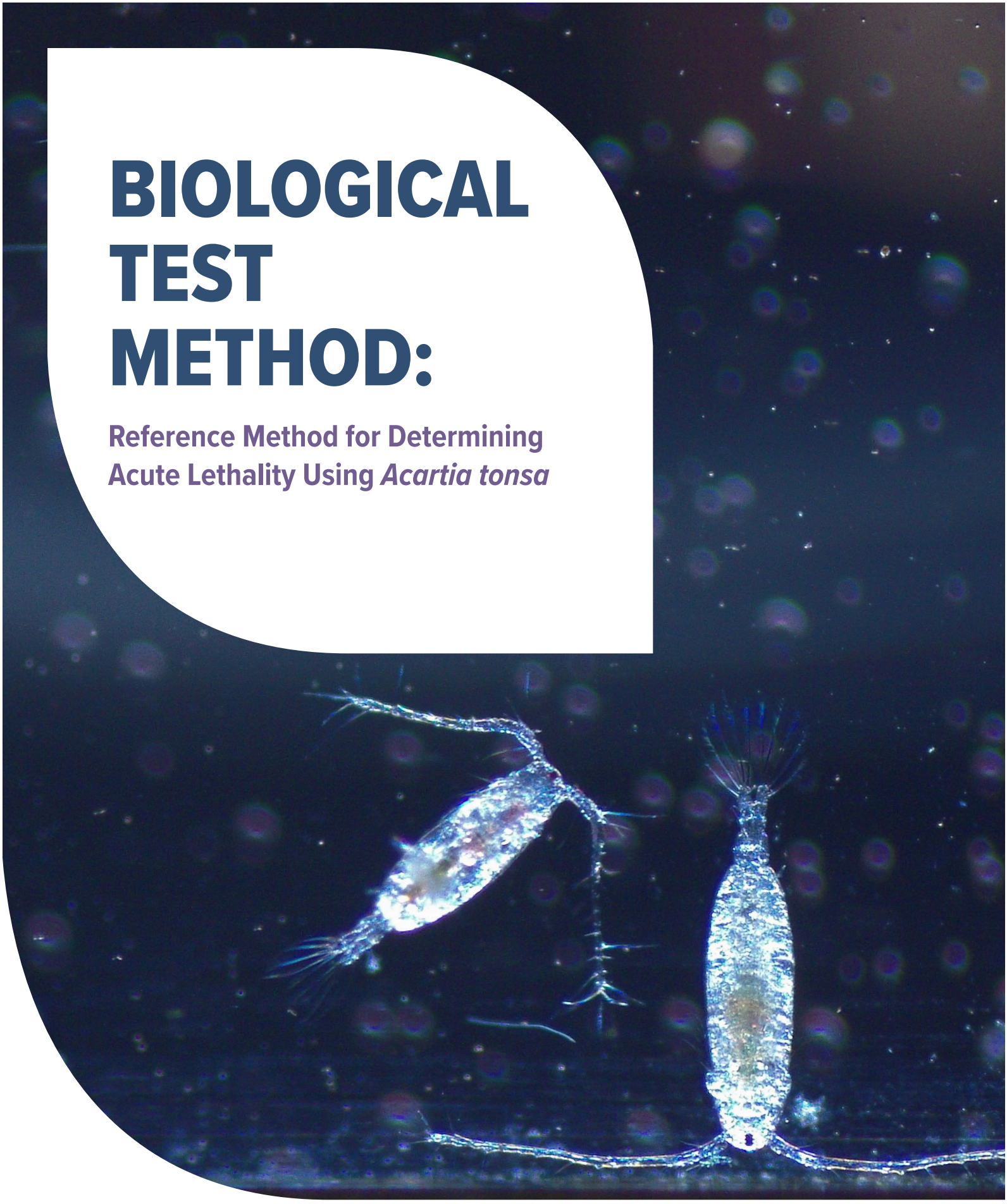
BIOLOGICAL TEST METHOD:

Reference Method for Determining Acute Lethality Using *Acartia tonsa*



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Reference Method for Determining Acute Lethality Using *Acartia tonsa*

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Reference Method
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Abstract

Explicit standard or reference methods for measuring the acute lethal toxicity of effluents to the marine copepod *Acartia tonsa* are described in this report. Specific instructions are provided for performing acute lethality tests with effluent samples having a salinity of > 4 g/kg discharging directly to estuarine or marine receiving waters.

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 culturing *A. tonsa* 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 that 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 that 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.

Table of Contents

Abstract ..... **iii**
Foreword ..... **iv**
Table of Contents ..... **v**
List of Abbreviations and Chemical Formulae ..... **vii**
Terminology ..... **viii**
Acknowledgements ..... **xiv**

Section 1
Introduction **1**

Section 2
Test Organisms **3**
2.1 Species and Source 3
2.2 Maintaining Cultures 3
2.2.1 Health Criterion 5
2.3 Water 6
2.4 Physicochemical Conditions 7
2.4.1 Temperature 7
2.4.2 Salinity 7
2.4.3 Dissolved Oxygen and pH 7
2.4.4 Lighting 8
2.4.5 Monitoring 8

Section 3
Facilities **9**

Section 4
General Procedure for Determining Acute Lethality of Effluent **10**
4.1 Sample Labelling, Transport, and Storage 10
4.2 Test Conditions 10
4.3 Preparing Test Solutions 12
4.4 Beginning the Test 13
4.5 Observations and Measurements 14
4.6 Validity Criteria 16

Section 5
Procedure for a Single-Concentration Test to Determine Percent Mortality at 48 Hours **17**

Section 6
Procedure for a Multi-Concentration Test to Determine the 48-h LC50 ..... **18**

Section 7
Procedure for Testing a Reference Toxicant **19**

Section 8
Procedure for Testing Chemicals 21
8.1 Properties, Labelling, and Storage of Sample 21
8.2 Preparing Test Solutions 21
8.3 Control/Dilution Water 22
8.4 Test Observations and Measurements 23
8.5 Test Endpoints and Calculations 24

Section 9
Reporting Requirements 25
9.1 Data to Be Reported 25
9.1.1 Effluent or Chemical 25
9.1.2 Test Facilities and Conditions 25
9.1.3 Results 26
9.2 Data to Be Held on File 26
9.2.1 Effluent or Chemical 27
9.2.2 Test Facilities and Conditions 27
9.2.3 Results 28

References 29

Appendix A
Biological Test Methods and Supporting Guidance Documents Published by Environment and Climate Change Canada's Method Development and Applications Unit 33

Appendix B
Members of the Inter-Governmental Ecotoxicological Testing Group (as of January 2019) 36

Appendix C
Environment and Climate Change Canada, Regional Environmental Testing Laboratories 39

Appendix D
General Characteristics of *Acartia tonsa* 40

Appendix E
Procedure for Preparing Algal Food (*Rhodomonas salina*) for *Acartia tonsa* Cultures 44

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
LED	light-emitting diode
m	metre(s)
mg	milligram(s)
min	minute(s)
mL	millilitre(s)
mm	millimetre(s)
mS	millisiemen(s)
nm	nanometre(s)
®	Registered Trade Mark
s	second(s)
SD	standard deviation
SI	International System of Units
™	Trade Mark
µg	microgram(s)
µL	microlitre(s)
µm	micrometre(s)
µmhos	micromhos
µmol	micromol(s)
×	times or magnification
÷	divided by
>	greater than
<	less than
≥	greater than or equal to
≤	less than or equal to
/	per; alternatively, “or” (e.g., control/dilution water)
~	approximately
=	equals
±	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.

Batch means a single group of *A. tonsa* received from a supplier at a particular time in order to start a laboratory *culture* to produce test organisms through reproduction. It *might* also refer to a volume of *seawater* (artificial or natural) intended for use for culturing/*acclimation* or in a particular *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 government 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 are typically temperature-adjusted to the standard temperature of 25°C, and are 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" (‰).

Copepod is a small aquatic crustacean. The copepod species used in this method is *Acartia tonsa*.

Copepodite is any of several stages in the life cycle of a *copepod* following the naupliar stage but prior to the sexually mature adult. *A. tonsa* goes through six copepodite stages prior to becoming a sexually mature adult. The term "copepodid" is equivalent to "copepodite".

Culture as a noun means the stock of animals raised in the laboratory under defined and controlled conditions through one or more generations to produce eggs for use as test organisms. For the purposes of this method, the word culture also refers to "age-class cultures", which are *copepods* separated out into specific age- or size-classes. As a verb, it means to carry out the procedure of raising healthy test organisms for one or more generations, under defined and controlled conditions.

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

Eurythermal is the ability of an organism to tolerate a wide variation in temperature without stress.

Light-emitting diode (LED) is a type of light source. It is a semi-conductor diode that glows when a voltage is applied. LEDs differ from fluorescent and incandescent light sources in the mechanism used to generate light.

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 are 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, 2014). The conversion 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 the 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*.

Nauplius (pl. nauplii) is a newly hatched *copepod* (i.e., eggs of *A. tonsa* usually hatch into nauplii within ~48 hours at 20 ±2°C). *A. tonsa* goes through six nauplius stages prior to becoming a *copepodite*.

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 < 7 indicating increasingly greater acidic reactions, and numbers > 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.

Refractometry is a technique that measures the extent to which light is bent (i.e., refracted) when it moves from air into a sample. It 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 culturing/acclimating organisms and for testing purposes (*control/dilution water*). Artificial seawater is also known as reconstituted seawater. 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 that enters 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 *culture* 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.

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 the *A. tonsa culture* is *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 > 4 g/kg and ≤ 35 g/kg, and salinity adjustment is carried out using commercially available dry ocean salts (see Section 2.3) or by dilution using fresh water.

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 lifespan of the test organism, usually taken as ≤ 48 hours for *marine copepods*. 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 *culture* 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.

Immobile for the purpose of this method is defined as the lack of any visible signs of movement (including antennae and appendages) during a 30-second observation period.

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., 48-h LC50).

Lethal means causing death by direct action. Death of *A. tonsa* test organisms is defined here as i) the egg is seen to be unhatched; or ii) the *nauplius* is *immobile* (as determined from a 30-second observation after locating the nauplius); or iii) the test organism is missing. Lethality is only definitively assigned at 48 hours in this test method, as an unhatched egg observed at earlier observation periods (e.g., 24 hours) may still hatch into a mobile nauplius.

