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Reference Method for Measuring the Toxicity of Contaminated Sediment to Embryos and Larvae of Echinoids (Sea Urchins or Sand Dollars)

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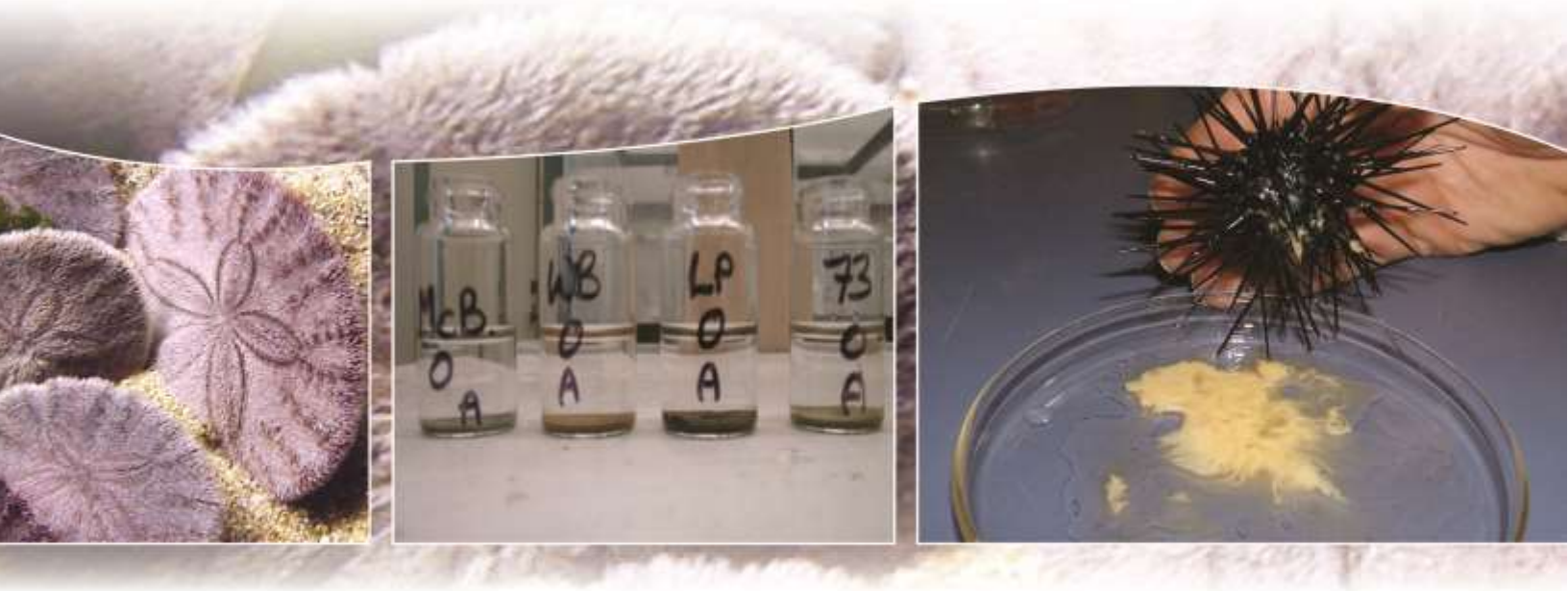

Canada

Reference Method for Measuring the Toxicity of Contaminated Sediment to Embryos and Larvae of Echinoids (Sea Urchins or Sand Dollars)

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

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Abstract

A reference method for measuring the toxicity of contaminated sediment to embryos and larvae of echinoids is described in this report. It is intended strictly for use with marine sediments having a minimum salinity of 15‰. Explicit instructions are provided for performing tests using one or more of the following three echinoid species: *Strongylocentrotus purpuratus* (Pacific purple sea urchin), *Lytechinus pictus* (white sea urchin) and *Dendraster excentricus* (eccentric sand dollar).

In the test, freshly fertilized eggs (embryos) are exposed to whole sediment samples. Each test includes a control sediment, test sediment(s) under investigation and a field-collected reference sediment. The test is started within 2 to 4 hours of fertilization, and a mean fertilization success rate of ≥ 90% must be achieved in order for the test to be initiated. Provided this fertilization rate is achieved, ~200 eggs (comprised of ≥ 90% newly fertilized eggs) are transferred to all test chambers. Test duration is species-dependent. The presumptive test end for each species is 48 h for *L. pictus*, 72 h for *D. excentricus* and 96 h for *S. purpuratus*. The test can be prolonged by 24 ± 1 hour, based on the percentage of normal larvae determined for the "water-only" controls included in the test for this purpose. If at presumptive test end, the mean % normal larva in the monitoring vials is less than 70%, then the test must be extended for an additional 24 h in order to ensure the test validity criteria [i.e., % normal larvae (Pn) ≥ 60%] will be met when the organisms are evaluated and scored.

Using a "total count" approach, at the end of the test, all embryos and larvae recovered from each replicate must be counted and scored (either in-vial using an inverted microscope or by use of a Sedgwick-Rafter cell). For each test replicate, the number of i) normal larvae (prism or pluteus), and ii) abnormal larvae (i.e., those with developmental anomalies) are counted and documented. Once the counting and scoring of all recovered organisms has been completed, the percentage of normal larvae is calculated for each treatment.

Specific conditions and procedures are stipulated that include instructions for acclimating and holding adult echinoids in the laboratory for extended periods of time, for holding adult echinoids in the laboratory for immediate use (for adults who are spawned within three days of arrival at the laboratory), and for obtaining sperm and eggs for a test. Also described are the required procedures and conditions for transporting, storing and manipulating samples of sediment to be used in the test; required physicochemical analyses of sediment and water; procedures and conditions to be followed in preparing for and conducting the test; criteria for acceptable performance and valid test results; measurements and observations to be made; required data analyses; instructions for interpreting test results; and minimum reporting requirements. Instructions on the use of reference toxicity tests are also provided.

Résumé

Le présent document décrit une méthode de référence pour mesurer la toxicité des sédiments contaminés chez les embryons et les larves des échinides. Cette méthode est uniquement destinée aux sédiments marins dont la salinité minimale est de 15 ‰. Des instructions explicites sont fournies pour effectuer des essais à l'aide d'au moins une des trois espèces d'échinides suivantes : *Strongylocentrotus purpuratus* (oursin violet du Pacifique), *Lytechinus pictus* (oursin blanc) et *Dendraster excentricus* (clypéastre excentrique).

Dans les essais, des œufs fraîchement fécondés (embryons) sont exposés à des échantillons de sédiments entiers. Chaque essai comprend un sédiment témoin, un ou plusieurs sédiments d'essai à l'étude et un sédiment de référence prélevé sur le terrain. L'essai débute dans les 2 à 4 heures suivant la fécondation, et un taux moyen de fécondation de ≥ 90 % doit être obtenu pour que l'essai soit initié. Si ce taux de fécondation est atteint, ~ 200 œufs (dont ≥ 90 % sont des œufs nouvellement fécondés) sont transférés dans toutes les enceintes d'essai. La durée de l'essai dépend de l'espèce. La fin présumée d'un essai pour chaque espèce est de 48 h pour le *L. pictus*, de 72 h pour le *D. excentricus* et de 96 h pour le *S. purpuratus*. L'essai peut être prolongé de 24 ± 1 h, selon le pourcentage de larves normales déterminé pour les témoins « en milieu exclusivement aqueux » qui font partie de l'essai à cette fin. Si, à la fin présumée de l'essai, le taux moyen de larves normales dans les flacons de surveillance est inférieur à 70 %, l'essai doit être prolongé de 24 h afin de s'assurer que le critère de validité de l'essai [c.-à-d. % de larves normales (Pn) ≥ 60 %] est satisfait lorsque les organismes sont évalués et notés.

En utilisant l'approche de « numération totale », à la fin de l'essai, tous les embryons et les larves recueillis dans chaque répétition doivent être comptés et notés (dans le flacon au moyen d'un microscope inversé ou en utilisant une cellule de Sedgwick-Rafter). Pour chaque répétition d'essai, i) les larves normales (au stade prisme ou pluteus) et ii) les larves anormales (c.-à-d. celles qui présentent des anomalies de développement) sont comptées et documentées. Une fois que tous les organismes recueillis ont été comptés et notés, le pourcentage de larves normales est calculé pour chaque traitement.

Des conditions et des méthodes précises sont stipulées, y compris des instructions pour acclimater et maintenir des échinides adultes en laboratoire pendant de longues périodes ou en vue de leur utilisation immédiate (adultes dont le frai est provoqué dans les trois jours suivant leur arrivée au laboratoire), et pour recueillir le sperme et les œufs nécessaires à l'essai. Les méthodes et les conditions nécessaires sont également décrites pour le transport, l'entreposage et la manipulation des échantillons de sédiment en vue de l'essai; les analyses physicochimiques nécessaires des sédiments et de l'eau; les méthodes et les conditions à respecter dans la préparation et l'exécution de l'essai; les critères relatifs à un rendement acceptable et à des résultats d'essai valides; les mesures et les observations à faire; les analyses de données requises; les instructions pour interpréter les résultats d'essai; et les exigences minimales à l'égard des rapports à produire. Des instructions sur l'utilisation d'essais toxicologiques de référence sont également fournies.

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 test materials or substances under controlled and defined laboratory conditions.

A **reference method** is defined herein as a specific biological test method for performing a toxicity test, i.e., a toxicity test method with an explicit set of test instructions and conditions which are described precisely in a written document. Unlike other multi-purpose (generic) biological test methods published by Environment Canada, the use of a **reference method** is frequently restricted to testing requirements associated with specific regulations (e.g., *Disposal at Sea Regulations* under the *Canadian Environmental Protection Act 1999*; CEPA 1999; Government of Canada, 2001).

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

- for regulatory use in the environmental toxicity laboratories of federal and provincial agencies;
- for regulatory testing which is contracted out by Environment 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 Canada's Method Development and Applications Unit in Ottawa, ON, along with other generic (more widely applicable) biological test methods and supporting guidance documents.

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

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List of Abbreviations and Chemical Formulae

°C	degree(s) Celsius
cm	centimetre(s)
Cu	copper
CV	coefficient of variation
d	day(s)
DNA	deoxyribonucleic acid
DO	dissolved oxygen (concentration)
g	gram(s)
g/kg	grams per kilogram
h	hour(s)
HSB	hypersaline brine
ICp	inhibiting concentration for a (specific) percent effect
KCl	potassium chloride
L	litre(s)
M	molarity (concentration)
m	metre(s)
mg	milligram(s)
min	minute(s)
mL	millilitre(s)
mm	millimetre(s)
mS	millisiemen(s)
N	Normal
rpm	revolutions per minute
s	seconds
SD	standard deviation
SI	International System of Units
sp.	species
TM (TM)	Trade Mark
µg	microgram(s)
µL	microlitre(s)
µm	micrometre(s)
µmhos	micromhos
V	volt(s)
× *g*	relative centrifugal force (times gravity)
>	greater than
<	less than
≥	greater than or equal to
≤	less than or equal to
/	per; alternatively, "or" (e.g., control/dilution water)
±	plus or minus
~	approximately
%	percentage or percent
‰	parts per thousand

Terminology

Note: The following definitions are given in the context of this test method. Additional definitions in the detailed generic method for testing with echinoids (Environment Canada, 2011) also apply here.[1]

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.

Batch means a single group of adult echinoids received from a supplier at a discrete time, in order to provide all of the gametes intended for use in a discrete *toxicity test* (including any associated reference toxicity test). It *might* also refer to the gametes collected from a single male and female or a group of males and females at one time, intended for use in a discrete toxicity test (including any associated reference toxicity test).

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

Conductivity is a numerical expression of the ability of an aqueous solution to carry an electric current. This ability depends on the concentrations of ions in solution, their valence and mobility, and on the solution's temperature. Conductivity in fresh waters is measured at 25°C, and is normally reported in the SI unit of millisiemens/metre, or as micromhos/centimetre (1 mS/m = 10 μmhos/cm). Conductivity is a standard method for measuring *salinity*, with the result read off as g/kg or "parts per thousand" (‰).

Embryo means an animal (organism) in the early stages of growth and differentiation (development), post (after)-fertilization of an egg. In this reference method, it is used to denote the stages between fertilization of the egg and the prism and pluteus larva; see Figures 2 and 3.

Gametes are the sperm or unfertilized eggs obtained from adult echinoids.

