caudal keel. Characteristic black spots are often used to distinguish *G. wheatlandi* from *G. aculeatus* (Mattern, 2007), but recent observations of wild-caught fish have revealed these black spots cannot be attributed exclusively to *G. wheatlandi* (Karen Marks, Harris Industrial Testing Service, personal communication, 2016; Envirosphere, 2017). Careful examination of other morphological features (e.g., in the pelvic fin and pelvic spine) are also used to distinguish these two similar species (Figure D-3; Wootton, 1976).

In live fish, the caudal keel is a morphological feature that can be used to distinguish threespine sticklebacks (caudal keel is present) from blackspotted sticklebacks (caudal keel is absent), and to distinguish complete-plated and partial-plated morphs (caudal keel is present; see Figure D-2, photos b, c, d, e, and f) from low-plated morphs (caudal keel is absent; see Figure D-1, photos c and d). The caudal keel (or caudal peduncle keel) is found on the sides of the fish, in the posterior region, just forward of the tail (or caudal) fin. It is a fleshy mound which runs horizontally along the body of the fish, and in complete-plated morphs, is typically 0.1–0.8 cm in length and 0.02–0.06 cm in width (Envirosphere, 2017). It can be observed unaided with the eye, and the minimal handling involved appears to have no adverse effects on the fish.

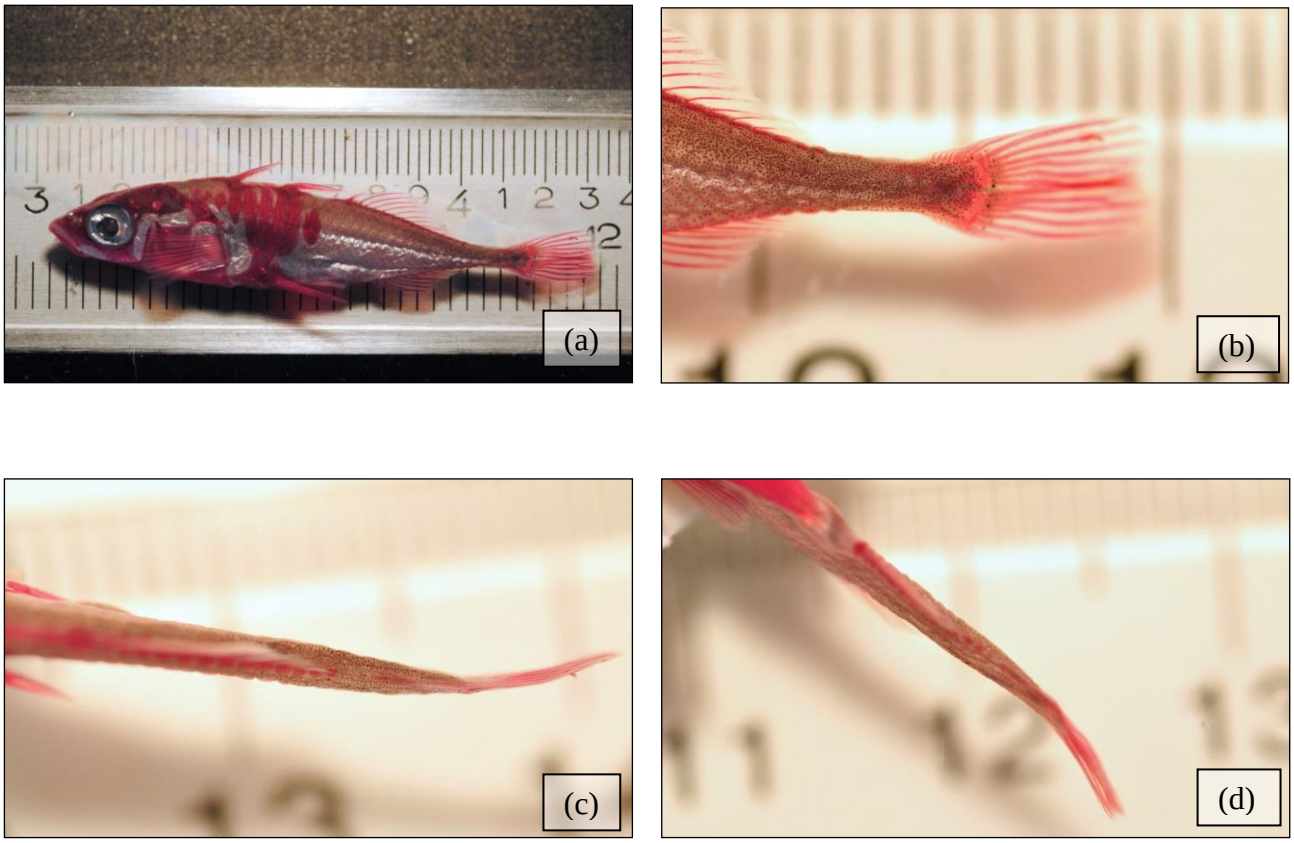


Figure D-1 Low-plated morph of *G. aculeatus*, stained with alizarin red. (a) lateral view, (b) lateral view of posterior region, (c) dorsal view of posterior region, (d) ventral view of posterior region. (photos: P. L. Stewart)