Overt means obviously discernible under the test/*culture* 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)*. For the purposes of this method document, a replicate refers to a single well of a microplate containing a single test organism 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 *culture* 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 test organism, 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 *marine copepods* 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 or agents 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 *endpoint* values 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 historical *geometric mean* of the *endpoint* values from *toxicity tests* with a *reference toxicant*.

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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, Moncton, NB (ALET) and Pacific & Yukon Laboratory for Environmental Testing, North Vancouver, BC (PYLET); AquaTox Testing & Consulting Inc., Puslinch, ON (AquaTox); AGAT Laboratories, Saint-Laurent, QC (AGAT); Maxxam Analytics, Burnaby, BC (Maxxam); and Nautilus Environmental Company Inc., Burnaby, BC (Nautilus). We gratefully acknowledge the contributions of all of the inter-laboratory participants: Lison Haché, Paula Jackman, and Megan McPherson from ALET; Craig Buday, Chris Le, Leah Purdey, and Grant Schroeder from PYLET; Paula Antunes, Reneta Dorosz, Carla Gibbs, Shawna Kirkpatrick, Lesley Novak, Anna Sobaszek, and Adam Wartman from AquaTox; Virginie Bérubé, Sophie Bélanger-Ricard, Caroline Leblanc-Houde, and Pierre Yves Robidoux from AGAT; Diana Cruz, Marriah Grey, Pam Howes, Donald Lai, Marie-Eve O'Toole, and Navpreet Shergill from Maxxam; and Eric Cheung, Emma Marus, and Armando Tang from Nautilus. Thanks also to the following people who reviewed a draft of this test method document and provided many useful comments: Lison Haché, Paula Jackman, and Megan McPherson from ALET; Craig Buday and Chris Le from PYLET; Paula Antunes, Lesley Novak, and Lisa Taylor from AquaTox; Virginie Bérubé, Sophie Bélanger-Ricard, and Caroline Leblanc-Houde from AGAT; Heather Roshon from CPCC; Dr. Gessica Gorbi from Università di Parma; and Dr. Isabella Buttino from Istituto Superiore per la Protezione e la Ricerca Ambientale. Thanks also to Sylvain Trottier and Charles Faille from Quebec Laboratory for Environmental Testing for their review of the French translation. 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 the *marine copepod* (*Acartia tonsa*), 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 > 4‰ discharging directly to *estuarine* or marine *receiving waters*. 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 and Diamond Mining Effluent Regulations* promulgated under the federal *Fisheries Act*. Another invertebrate (*Daphnia magna*) reference method, published by Environment Canada (2000), is used for assessing effluents containing fresh water (i.e., having salinities of ≤ 4‰), as well as those effluents that are saline (i.e., > 4‰) 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 method development research conducted by Environment and Climate Change Canada's Atlantic Laboratory for Environmental Testing (ALET, 2018, 2019), Pacific and Yukon Laboratory for Environmental Testing (Craig Buday, PYLET, Environment and Climate Change Canada, personal communication, 2017), and AquaTox Testing and Consulting Inc. (AquaTox, 2017, 2018). Procedures and conditions stipulated in this reference method must be taken as definitive 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, 2018). Nickel and phenol were the two *toxicants* evaluated. Results from the first *reference toxicant* round using nickel yielded a *Coefficient of Variation* (CV) of 48.6%, which is within an acceptable range of variability for inter-laboratory tests. The second round, which tested phenol as the reference toxicant, produced results that were more variable (AquaTox, 2018). A number of recommendations provided by AquaTox (2018) concerning the inter-laboratory evaluation were adopted in this test method document, and are expected to reduce the overall variability.[1] 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 in a single laboratory, and that inter-laboratory precision could be expected to be reflected in CVs ranging from 30% to 50% (EC, 1999). As a follow-up to the inter-laboratory study, an effluent sample was divided and tested concurrently in two laboratories, and the results were in good

[1] A number of changes to this test method document were implemented following two rounds of inter-laboratory testing. They were based on recommendations for reducing variability following an examination and evaluation of the test results (AquaTox, 2018). These recommendations included: (1) specifying the optical quality of the microscope used to assess *Acartia* hatching, mobility, and mortality (Section 4.5 and footnote 16); (2) providing further clarification and guidance on assessing mobility (Section 4.5); (3) providing a recommendation for a maximum number of missing test organisms in a given test (Section 4.5); and (4) increasing the number of replicates required for a single-concentration test (Section 5).

agreement (Paula Jackman, ALET, Environment and Climate Change Canada, personal communication, 2019).

The calanoid copepod *Acartia tonsa* is a free-swimming planktonic crustacean most commonly found in shallow coastal waters at depths of < 50 m. It is *euryhaline* and *eurythermal*, inhabiting waters with salinities ranging from 5‰ to 35‰ (Miller and Marcus, 1994) and temperatures ranging from 1 to 32°C (Gonzalez, 1974). It is widely distributed in temperate seas, being the dominant copepod in many subtropical and temperate coastal marine and estuarine areas (Peck and Holste, 2006). It is ecologically important, playing both top-down and bottom-up roles in pelagic coastal food webs. Distributed worldwide, *A. tonsa* has been found in the Baltic, Black, Caspian, Mediterranean, and North Seas as well as the Indian, Atlantic, and Pacific Oceans, and the Gulf of Mexico (Mauchline, 1998). In Canada, *A. tonsa* is found on both the Atlantic and Pacific coasts as far north as the Gulf of St. Lawrence and Graham Island (200 km north of Vancouver Island), respectively (McAlice, 1981; Jepsen, 2014). General aspects and illustrations of the various developmental stages of *A. tonsa* are given in Appendix D. The extensive geographic reach and critical role in the food web played by *A. tonsa*, as well as the ease with which it can be cultured in a laboratory setting, has made it the subject of myriad studies for over half a century. Method development and toxicity testing completed during these decades of research have led to standardized approaches to testing and provide baseline values for its sensitivity to many toxicants (Parrish and Wilson, 1978; Sosnowski and Gentile, 1978; Ward, 1995; Kusk and Petersen, 1997; Tsui and Chu, 2003; Medina and Barata, 2004; Pedroso *et al*., 2007; Lauer and Bianchini, 2010; Gorbi *et al*., 2012; Vitiello *et al*., 2016; Zhou *et al*., 2016). Studies have also been undertaken to directly compare chemical toxicities in *D. magna* to *A. tonsa* (Sverdrup *et al*., 2002). In addition, several national and international method standardization agencies have designed standardized test methods employing *A. tonsa*, including the International Organization for Standardization (ISO, 1999, 2014) and the Italian Ministry of the Environment and Territory and Sea Protection (Associazione per L'unificazione Nel Settore dell Industria Chimica, 2012a, 2012b). With its naturally high nutritional quality, and as a natural prey organism for many marine fish species, *A. tonsa* has also gained international popularity as a live feed organism for larval fish in marine aquaculture (Drillet *et al*., 2006; Peck and Holste, 2006; Marcus and Wilcox, 2007; Hagemann, 2011; Jepsen, 2014; Hagemann *et al*., 2016a, 2016b; Zhang *et al*., 2015).

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 organism 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 (> 4‰) 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 marine copepod *Acartia tonsa* must be used as the test organism in this reference method. The test must be initiated using eggs that are ≤ 24 hours old. These eggs should be obtained from laboratory *cultures* that are 14–28 days old. Older cohorts (i.e., "28–35 days" or "35–42 days") may be used if they continue to meet the culture health criteria (see Section 2.2). Eggs are obtained by transferring adult copepods from the culture to smaller vessels of culture/*control/dilution water* ≤ 24 hours prior to the start of the test (see Section 2.2).

All eggs used in a test must be derived from the same population, and must originate from cultures that have historically met health criteria. Test organisms must be cultured and maintained in the laboratory facility carrying out the testing.

Cultures of *A. tonsa* are available from commercial suppliers and from Canadian government laboratories.

For information on suppliers for *A. tonsa* 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.

Very few organisms are required to start a culture, and starter cultures often consist of eggs, juveniles, and adults shipped in artificial or *natural seawater* containing live algae as food. Regional, provincial, or federal authorities (e.g., Federal-Provincial Introductions and Transfers Committee) might require approval for the procurement, shipment, or transfer of *A. tonsa* and its feeder algae, *R. salina*. For further information on federal or provincial permit requirements, contact Environment and Climate Change Canada's regional environmental testing laboratories (see Appendix C).

Taxonomic identification and documentation of the species of test organisms must be made by a qualified taxonomist for each new *batch* of *A. tonsa* introduced into the laboratory 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 or obtained from another laboratory may be supplied with certification of the organisms' species identification, and the taxonomic reference or name(s) of the taxonomic expert(s) consulted. Records accompanying each batch of *A. tonsa* must include, at a minimum, the approximate quantity and source of organisms in each shipment; the supplier's name; the date of shipment; the date of arrival at the testing laboratory; and the arrival condition (i.e., temperature, DO, *pH,* salinity, and general visual observations related to water quality and copepod behaviour).

2.2 Maintaining Cultures

Culture vessels and all accessories contacting the organisms, water, or culture media must be made of non-toxic materials (e.g., glass, stainless steel, Nalgene®, porcelain, polyethylene, polypropylene). Glass aquaria, beakers, or wide-mouth jars (e.g., 500 mL to 2 L) are recommended as culture vessels, each loosely covered to exclude dust and reduce evaporation. Culturing should be isolated from any physical disturbances, and preferably in a location separate from testing areas.

After a new batch of *Acartia tonsa* has been transported to the testing laboratory, they should be acclimated to the conditions specified in Section 2.4 and fed according to the procedures described below. *Acclimation* to test conditions,

as specified in Section 2.4, is required prior to testing and this acclimation period must immediately precede their use in a test. To avoid stressing the animals, cultures are not renewed or sorted by size/age class during the first week following their arrival at the laboratory.

Culture vessels must be renewed once per week, at which time new culture vessels are started with complete (100%) renewal of the culture water. On the day of culture renewal, copepods must be separated out into age- and size-classes (i.e., age-class cultures). Stacked sieves, with a large sieve (mesh size of 180 to 200 µm), stacked on top of a small sieve (mesh size of 45 to 64 µm) can be used for this purpose. Alternatively, sieves can be used sequentially (i.e., one at a time, starting with the larger mesh size), and the organisms retained on the sieve collected before passing the culture water through the next sieve. Sieves should be kept moist or submerged in water during culture renewal as *A. tonsa* individuals dry out and perish easily if left exposed to the air. In addition, water velocity passed through the sieves should be kept at a minimum so as to not crush the copepods against the mesh (Marcus and Wilcox, 2007).