[1] For full definitions of the following general terms see EPS 1/RM/27 (Environment Canada, 2011): lux, monitoring, protocol, reference method, turbidity, artificial sediment, dechlorinated water, deionized water, distilled water, estuarine water, marine water, material, sediment, stock solutions, substance, normality, precision, quantal, quantitative, sublethal, toxic, toxicology.

Larva (plural, larvae) is a recently hatched organism which has physical characteristics other than those seen in the adult of the species. In this reference method, larvae refer to the prism and pluteus stage of echinoid development (see Figures 2 and 3).

Percentage (%) is a concentration expressed in parts per hundred. With respect to test substances, 10 percent (10%) represents 10 units of substance diluted with sediment or water to a total of 100 parts. Depending on the test substance, concentrations *can* be prepared on a weight-to-weight, weight-to-volume, or volume-to-volume basis, and are expressed as the percentage of test substance in the final sediment mixture or solution.

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

Photoperiod is the duration of illumination and darkness within a 24-hour period.

Salinity is the total amount of solid substance, in grams, dissolved in 1 kg of water. It is determined after all carbonates have been converted to oxides, all bromide and iodide have been replaced by chloride, and all organic matter has been oxidized. Salinity can be measured directly using a salinity/*conductivity* meter or by other means (see APHA *et al.*, 1989, 2005). Salinity is reported here as g/kg. The term "parts per thousand" (‰) is synonymous with g/kg.

Terms for Test Materials or Substances

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 sediment or water.

Clean sediment is sediment that does not contain concentrations of any substance(s) causing discernible distress to the test organisms or reducing their survival or development during the test.

Contaminated sediment is sediment containing *chemical* substances at concentrations that pose a known or potential threat to environmental or human health.

Control is a *treatment* in an investigation or study that duplicates all the conditions and factors that might affect the results of the investigation, except the specific condition that is being studied. In toxicity tests, the control *must* duplicate all the conditions of the exposure treatment(s), but must contain no contaminated test material or substance. The control is used as a check for the absence of measurable toxicity due to basic test conditions (e.g., quality of *dilution water*, health of test organisms or effects due to their handling).

Control/dilution water is the seawater used for preparing a series of concentrations of a test substance, or that is used as *overlying water* in a sediment toxicity test or as *control* water in a "*water-only*" test (e.g., in a *reference toxicant* test). Control/dilution water is frequently identical to the culture and test (overlying) water.

Control sediment is uncontaminated (clean) sediment which does not contain concentrations of one or more contaminants that could affect the survival or development of the test organisms. This sediment might be natural sediment from an uncontaminated *site*, or formulated (reconstituted) sediment. This sediment must contain no added test material or substance, and must enable an acceptable rate of

echinoid development according to the test conditions and procedures. Control sediment is used to confirm that the test has met the validity criterion and provides a basis for interpreting data derived from toxicity tests using *test sediment*(s).

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

Dredged material is sediment and/or settled particulate waste (e.g., solids from the sea bed of a harbour or channel) that has either been dredged from a water body or is being considered for dredging and subsequent ocean disposal.

Hypersaline brine is a solution of sea salts in water, in stronger concentration than in oceanic water. It can be obtained from high quality filtered seawater by partial freezing and draining off the unfrozen liquid, freezing and partially thawing, or slow heating and evaporation. Hypersaline brine can also be prepared by adding commercially available ocean salts or reagent-grade salts to fresh or distilled water. The strength of brine used for this fertilization assay *should* be 90 ± 1 g/kg.

Overlying water is water placed over sediment in a test chamber or holding/acclimation chamber.

Pore water (also called interstitial water) is the water occupying space between sediment particles. The amount of pore water is expressed as a *percentage* of the wet sediment, by weight.

Reconstituted seawater is fresh water (deionized or glass distilled) to which commercially available dry ocean salts, reagent-grade salts, or *hypersaline brine* has been added, in a quantity that provides the seawater *salinity* (and *pH*) desired for holding organisms and for testing purposes (control/dilution water).

Reference sediment is a field-collected sample of presumably clean (uncontaminated) sediment, selected for properties (e.g., particle size, compactness, total organic content) representing sediment conditions that closely match those of the sample(s) of test sediment except for the degree of chemical contaminants. It is often selected from a site that is uninfluenced or minimally influenced by the source(s) of anthropogenic contamination but within the general vicinity of the site(s) where samples of test sediment are collected. One or more samples of reference sediment should be included in each series of toxicity tests with test sediment(s). This sediment might or might not prove to be toxic due to the presence of naturally occurring chemicals such as hydrogen sulphide or ammonia, or the unanticipated presence of contaminants from human influence at harmful-effect concentrations. The use of such (toxic) sediment as reference sediment in future toxicity tests should be avoided, unless this is recognized in the experimental design and the investigator(s) wish to compare test results for this material with those for one or more samples of test sediment.

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

Reference toxicity test is a test conducted using a reference toxicant in conjunction with a sediment toxicity test, to appraise the sensitivity of the organisms and the precision and reliability of results obtained by the laboratory at the time the test material is evaluated. Deviations outside an established normal range indicate that the sensitivity of the test organisms, and the performance and precision of the test, are suspect. For this reference method, a reference toxicity test is performed in the absence of sediment (i.e., as a water-only test).

Sampling station means a specific location, within a site or sampling unit (depending on the study design), where samples of field-collected sediment are obtained for toxicity tests and associated physicochemical analyses. See also "site."

Site means a delineated tract of sediment that is being used or considered as a study area, usually from the perspective of it being contaminated or potentially contaminated by human activity.

Test sediment is a field-collected sample of whole sediment, taken from a marine, estuarine, or freshwater site thought to be contaminated (or potentially so) with one or more chemicals, and intended for use in this reference method. In some instances, the term also applies to any solid-phase sample (including *reference sediment*, artificial sediment, or *dredged material*) used in the test. See also "*contaminated sediment*," "reference sediment," "artificial sediment," and "*control sediment.*"

Water-only (toxicity test) refers to a (toxicity) test which does not include any sediment or other solid-phase material (e.g., a test using an aqueous solution of a reference toxicant). The term "water-only" (toxicity test) is synonymous with liquid-phase (toxicity test). In this reference method, the "water-only" vials are used to determine the fertilization success rate in the seawater used as overlying water for all *replicates* and treatments. The data are also used to confirm that the fertilization success rate at the start of the test for each treatment, was ≥ 90%, and to confirm that the test has met the validity criterion. The reference toxicity test is also conducted as a "water-only" test.

Statistical and Toxicological Terms

Acute means within a short period of exposure in relation to the life span of the test organism. This would be within a few days for echinoids, which generally have a life span of several years, e.g., four to eight years for sea urchins. An acute toxic effect would be induced and observable within the short period.

Acute toxicity is an adverse effect (lethal or sublethal) induced in the test organisms within a short period (for purposes of this document, within a few days) of exposure to *test sediment*(s).

Battery of toxicity tests is a combination of several toxicity tests, normally using different species of test organisms (e.g., a series of sediment toxicity tests using one or more species of echinoids, *Vibrio fischeri*, one or more species of marine or estuarine amphipods, and a polychaete worm).

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 variable(s) (i.e., time, reaction of the organisms, etc.) that indicate(s) the termination of a test. It also means the measurement(s) or derived value(s) that characterize the results of the test (e.g., mean percent normal *larvae*).

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

ICp is the inhibiting concentration for a (specified) percent effect. It represents a point estimate of the concentration of test substance or material that causes a designated percent impairment in a quantitative biological function such as a larval development, growth rate, or number of young per brood, compared to the control. For example, an IC50 could be the concentration estimated to cause a 50% reduction in

larval development, relative to the control. This term should be used for any toxicological test which measures a quantitative effect or change in rate, such as growth, respiration, or reproductive rate. In this reference method, the *endpoint* is a count of "normal larvae" expressed as a percent of all observations. The observations are binomial and expressed as a percent. However, the numbers are large (~200 observations) and the change in percent effect caused by one individual reacting would be low enough that the data could be treated as if they represent a continuous distribution. Environment Canada (2005), therefore, recommends estimating the ICp, a quantitative endpoint, for the reference toxicant test results generated for this reference method.

Normality (or normal distribution) is a symmetric bell-shaped array of observations. The array relates frequency of occurrence to the magnitude of the item being measured. In a normal distribution, most observations will cluster near the mean value, with progressively fewer observations toward the extremes of the range of values. The shape is determined by the mean and standard deviation, with 68.3%, 95.4%, and 99.7% of the observations included within plus or minus one, two, and three standard deviations of the mean, respectively.

Replicate (*test vessel or vial*) refers to a single test chamber containing a prescribed number of organisms in either one concentration of the test material or substance, or in the control or reference treatment(s). A replicate in a treatment must be an independent test unit; therefore, any transfer of organisms or test substance or material from one test chamber to another would invalidate a statistical analysis based on replication. The term is also used to refer to more than one sample of test material taken at one time from a particular location and depth (i.e., field replicates), or for subsamples of a particular test material taken for multiple (duplicate or more) toxicity tests using identical procedures and conditions (i.e., laboratory replicates).

Replicate samples are field-replicated samples of sediment collected from the same *sampling station*, to provide an estimate of the sampling error or to improve the precision of estimation. A single sediment sample from a sampling station is treated as one replicate. Additional samples are considered to be additional replicate samples when they are treated identically but stored in separate sample containers (i.e., not composited).

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

Toxicant is a toxic substance or material.

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

Toxicity test is a determination of the effect of a substance or material on a group of selected organisms, tissues, cells or other living material, under defined conditions. An aquatic toxicity test usually measures either (a) the proportions of organisms affected (quantal), or (b) the degree of effect shown (quantitative or graded), after exposure to specific test substance or material (e.g., a sample of sediment).

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 specific concentration of a potentially toxic material or substance. Alternatively, a treatment might be a particular test material (e.g., a particular sample of sediment, chemical, effluent, elutriate, leachate, receiving water or control water). Samples or subsamples of test sediment representing a particular treatment are typically replicated in a toxicity test. See also "replicate."

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

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

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This method was based on research conducted by Environment Canada's Atlantic Laboratory for Environmental Testing (ALET) and Pacific & Yukon Laboratory for Environmental Testing (PYLET). The tremendous efforts of Paula Jackman (ALET), Ken Doe (Ret. Environment Canada, ALET), and Craig Buday (PYLET) are gratefully acknowledged. The inter-laboratory studies undertaken to validate the test method described herein were coordinated by Lesley Novak (AquaTox Testing & Consulting Inc.) and performed by the following participating laboratories: Environment Canada's ALET and PYLET, AquaTox Testing & Consulting Inc., EA Engineering, Science, and Technology Inc., Maxxam Analytics, USEPA Region 9 Laboratory, Nautilus Environmental and Southern California Coastal Water Research Project.

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Introduction

This reference method specifies the procedures and conditions to be used when preparing for and undertaking an echinoid *embryo*/larval sediment-contact test for measuring the toxicity of samples of contaminated or potentially contaminated marine or estuarine sediments. The present test method is intended strictly for use with marine sediments, having a minimum salinity of 15‰. The reference method herein is to be applied to one or more of the following three echinoid species: *Strongylocentrotus purpuratus* (Pacific purple sea urchin), *Lytechinus pictus* (white sea urchin), and *Dendraster excentricus* (eccentric sand dollar). This reference method represents one of the biological test methods to be used as part of sediment assessments consistent with the federal regulations on Disposal at Sea under the *Canadian Environmental Protection Act, 1999* (CEPA 1999; Government of Canada, 2001). It can also be used to measure the toxicity of sediment samples being considered for disposal at any estuarine or marine site for which regulatory appraisals or stringent testing procedures apply. Two other reference methods, intended for use with sediment samples, have been published by Environment Canada (1998; 2002). Other federal (Environment Canada) biological test methods for measuring sublethal toxicity, using *gametes* obtained from echinoids, as well as for measuring sediment toxicity are also available (see Appendix A).