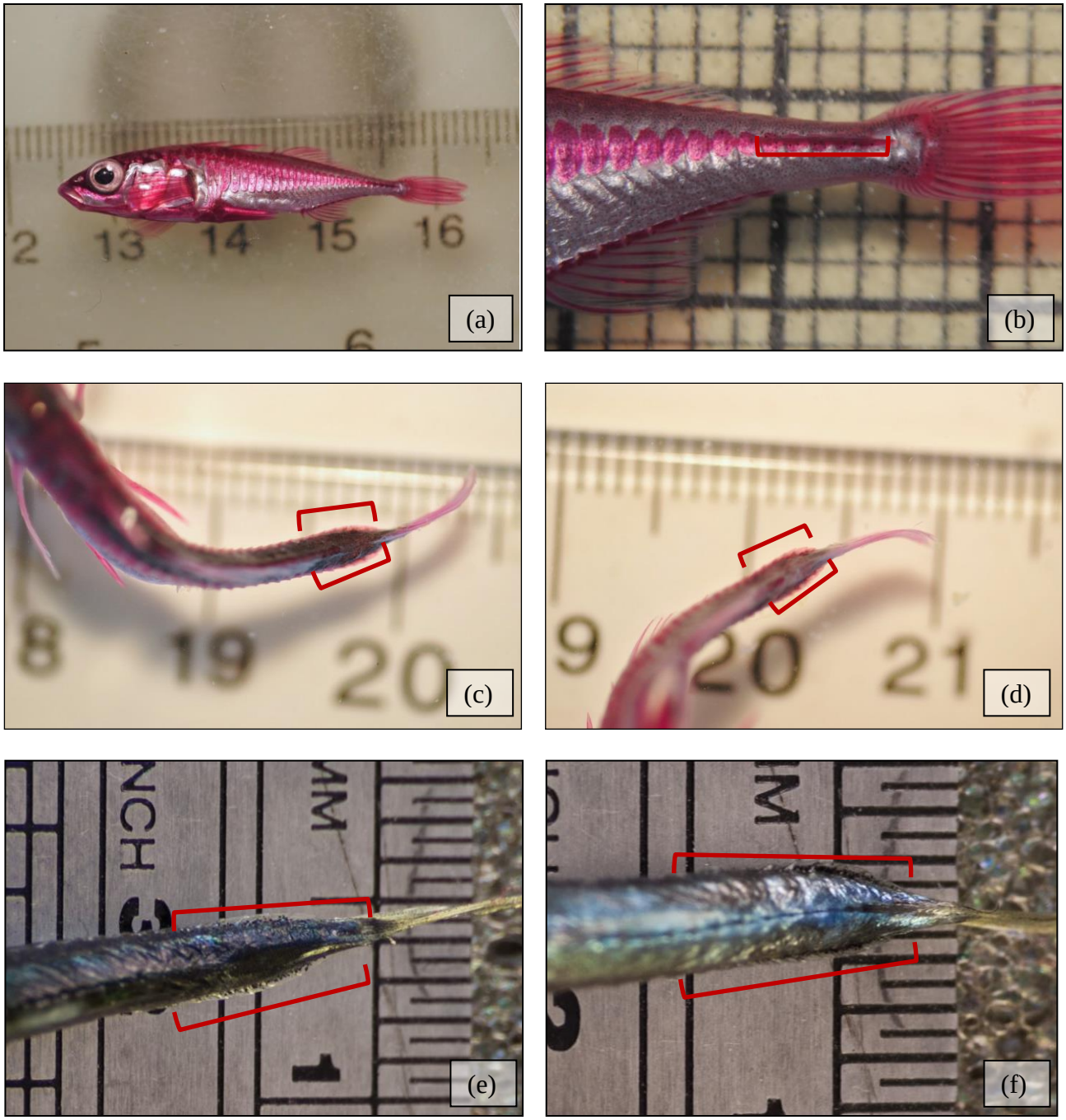
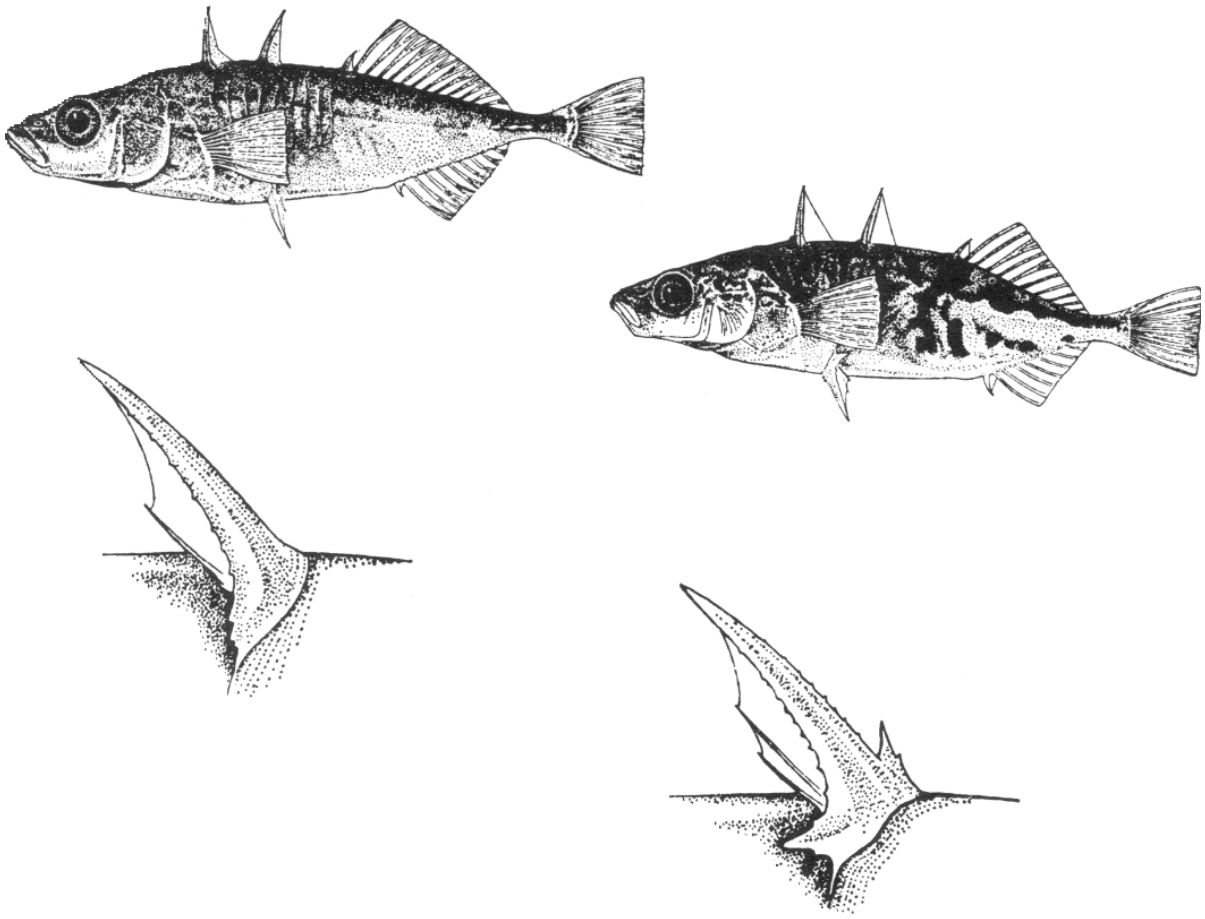


Figure D-2 Complete-plated morph of *G. aculeatus*, stained with alizarin red (a-d) and unstained (e and f). (a) lateral view, (b) lateral view of posterior region, (c) dorsal view of posterior region, (d) ventral view of posterior region, (e) dorsal view of posterior region, (f) ventral view of posterior region. Red brackets indicate caudal keel. (photos a, b, c, and d: P. L. Stewart; photos e and f: C. Kidd)



Gasterosteus aculeatus
Dorsal spines three (rarely four); last spine short; pelvic fin of one spine and one soft ray, spine with one pointed cusp at base; caudal peduncle with a keel; body with or without round black spots; colour in life green, blue silvery.

Gasterosteus wheatlandi
Dorsal spines three (rarely two); pelvic fin of one spine with two soft rays, spine with two well-developed pointed cusps at base; caudal peduncle keel-less; many round black spots along sides; colour in life lemon-yellow.

Figure D-3 Key for distinguishing threespine stickleback (*G. aculeatus*) from blackspotted stickleback (*G. wheatlandi*); enlargement shows pelvic fin region. (Adapted from Scott and Crossman, 1973; and Scott and Scott, 1988)