The following procedure may be used:

i) Pour the contents of the "0–7 days" old culture container through the stacked sieves.

ii) Discard the culture water that passes through the sieves. Gently separate the sieves. Invert the large sieve (on the top) onto a crystallizing or Petri dish and gently rinse the underside of the sieve with new culture water. Collect the rinse water as well as juveniles and adults displaced from the large sieve in a clean culture vessel labelled "7–14 days", containing new aerated, filtered seawater.

iii) Invert the small sieve onto a crystallizing or Petri dish and gently rinse the underside of the sieve with new culture water. Collect the rinse water, eggs, and nauplii displaced from the small sieve and place them into a clean culture vessel labelled "0–7 days" containing new aerated, filtered seawater.

For sieves used one at a time in sequence, the same procedure may be used except the culture water that passes through the larger sieve is collected and then poured through the smaller sieve.

These steps should be repeated for each culture vessel, and organisms from each size class (i.e., separated using sieves) must be placed in a new culture vessel and labelled appropriately. Juvenile and adult copepods maintained in the "7–14 days" culture vessel for 1 week should also be transferred (i.e., sieved, as described above) to a new vessel labelled "14–21 days" containing renewed culture water; and the eggs and nauplii displaced from the small sieve are placed into a clean culture vessel labelled "0–7 days", as described in Step (iii) above. This process is also repeated for copepods already maintained in the "14–21 days" culture vessels, with juveniles and adults being transferred to new vessels labelled "21–28 days". Copepods may be discarded once they reach the ≥ 28 day-old age class or held as a back-up culture. Alternatively, they may be used as a continued source of eggs for testing (i.e., transferred to new vessels labelled "28–35 days"); however, the culture health check should be repeated to ensure that they continue to meet the health criteria.

All eggs collected on the 45 to 64 µm sieve (i.e., from the age-class culture vessels for all of the different age classes) can be combined and then divided among one or more "0–7 days" culture vessel(s). Juveniles and/or adults collected from multiple culture vessels representing the same age-class (e.g., "7–14 days") may be combined or divided among several age-class culture vessels to maintain organism density. Under routine laboratory practice, approximate organism density is typically between 100 and 500 organisms per L; however, higher densities

can be maintained without any adverse effects on culture health.[2]

Additional sieve sizes between those described above (e.g., 105 and 150 µm) may be used if there is a preference to segregate organisms into further size classes or to filter out some of the debris present in the culture vessels.

Mass culture vessels containing mixed-age cultures may be maintained as a backup. For these cultures, vessels may be larger (≥ 1.5 L). Renewal of mass cultures can be accomplished by passing the contents through a small sieve (45 to 64 µm) and combining all age classes in a clean vessel containing new culture water.

The fragile nature of *A. tonsa* renders this organism extremely susceptible to damage caused by excessive or improper handling stress. *A. tonsa* adults can be handled by gently pouring them from one container to another or by careful pipetting or siphoning. Eggs can be transferred using a 1–2 mL pipette with a narrow opening (~1 mm). Adult copepods are susceptible to drying out and being crushed, and minimal handling should be practised. A disposable glass pipette cut off and fire-polished to provide a 3- to 5-mm opening can be used to transfer adults. The tip of the pipette should be under the surface when copepods or eggs are released. Transfers should be quick, with minimal carryover of "old" water to the new container.

Copepods in all culture vessels must be fed with *Rhodomonas salina*. Culture vessels should be fed daily with 6–60 million *Rhodomonas salina* cells per L of *A. tonsa* culture water. At a minimum, all culture vessels must be fed three times a week with an amount of *Rhodomonas salina* that supports continual growth and reproduction. The ration of algae for three times a week should be approximately 14-140 million cells per L of *A. tonsa* culture water. Vessels fed with *R. salina* typically have a slight pink or red colour, and this visual assessment may be used to adjust the food ration. The algal food ration is one of the primary factors in limiting egg production (Drillet *et al*., 2011; Zhang *et al*., 2015) and the overall health of *A. tonsa* cultures (Parish and Wilson, 1978). Increasing the algal food ration can be one of the first steps taken to improve culture health and/or culture density. Guidance for culturing *R. salina* as food for *A. tonsa* cultures is provided in Appendix E.

A double feed ration may be provided to the culture vessel the day prior to initiation of the toxicity test, as this typically promotes a larger production of eggs that can be used for testing.

2.2.1 Health Criterion

The health of the *A. tonsa* culture is judged by the following health criterion that must be met if eggs from the culture are to be used in a toxicity test:

- Survival of test organisms must be ≥ 80%.

This culture health check must be based on individual eggs (≤ 24 hours old) placed in each of 20 wells (of a 24-well microplate; see Section 3.0). [3] After 48 hours of incubation egg hatching, naupliar mobility, and missing egg and/or *nauplius* must be assessed and recorded for each well. The test organism is considered dead if the egg is seen to be unhatched, the nauplius is *immobile* (as determined from a 30-second observation after locating the nauplius), or the test organism is missing (see

[2] During the inter-laboratory evaluation, many laboratories experienced success in culturing *A. tonsa* at higher densities (i.e., up to 2,000 copepods per L). The average across all laboratories was approximately 500 copepods per L in the "14–21 days" and "21–28 days" cultures (AquaTox, 2018).

[3] On the day prior to initiating the culture health check, several adult copepods can be transferred from an age-class culture vessel to a clean "egg laying" vessel (e.g., crystallizing dish, Petri dish, or glass beaker) containing fresh culture water. Eggs produced by the adult copepods on the following day are ≤ 24 hours old and can be used to initiate the culture health check.

Section 4.5). During this culture health check, microplates must be kept under the same conditions as those used for testing. The health of an age-class culture (e.g., the "14–21 days" culture) must be assessed at least once, and must meet the health criteria before the eggs from that culture can be used in a definitive test.[4] In the case in which there are multiple culture vessels of the same age-class (e.g., two or more vessels representing the "14–21 days" culture), the health check may be carried out using only one of the culture vessels representative of the age-class culture that will be used as the source of eggs for the definitive test.

To monitor the health indices, eggs from adult copepods are transferred one at a time to 20 individual microplate wells each containing 1.5 mL of fresh culture water. A microscope must be used to confirm that each well contains a single egg.[5] The eggs must be ≤ 24 hours old. The adult copepods used to produce eggs for use in a culture health check must be cultured under similar loading conditions and feeding rates as those used to produce eggs for use in a definitive test. Eggs used in a definitive test must be traceable back to a valid culture health check. If there is no traceability, it cannot be assured that the health criterion pertaining to the specific test organisms used in that test was met. Adult copepods used to produce eggs for the culture health check may be returned to their original culture vessel.

Cultures should also be observed periodically to ensure that copepods are swimming in the usual manner and that their body size is reasonable. The results of the control(s) in a test and the findings of a test with a reference toxicant (see Section 7) give further indication of suitability of the copepod culture used for testing.

2.3 Water

Water to be used for culturing *A. tonsa* and as control/dilution water may be either an uncontaminated supply of natural seawater or *artificial seawater* made up to a desired salinity using commercially available dry ocean salts. If natural seawater is to be used for culturing, it must be filtered (e.g., ≤ 1 µm) to remove particulates and indigenous organisms, and aerated, if necessary. If artificial seawater is to be used for culturing *A. tonsa*, 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 (1 µm) 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, reproduction, and health of *Acartia tonsa* (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

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[4] It is important to note that if the culture health check is conducted 1 week prior to testing using eggs produced by adults in the "14–21 days" culture, the same copepods used to produce eggs for the definitive test will be 21–28 days old at the time of the test. This is acceptable practice. Once a specific age-class culture has met the culture health criteria, it may be used to produce eggs for definitive testing until the copepods are no longer suitable for egg production (e.g., too old or fail a repeated health criteria check).

[5] If more than one egg (or no egg) is found in a given well, the extra egg can be removed (or added if no egg was present) using a pipette; the well may be emptied and the process of setting up the well can be repeated; or the well containing multiple eggs can be marked as "void" on the microplate and a new well can be set up.

2 weeks, in covered containers protected from light.

The culture/control/dilution water supply must consistently support good survival, reproduction, and health of *A. tonsa*. 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. The water must not be supersaturated with gases. Any supersaturation with gases should be remedied (see Section 2.4.5 in EC, 1990a).

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 must be free of any harmful concentration of chlorine or chlorinated compounds upon the organisms' exposure (see Section 2.4.5 in EC, 1990a). A readily measurable total residual chlorine value of 20 µg/L has been shown not to affect *A. tonsa* health (Heinle and Beaven, 1977; Hall *et al*., 1982).[6]

2.4 Physicochemical Conditions

2.4.1 Temperature

A. tonsa cultures must be held for ≥ 2 weeks at 20 ± 2°C before eggs are used in tests. For acclimation, the recommended rate of temperature change is ≤ 3°C/day.

2.4.2 Salinity

A. tonsa must be acclimated for ≥ 2 weeks to a salinity within 5 g/kg of that used for the control/dilution water to be used in the test. 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 adult copepods supplying eggs for the test have been acclimated (see Section 4.2). For testing samples with salinities of > 4 to ≤ 10 g/kg, *A. tonsa* must be acclimated to a lower salinity; however, the target salinity and length of acclimation period can vary (e.g., according to test objectives and performance in the culture health check).[7] These salinity-adjusted cultures must meet the health criteria in a culture health check (see Section 2.2) prior to being used to produce eggs for a test.

2.4.3 Dissolved Oxygen and pH

The dissolved oxygen (DO) content of the water within culture containers should be maintained at 80% to 100% saturation. Aeration of the

[6] 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/chloramine toxicity with the contaminant(s) being tested. The limit of detection for the analytical technique used to measure total residual chlorine or chloramines in the treated supply of dechlorinated water should ideally be low enough to assure that total 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.