This reference method is based on method-development research conducted by Environment Canada's Atlantic Laboratory for Environmental Testing (ALET) and Pacific and Yukon Laboratory for Environmental Testing (PYLET) (McLeay, 2010). Many components of the procedures and conditions specified herein are consistent with guidance and approaches for conducting echinoid sediment-contact toxicity tests described in other methodology documents or laboratory Standard Operating Procedures (SOPs) including: United States Environmental Protection Agency and Puget Sound Water Quality Authority (USEPA and PSWQA, 1995); Chapman, 1995; Southern California Coastal Water Research Project (SCCWRP, 2004); American Public Health Association (APHA *et al*., 2005); American Society for Testing and Materials (ASTM, 2007). In particular, this reference method is similar in concept to the echinoid embryo/larval test method developed by the USEPA and PSWQA (1995) and described in Annex A1 of ASTM (2007), when testing samples of field-collected sediment for toxicity. Appendix E provides a review of the similarities and differences associated with various procedures and conditions specified in those documents. The contribution of those methods and SOPs to all parts of this reference method is acknowledged, and they are recommended as sources of supporting rationale. Procedures and conditions stipulated in this reference method must, however, be taken as the definitive ones when planning and undertaking an assessment of sediment toxicity using an echinoid embryo/larval sediment-contact assay for regulatory purposes in Canada.

Before finalizing this reference method, three inter-laboratory (round robin) studies were performed to assess inter-laboratory precision and to validate the test method. Samples evaluated included a reference toxicant (copper) as well as field-collected reference and contaminated sediments. The first series of tests consisted of an evaluation of a reference toxicant using the Pacific purple sea urchin, *Strongylocentrotus purpuratus*, and was designed to allow laboratories to gain familiarity and experience with the method. The second series of tests consisted of an evaluation of contaminated, reference, and laboratory control sediments, as well as a reference toxicant using *S. purpuratus*. In the third series of tests, the sediment samples used for the previous series were retested using the white sea urchin, *Lytechinus pictus*. A reference toxicant was also included in the third test series. Results from the three reference toxicant rounds yielded *Coefficients of Variation* (CVs) ranging from 23.9% to 32.4%, values that were within an acceptable range of variability for inter-laboratory tests. Environment Canada (2005) has suggested that CVs of 20% to 30%

would be a reasonable range of variability expected in repeated tests for a reference toxicant. When evaluated using sediment samples, this reference method also showed good agreement and comparable sensitivity to results from the luminescent bacteria and amphipod tests, both of which are used to judge sediment toxicity for ocean disposal. All three test methods showed one contaminated sediment sample would have been judged to be toxic.

Test Organisms

2.1 Species

One or more of the following three echinoid species must be used with this reference method:[2]

Strongylocentrotus purpuratus (Pacific purple sea urchin),

Lytechinus pictus (white sea urchin), or

Dendraster excentricus (eccentric sand dollar).

For a given test, animals representing a single species must be used, and all test organisms must be derived from gametes retrieved from the same population of sexually mature adults of that species. An overview of conditions for spawning, holding, and acclimating echinoid adults (to be used as the source of gametes in this reference method) is provided in Table 1. However, Sections 1.2 and 2 of Environment Canada (2011) should be consulted for further details regarding the information provided in Table 1, as well as background information on the geographical distribution of these test organisms, availability for testing, and their past use in laboratory toxicity tests.

2.2 Life Stage and Source

Gametes (i.e., unfertilized eggs and sperm) to be used to provide the newly fertilized embryos required to start the test must be obtained from mature and gravid adults. Sperm and eggs obtained outside the main period of maturation can give poor fertilization rates, and subsequently, poor test results that do not meet validity criteria or minimum mean fertilization rates. Inspections for state of maturity requires some experience on the part of the investigator, but can be assessed by spawning a sample of echinoids (see Section 4.5.1) and examining the gametes. Mature sperm are minute and quickly become active in seawater. Mature eggs rapidly become spherical in seawater. Immature eggs have a clear spot in the cytoplasm.

Adults used as the source of gametes for this reference method can be obtained from commercial suppliers or be field-collected by laboratory personnel. Section 2.2 of Environment Canada (2011) provides detailed guidance on organism availability (spawning seasons), collection, and laboratory holding temperatures for the echinoids to be used in this reference method. With the exception of the white sea urchin *(L. pictus)*, the test species can be collected on one or more Canadian coasts. *Lytechinus pictus* and *S. purpuratus* can be purchased from a biological supply house and shipped to the test laboratory, and are available year-round for reliable spawning. *Strongylocentrotus purpuratus* is generally available January to May (optimally January to March for field-collected organisms; late October to April for those collected on the California coast). *Dendraster excentricus* is generally available May to October.

Adult echinoids must be positively identified to species. Confirmation and documentation of the species of test organisms received from a supplier must be made by a qualified taxonomist, at least once for any shipment of echinoids provided by that supplier, 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 should be supplied with certification of the organisms' species identification, and the taxonomic reference or name(s) of the taxonomic expert(s) consulted. After the initial taxonomic identification of each species provided by a given supplier, confirmation of the species of test organisms in a shipment can be conducted by the testing laboratory. All information needed to properly identify the adult echinoids transported to a testing laboratory must be provided with each shipment.

[2] Environment Canada currently has another multi-species test in its publication "Biological Test Method: Reference Method for Determining *Acute* Lethality of Sediment to Marine or Estuarine Amphipods" (Environment Canada, 1998).

Table 1 Summary of conditions for source, spawning, holding and acclimating echinoid adults (Environment Canada, 2011)

Description	*S. purpuratus*	*L. pictus*	*D. excentricus*
Source of adults	Biological suppliers or field collection; adult echinoids must be positively identified to species; all information needed to properly identify the adult echinoids transported to a testing laboratory must be provided with each shipment (must include, as a minimum: the quantity and source of test organisms in each shipment, supplier's name, date of shipment, date of arrival at the testing laboratory, arrival condition, and species identification)		
Spawning season	Generally January to May, optimally January to March for feral animals; late October to April (California coast)—possible to extend spawning in the laboratory by holding at constant temperature (12°C to 15°C) in the dark	March to November	May to October (February to December—possible extended spawning in the laboratory by holding at appropriate temperature)
Holding temperature in laboratory (°C)	10 ± 2	13 ± 2	13 ± 2
Acclimation for organisms held in laboratory for > 3 days	Gradual acclimation and a minimum holding time of 3 to 4 d at test temperature, salinity, and in *control/dilution water* used for testing.		
Acclimation for organisms to be spawned ≤ 3 days of receipt in laboratory	Minimum holding period of 3 h is required to allow for observation of the general health of the adults and to move the adults from their shipping conditions (i.e., temperature and water) to testing conditions.		
Holding containers/conditions	Held in tanks, aquaria, troughs, or trays with a water depth of ≥ 20 cm; the bottoms of the containers should be covered with 2 to 3 cm of sand, sediment or gravel.		Trays are frequently used with a water depth of 10 cm; bottom of container should be covered with 2 to 3 cm of sand, sediment, or gravel and it should be rich in detritus, including settled algal cells.
Water	Uncontaminated natural seawater or *reconstituted seawater*; flow-through or *static-renewal* replacement (e.g., once every 24 h); average salinity from 28 to 34 g/kg, and individual measurements must not be outside 25 to 36 g/kg; rate of salinity change must be < 5 g/kg per day for adults to be held for > 3 d; as a general guideline, volume of flow should provide 5 to 10 L/d for each animal and equal the volume of tank in 6 to 12 h; in practice, lower or fewer water exchanges have also been successful in static-renewal systems		
Holding water dissolved oxygen (DO) and pH	DO 80% to 100% saturation; pH must be 7.5 to 8.5		
Water quality monitoring	Temperature, salinity, DO, pH, and flow to each tank should be measured, preferably daily.		
Lighting	Lighting conditions not considered critical; normal laboratory lighting (100 to 500 lux) and a 16-h light:8-h dark *photoperiod*		
Feeding (for organisms to be spawned within 3 days of receipt)	No feeding required		

Table 1 (continued)

Description	*S. purpuratus*	*L. pictus*	*D. excentricus*
Feeding (for organisms held in laboratory for extended period of time; > 3 days)	For sea urchins: kelp, other macroalga, or romaine lettuce, spinach, and carrots; ad libidum		Provide sediment with detritus and alga, use lighting to encourage growth of algae, and if necessary add cultured alga or algal paste; a steady stream of unfiltered water may also provide adequate food
Cleaning	Removal of old algae, fecal material, and debris, daily or as required, unless intended as food		
Culture health criteria	Monitor mortality daily; for adults held > 3 d, mortality should be ≤ 2%/d averaged over 7 day preceding collection of gametes, and cumulative mortality over the same 7-day period must be ≤ 20%; for adults held ≤ 3 d, cumulative mortality must be ≤ 20%; remove diseased or moribund animals; groups of diseased animals should be discarded		

Records accompanying each *batch* of test organisms must include, at a minimum: the quantity and source of test organisms in each shipment, supplier's name, date of shipment, date of arrival at the testing laboratory, arrival condition (i.e., mortality, temperature, DO, pH if shipped in water), and species identification.

Shipping extremely ripe or gravid individuals (particularly under stressful conditions; e.g., extreme temperature changes) might cause spawning or mortality during shipment or upon receipt. This can sometimes be avoided by having the animals acclimated to laboratory conditions, as much as possible, prior to being shipped.[3]

Moving animals from one location to another marine location also raises serious questions of introducing non-native species or transporting diseases and parasites. Any proposed procurement, shipment, or transfer of echinoids are submitted for the approval of federal, provincial, or regional authorities. Testing laboratories might be required to establish and use a quarantine section within their facilities, where imported organisms can be isolated and all equipment and fluids that come in contact with the test organisms or gametes can be sterilized and disposed of according to provincial or federal regulations.[4]

[3] Adults can be shipped dry with cold packs to keep the temperature at 10 ± 2°C. The Pacific purple sea urchin can be shipped surrounded by algae or some other moist material. The eccentric sand dollar should be shipped in a small amount of chilled seawater. See Environment Canada (2011) for additional guidance.

[4] Provincial governments might require a permit to import organisms whether or not the species is native to the area, and movements of aquatic organisms might be controlled by a Federal-Provincial Introductions and Transplant Committee. Advice on contacting the committee or provincial authorities, and on sources of echinoids, can be obtained from the regional Environmental Protection Office. See Appendix C.

2.3 Holding and Acclimating Adults in the Laboratory

Guidance provided in Section 2.3 of Environment Canada (2011) must be followed when holding and acclimating adults to be used as the source of gametes to provide freshly fertilized eggs and sperm for use with this reference method.

2.3.1 General

All three test species employed in this reference method have been successfully maintained in spawning condition in the laboratory for extended periods of time (i.e., 3 months to 1 year), although one species (i.e., the white sea urchin) is reportedly more easily maintained than others. The white sea urchin can often be sexed and housed in separate aquaria to facilitate the quick selection of the appropriate numbers of males and females required for testing. However, sex determination based on external features may not be consistently accurate. Males and females can be stimulated to release gametes and can then be identified as males by their white sperm/semen or females (pink-yellow eggs in fluid). Most laboratories report very little mortality with *L. pictus* during acclimation and holding, and this species can be easily acclimated and maintained in closed, recirculating, temperature-controlled aquariums (Environment Canada, 2011). For the Pacific purple sea urchin and the eccentric sand dollar, there are varying reports on the ease with which these species can be held in the laboratory for extended periods of time. Many Canadian laboratories have resorted to purchasing these two test species from a commercial supplier when tests are requested, and spawning adults on the day of, or within a few days after arrival at the laboratory (i.e., without a thorough acclimation). As a result of reported problems associated with holding the Pacific purple sea urchin and the eccentric sand dollar for extended periods of time, the second edition of EPS 1/RM/27 included an option for "holding adults for immediate use," in which gametes may be collected within a short period of time (≤ 3 days) after the adults are received at the laboratory, an option that can also be employed herein. An overview of holding and acclimating conditions for adults is provided in the following sections. However, Section 2.3.1 of Environment Canada (2011) must be consulted for additional information and guidance when "holding adults for immediate use" and when holding for extended periods (i.e., > 3 days).