[7] As an example, *A. tonsa* cultured at 28 g/kg salinity can be acclimated to and held at 15 g/kg for approximately 2 weeks prior to introducing them into culture solutions of 10 g/kg. These cultures at a salinity of 10 g/kg can be held for at least two weeks prior to use in a culture health check. Provided health criteria are met, eggs can then be used in testing. (Paula Jackman, ALET, personal communication, 2018).

culture water should be carried out using filtered, oil-free compressed air. Continuous gentle aeration (e.g., 2–3 bubbles per s) must be applied to the culture. This may be achieved by extending a pipette connected to an aeration tube to approximately 1–2 cm from the bottom of each culture vessel. Overly vigorous aeration should be avoided.

The pH of water used for culturing *A. tonsa* should be in the range of 7.5 to 8.5, assuming seawater with approximate salinity of 26–31 g/kg is used.

2.4.4 Lighting

Lighting should be cool white, using a *light-emitting diode* (LED) or fluorescent source, with 400 to 800 *lux* intensity at the water surface. For at least 2 weeks before a test, the *photoperiod* must be a constant at 16 ± 1 hours of light and 8 ± 1 hours of darkness.

2.4.5 Monitoring

Water temperature, DO, salinity, pH, aeration, culture density, and light intensity must be monitored for each culture vessel at regular intervals (e.g., at the time of culture water renewal).

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. Tests 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.

Test vessels, equipment, and supplies that might come into contact with 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 a 24-well flat-bottom polystyrene microplate that accommodates a 1.5 to 2.2 mL per well working volume.[8] All containers (i.e., type, size, and shape) used for a test vessel must be identical, and the volume of test solution in each well must be 1.5 mL and identical for each test solution. Non-disposable 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, DO, and pH), and must be prepared to undertake prompt and accurate analysis of other variables such as ammonia. The laboratory must have a microscope and lens that allow for clear observation of nauplii and copepod eggs.

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 culturing the test organisms.

[8] Microplates acceptable for use include Falcon™ Fisher Scientific, Catalogue No. 08-772-51, with a non-treated surface; 3.5-mL well volume. Equivalent microplates may be used; however, the difference in their surface coating, or lack thereof, may affect the surface reactivity of ionic toxicants.

Section 4

General Procedure for Determining Acute Lethality of Effluent

4.1 Sample Labelling, Transport, and Storage

Sample volume requirements depend on the number of test concentrations and the number of *replicates*. Sample volumes of ≥ 500 mL (depending on chemical-analytical requirements) are normally required for either single-concentration tests or determination of an LC50.

Containers for transportation and storage of samples must be made of non-toxic material (e.g., polyethylene or polypropylene carboys or pails, or bags in 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 the 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 they spend more than 2 days 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 20 ± 2°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 adjustments 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 period specified below. Using this option, the sample(s) must be stored in full, sealed containers that 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., 20 ± 2°C), in which instance the test must be started the next day. If a sample is warmed or cooled at 20 ± 2°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.

4.2 Test Conditions

This is a 48-hour *static* test (i.e., there is no replacement of solutions during the test). Test organisms must not be fed during the test. The test is not valid if > 20% of the control organisms die (Sections 4.5 and 4.6).

The test must be conducted at 20 ± 2°C (as measured in test solutions). Test solutions must not be aerated during the test. The lighting and photoperiod must be the same as those defined for culturing (see Section 2.4.4).

The test must be conducted without adjustment of the 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)[9] 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. A rationale and procedural details regarding pH adjustment are provided elsewhere (see Section 4.3.2 in EC, 1990a). 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 4 parts per thousand salinity (‰). The salinity of the effluent must be measured before testing commences. There are two acceptable methods of measuring 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‰.

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*., 2017) are recommended. Both automatic conversion using instruments and the use of online 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‰.

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

[9] 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 (environmentally) atypical.

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 the *A. tonsa* culture has been acclimated (see Section 2.4.2), 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 > 4‰ (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 culture water) must also be used as the dilution-water control. In instances in which 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 in which 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, DO, and salinity must be made in the unadjusted, undiluted effluent just before the test solutions are prepared. Adjustment of the effluent sample and control/dilution water to 20 ± 2°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 non-toxic 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.

Subsamples (i.e., aliquots of a sample divided between two or more containers) must be combined prior to solution preparation. The contents of each sample container must be agitated thoroughly (i.e., to resuspend settleable solids) just before aliquots are poured to prepare solutions. Filtration of the sample is normally not recommended. However, if the sample contains organisms that might be mistaken for test organisms, or that might predate on the test organisms, or if suspended or settleable solids prevent observation of the test organism, the sample must be filtered. The recommended filter size is 1 µm. If the sample is filtered because of suspended or settleable solids, parallel tests using both filtered and unfiltered samples is highly recommended.

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 culturing. 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 or glass diffusers. Air stones acceptable for use are:

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

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

iii) alternate air stones that have been shown to perform equivalently to the Marina® or AS1 air stone.[10]

If artificial seawater is to be used as the dilution and control water, it must be prepared as described in Section 2.3. All non-disposable test vessels, measurement devices, stirring equipment, and copepod-transfer equipment must be thoroughly cleaned and rinsed in accordance with standard laboratory practice.

Dissolved oxygen must be measured in the sample just before the test is begun. If DO is between 70% and 100%, the sample must not be pre-aerated. If (and only if) oxygen in the sample is < 70% or > 100% of air saturation, then the sample must be pre-aerated (i.e., aerated before test organism exposure) for a period not exceeding 30 minutes, at a rate within the range of 25 to 50 mL/min · L. Any pre-aeration of the test sample must be provided by bubbling compressed air through the clean air stones described earlier. Aeration of the sample is then stopped, the test solutions prepared, the organisms introduced, and the test initiated immediately, regardless of whether 70% to 100% saturation was achieved in the sample. During the 48-hour duration of the test, there must be no aeration of test solutions or the control.

Each test solution must be prepared and well mixed just before its use (e.g., mixing by inversion, with a glass rod, Teflon™ stir bar, or other device made of non-toxic material). Immediately thereafter, 1.5 mL of each concentration-specific test solution must be placed into each of a minimum of 10 wells of a microplate. Each microplate can accommodate two test concentrations (10 wells per test concentration), with 4 empty wells left over. In a typical test design in which 10 replicates are used per concentration, the four empty wells should be in the middle two columns of the microplate, so that there is some spatial separation between concentrations (AquaTox, 2018). In addition, a separate beaker (e.g., 100 mL), containing test solution (e.g., 50 mL) must be prepared for each test solution for measurement of required water quality parameters (temperature, DO, pH, and salinity) at test initiation and test end (see Section 4.5).

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 test organisms 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. If a multi-concentration test is being performed (Section 6), the order of concentrations on the microplates must be randomized.[11]

Eggs (≤ 24 hours old) must be used for the test. Less than 24 hours before the test, adult copepods are removed (i.e., sieved or pipetted; see Section 2.2) from the cultures that have met the required health criteria.[12] These adults can be

[10] 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 www.hagen.com and search for product A960, A961, or A962. 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.

[11] Block randomization can be used to determine the order of concentrations on the plate.

[12] Experience has shown that using adults ranging in age from 14 to 28 days avoids the use of both young and senescent females.

transferred to clean glass beakers or crystallizing dishes containing 100 mL of control/dilution/culture water and an inoculum of prepared food. The feed may be at the concentration double that used in culturing as this typically promotes a large production of eggs that can be used for testing.[13, 14] Water must be adjusted to 20 ± 2°C and 90% to 100% air saturation with dissolved oxygen before adults are added. Stocking density in the beakers/crystallizing dishes should be approximately 20–200 copepods/100 mL. Adults can be transferred back to their original culture vessels once all of the required eggs are removed.

To begin the test, a single egg must be placed into each of a minimum of 10 wells for every test concentration. To begin this process, eggs that are ≤ 24-hours old are collected using a narrow-mouth pipette and placed into a Petri dish containing culture/control/dilution water. Once enough eggs are obtained for a test (i.e., the pool of test eggs), a portion of the test eggs should be transferred to a smaller Petri dish containing test solution (i.e., concentration-specific test solution).[15] From this concentration-specific Petri dish, one egg must then be randomly selected and transferred to each of the microplate test wells containing the matching test solution (i.e., test concentration). The time at which eggs have been added to all wells for a given concentration must be recorded. (Note this will result in a rolling start time, with exposure to each concentration beginning approximately 30 minutes apart.) All wells must be checked using a microscope to confirm that only a single egg has been added to each well. If more than one egg (or no egg) is found in a given well, the extra egg must be removed from the well using a pipette (or added if no egg was present) with a minimal amount of test solution (≤ 90 µL). Alternatively, the well must be emptied and the process of setting up the well repeated; or the well containing multiple eggs must be marked as "void" on the microplate and a new well set up, again confirming that only one egg has been added to the well. The process of adding eggs to wells is repeated, starting with the control(s) and working towards the highest test concentration to avoid cross-contamination. The time of test initiation (one egg has been added to each of 10 wells for a given concentration) must be recorded for each test concentration. The microplates must then be covered and randomly positioned within the test facility.

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 DO, pH, and temperature must be made in representative vessels set up for each test solution, including the control(s), at the start and end of the test as a minimum. Temperature should be measured every 24 hours. DO, pH, and salinity can also be measured during the test (e.g., every 24 hours). Initial measurements on each test

[13] This can be a simple doubled-up volume of the daily feed normally given to copepods in the 1-L culture vessels (e.g., if all copepods in that culture vessel are transferred to the 100-mL volume via sieving) or a double-up feed that is reduced by 1/10 to take into consideration the 1/10 difference in culture water volume (e.g., if only a subsample of copepods are transferred out of the main culture vessel via pipetting).

[14] Debris can occur in the egg-laying vessel, which might obscure eggs and can hamper the retrieval of eggs. This can be overcome by minimizing the disturbance of the culture vessel when transferring adults or by adjusting the amount of food to either encourage an overabundance of eggs, or reduce the amount of debris.

[15] This step prevents excessive dilution of the test concentration in the microplate. With test solutions that are not transparent (e.g., due to colour or suspended solids), however, the eggs would be very difficult to locate in the concentration-specific Petri dish. In this case, eggs can be transferred directly from the pool of test eggs into the microplate wells with a minimal amount of test solution (≤ 90 µL); however, this may result in some dilution of the test concentration in the microplate.

solution should be carried out after the pre-aeration period, if pre-aeration was applied (see Section 4.3). The salinity of each test solution must be measured at the start of the test as a minimum.

Routine observation of eggs in each test well is required to obtain information regarding survival. Copepods in each test well must be inspected at least at 24 and 48 hours. Observations must be made using a microscope and appropriate lens that allow for the clear observation of nauplii and copepod eggs.[16] During each observation period, egg hatching, copepod mobility, and missing eggs and/or nauplii must be recorded. Eggs are considered hatched when a clear perforation in the egg (i.e., where the nauplius was released) is observed. A nauplius is considered mobile if it can be seen exhibiting any form of movement (e.g., any movement or twitching of the antennae or appendages). A nauplius is considered immobile if it lacks any form of movement within 30 seconds of observation once located within the test well. If an immobile nauplius is observed adhering to, or settling adjacent to, the wall of the microplate well, the microplate should be gently agitated, and the well observed a second time to confirm that the nauplius is immobile. The test organism is considered dead if:

i) the egg is seen to be unhatched; or

ii) the nauplius is immobile (as determined from a 30-second observation after locating the nauplius); or

iii) the test organism is missing.[17]

The number of missing test organisms should be ≤ 10% of the total number of test organisms introduced at the beginning of the test (AquaTox, 2018).

If more than one test organism is found in a given well at the end of the test, each test organism must be evaluated independently, and both must be included in data analysis. This may result in more than 10 organisms for the affected test concentration. Any additional test organisms in a microplate well must be reported as such.

Any differences in appearance or behaviour from control organisms should be noted. With some substances (e.g., narcotics), mobility of the nauplii will be clearly impaired, and the characteristic jumping motion of healthy nauplii will be absent. However, movement may still be observed in the form of twitching appendages or antennae. In this case, the nauplii must be scored as "mobile", but the *overt sublethal* toxic effect should be recorded. For test solutions that are non-transparent (e.g., due to colour, turbidity, or suspended solids), observations of the egg/nauplius can be challenging.

Results for each of the individual wells must be pooled so that for each concentration with 10 replicate wells there is a score out of 10 (e.g., 8/10 dead), and for each concentration with 30 replicate wells (see Section 5) there is a score out of 30 (e.g., 24/30).

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[16] An inverted microscope with a 2–4× magnification lens, or a dissecting scope with a 10× ocular lens and a 0.63–4× magnification has been used by Canadian laboratories for this purpose. The higher magnification lenses listed as examples may be required to observe microplate wells when there is uncertainty as to whether the egg hatched or the nauplius is present and/or mobile. When initially locating nauplii, the set-up chosen should allow for visualization of the entire test volume. The optical quality of the microscope must allow for observation of the egg or nauplius at higher magnifications for accurate assessment of hatching (i.e., subtle changes in colour and shape of the egg) or any nauplius movement (i.e., twitching of antennae or appendages).

[17] Test organism refers to the egg and/or nauplius. If a nauplius is missing, but the remnants of the hatched egg are found, the test organism is considered dead. Highly toxic test substances may result in disintegration of the egg (Paula Jackman, ALET, Environment and Climate Change Canada, personal communication, 2019).

All surviving copepods (including controls) used in the test must be disposed of at the end of the test.

4.6 Validity Criteria

The test is not valid if > 20% of the control organisms die (see Section 4.5).

For the results of any toxicity test that 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 48 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 culture water. If the salinity of the effluent and the salinity to which the adult copepods supplying eggs for the test 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). A minimum of 30 replicates for the 100% concentration and 30 replicates for each control solution is required for this test to provide greater confidence in the test results and their interpretation.

The test is invalidated if > 20% of the control organisms exhibit mortality (see Sections 4.5 and 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 48 hours, and must be reported for the 30 replicates of effluent and the 30 replicates of control(s). Mortality of > 50% is commonly used to define whether or not a sample would receive a "pass" or "fail" rating.

Section 6

Procedure for a Multi-Concentration Test to Determine the 48-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 culture water, must be used in tests to estimate an LC50. If the salinity of the effluent and the salinity to which adult copepods (i.e., supplying eggs for the test) 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 10 eggs (i.e., 10 replicates) 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, 1990a).

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 48 hours (see Section 5) can also be determined from the results of this test.

Additional replicates of each concentration may be used. The use of additional replicates (i.e., exposing a greater number of eggs) could provide a more accurate representation of the concentration-response curve (AquaTox, 2018), and therefore greater confidence in the test results and their interpretation. The 48-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 the LC50 and confidence limits are available (EC, 2005) and should be used.

The test is invalidated if > 20% of the control organisms exhibit mortality (see Sections 4.5 and 4.6). For the results of any toxicity test that 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 culture of copepods 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 using the laboratory's established cultures, and upon acclimation of a new batch of *A. tonsa*. 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 culture/control/dilution water used routinely in effluent tests must also be used for the *reference toxicity test*.

Nickel (e.g., as nickel chloride) is recommended for use as a reference toxicant with *A. tonsa*. The 48-h LC50 should be calculated for the reference toxicant used and expressed as mg/L based on nickel. Based on results generated during inter-laboratory testing, the mean 48-h LC50 for *A. tonsa* was 0.43 mg nickel/L (CV = 48.6%; n = 6) (AquaTox, 2018). Stock solutions of nickel should be made up on the day of use, or can be stored if shown to remain stable over time.

Concentrations of reference toxicant in all stock solutions should be measured chemically using appropriate methods (APHA *et al*., 2017). 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. Nickel solutions should be acidified before storage (APHA *et al*., 2017). 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. 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* that 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 based on the logarithm of concentrations. The *mean* of log(LC50), together with its upper and lower warning limits (± 2 SD), as calculated 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 organisms 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 copepod eggs or the precision of the toxicity data produced by the laboratory is in question. Rather, it provides a warning that this might be the case. A thorough check of all culture and test conditions, as well as technical proficiency, is required at this time.

Depending on the findings, further acclimation and re-evaluation of the copepod culture with one or more reference toxicant(s) should be undertaken, or a new culture of copepods should be established 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 an 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 Samples

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.[18] 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 data sheet 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 additional replicates (e.g., 20 or 30) of each test concentration for the purpose 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 48-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 the chemical can be added to the control/dilution water to give the nominal strengths for testing. For aqueous samples (e.g., chemical formulations in water), test solutions can

[18] 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.

also be prepared by adding appropriate quantities of commercially available dry ocean salts (see Section 2.3) directly to the sample or to each of the test solutions in order to adjust the salinity. 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 the copepods are 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 the 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 in which 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 *A. tonsa* 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, 2016): 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 copepod eggs 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 the eggs added. In most instances, the pre-aeration of test solutions is not necessary nor warranted.[19] 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

[19] Aeration can strip volatile chemicals from a solution or can increase the rate of chemical oxidation and degradation to other substances. However, aeration of test solutions before copepod egg 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.

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 water.[20, 21] 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 culture/control/dilution water that is normally used for the *A. tonsa* 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.

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[20] 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 *A. tonsa* lethality tests; and as a maximum, another series of concentrations using this same water source as the diluent.

[21] 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, 1990a.

It is desirable and recommended that aliquots of test solutions be analyzed to determine the concentrations of chemicals to which test organisms are exposed.[22] In instances in which chemicals are to be 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 the best proven, validated methods with acceptable detection limits available for determining the concentration of the particular chemical in an aqueous (seawater) solution.

Normally, if chemical measurements indicate that the concentrations declined by more than 20% during the test period, the test would be repeated with a *flow-through* or *static-replacement* design. However, at the time of writing, there was no suitable flow-through or static-replacement design available for this test.

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 the test organisms were exposed.

8.5 Test Endpoints and Calculations

The endpoint for tests performed with chemicals will usually be a 48-h LC50 for *A. tonsa* 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 (Sections 4.5 and 4.6). The test is rendered invalid if > 20% of the control organisms exhibit mortality (see Sections 4.5 and 4.6) in any additional control or in the untreated dilution-water control. 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(s), the percent mortality for the test organisms at the end of the test must be calculated and reported if the test is performed using more than 10 replicate wells.

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[22] 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 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 set of regulations, 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 5 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 if 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-hour composite with sub-samples at 1-hour 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 EPS 1/RM/60";
- name and city of testing laboratory;
- 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;

- indication if sample or solution was filtered; indication if any parallel tests with unfiltered sample or solution were performed (see Section 4.3);

- 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 sample (rate and time) before introduction of test organisms;

- concentrations and volumes tested, including control(s);

- number of eggs added to each microplate well; number of microplate wells per concentration;

- indication if any additional test organisms were observed in a microplate well at the end of the test and, if so, how the data were analyzed;

- 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;

- results of culture health check(s) (i.e., % mortality) conducted for the age-class culture to be used as the source of eggs for use in the definitive test; and

- age of adults (i.e., age-class culture) used as source of eggs for the test and age of eggs at the start of the test;

9.1.3 Results

- numbers of unhatched eggs, immobile nauplii, and missing test organisms in each concentration, including the control(s), at 24 hours;

- number of dead test organisms (report numbers of unhatched eggs, immobile nauplii, and missing test organisms) in each concentration, including the control(s), at 48 hours;

- percent mortality of *A. tonsa* in test concentration(s) and control(s), at 48 hours, for a single-concentration;

- estimate of 48-h LC50 and 95% confidence limits in multi-concentration tests, if statistically achievable; methods used for calculating statistical endpoints;

- most recent 48-h LC50 (with 95% confidence limits) for reference toxicity test(s); reference chemical(s); date test initiated; historical 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 5 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 *Acartia tonsa* 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 or partially 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 culturing and test facilities, including general layout of each and means of isolation;
- source of test species, date obtained, and records of taxonomic confirmation of species;
- normal culturing and acclimating conditions (containers; location; lighting; temperature, including maximum rate of change; salinity, including maximum rate of change; aeration; volumes; procedure and frequency for water renewal; procedure for separation into age-class cultures; maximum densities of various cultures; handling procedures; food type, ration, and frequency of feeding);
- duration of acclimation immediately preceding the test, if any;
- brief history of test-specific conditions and procedures for culturing and handling *A. tonsa* (e.g., times; water source; loading density; characteristics such as temperature, salinity, pH, and DO; food type and ration) if different from usual practice;
- description of source(s) of water used for culturing 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 culture/control/dilution water and salinity-control water, if used; and/or test solutions for chemical testing;
- pre-treatment of culture/control/dilution water, if any (e.g., filtration, adjustment of temperature and salinity, aeration rate and duration, de-chlorination, type and quantity of any chemical added, storage details);
- quality (mean and range values) of culture/control/dilution water; to include pH, salinity, DO, and total residual chlorine (if dechlorinated municipal drinking water is used to prepare culture/control/dilution water); preferably also suspended solids, total organic carbon, ammonia, metals, 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 surface of culture and test vessels;
- description of test vessels (size, shape, and material), and covers; routine cleaning procedures for each;
- procedures used to randomize the introduction of test organisms to microplate wells and to randomize the positioning of the test concentrations within the testing facility;
- description of the microscope used for observations of test organisms;
- 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;
- method of obtaining eggs for use in tests;
- method used to monitor health criteria of copepods producing eggs for use in a test;
- characteristics of copepods used for culture health check relative to those used to produce eggs for testing (i.e., age of test organisms used for health check; date of health check relative to date of test start, tested under similar conditions as those for definitive test);
- 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