For adults that are to be spawned and gametes tested within a 3-day period after adult arrival at the testing laboratory, confirmation should be obtained from the supplier that adults are mature and that the eggs are viable prior to shipping. The temperature at which the test organisms are shipped should be maintained at or near the required test conditions, since there is little time for acclimation upon arrival. Even with "holding for immediate use," the adults should be moved to laboratory holding conditions as gradually as possible. Gradual exposure of the adult echinoids to the testing laboratory's control/dilution water is recommended in all cases, but especially in instances where there is a marked difference in quality (i.e., temperature, *salinity, pH*) from that to which they were previously acclimated. For adults that are to be spawned for testing on the same day that they arrive at the laboratory, a minimum holding period of 3 h is required to allow for observation of the general health of the adults and to move the adults from their shipping conditions (i.e., temperature and water) to testing conditions. Procedures for moving adults from their shipping water to control/dilution water prior to spawning are provided in Section 2.3.1 of Environment Canada (2011). Adults that are shipped "dry" (i.e., wrapped in moist paper towel or seaweed), do not have to be placed in control/dilution water prior to spawning; however, they must be held for a 3-hour observation period, prior to spawning, and any adjustment of their temperature (i.e., air temperature) to the test temperature should be made as gradually as possible, if necessary. The shift of adults from shipping conditions to test conditions should be started as soon as possible after the sexually mature adult echinoids arrive at the testing facility.

For adult echinoids that are going to be held in the laboratory for extended periods of time (i.e., > 3 days), it is desirable to provide a gradual acclimation at the test temperature, salinity, and in the water to be used for controls and dilution, prior to gamete collection. Acclimation should be started as soon as possible, upon arrival of the adults at the testing

facility. The need for appropriate procedures for "holding for immediate use" or gradual acclimation and satisfactory long-term holding conditions, is dependent on the requirement for the delivery of viable gametes that meet the needs and validity criteria of the test.

Echinoids must be handled with care and should not be subjected to sudden shocks or changes in holding conditions. In particular, changes in temperature or hydrostatic pressure can stimulate spawning. Furthermore, spawning by individual organisms can induce others to spawn. Therefore, any spawning echinoids should be immediately isolated on detection. To further avoid mass spawning, adults should be separated into small (e.g., ≤ 20 individuals per tank) male and female groups. The holding containers should be labelled with the date spawned.

Recommended conditions for holding adult echinoids in the laboratory for an extended period of time (i.e., > 3 days) are outlined in Sections 2.3.2 to 2.3.8, as well as in Section 2 of Environment Canada (2011). The guidance allows some degree of flexibility within a laboratory, while at the same time standardizing those elements which, if uncontrolled, might affect the health of animals or viability of their gametes.

2.3.2 Holding Containers

Groups of male and female echinoids are held in tanks, aquaria, or troughs. For sea urchins, the water depth should be ≥ 20 cm. For sand dollars, trays (e.g., 1 × 2 m) are frequently used with a water depth of 10 cm. The bottom of the containers used to hold the echinoids should be covered with 2 to 3 cm of sand, sediment, or gravel (and in the case of sand dollars, it should be rich in detritus, including settled algal cells).

2.3.3 Lighting

Lighting conditions (including photoperiod and intensity) during holding of adult echinoids do not appear to be of major importance, and normal laboratory lighting at low intensity (100 to 500 lux) and a 16-hour light:8-hour dark photoperiod is generally considered acceptable. For sand dollars, overhead fluorescent lighting at the equivalent of bright office lighting encourages algal growth on the sediment, which might result in desirable nutritional self-sufficiency for the tray of sand dollars.

2.3.4 Water

Guidance on water type (i.e., natural seawater or reconstituted seawater), appropriate exchange rates, and water quality found in Section 2.3.4 of Environment Canada (2011) and summarized in Table 1, should be followed.

The water used for holding adult echinoids may be either an uncontaminated supply of natural seawater or "reconstituted" seawater (also known as artificial seawater) made up to a desired salinity according to Environment Canada's recommended procedure (Environment Canada, 2001). Any commercially available sea salts or appropriate mixture of reagent-grade salts used to prepare the reconstituted water, should have previously been shown to consistently and reliably support good survival and health of echinoids. The water supply should be monitored and assessed as frequently as required to document its quality. Temperature, salinity, dissolved oxygen, pH, and the volume of flow to each tank should be measured, preferably daily.

The water in containers holding adults should be renewed continuously (i.e., flow-through system) or periodically (i.e., static-renewal system) to prevent a build-up of metabolic wastes. General guidelines for the optimal maintenance of high-quality water, including flow/exchange rates and loading densities, are provided in Environment Canada (2011).

The average salinity of the holding water should be 28 to 34 g/kg, but preferably 30 to 32 g/kg. Extreme salinity values must not be < 25 g/kg or > 36 g/kg during holding of echinoids. For organisms that are to be held in the laboratory for extended periods of time, the rate of any salinity adjustment should be ≤ 3 g/kg per day and must be ≤ 5 g/kg per day. For adults "held for immediate use" (e.g., spawning adults for test purposes within 3 days of arrival at the laboratory), salinity adjustments should be made as gradually as possible; however, a daily shift of > 5 g/kg may be made if the criteria for test

validity can be met and the sensitivity of the gametes in reference toxicant tests is not affected (see Environment Canada 2011 for additional information).

If reconstituted (artificial) seawater is to be used for holding of organisms, it must be made up to the desired salinity by adding hypersaline brine (HSB) and/or commercially available dry ocean salts or reagent-grade salts to the appropriate quantity of suitable fresh water. The HSB should have a salinity of 90 ± 2 g/kg. Any reconstituted water prepared by the direct addition of dry salts must be aerated vigorously for a minimum of 24 h before being used; however, longer periods of aging (i.e., ≥ 3 days) with aeration are recommended. Guidance in Environment Canada (2011) should be followed when preparing, aging, and storing HSB. Sources of water used for preparing reconstituted seawater may be deionized water, distilled water, an uncontaminated supply of groundwater or surface water, or dechlorinated municipal drinking water.

2.3.5 Temperature

During the holding period preceding acclimation to test conditions, adults should be held within the temperature range shown previously to be suitable for the species based on species-specific temperature ranges recommended in Environment Canada (2011; also see Table 1). Gradual acclimation to test temperature before spawning the animals is advised, even if the gametes are to be collected on the day of, or the day after, the gravid adults are received in the laboratory. When the adults are first brought into the laboratory, the temperature to which they are adapted should be changed as necessary, but at a rate that should not exceed 3°C/day (Environment Canada, 2011).

2.3.6 Dissolved Oxygen and pH

The dissolved oxygen (DO) content of the water within holding containers should be maintained at 80% to 100% saturation. If required, gentle aeration of the water should be carried out using filtered, oil-free compressed air. Overly vigorous aeration should be avoided. As per Section 2.3.7 in Environment Canada (2011), the pH of water used for holding adults should be in the range of 8.0 to 8.2, and must be within pH 7.5 to 8.5.

2.3.7 Feeding

Feeding is not necessary for adult echinoids that are spawned for testing within 3 days of arrival at the laboratory. Sea urchins that are held in the laboratory for an extended period of time (i.e., > 3 days) should be fed with kelp or macroalgae (*Laminaria*, *Nereocystis*, *Macrocystis*, *Egregia*, *Hedophyllum*) or, alternatively, with romaine lettuce, spinach, or shredded carrots. Food should be added frequently enough to ensure consistent availability, while old and decomposing food should be removed. Sand dollars typically selectively ingest particles from the bottom of their holding containers. For this reason, natural and uncontaminated sediment used on the bottom of their holding containers should contain detritus, and possibly microalgae (i.e., diatoms), if necessary, cultured alga or algal paste may be added (also see Section 2.3.3). A steady stream of unfiltered water may also provide adequate food for sand dollars. In general, individual laboratory experience holding adult echinoids (intended to provide gametes or embryos for use in toxicity tests) will determine those feeding conditions and rates that promote low mortality rates, acceptable fecundity, and spawning success.

2.3.8 Culture Health Criteria

Adults must be inspected upon arrival at the laboratory, and thereafter daily to monitor mortality and check for signs of disease. Moribund individuals should be removed immediately. Specific culture health criteria, based on laboratory holding times for adult echinoids are as follows:

- In groups of animals, which are held in the laboratory for an extended period of time (i.e., > 3 days) before their gametes are collected for use in a test, mortality should not exceed 2% per day, averaged over the 7 days preceding (i.e., including the day of spawning) collection of gametes. The cumulative mortality over the same 7-day

period must not exceed 20%. If a number of organisms from a given batch die after spawning is induced for testing purposes, those individuals may be excluded in the calculations of daily/weekly mortality. Adults spawned for use in a test may be separated from the remainder of the batch and may be excluded from mortality calculations, unless they are intended to be used for future testing (following a post-spawning recovery period).

- For adults that are to be spawned for testing ≤ 3 days of arrival at the laboratory, the cumulative mortality data for the 7-day period prior to shipment (and including the day of receipt at the laboratory) should be obtained for the batch of organisms shipped from the supplier, and must not exceed 20%.

- For those groups of adults with a high mortality rate (i.e., exceeding any of the criteria described herein), surviving echinoids should be either euthanized or held for an extended period until the mortality rate is acceptably low. Euthanize any moribund animals, sea urchins with significant loss of spines, and sand dollars with patches of fungus. Treatment of diseased adults with chemicals should not be attempted; it is strongly recommended that groups of animals showing a high incidence of disease be euthanized.

Facilities, Equipment and Supplies

The test can be conducted in a clean laboratory with standard ambient laboratory lighting. Photoperiod to which test chambers are exposed on Day -1 as well as throughout the test should be 16-hours light: 8-hours dark.

The need for any special facilities would be governed by the degree of hazard associated with the sediment samples that are to be tested, and by the risk of sample and apparatus contamination. Facilities should be well-ventilated, free of fumes, and isolated from physical disturbances or airborne contaminants that might affect the test organisms. The testing facilities should also be isolated from areas in which test sediments are prepared, and removed from areas in which equipment is cleaned.

Equipment and supplies that contact sediments, water, or stock solutions must not contain substances which 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. The laboratory must have the instruments to measure the basic variables of water quality (temperature, salinity, dissolved oxygen, and pH), and must be prepared to undertake prompt and accurate analysis of other variables such as ammonia and particle size.

A mechanical vortex shaker or mixer (for flat bottom scintillation vials) is needed to ensure the contents of each vial (containing sediment and water) are mixed at a rate of 1800 *rpm* for 10 seconds.

Disposable glass scintillation vials with a capacity of 20 mL are used as the test chambers in which organisms are to be scored using a Sedgwick-Rafter cell. Inter-laboratory test results have also shown that straight-sided 20-mL borosilicate shell vials are acceptable as test vessels to be used in cases where organisms are to be scored in-vial using an inverted microscope (AquaTox, 2013; also see Section 4.7.2). All vials should be new and unwashed before use. However, rinsing of vials (before use) with de-ionized or reverse osmosis water is recommended. When conducting the test, all vials must be loosely covered with a clean (new) plastic film (e.g., plastic derived from a clear plastic bag) or a sheet of transparent Plexiglass™.[5]

[5] Loose-fitting covers on vials are desirable, to enable some air exchange and thus higher dissolved oxygen concentrations in the seawater within each test chamber. Although volatiles are not likely to be an issue (because of agitation of the sediment and overlying water on Day -1), if the test sediment is known to contain appreciable volatiles (e.g., detectable odours of hydrogen sulphide or hydrocarbons), it may be useful to have a tighter seal on individual vials to reduce the risk of cross-contamination.

Procedure for Testing Sediment

4.1 Sample Collection

Environment Canada (1994) provides guidance on field sampling designs and appropriate techniques for sample collection; this guidance document should be followed when collecting samples of sediment to be tested for toxicity using this reference method.

Procedures and equipment used for sample collection (i.e., core, grab, dredge, or composite) will depend on the study objectives or regulatory requirements, and on the nature of the material being sampled. Samples of dredged material should be taken at all depths of interest. Samples of field-collected test or reference sediment, including those taken from or adjacent to ocean disposal sites, frequently represent the upper 2-cm depth. Sites for collecting samples of reference sediment should be sought where the geochemical properties of the sediment, including grain size characteristics, are similar to those at the site(s) where samples of test sediment are collected. Ideally, reference sediment should be collected from a site uninfluenced by the source(s) of contamination but within the general vicinity of the site(s) where samples of test sediment are taken. It is recommended that reference sediment from more than one site be collected to increase the likelihood of a good match with grain size and other physicochemical characteristics of the test sediments. Test validity criteria are based on an acceptable number of normal larvae in both the "water-only" controls and the lab control sediment. In addition, determining if a sediment sample "passes" or "fails" the test is based on a comparison to field-collected reference sediment, or if not available, a lab control sediment (Section 6). Therefore, selection of appropriate lab control and reference sediments will be crucial to successfully conducting the test, as well as interpreting the test results. Furthermore, with repeated evaluation, a non-toxic field-collected reference sediment could eventually be used as a suitable laboratory control sediment.