- observations of test organism 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 Alga	EPS 1/RM/25 2nd Edition	March 2007	–
Acute Test for Sediment Toxicity Using Marine or Estuarine Amphipods	EPS 1/RM/26	December 1992	October 1998
Fertilization Assay Using Echinoids (Sea Urchins and Sand Dollars)	EPS 1/RM/27 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 the 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 available free of charge in PDF format 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 3rd Edition	September 2017	–
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 Using Threespine Stickleback	EPS 1/RM/10 2nd Edition	December 2017	–
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	–
Reference Method for Determining Acute Lethality Using *Acartia tonsa*	STB 1/RM/60	June 2019	–

[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 that 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	–
Procedure for pH Stabilization During the Testing of Acute Lethality of Pulp and Paper Effluent to Rainbow Trout	STB 1/RM/59	March 2018	–
Supplementary Guidance for Investigating Acute Lethality of Pulp and Paper Mill Effluents due to Ammonia	–	March 2018	–

Appendix B

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Appendix D

General Characteristics of *Acartia tonsa*

Several resources were consulted and used in the preparation of this appendix and include: Mauchline, 1998; Drillet *et al*., 2006, 2008, 2011; Medina and Barata, 2004; Jepsen *et al*., 2007; Hagemann, 2011; Marcus and Wilcox, 2007; Gorbi *et al*., 2012; Gonzalez, 2013; ISO, 2014; Zhang *et al*., 2015; Hagemann *et al*., 2016a; Vitiello *et al*., 2016; Paula Jackman, ALET, Environment and Climate Change Canada, personal communication, 2018; Craig Buday, PYLET, Environment and Climate Change Canada, personal communication, 2017; Isabella Buttino, Istituto Superiore per la Protezione e la Ricerca Ambientale, personal communication, 2018; and AquaTox, 2018.

Acartia tonsa (Dana 1849) is a free-swimming planktonic copepod belonging to the family Acartiidae. It is a cosmopolitan species in temperate regions, distributed worldwide in coastal waters, and is often the dominant zooplankton in nearshore environments above 200 metres. It is a large-sized filter feeder, feeding primarily on phytoplankton, and thus forms an important link between the primary producers and higher trophic levels like larger zooplankton, crustaceans, and fish. It is omnivorous, feeding on dinoflagellates, ciliates, protozoans, bacteria, phytoplankton, algae, diatoms, as well as eggs and nauplii of its own and other species. In daytime, individuals are more often found in deeper waters to avoid predators, but they move to shallower waters at night to feed. It generally moves by "jumping", using its large antennae and swimmerets. *Acartia tonsa* originating from different geographic areas have distinct mitochondrial clades and distinct life history traits such as generation times and productivity.

Adults range in size from 0.5 to 1.5 mm in length. They have translucent bilaterally symmetrical bodies divided into three segments: prosome (head and antennae), metasome (legs and swimmerets), and urosome (containing sexual organs). Male and female *A. tonsa* are sexually dimorphic with females being slightly larger than males. The most readily distinguishable feature among sexes are the antennae, which are bent and asymmetric (geniculate) in males and straight in females (Figure D-1). In addition, the females have a more rounded head form and the second urosome segment (of four) is bigger and prolonged, whereas males have a more squared head form and the second urosome segment (of five) is round. Females survive longer than males, 70–80 days versus 15 days.

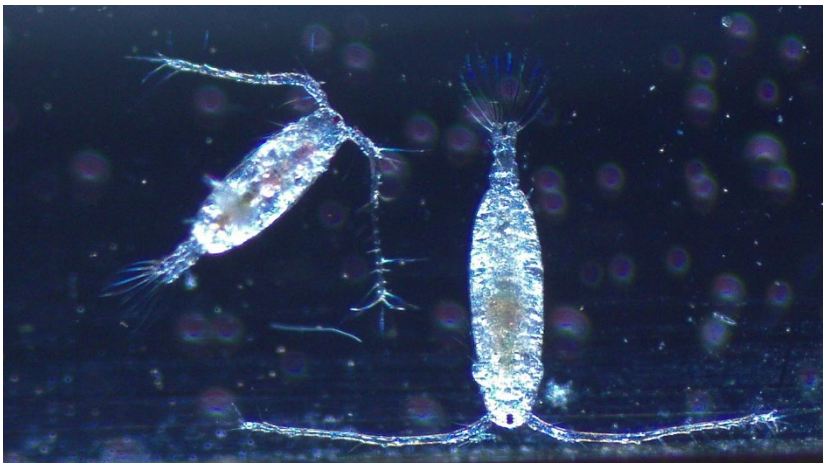

Figure D-1 Adult male (left) and female (right) *Acartia tonsa* illustrating the sexual dimorphism, and the difference in antennae and head shape of the species. (Photos: L. Haché and P. Jackman)

Acartia tonsa progresses through six naupliar stages (N1 through N6) to become a *copepodite*, which then progresses through six stages (C1 through C6), before becoming a sexually mature copepod adult (Figure D-2). This occurs in approximately 2 to 3 weeks at 20°C.

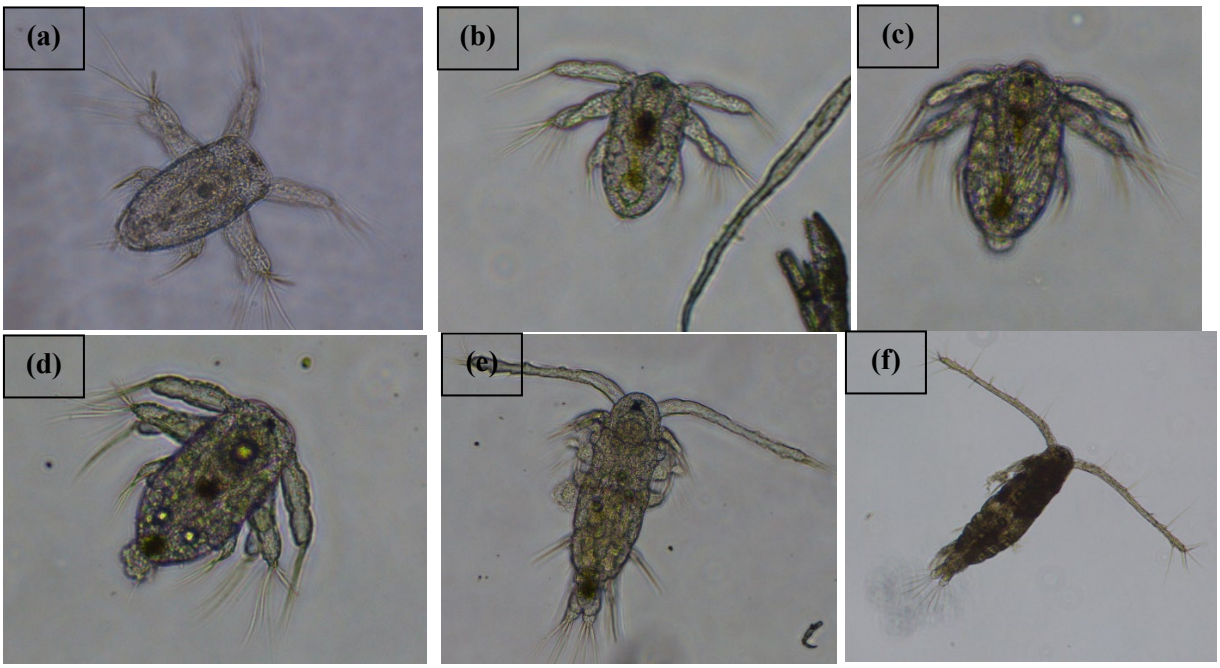

Figure D-2 Developmental stages of *Acartia tonsa*, illustrating: (a) nauplii stage II, (b) nauplii stage III, (c) nauplii stage V, (d) nauplii stage VI, (e) copepodite (unknown stage), and (f) adult. (Photos: L. Haché and P. Jackman)

N1 nauplii are approximately 150 µm in length (Figure D-3c). The eggs of *A. tonsa* are 70–80 µm in diameter, spherical, slightly opaque (Figure D-3a), and covered with short spines (visible only with high magnification). When hatched, eggs are more translucent (i.e., demonstrate a colour change), and often the perforation in the egg where the nauplius exited is visible (Figure D-3b). The optical quality of the microscope used to make observations at the end of the test can affect the ability to detect movement (i.e., twitching of appendages or antennae) and also the ability to discern the characteristics that distinguish a hatched versus an unhatched egg (see Section 4.5). The use of lower-quality equipment or lower magnifications only could lead to artifacts being mistaken for *A. tonsa* eggs (Figure D-3d).