The number of stations to be sampled at a study site and the number of *replicate samples* per station will be specific to each study. This will involve, in most cases, a compromise between logistical and practical constraints (e.g., time and cost) and statistical considerations. Additional guidance on sampling for disposal-at-sea applications is found in Environment Canada (1995) and in Chevrier and Topping (1998).

Where practical and consistent with the study design and objectives, a minimum of five replicate samples (i.e., field replicates) of sediment must be taken from each discrete *sampling station* and depth of interest. Where practical and appropriate, sample collection must also include ≥ 5 samples (i.e., field replicates) from each of one or more reference stations (i.e., sites where uncontaminated sediment, having physicochemical properties similar to that of the test sediments, can be found) within the vicinity. The objective of collecting replicate samples at each station is to allow for quantitative statistical comparisons within and among different stations (Environment Canada, 2005). Accordingly, each of these "true replicate" samples of sediment must be tested for its toxicity to echinoid embryo/larvae, using a minimum of six test chambers per sample (i.e., laboratory replicates).

The collection of replicate samples at a given sampling station is often not necessary for certain dredging projects (Environment Canada, 1994; 1995). If the objective is to obtain a "cost-effective" assessment of toxicity within the project area, sampling as many stations as possible (subject to cost constraints) with a single sample from each station might be the best way to achieve this. In this instance, testing might be restricted to six laboratory replicates (i.e., 6 subsamples) per sample (and no replication of samples from each station), each of which is prepared in the laboratory.

A benthic grab (i.e., Smith-MacIntyre, Van Veen, PONAR) or core sampler should be used to sample sediment rather than a dredge, to minimize

disruption of the sample. Care must be taken during sampling to minimize loss of fine particles. The same collection procedure should be used for all field sites sampled.

The volume of sample required to perform an echinoid embryo/larval sediment-contact test is small (see Section 4.6.2). A sample volume of ~100 mL should be submitted specifically for the performance of this test. Larger sample volumes (e.g., 5 to 7 L of whole sediment) are frequently required depending on the study objectives/design, the nature of the associated physicochemical analyses, and the *battery of toxicity tests* to be performed. To obtain the required sample volume for a battery of toxicity tests, it is frequently necessary to combine subsamples retrieved using the sampling device. Guidance provided in Environment Canada (1994) for compositing subsamples in the field should be followed.

4.2 Sample Labelling, Transport, and Storage

In addition to the following, more detailed and useful guidance pertaining to sample labelling, transport, and storage is found in Environment Canada (1994). Persons undertaking these procedures should be familiar with this guidance document.

Containers for transporting and storing samples must be new or thoroughly cleaned, and rinsed with clean water. Environment Canada (1994) should be consulted for guidance in selecting suitable containers. Each sample container should be filled completely, to exclude air. Immediately after filling, each sample container must be sealed, and labelled or coded. Labelling and accompanying records made at this time must include at least a code which can be used to identify the sample or subsample. A cross-referenced record, which might or might not accompany the sample or subsample, must be made by the field personnel identifying the sample type (e.g., grab, core, composite), source, precise location (e.g., water body, latitude, longitude, depth), replicate number, and date of collection. This record should also include the name and signature of the sampler(s). Sediment sample collectors should also keep records describing:

- the nature, appearance, volume and/or weight of each sample;
- the sampling procedure and apparatus;
- any procedure used to composite or subsample grabs or cores in the field;
- the number of replicate samples taken at each sampling station;
- the sampling schedule;
- the types and numbers of containers used for transporting the samples;
- any field measurements (e.g., temperature, salinity, pH, DO) of the overlying water or sediment at the collection site; and
- procedures and conditions for cooling and transporting the samples.

Upon collection, warm (> 7°C) samples should be cooled to between 1°C and 7°C with regular ice or frozen gel packs, and kept cool (4 ± 3°C) in darkness throughout transport. As necessary, gel packs, regular ice, coolers, or other means of refrigeration should be used to assure that sample temperatures range within 1°C to 7°C during transit. Samples must not freeze or partially freeze during transport or storage, and must not be allowed to dry.

Upon arrival at the laboratory, the sample temperature and date of receipt must be recorded on a bench sheet or chain-of-custody form. Samples to be stored for future use must be held in airtight containers and in darkness at 4 ± 2°C. It is recommended that samples of sediment or similar particulate material be tested as soon as possible after collection. The sediment toxicity test should begin within two weeks of sampling, and preferably within one week; the test must start no later than six weeks after sample collection. However, the decision on exact timing for test initiation will depend on the suspected type of contaminant [e.g., samples suspected of containing less persistent or volatile contaminants (sulphides) might be tested sooner (e.g., within 1 week of sample collection) than a sample containing more stable/persistent substances, such as metals].

4.3 Sample Manipulation and Characterization

Samples of field-collected test sediment and reference sediment must not be wet-sieved. Particles ≥ 2 mm should be removed along with large debris or large indigenous macro-organisms. Depending on the sample, this may be accomplished by using forceps or a gloved hand. Forceps or gloves contacting each sample should be rinsed or replaced thereafter, to prevent cross-contamination. If a sample contains a large number of particles ≥ 2 mm and/or a large number of indigenous macro-organisms which cannot be removed using forceps or a gloved hand, the sample may be press-sieved (dry, not washed) through one or more suitably sized (e.g., < 2 mm) mesh stainless steel screens. Such manipulation should include all portions of the sample used for physicochemical (including grain size) analyses as well as those used for toxicity testing. Procedures used to manipulate each sample must be recorded on a bench sheet.

Any *pore water* that has separated from the sample during shipment and storage must be mixed back into the sediment.[6] To achieve a homogeneous sample, either mix it in its transfer/storage container, or transfer it to a clean mixing container. The sample should normally be stirred using a nontoxic device (e.g., stainless steel spoon or spatula), until its texture, consistency, and colour are homogeneous. Alternatively, a mechanical method (Environment Canada, 1994; 1998; 2002) may be used to homogenize the sample. For each sample included in a test, mixing conditions including duration and temperature must be as similar as possible.

If there is concern about the effectiveness of sample mixing, subsamples of the sediment should be taken after mixing, and analyzed separately to determine homogeneity.

Immediately following sample mixing, subsamples of test material required for this and other toxicity tests (e.g., Environment Canada, 1998; 2002) and for physicochemical analyses must be removed and placed in labelled test chambers, and in the labelled containers required for storage of samples for subsequent physicochemical analyses. Any remaining portions of the homogenized sample that might be required for additional toxicity tests using luminescent bacteria (Environment Canada, 2002), amphipods (Environment Canada, 1998) or other test organisms should also be transferred at this time to labelled containers. All subsamples to be stored should be held in sealed containers with no air space, and must be stored in darkness at 4 ± 2°C until used or analyzed. Just before physicochemical analysis or use in a toxicity test, each subsample must be thoroughly re-mixed to ensure that it is homogeneous.

Each sample (including all samples of reference sediment and control sediment) must be characterized by analyzing subsamples for at least the following (Environment Canada, 1998; 2002): for whole sediment-percent coarse-grained sediment (e.g., particles > 2.0 mm), percent sand (e.g., particles > 0.063 to ≤ 2.0 mm), percent silt (e.g., particles > 0.002 to ≤ 0.063 mm), percent clay (e.g., particles ≤ 0.002 mm), percent water content (moisture), total organic carbon (TOC) content, total ammonia, sulphide, and pH.

Other analyses could include: total inorganic carbon, total volatile solids, biochemical oxygen demand, chemical oxygen demand, cation exchange capacity, acid volatile sulphides, metals, synthetic organic compounds, oil and grease, and petroleum hydrocarbons.

Analyses for particle size distribution must be undertaken as soon as possible after sample collection, and preferably before sample collection for toxicity testing, to enable the selection of the appropriate sample(s) of reference sediment and control sediment.

4.4 Test Conditions

4.4.1 Outline of Test

This reference method is a static, echinoid embryo/larval sediment-contact test for measuring the toxicity of samples of contaminated or potentially contaminated marine or estuarine sediments. Under standardized test conditions, replicate groups of freshly fertilized eggs (embryos) are exposed to each treatment for the same period of time

[6] Pore water salinity could be a useful measurement to determine if the sediment sample(s) to be evaluated fall within the minimum salinity requirements (15‰) for this method.

(see Section 4.6.5). Thereafter, embryos/larvae are preserved for subsequent examination and determination of numbers of normal and abnormal larvae in each replicate, followed by a determination of the % normal larvae in each treatment (see Section 4.8). Table 2 provides a checklist of the conditions that are required or recommended for this reference method. Further details are given in Sections 4.4.2 to 4.4.5.

4.4.2 Test Water

Control/dilution water used in any given test must be from the same source. The seawater must be filtered (~ 60 µm) prior to use and must be from a natural uncontaminated source (with known water quality characteristics). Water should be used within three days or less of filtration. Additional details on control/dilution water can be found in Section 2.3.4 herein, and in Section 3.4 of Environment Canada (2011). The salinity of the seawater used for testing must range within 30 ± 2 g/kg.[7]

Test water must be adjusted to the required test temperature (i.e., 15 ± 1°C for *S. purpuratus* and *D. excentricus*; 20 ± 1°C for *L. pictus*) (see Section 4.4.3). Dissolved oxygen concentration must be 90% to 100% of the air-saturation value for that temperature and salinity. As necessary, the required volume of water should be aerated vigorously (using oil-free compressed air passed through one or more air stones) immediately before use, and its DO content checked to confirm that 90% to 100% saturation has been achieved prior to test initiation. The pH of control/dilution water must range within 7.5 to 8.5, and should normally be 8.0 ± 0.2.

[7] Method development tests with *L. pictus* and *S. purpuratus* were conducted to determine their tolerance to salinities ranging from 15 to 35 g/kg. Results showed that salinities of ≤ 20 g/kg resulted in 0% normal larvae, and that a salinity of 25 g/kg was also stressful and reduced the percentage of normal larvae exposed to that treatment. *Lytechinus pictus* also showed intolerance to high (35 g/kg) salinity in two of three salinity tolerance tests (McLeay, 2010). Additional tests with *D. excentricus* indicated that sand dollars were intolerant to salinities ≤ 20 g/kg or ≥ 35 g/kg (C. Buday, Pacific and Yukon Laboratory for Environmental Testing, personal communication, 2012).

4.4.3 Temperature

Test temperature is species-dependent. Daily mean values must be held within the following ranges:

- 15 ± 1°C for *S. purpuratus* and *D. excentricus*
- 20 ± 1°C for *L. pictus*

Additionally, the instantaneous temperature must be within 3 °C of the daily mean temperature at all times.

4.4.4 Manipulations and Adjustments

- Test sediments, including sample(s) of reference sediment recommended for inclusion in each test series, must not be wet-sieved and no adjustments of pore water salinity are permitted.
- The pH of the overlying water in each replicate test chamber must not be adjusted before or during the test.
- The overlying seawater must be from a natural source and must have a salinity within the range of 30 ± 2 g/kg. Salinity must not be adjusted on Day -1 (i.e., the day preceding the start of the test), at the start of the test (Day 0), nor at any time during test progression.
- Aeration of the overlying water in each test chamber is not permitted during the test.
- Food must not be added to the test chambers at any time during the test.

4.4.5 Timing of Events

Table 3 provides an overview of the timing of events for conducting this reference method, while details are provided in Sections 4.5 to 4.7. In keeping with APHA *et al*. (2005) and ASTM (2007), this echinoid embryo/larval test should be started within 2-h of fertilization, and must begin within 4-h of fertilization. The test duration is 48 to 120 h, depending on species and developmental rate in the "water-only" control (see Section 4.6.5).