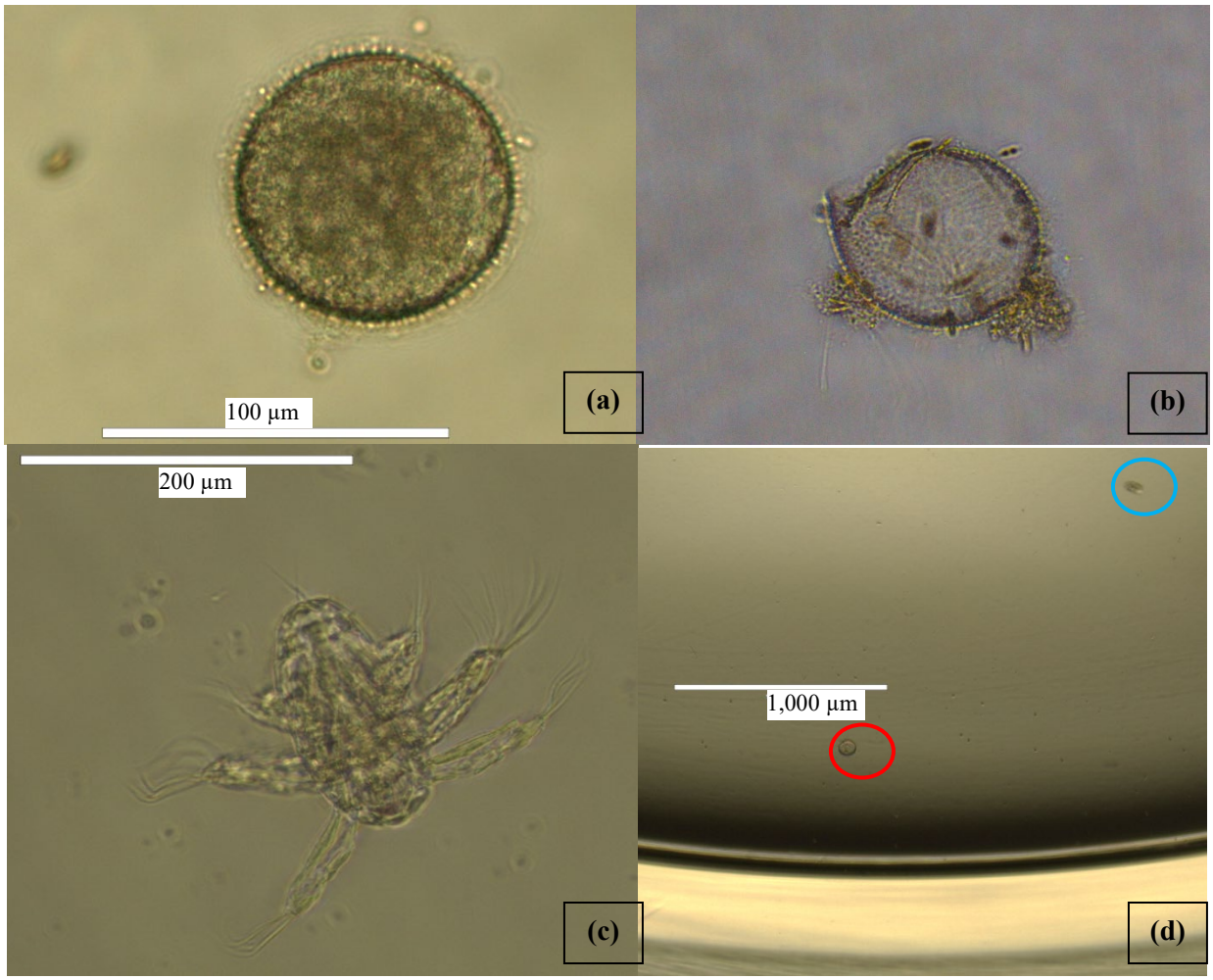

Figure D-3 **Images of *Acartia tonsa*: (a) unhatched egg (uniformly round and somewhat opaque), (b) hatched egg (more translucent and visibly ruptured/deformed), (c) nauplius (approximately 24 hours old), and (d) hatched egg in a test well (red circle) with the microplate wall visible as the lighter area at the bottom of the image. Note that without adequate magnification, equipment imperfections (blue circle) visible in the upper right corner can be mistaken for an *A. tonsa* egg.** (Photos: L. Haché and P. Jackman)

A. tonsa reproduces by gamogenesis, where the male deposits spermatophores on the urosome of the female. After mating, the female can store the semen for fertilization of eggs for some time. *A. tonsa* is a broadcast spawner and does not have an egg bag or brood pouch. Eggs are released singly and continuously into the water and being slightly heavier than seawater, they sink. At 20°C, the eggs will hatch after approximately 24 hours. In the laboratory under good conditions, a female can produce from 18 to 50 eggs per day at 15°C, and 10 to 60 eggs per day at 20°C. During the inter-laboratory investigation for this method, eggs that were opaque and of average size (i.e., not too small, not too large) were reported to have better hatching success, and therefore it was suggested that these characteristics be selected for use in culture health and definitive tests. In addition, entire groups of eggs were sometime found to be "sticky". The reason for "sticky" eggs is unknown; however, these eggs were avoided for use in tests as they were found to be harder to handle and often resulted in poor hatching success.

There is a positive relationship between algal concentrations and egg production, confirming that food quantity can limit *A. tonsa* fecundity. Culture density can also affect copepod development and egg production. Egg hatching success, however, is not dependent on culture densities or the quantity of food used. At very high culture densities (e.g., 2,000 individuals/L), development was found to be delayed and egg production was low compared with lower culture densities (e.g., 500 and 100 individuals/L). In one study, it was shown that egg production was not significantly different in *A. tonsa* cultures of 100 to 600 adults/L and an average 22.5 ± 8.8 eggs/female/day in all densities. In addition, there was no significant difference in egg viability across all stocking densities (mean 84.7 ± 4.8%). Under controlled laboratory conditions, eggs can be stored cold (e.g., 3 ± 1°C) for prolonged periods of time (3–11 months), although hatching success decreases as storage time increases. Cold storage of *A. tonsa* eggs could ensure sufficient quantities for testing at times of the year when adults are not readily available.

Appendix E

Procedure for Preparing Algal Food (*Rhodomonas salina*) for *Acartia tonsa* Cultures

Background

Rhodomonas salina (previously known as *Pyrenomonas salina*, *Chroomonas salina*, or *Cryptomonas salina*) is a motile marine red alga that is used as a food source for the marine copepod *Acartia tonsa*. The algae can be obtained as a small "starter" culture from the Canadian Phycological Culture Centre (CPCC) at the University of Waterloo, the Canadian Center for the Culture of Microorganisms (CCCM) at the University of British Columbia, or the Provasoli-Guillard National Center for Marine Algae and Microbiota (NCMA) at the Bigelow Laboratory for Ocean Sciences in Maine, USA. A series of small "stock" cultures can be prepared and then used to inoculate larger "feeding" cultures to be used for feeding *A. tonsa*. Many strains of *R. salina* are available; however, two strains (CPCC 714 and CPCC 715) were investigated for use in this method and are described herein. CPCC 714 *Rhodomonas salina* was originally obtained from the CCCM as CCCM 427 *Pyrenomonas salina* and has bacterial contamination. CPCC 715 *Rhodomonas salina* was originally obtained from the NCMA as CCMP 1319 *Rhodomonas salina* and is axenic. There are a number of successful culturing methods described for *R. salina* (Marcus and Wilcox, 2007; ISO, 2014; AquaTox, 2018). The procedures and conditions described below are one set of guidelines only, allowing flexibility in a given laboratory's approach to culturing *R. salina*.

Preparing Algal Culture Medium (F/2 Medium) from Purchased F/2 Concentrate

1. Sterile F/2 medium concentrate (hereafter referred to as F/2 concentrate) can be purchased from the University of Waterloo's CPCC. The sterile F/2 concentrate can be used to prepare F/2 medium. It can also be used as a nutrient spike that may be added to both "stock" and "food" cultures weekly. It must be kept sterile and is stored at 4 ± 2°C.
2. To prepare 1 L of F/2 medium, add 10 mL of F/2 concentrate to 990 mL of artificial seawater (e.g., Instant Ocean or ESAW) or filtered (0.45 µm) natural seawater and mix well. [23] Adjust the pH to 8.0 ± 0.1, using 1 *N* NaOH or HCl, as necessary.
3. Divide the media into clean glass flasks or reusable glass media storage bottles and sterilize by autoclaving (e.g., 121°C for 15 minutes or longer; 30–60 minutes for larger volumes). The medium may precipitate when removed from the autoclave, but will likely dissolve again when cooled.[24]
4. Store the sterile medium for a minimum of 1 day prior to use to allow the chemical moieties and pH to equilibrate. The sterile F/2 medium can be stored indefinitely in the fridge (e.g., 4°C) or for a shorter time at warmer temperatures (e.g., 16 ± 1°C for up to 3 months, or at room temperature).

[23] To prepare larger volumes of F/2 medium, the volumes described can be doubled or tripled.

[24] If precipitation is problematic, it can be minimized by adding 0.12 g of $NaHCO_3$ and 1.44 mL of 1 *N* HCl prior to autoclaving. Filter-sterilization of the medium using a 0.22 µm filter may also be used to eliminate precipitation.

Preparing Algal Culture Medium (F/2 Medium) from Stock Solutions

1. Prepare five stock nutrient solutions using reagent-grade chemicals as described in Table E1.

Table E1 Nutrient stock solutions for maintaining stock cultures of algae

Nutrient Stock Solution[a]	Compound	Stock or Sub-Stock Concentration	Stock Volume (mL) added per 1L of Medium
1	$NaNO_3$	75 g/L	1
2	$NaH_2PO_4 \cdot H_20$	5 g/L	1
3	$Na_2SiO_3 \cdot 9H_20$	30 g/L	1
4[b]	$FeCl_3 \cdot 6H_20$	3.15 g/L	1
	$Na_2EDTA \cdot 2H_20$	4.36 g/L	
	$CuSO_4 \cdot 5H_20$	9.8 g/L[c]	
	$Na_2MoO_4 \cdot 2H_20$	6.3 g/L[c]	
	$ZnSO_4 \cdot 7H_20$	22 g/L[c]	
	$CoCl_2 \cdot 6H_20$	10 g/L[c]	
	$MnCl_2 \cdot 4H_20$	180.0 g/L[c]	
5[d]	Vitamin B_{12}	5 mg/5 mL[e]	0.5
	Biotin	1 mg/10 mL[f]	
	Thiamine	20 mg/100 mL[g]	

[a] Stock and sub-stock solutions are prepared using distilled or Milli-Q®/Nanopure® water.
[b] Stir and heat (if necessary) on stirring magnetic hot plate until all reagents are dissolved. If heated, then once cooled, top back up with distilled or Milli-Q® water.
[c] Concentration in sub-stock solution; add 1 mL of sub-stock to 1 L nutrient stock solution 4.
[d] Nutrient solution 5 can be stored as small aliquots in the freezer (e.g., 1 mL in 2-mL cryovials, or 25 mL scintillation vials).
[e] Concentration of Vitamin B_{12} sub-stock solution; add 0.1 mL of sub-stock solution to 100-mL nutrient stock solution 5.
[f] Concentration of Biotin sub-stock solution; add 1 mL of sub-stock solution to 100-mL nutrient stock solution 5.
[g] Add 20 mg of Thiamine to 100 mL nutrient stock solution 5.

2. To prepare 1 L of F/2 medium, add 1 mL of each of stock solutions 1, 2, 3, and 4; and 0.5 mL of stock solution 5 to 900 mL of artificial seawater or filtered (0.45 µm) natural seawater. Mix well after each solution is added. Dilute to 1 L, mix well and adjust the pH to 8.0 ± 0.1, using 1 *N* NaOH or HCl, as necessary. The final concentration of compounds in the culture medium is given in Table E1.
3. Divide the medium into clean glass flasks or reusable glass media storage bottles and sterilize by autoclaving (e.g., 121°C for 15 minutes or longer; 30–60 minutes for larger volumes). The medium may precipitate when removed from the autoclave, but will likely dissolve again when cooled.[24]
4. Store the sterile medium for a minimum of 1 day prior to use to allow the pH to equilibrate. The sterile F/2 medium can be stored indefinitely in the fridge (e.g., 4°C) or for a shorter time at warmer temperatures (e.g., 16 ± 1°C for up to 3 months or at room temperature).
5. To prepare F/2 concentrate, add 10 mL of F/2 stock solutions 1, 2, 3, and 4; and 5 mL of F/2 stock solution 5 per 100 mL. Cap and freeze until use. The F/2 media concentrate can be stored in the dark at -20 to -25°C for up to 1 year and thawed just prior to use.
6. Stock solutions 1, 2, 3, and 4 can be stored in the dark at 4 ± 2°C for up to 1 year; and stock solution 5 can be stored in the dark at -20 to -25°C for up to 1 year and thawed just prior to use.[25]

Establishing and Maintaining "Stock" Cultures of Algae

1. Small "stock" cultures can be initiated from a purchased "starter" culture (usually about 30 mL or more), or from an older (e.g., usually the oldest, but ≤ 3 weeks old), previously established stock culture. Stock cultures are initiated by aseptically transferring ~1–5 mL or more to each of several 250-mL culture flasks containing ~100 mL of sterile algal culture medium (F/2; prepared as described above). The stock culture should have a pink (for CPCC 715) or tan (CPCC 714) colour following inoculation of the stock culture medium. At inoculation, an extra 1 mL of F/2 concentrate may be added to the culture for a quick growth start. The purchased starter culture does not store well in shipping tubes and therefore it should be used within a few days of receipt, and any unused portion discarded.
2. The stock cultures can be used as a source of algae to initiate "food" cultures (see following section). The volume of stock culture maintained at any one time will depend on the amount of algal food required for the *A. tonsa* cultures. Stock culture volume may be rapidly "scaled up" to several litres, if necessary, by inoculating more culture flasks.
3. Stock cultures can be incubated until used to start "food" cultures, or can be aseptically transferred to a new medium (e.g., every 7 to 10 days) to maintain "stock" cultures. Incubation temperature depends on the strain of *R. salina*. The CPCC 714 strain grows best when incubated at 16 ± 2°C and the CPCC 715 strain at 20 ± 2°C; however, both strains can be maintained at either 16 ± 2°C or 20 ± 2°C. Stock cultures may be maintained in environmental chambers with cultures of other organisms if the illumination is adequate. The photoperiod may be 16 hours light:8 hours dark for both strains or 12 hours light:12 hours dark for CPCC 714, and cool-white or full-spectrum (LED[26] or fluorescent) lighting with an intensity of 3,500–4,000 lux for the CPCC 714 strain, and 1,500–2,000 lux for the CPCC 715 strain. Several days after inoculation, the solution should develop a pink/orange colour for the CPCC 714 strain, and a pink/brown colour for the CPCC 715 strain; these are indicative of healthy cultures and actively growing algal cells.

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[25] Stock solutions may be stored for longer than a year; however, if the performance of the algae declines, the potency of the stock solutions might be suspect, and preparation of new solutions might be necessary.

[26] Full-spectrum, daylight, plant-growth, and cool-white bulbs are all acceptable. LEDs with mostly blue spectrum and some red spectrum wavelengths are also acceptable. Warm-white, soft-white, and other yellow spectrum bulbs are not usually acceptable since most algae are unable to make use of yellow wavelengths.

4. Stock cultures can be mixed daily by hand to resuspend cells.
5. If stock cultures start to turn green due to lack of nutrients, 1 mL of F/2 concentrate may be added as a nutrient boost. Alternatively, the concentrate may be added to the stock cultures weekly to prevent the cultures from running out of nutrients and turning green.
6. Stock cultures can be examined microscopically at transfer to ensure that the *R. salina* cells are still looking normal and motile and have not become contaminated with other microscopic organisms, such as other types of algae, fungi, or protozoa. Reserve quantities of cultures can be maintained at the temperature described above, a 12 hours:12 hours light:dark cycle at 1,850 to 3,000 lux (cool white) for a period of 5 weeks without transfer.[27]

Establishing and Maintaining "Food" Cultures of Algae

1. To produce enough algae to feed *Acartia tonsa*, larger cultures of *Rhodomonas salina* are necessary. "Food" cultures can be started from previously established "stock" cultures or from purchased "starter" cultures, 10 days to 3 weeks prior to use for feeding *A. tonsa*. Approximately 500–900 mL of sterile F/2 solution is inoculated with ~100 mL of an older (i.e., 2 to 3 weeks old with dark red/brown/orange colour; see Figure E-1a) algal "stock" culture (described in the previous section) or 1–2 mL of the starter culture. The large cultures do not have to be inoculated under sterile conditions; however, aseptic techniques are recommended for use in preparing and maintaining the cultures, and care should be exercised to avoid contamination by other microorganisms.
2. Food cultures should be maintained at 16 ± 2°C or 20 ± 2°C (depending on the strain; described in the previous section), in environmental chambers with the algal stock cultures or cultures of other organisms if the illumination is adequate (described in the previous section).
3. Cultures should be covered and lightly aerated (e.g., 2–3 bubbles per s) using a sterile pipette. Cultures can be swirled daily by hand to resuspend any settled algal cells. Caution should be exercised to prevent the culture temperature from rising or falling by more than a few degrees as this can affect growth or result in mortality.
4. Cultures can be spiked with F/2 concentrate (e.g., 2 to 5 mL) as needed (i.e., if they turn green due to lack of nutrients), or once per week to prevent the cultures from running out of nutrients.
5. Algal cultures are ready to use as food when they are opaque and dark in colour (usually 10 days to 3 weeks old). Cell density is determined in order to calculate the volume to be used for feeding the copepods and should be a minimum of 4.0×10^5 cells/mL (note: a more typical cell count in a healthy culture would be ~1.0 to 3.0×10^6 cells/mL). *A. tonsa* cultures are fed daily at a rate of 6.0×10^6 cells/L to 6.0×10^7 cells/L of *A. tonsa* culture water, depending on the age and density of the *A. tonsa* cultures (see Section 2.2). Feeding three times a week using a higher ration of algae is also acceptable (see Section 2.2). If the algae culture is used for more than a week, the algae cells should be recounted to ensure the cell density has not changed or to readjust the volume if it has.
6. The health of the algae cells and the cell density of the cultures should be assessed on a regular basis. A small quantity of the algae culture is examined under a microscope (20–400× power) to ensure the cells look normal (i.e., actively moving, intact, and normal size/shape). The cell density (cells/mL) can be measured with an electronic particle counter, microscope and haemocytometer, fluorometer, or spectrophotometer, and used to determine the volume required for feeding. A 50/50 mix of the algae and a 0.5% Gluteraldehyde solution (usually 1 mL of each) is prepared to stop the algae from moving prior to performing cell counts using a haemocytometer. Alternatively, the bottom of the haemocytometer containing the cells can be briefly heated to slow down or stop the algae from moving.

[27] Cultures will not remain pink during the entire 5-week period between transfers, but they should remain tan and mostly motile.

7. Algae cultures (stock and/or food) can be renewed weekly to maintain a supply of healthy, growing cultures.
8. During culturing or storage, the appearance of the algae may change. Algae that have settled into clumps, have turned completely green, or have green clumps at the bottom should not be used to feed *A. tonsa* cultures (see Figure E-1b). If the algae have changed colour or have a strong odour, the cultures should be examined under a microscope to determine if there are active cells and whether or not the culture has been contaminated.

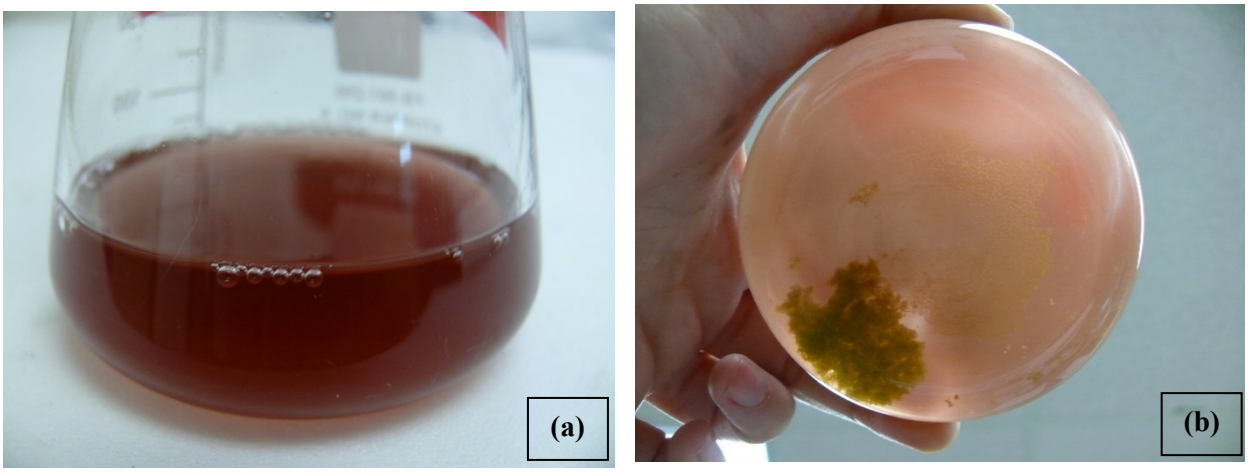

Figure E-1 **Healthy (a) and contaminated (b) cultures of *Rhodomonas salina.*** (Photos: L. Haché and P. Jackman)