Table 2 Checklist of required and recommended test conditions

Test type	– static; freshly fertilized eggs (embryos) are exposed to each treatment for the same period of time (48- to 120-h, depending on species and developmental rate in the "water-only" control), then preserved using glutaraldehyde for subsequent examination and determination of numbers of normal (pluteus or prism) and abnormal larvae
Control/dilution (overlying) water	– filtered (~60 µm) uncontaminated natural seawater; DO content 90% to 100% saturation at time of use; salinity must range within 30 ± 2 g/kg; pH 7.5 to 8.5, preferably 8.0 ± 0.2
Organisms	– *Strongylocentrotus purpuratus* (the Pacific purple sea urchin), *Lytechinus pictus* (the white sea urchin), or *Dendraster excentricus* (the eccentric sand dollar).
	– a target embryo (i.e., newly fertilized eggs) density of ~200 eggs per 200 µL aliquot (in 10-mL exposure volumes) is required; sperm:egg ratio is ascertained to target a mandatory mean fertilization rate ≥ 90% at test initiation
Test design	– a minimum of eight *replicate vials* containing control sediment;
	– a minimum of eight replicate vials containing each test sediment under investigation;
	– a minimum of 23 "water-only" vials; and
	– if available, a minimum of eight replicate vials containing a suitable reference sediment.
Test vessel	– 20 mL disposable glass scintillation or shell vials; vials must be loosely covered with a clean (new) plastic film; if volatiles are present, individual vials should be tightly sealed
Volume of wet sediment per test vessel	– 0.5 ± 0.05 g
Volume of test (overlying) water per test vessel	– 10 mL of filtered (~60 µm), uncontaminated natural seawater
Mean temperature	– 15 ± 1°C for *S. purpuratus* and *D. excentricus*
	– 20 ± 1°C for *L. pictus*
	– instantaneous temperature must be within 3°C of daily mean temperature
Oxygen/aeration	– none required
pH and salinity	– overlying water must not be adjusted before or during the test
Lighting	– normal laboratory lighting (500 to 1000 lux adjacent to water surface is recommended) and photoperiod (16-hour light:8-hour dark)
Duration	– duration is species-dependent; test can be prolonged by 24 h, based on the percentage of normal larvae determined for the six "water-only" controls included in the test for this purpose; presumptive test end for each species is: 48 h if *L. pictus*; 72 h if *D. excentricus*; 96 h if *S. purpuratus*
Observations	– each organism scored as either normal (pluteus or prism) or abnormal larvae; any unfertilized eggs must not be counted or scored

Table 2 (continued)

Measurements	– at the start and end of the test in all control and exposure solutions (reference toxicant and sediments): temperature, salinity, dissolved oxygen content, pH must be measured in the overlying water in the two replicates dedicated for this purpose; total ammonia must be measured (and un-ionized ammonia calculated) in the control at the start and end of the reference toxicant test, and in all sediment exposures at test start and end.
Endpoints	– percentage of embryos (i.e., newly fertilized eggs) developing to normal larvae during the test (i.e., Pn) calculated for each treatment according to the formula Pn = 100(Ln/En), where: Ln = number of normal larvae at test end, and En = number of embryos at start of test in "water-only" controls
Reference toxicant	– copper (as copper sulphate or copper chloride) is recommended for use as the reference toxicant as a water-only test; minimum 5 exposure concentrations and 3 replicates per concentration
Test validity	– average of ≥ 60% of the embryos maintained in the "water-only" control chambers must be judged to be normally developed larvae at the end of the test
	– average of ≥ 60% of the embryos maintained in the control sediment chambers must be judged to be normally developed larvae at the end of the test
Field-collected sediment	
Transport, storage	– if sample > 7°C, cool to 7°C (ice or frozen gel packs); transport in dark at 1°C to 7°C; store in dark at 4 ± 2°C; samples must not freeze or partially freeze during transport or storage, and must not be allowed to dry; test should begin within two weeks of sampling and must start within six weeks
Reference sediment	– parallel test with *clean sediment* of similar physicochemical properties (uncontaminated sediment), if possible; otherwise use control sediment
Sample characterization	– all samples (i.e., reference and control sediment) must be characterized by analyzing subsamples of whole sediment for percent coarse-grained sediment (e.g., particles > 2.0 mm), percent sand (e.g., particles > 0.063 to ≤ 2.0 mm), percent silt (e.g., particles > 0.002 to ≤ 0.063 mm), percent clay (e.g., particles ≤ 0.002 mm), percent water content (moisture), TOC, total ammonia, sulphide, and pH
Preparation of sample	– field-collected test sediment and reference sediment must not be wet-sieved; using forceps or a gloved hand, particles ≥ 2 mm should be removed along with large debris or large indigenous macro-organisms; if a sample contains a large number of particles ≥ 2 mm or a large number of indigenous macro-organisms, sample may be press-sieved (not washed) through suitably sized (e.g., ≤ 2 mm) mesh stainless steel screens; any pore water that has separated from the sample during shipment and storage must be mixed back into the sediment.

4.5 Spawning and Fertilization

4.5.1 Collecting Gametes for the Test

Achieving a high rate of fertilization is critical to this reference method, as it assures an acceptably high percentage of newly fertilized eggs in each test chamber at the start of the test. In this regard, the mean fertilization success rate must be ≥ 90% for the test to proceed (APHA *et al*., 2005; McLeay, 2007). Up-to-date guidance on spawning adult echinoids and fertilizing procedures are provided in Sections 4.2.1, 4.2.2, and 4.2.3 of Environment Canada (2011) and must be consulted before commencing this reference method. However, the prerequisite for a mean fertilization success rate of ≥ 90% at test initiation necessitated some reiteration of the text from EPS 1/RM/27 procedures in this reference method.

Table 3 Overview of test activities and timing of events[a]

Day -2 (i.e., two days preceding the start of the test)	– filter (60 µm) and aerate seawater
Day -1 (i.e., the day preceding the start of the test)	– 0.5-g aliquots of (wet) homogenized sediment are added to each 20 mL test chamber (minimum of eight replicates per treatment, including test sediment, control sediment and reference sediment, if available); a 10-mL volume of filtered (~60 µm), uncontaminated seawater is added to each vial; salinity must be 30 ± 2 g/kg; see Section 4.6.2 – each vial must be mixed by agitating individual vials on a vortex mechanical shaker at a rate of 1800 rpm for 10 seconds – vials are transferred to testing area, randomly distributed (see Figure 1), covered, and held undisturbed overnight under the temperature and lighting conditions to be employed during the test (see Section 4.4 and Table 1). – prepare reference toxicant test solutions (3 replicates per concentration) and transfer to testing area
Day 0 (starting the test)	– adult echinoids are spawned and gametes collected (Section 4.5.1); appropriate sperm:egg ratio is determined and fertilization is started (Section 4.5.2 and 4.5.3) – within 15 to 30 minutes of fertilization, determine that mean fertilization success rate was ≥ 90% (see Section 4.5.4); provided this fertilization rate was achieved, embryos are transferred to all test chambers, including those used for water quality monitoring; transfers should be completed within 2 h of fertilization, and must be completed within 4 h of fertilization – count number of fertilized and unfertilized eggs in each of the six (or more) of the "water-only" vials included in the test for this purpose (see Section 4.6.4) – temperature, salinity, dissolved oxygen content, pH, and ammonia must be measured in the overlying water in one of the two replicates (i.e., "the seventh replicate") dedicated for this purpose for each treatment (e.g., each sample under evaluation); see Section 4.6.3
Presumptive test end Day 2 (48 -h): *L. pictus* Day 3 (72 -h): *D. excentricus* Day 4 (96 -h): *S. purpuratus*	– one hour immediately preceding these species-specific test times terminate and determine the % normal larvae in six of the remaining "water-only" controls ("monitoring vials"); if % normal larvae is less than 70%, the test must be extended for an additional 24-h in order to ensure the test validity criteria (i.e., Pn ≥ 60%) will be met when the organisms are evaluated/scored (see Sections 4.6.5 and 4.7) – if normal larvae in the six "water-only" "monitoring vials" is ≥ 70%, the test must be ended; recover and preserve (using glutaraldehyde) all embryos and larvae within each of the test replicates and treatments (replicates 1 to 6) – temperature, salinity, dissolved oxygen content, pH, and ammonia must be measured in the overlying water in one of the two replicates (i.e., "the eighth replicate") dedicated for this purpose for each treatment (e.g., each sample under evaluation); see Section 4.6.6

[a] A "water-only" reference toxicity test must be conducted at the same time as the definitive test, using replicate groups of newly fertilized eggs from the same batch as those used to conduct the definitive test.

Adult echinoids are stimulated to spawn by injecting potassium chloride (KCl).[8] Sea urchins are injected with 0.5 to 1.0 mL of 0.5 *M* KCl through the peristomial membrane (i.e., between Aristotle's lantern and the "test" or the hard outer shell) on an angle pointing toward the outer shell into the coelom.[9] The KCl injection can be divided and injected in several different locations around Aristotle's lantern, and/or the sea urchin can be gently shaken to distribute the KCl within the organism. Sand dollars are injected with 0.5 mL of the same solution at an angle through the mouth. A tuberculin syringe with 25-gauge needle is satisfactory for this manoeuvre.

The preferred and recommended technique for collecting semen from male sea urchins is called "dry spawning." Once sperm is wetted, it has limited viability, so in order to complete both a gamete check and a pre-test and still have viable sperm for use in testing, sperm should be collected "dry." Care must be taken when collecting "dry" sperm from the males to avoid the sperm becoming contaminated with water or KCl solution from the animal while spawning. One technique for dry-spawning male urchins is to place an individual in a dry beaker or petri dish, with its aboral surface down. Semen is then collected from the bottom of the container (as opposed to from the surface of the animal). Another technique is to place the animal in a beaker with its aboral surface up (i.e., the opposite side of the body from the mouth facing upwards), and with control/dilution water covering only the lower half of the shell. Extruded semen which accumulates on the animal's surface by the pores is gathered with a micropipet, transferred to a small capped or covered tube, and stored on ice. Care must be taken to ensure that the surface onto which the sperm is extruded (i.e., the bottom of a petri dish or the surface of the sea urchin) is dry, in order to avoid wetting the sperm and thereby activating it.

Male eccentric sand dollars might produce insufficient volumes of sperm when spawned "dry." Sand dollars can be spawned in a minimal amount of seawater (5 mL)[10]; however, they should be placed on top of a beaker such that they are suspended over the water column. Experience indicates that sand dollars will not spawn if placed in a seawater-rinsed petri dish with their aboral surface in direct contact with the bottom of the dish.

Semen collected "dry" may be held on ice[11] for 4 h before "activation" in seawater, then used in a test in the subsequent 2- to 4-hour period. If sperm are collected in beakers of seawater, they should be used to start the test in a period ≥ 0.5 to ≤ 2 h after collection is completed. In the interim, they are to be stored in a minimum amount of control/dilution water, on ice.

For the alternative "wet spawning" method (which is preferred for the sand dollar and females of other species), each sea urchin or sand dollar is placed aboral side down on a small beaker, 50 to 250 mL or other size as appropriate, filled to the brim with control/dilution water at the test temperature. After spawning is terminated, as much water as possible is decanted from the gametes. Alternatively, females can be placed aboral side up in a vessel with just enough control/dilution water to cover the test (shell) of the urchin by about 1 cm. Eggs can be collected off the surface of the test and placed in a small beaker or other appropriate vessel. The collected eggs are washed three times by diluting with 100 mL of control/dilution water, mixing, settling for 10 min, and decanting. If a pigmented substance is obtained with the eggs, it might be important to rinse the eggs soon after collection, since the substance might be toxic to the Pacific

[8] A solution of 0.5 *M* KCl is prepared by dissolving 3.75 g KCl in 100 mL of distilled or deionized water.

[9] See Figure 2 in Environment Canada 2011. In addition, the following website provides a good description and an animated diagram for injecting sea urchins to induce spawning: www.stanford.edu/group/Urchin/inject.htm

[10] Gamete viability for sand dollars is greatly enhanced when organisms are spawned into seawater with a salinity of > 30 g/kg (Pickard, Maxxam Analytics Inc., personal communication, 2008).

[11] Vials of semen may be wrapped in paper towel before being stored on ice to ensure that the semen does not freeze (Carr, Nipper, and Biedenbach, Columbia Environmental Research Centre, TAMU-CC, Centre for Coastal Studies, personal communication, 2008). Vials of semen can also be held in a small cooler with frozen ice packs.

purple sea urchin and perhaps toxic to other species.[12] Eggs may be held in the final addition of control/dilution water, at the test temperature, for 4 h until use. It is recommended that eggs be gently aerated using a Pasteur pipette during holding.

If there is no spawning in 5 or 10 min, a second injection may be used; however, this might cause the organisms to extrude gametes that are immature and of poor quality. Semen or eggs should be produced by the adults in a steady stream, within half an hour of the final injection, as a maximum. Semen appears as a compact white string when shed into water, whereas eggs appear as a somewhat granular material, usually pastel in colour (pinkish in sand dollars). Coloured substances are sometimes extruded before or during the spawning, and should not be mistaken for gametes.

Collection of spawn should be terminated within 15 min of the start of steady spawning. Enough gametes should be collected from the same individuals for the gamete check, the pre-test, and the definitive test.

Multiple collections of gametes from the same adult are normally pooled using a pipette. For manipulations of eggs, many investigators use a standard 1-mL plastic micropipette with 2 to 3 mm cut off the tip by means of a scalpel, to provide a bore diameter of approximately 1 mm and to reduce damage to the eggs.

The sperm collected for this reference method should represent three or more male adult echinoids of the selected species, and the eggs should represent three or more adult females. Since it is possible that sperm or eggs from one adult might be particularly sensitive or particularly tolerant, an attempt should be made to achieve homogeneity of the experimental units (i.e., to avoid any differences among vessels that are related to the parent). The only practical way to do this is to pool the male or female gametes from different parents before transferring them to the test vessels; however, pooling good quality gametes, with poor quality gametes can result in poor fertilization success. Therefore, a gamete check (see following paragraph) of individual males and females should be performed to ensure that only good-quality gametes are being selected for use in the test. It is permissible to use gametes from only one adult from each gender (i.e., 1 male and 1 female) whose gametes yield good fertilization success; however, under these conditions a gamete check and pre-test must be conducted. A gamete check is required to ensure that a subsample of gametes from each of the adult males and females chosen as likely sources of sperm and eggs to be used in the test have a high degree of viability. In this procedure, at least three females and three males are selected for microscopic examination of each individual's gametes. Each of these individuals is spawned, and their gametes placed in a separate container. The samples of semen from each male are stored separately on ice. A small portion (e.g., 0.1 mL) of each male's sperm is then diluted with control/dilution water (e.g., 10 mL) and then pipetted onto a microscope slide, so that the motility of the sperm can be judged. Eggs from each individual female are similarly examined under a microscope. Poor quality eggs are small in size, irregular in shape, and display vacuolization (i.e., formation of small cavities in the cytoplasm bound by a single membrane).

Small aliquots (e.g., 0.1 mL) of eggs from each female having "good-quality" eggs are then placed in several scintillation vials.[13] Separate groups of eggs representing each "good-quality" batch are then fertilized with a few drops of diluted sperm from one of each of the "good-quality" batches of sperm. For example, if gametes from four females and three males are being examined, three vials of eggs are prepared with 10 mL of seawater for each

[12] Excess handling might decrease fertilization success rate; therefore,washing the pigment from the eggs might be unnecessary (Buday, Pacific & Yukon Laboratory for Environmental Testing, personal communication, 2008).

[13] At this stage it is important to at least qualitatively equalize egg density since differences in egg densities (i.e., the 0.1 mL placed into each scintillation vial) may impact the outcome of the gamete check. Different egg densities mixed with less variable (more consistent) sperm densities will result in variable sperm:egg ratios, thus biasing the outcome of the gamete check. It is generally sufficient to visually equalize the egg densities during the washing stage described earlier in this section [P. Jackman (Environment Canada) and E. Jonczyk (AquaTox Testing & Consulting Inc.), personal communication, 2012].

female to be spawned (i.e., for every female spawned, one vial of eggs is prepared for each male spawned). Each vial is fertilized with 5 to 7 drops of slightly diluted sperm (i.e., 20 to 50 µL of concentrated or "dry" sperm in 10 mL of filtered seawater) from one of the three different males (i.e., each vial of eggs is fertilized by the sperm from a different male). After 10 min each mixture of sperm and eggs in each vial are observed under a microscope for percent fertilization. Sperm quality is assessed by looking at motility, activity, clumping, and fertilization success. Egg quality is assessed by looking at shape, colour, size, and fertilization success.

4.5.2 Preparing Standard Suspensions of Gametes

Semen from the male sea urchins or sand dollars chosen following the gamete check (see Section 4.5.1), is pooled to produce a concentrated suspension of quality sperm. If sperm were collected in beakers of water, pipette them from the bottom of the water and combine sperm from the various beakers. Semen should be transferred by drawing it slowly (without cavitation) into a micropipette (orifice ≥ 1 mm), and then delivering it by multiple expulsions and refills, to rinse it into the water receiving it.

Sperm density in the "initial suspension" is estimated with a hemocytometer or other counting chamber under 400 × magnification.[14] Dilute a small sample (e.g., 0.1 to 1 mL) of the mixed suspension 100-fold to 1000-fold (depending on concentration of sperm), using 10% glacial acetic acid made up with control/dilution water. Mix by inverting ten times and allow bubbles to clear for a minute or two. Add a drop of the mixture to the hemocytometer counting chamber and let the sperm settle for 15 min. Count the sperm in the middle 400 small squares. Calculate the number of sperm per mL in the "initial suspension." This is done by multiplying: (dilution factor) × (number of sperm counted in 400 squares) × (hemocytometer conversion factor) × (conversion of mm^3 to mL) ÷ (the number of squares counted).

For a standard hemocytometer (Neubauer), the formula becomes:

Number of sperm/mL = 100 × (number of sperm counted) × 4000 × 1000 ÷ 400

The initial suspension of sperm is adjusted to the desired concentration in a "standard sperm suspension", using control/dilution water.[15] The concentration of this "standard sperm suspension" is determined by the sperm:egg ratio that is selected (Section 4.5.3).[16] Calculations of proper dilution are easily done by the following a standard chemistry formula:

C1 × V1 = C2 × V2

"concentration one × volume one = concentration two × volume two."

For example, if a count of 125 million sperm/mL were obtained for the "initial suspension", and if 5 mL of "standard sperm suspension" of 40 million/mL were desired, then the volume of "initial suspension" to be made up to 5 mL would be calculated as V1:

125 × V1 = 40 × 5; therefore, V1 = 1.6 mL

The density of the mixed suspension of eggs is then determined. For this reference method, a target embryo (i.e., newly fertilized eggs) density of ~200 eggs per 200 µL aliquot (in 10 mL exposure volumes) is required (equivalent to a density of 20 eggs per 20 µL or 100 000 eggs per 100 mL). This represents the addition of ~200 eggs (≥ 90% fertilized and < 10% unfertilized) to each 10 mL vial. Counting

[14] A very detailed explanation of the hemocytometer and its use for counting sperm is provided by Chapman (1992).

[15] The instructions for the amounts of water used for the initial suspension are necessarily indeterminate. The particular technique used to collect semen will govern the concentration of sperm that is obtained in the initial suspension and the required dilution for a standard suspension. A given laboratory and investigator will normally develop standardized methods of collection and dilution that achieve somewhat predictable concentrations and dilutions that are satisfactory for counting.

[16] An alternative counting technique that may be used is turbidity or optical density as an indication of the number of sperm/mL, without a hemocytometer count. Details on this technique are provided in Environment Canada (2011).

can be done by adding to a Sedgwick-Rafter cell, 20 µL (or other known volume) of the mixed suspension as required, then observing at 20 to 100 × magnification. Other techniques of counting may be used if they are effective. Egg density can be adjusted by adding control/dilution water to reduce the density, or by settling the eggs and decanting water to increase the density.

4.5.3 Ratio of Sperm to Eggs

The optimum sperm-to-egg ratio must be determined in each laboratory, such that the mean fertilization success ≥ 90% is obtained.

The following sperm:egg ratios have been reported by Canadian and US laboratories to achieve a mean fertilization success of ≥ 90% for the test organisms to be used in this reference method: 20 000:1 for *L. pictus*, 500:1 to 20 000:1 for *S. purpuratus*, and 2000:1 for *D. excentricus*. Such general guidance must not, however, be depended on to yield satisfactory test results in any given laboratory or season.

The appropriate sperm:egg ratio should be determined immediately before each test, and with the gametes to be used in that test. The pre-test could use one or two (possibly more) sperm:egg ratios, such that results could be used to position the gametes that are to be used on a "curve of fertilization success" from past experience in the laboratory, allowing an appropriate ratio to be selected for the test.

An alternative pre-test procedure may be used to determine the sperm:egg ratio to be used in order to target ≥ 90% fertilization (Carr and Chapman, 1995). This pre-test uses two replicates of control/dilution water and one replicate of each of three concentrations of a reference toxicant, tested with each of several sperm:egg ratios (i.e., five) in order to determine the "optimum" sperm:egg ratio to be used in the test. The sperm:egg ratios used in the pre-test, should cover a wide range (e.g., 10-fold difference in sperm concentration). After counting the % fertilization in all of the sperm:egg ratios for each treatment, a sperm:egg ratio is chosen based on the % fertilization results in the control/dilution water (targeting ≥ 90% mean fertilization), and that which maximizes the potential for the reference toxicant result to fall within the *warning limits* of a control chart. Using this method, a sperm:egg ratio can be chosen which demonstrates the appropriate sensitivity at the required mean fertilization rate of ≥ 90%.

4.5.4 Preparation of Fertilized Eggs for Testing and Check on Fertilization Success Rate

On Day 0 (immediately following the sperm:egg ratio determination), within 15 to 30 min of fertilization of the batch of eggs intended for use with this reference method, a minimum of five replicate subsamples must be examined to assess the fertilization success rate in the seawater used as overlying water for all replicates and treatments included in the test. Data from these five replicates serve to confirm that the mean fertilization success rate at the start of the test, under the defined test conditions for each treatment, was ≥ 90%.

For each of five replicates, a minimum of 100 eggs from each replicate should be transferred to a Sedgwick-Rafter or similar cell. One hundred eggs (fertilized or unfertilized) from each replicate should then be counted, scored, and documented as either fertilized or not. Fertilization is determined by the presence or absence of a fertilization membrane. Absence of a membrane indicates the egg is unfertilized.

If the mean percent fertilization is ≥ 90% testing may proceed. However, if the mean fertilization rate is < 90%, more sperm may be added to the sperm:egg mixture, and the fertilization success rate revisited as per the preceding section. A mean fertilization rate of < 90% after the second addition of sperm indicates that the gametes are of poor quality (APHA *et al*., 2005), and must not be used to provide newly fertilized eggs for this reference method. In this instance, investigators must spawn additional animals in an attempt to obtain better quality gametes and achieve an associated mean fertilization success rate of ≥ 90%.

An overview of the procedures used to determine fertilization success rate prior to test initiation follows:[17]

1. Spawn echinoids, collect gametes, and check gamete quality.

2. Conduct pre-test to determine required sperm: egg ratio in order to achieve a mean fertilization success ≥ 90% (as per Section 4.5.3).

    a. In this example, the pre-test indicated a sperm: egg ratio of 2000:1 yielded a mean fertilization of ≥ 90%.

3. Pipette 100 µL of concentrated eggs into a vial containing 10 mL of dilution/control water.

4. Determine the egg density in a 20-µL volume from step 3 (target is ~200 eggs per 200 µL).

    a. Counted 18 eggs per 20-µL volume.

5. Calculate the volume of concentrated eggs required to achieve a density of 200 eggs/ 200 µL in a 100 mL egg suspension volume.

    a. Based on step 4, ratio increase = 1.11.

    b. Therefore, 110 µL eggs in 10 mL or 1.1 mL eggs in 100 mL (X).

6. Add ~ 98 mL of control/dilution water to a 200 mL beaker.

7. Add calculated egg volume (X = 1.1 mL).[18]

8. Add the calculated sperm volume.

    a. Based on formulae in Section 4.5.2, and assuming the initial sperm suspension was 200 million/mL, then add 1 mL of initial sperm suspension to the 200 mL beaker obtain a sperm:egg ratio of 2000:1 in 100 mL volume.

9. Stir with Pasteur pipette and gently aerate.

10. Allow a 15- to 30-minute fertilization period.

11. Transfer 200 µL to 5 vials containing 10 mL of control/dilution water.

12. Add 1 mL of 0.5% glutaraldehyde to preserve the newly fertilized eggs for examination.[19]

13. Count, for each of five replicates, a minimum of 100 eggs (fertilized and unfertilized) and score as fertilized or not.

14. Record the number and % fertilized eggs in each of the five replicates, and calculate the mean.

    a. If the mean fertilization success was ≥ 90%, an identical aliquot of eggs (fertilized and unfertilized) from the batch is then transferred to each test vial. Transfers should be completed within 2 h of fertilization, and must be completed within 4 h of fertilization (see Section 4.6.4).

    b. If the mean fertilization rate is < 90%, more sperm may be added to the sperm:egg mixture, and the fertilization success rate reassessed (see Section 4.5.4).

[17] Environment Canada can also be contacted to obtain Excel templates for various aspects of this test, including determining fertilization success rate. Requests can be emailed to methods@ec.gc.ca

[18] Prior to mixing of eggs with sperm, a re-check on egg density is suggested in order to have a final opportunity to correct for possible errors (e.g., resulting from pipetting or calculation errors).

[19] Glutaraldehyde must be used as the preservative in this test method (AquaTox, 2013).

4.6 Test Procedures

4.6.1 Overview of Test Design

The test design requires the following to be included in each test performed:

1. a minimum of eight vials containing control sediment;

2. a minimum of eight vials containing each test sediment under investigation;

3. a minimum of 23 "water-only" vials are required when conducting sediment tests in conjunction with a reference toxicant test

    - 14 "water-only" vials are required when conducting a reference toxicant test **alone**: 6 vials for En, 2 vials for water quality monitoring at start and end, 3 "monitoring" vials for presumptive test end; 3 vials for judging test validity;

4. if available and included as part of the field sampling program, a minimum of eight vials representing a suitable reference sediment should also be incorporated as part of the test design. Some study designs and field-sampling programs might incorporate more than one reference sediment, in which case eight vials are required for each of these separate treatments.

In each instance where eight or more vials representing a single treatment (sediment sample) are set up, test organisms (i.e., newly fertilized eggs) are placed in all vials including those used for water quality monitoring. Six (or more) of these vials will be used as replicates to evaluate larval development. The seventh and eighth (or more, depending on analytical requirements) replicates will be used for measuring the chemistry of the overlying water at the beginning and end of the test. For each of these replicate vials, a measured aliquot of sediment and seawater is added, followed by standardized mixing of the contents in each vial (see Section 4.6.2) and their overnight equilibration under test conditions of temperature and lighting.

Twenty-three (or more) "water-only" vials (to which organisms are added) are to be set up and used for the following purposes:

- Six vials are used to determine the fertilization success rate in the seawater used as overlying water for all replicates and treatments included in the test, at the start of the test (0 h). Data from these replicates serve to confirm that the mean fertilization success rate at the start of the test, under the defined test conditions for each treatment, was ≥ 90%. These data also provide an estimate of the number of fertilized eggs (embryos) in each treatment at the start of the test (En), which is used when calculating % normal larvae for each treatment at the end of the test.

- Two vials are used for monitoring the quality of water representing the "water-only" control, at the beginning and end of the test.

- Six "monitoring" vials are used to determine the % normal larvae in the seawater used as overlying water for the "water-only" control, during 1 h immediately preceding the presumptive end of the test. Data from these replicates are used to assess whether the test can be terminated at the presumptive test end with a high likelihood of achieving the criterion for test validity (see Section 4.9), or whether the test should be continued for an additional 24.[20]

- The "final" nine "water-only" vials to be included in the test are used to confirm the test met the validity criterion (see Section 4.9). One set of vials (six final "water-only" controls) is paired with the sediment samples and must be transferred to new vials (as if it was a sediment sample) prior to preservation with glutaraldehyde. The second set of three "water-only" controls must be paired with the reference toxicant and preserved without transfer to a new vial (as would occur with the reference toxicant exposures).

[20] Percent normal larvae in one or two of the "monitoring vials" could be checked the morning of the presumptive test end, and the remaining 3 or 4 "monitoring vials" checked 1 h before the actual presumptive test end to determine the likelihood of test termination.

4.6.2 Preparing Test Solutions

On Day -1 (i.e., the day preceding the start of the test), a minimum of eight replicate test chambers per treatment (i.e., for each test sediment and the control sediment) are set up in the testing facility.

Following mixing (for homogenization) of each test sediment (see Section 4.3), a 0.5-g aliquot of (wet) sediment is added to each 20-mL test chamber (minimum of eight replicates per treatment). Thereafter, a 10-mL volume of filtered (~60 µm), uncontaminated natural seawater is added to each pre-labelled vial (see Section 4.4.2). The seawater must be from a natural source with salinity in the range of 30 ± 2 g/kg.

The contents of each vial must then be mixed by agitating individual vials on a vortex mechanical shaker (appropriate for flat bottom scintillation or shell vials) at a rate of 1800 rpm for 10 seconds.[21] Thereafter, all vials (including the 23 "water-only" vials) to be used in the test must be transferred to the testing area, where they are randomly distributed, covered, and held overnight undisturbed using the temperature and lighting conditions to be employed during the test (see Section 4.4 and Table 2). Individual vessels are positioned for the exposure in a test tube rack or other rack, held in the water bath or other temperature-controlled facility. Vessel positions in the rack must be randomized in "columns" of the rack, each column representing one sample/treatment and control (e.g., samples/treatments are randomized, but replicates are kept together). Each vessel must be clearly labelled or positions coded such that samples/treatments and replicates can be identified. An example of randomization within a test is provided in Figure 1.

4.6.3 Water Quality on Day 0

At the start of the test, temperature, salinity, dissolved oxygen content, and pH must be measured in the overlying water in one of the two replicates (i.e., "the seventh replicate;" see Section 4.6.1) dedicated for this purpose for each treatment. For the reference toxicant test, total ammonia must be measured at the start of the test in the corresponding "water-only" control tested in conjunction with the reference toxicant. Total ammonia must also be measured in all sediment exposures at the start of the test, including in the corresponding "water-only" control. Values for ammonia are to be expressed as both total ammonia (as measured) and, by calculation, un-ionized ammonia. The percentage of un-ionized ammonia in total ammonia is determined by pH and temperature. The following formulae can be used to calculate the un-ionized ammonia concentration:

Un-ionized ammonia (mg/L) = total ammonia (mg/L) × $[1 / (1 + 10^{pKa - pH})]$
(Emerson *et al*., 1975)

where: pH is that measured in the overlying water; and

pKa (the acid dissociation constant of NH_4^+)

= 0.09018 + 2729.92/T

T = temperature in Kelvin[22]

A comparison of species sensitivity to ammonia (based on testing conducted during method development) is provided in Appendix F.

4.6.4 Exposure (Starting the Test)

On Day 0, the adult echinoids intended to provide the test organisms (i.e., newly fertilized eggs) are spawned, appropriate sperm:egg ratio are determined, and fertilization is started (Section 4.5).

[21] A 10-second period for mixing was chosen for consistency with USEPA and PSWQA (1995) and Annex A.1 in ASTM (2007) in this regard.

[22] Temperature in Kelvin = °C + 273.2

WO-6	C-2	A-8	G-1	D-4	B-5	E-8	WO-16	F-5	WO-21
WO-3	C-1	A-4	G-6	D-3	B-2	E-3	WO-9	F-5	WO-15
WO-7	C-3	A-6	G-8	D-7	B-7	E-1	WO-11	F-2	WO-17
WO-5	C-4	A-7	G-2	D-2	B-1	E-2	WO-14	F-1	WO-22
WO-1	C-8	A-2	G-7	D-8	B-6	E-7	WO-15	F-7	WO-20
WO-2	C-5	A-5	G-5	D-5	B-3	E-4	WO-13	F-3	WO-19
WO-8	C-3	A-1	G-4	D-1	B-4	E-5	WO-10	F-4	WO-23
WO-4	C-7	A-3	G-3	D-6	B-8	E-7	WO-12	F-6	WO-18

Figure 1 **Example of sample randomization within a test.** Treatments are labelled A-F with WO indicating "water-only" controls. Replicates are labelled 1-8. Sample treatments are arranged randomly such that each column of a rack holds one treatment. Replicate samples of each treatment are also randomized but kept within the confines of their appropriate column. The entirety of treatment A is highlighted illustrating how all of treatment "A" replicates are located in the same column.

Thereafter, and within 15 to 30 min of fertilization, the batch of eggs is checked to determine that a mean fertilization success rate of ≥ 90% was achieved (see Section 4.5.4). Provided this fertilization rate is achieved, a 200-µL aliquot of embryos is transferred to all test chambers, including those to be used for water quality monitoring. Each 200-µL aliquot will represent the addition of ~200 eggs, comprised of ≥ 90% newly fertilized eggs, along with a small percentage (< 10%) of unfertilized eggs.

Transfers to the test vials should be completed within 2 h of fertilization, and must be completed within 4 h of fertilization. During transfers of individual aliquots from the newly fertilized pool of eggs, the eggs in that pool must be kept in a homogeneous suspension. This can be achieved by gently swirling them by hand using a glass rod immediately before removing each aliquot. Alternatively, a perforated plunger (plastic disk containing numerous holes, attached to a plastic rod) may be used to gently mix the egg pool thoroughly during each transfer (APHA *et al*., 2005). Thereafter, a pipette (a repeater pipette is preferred) with a wide-bore (~2 mm orifice) tip is used to transfer a 200-µL aliquot to each vial. Vials must be covered (see Section 3) following the completion of all transfers.

Six of the "water-only" vials are then checked to determine the fertilization success rate in the seawater used as overlying water for all replicates and treatments included in the test, at the start of the test (0 h). A 1-mL aliquot of 0.5% glutaraldehyde is added directly to each test vial and the procedures described in Section 4.7.2 are followed to:

i) confirm that the mean fertilization success rate at the start of the test, under the defined test conditions for each treatment, was ≥ 90%; and

ii) provide an estimate of the number of fertilized eggs (embryos) in each treatment at the start of the test (En), which is used when calculating % normal larvae for each treatment at the end of the test (see Section 4.8).

The number (and percentage) of fertilized and unfertilized eggs in each replicate must be recorded, and the mean (± SD) for the six replicates calculated and recorded. This (mean) number represents the "En" value used when calculating the test endpoint for each treatment (see Section 4.8), as well as when determining if the criterion for test validity was met (see Section 4.9).

4.6.5 Test Duration

Test duration is species-dependent. The test duration can be prolonged by 24 ± 1 h, based on the percentage of normal larvae determined for the six "water-only" controls included in the test for this purpose (see Section 4.6.1). The presumptive test end for each species is:

- at 48 h, if *L. pictus*
- at 72 h, if *D. excentricus*
- at 96 h, if *S. purpuratus*

During the hour immediately preceding these species-specific times,[20] six of the remaining seventeen "water-only" controls should be preserved and examined immediately thereafter (see Sections 4.7.1 and 4.7.2) to determine the % normal larvae in each vial.

If mean normal larvae in the six "water-only" "monitoring vials" is ≥ 70%, the test must be terminated and all embryos and larvae within each replicate and treatment recovered and preserved (using glutaraldehyde) for subsequent scoring and counting (see Sections 4.7.1 and 4.7.2).[23] One set of "water-only" vials (six final "water-only" controls paired and scored in conjunction with the sediment samples) must be transferred to new vials (as if it was a sediment sample) prior to preservation with glutaraldehyde. Three "water-only" controls must be paired with the reference toxicant and preserved without transfer to a new vial (as would occur with the reference toxicant exposures). If at presumptive test end, mean % normal larva in the "monitoring vials" is < 70%, the test must be extended for an additional 24 h to ensure the test validity criteria (i.e., Pn ≥ 60%) will be met when the organisms are evaluated/scored. At that time (i.e., at 72 h if *L. pictus*; at 96 h if *D. excentricus*; and at 120 h if *S. purpuratus*), the test must be terminated and all embryos and larvae within each replicate and treatment recovered and preserved for subsequent scoring and counting (as previously described).

4.6.6 Water Quality at Test End

At the end of the test, temperature, salinity, dissolved oxygen content, and pH must be measured in the overlying water in one of the two replicates (i.e., "the eighth replicate;" see Section 4.6.1) dedicated for this purpose for each treatment (these parameters are also measured at test start). For the reference toxicant test, total ammonia must be measured at the end of the test in the corresponding "water-only" control tested in conjunction with the reference toxicant. Total ammonia must also be measured in all sediment exposures at the end of the test, including in the corresponding "water-only" control tested in conjunction with the sediment samples. The water-quality variables should be re-examined to determine the characteristics of the overlying water representing each treatment at the end of the test. To assist in interpreting the test results, these measurements should be tabulated alongside the corresponding initial (Day-0) water quality measurements for each treatment and the tabulated data included as part of each report on the test results.

4.7 Test Observation and Measurements (Ending the Test)

4.7.1 Recovering Embryos and Larvae

To end a test, the water overlying sediment in each test vial is transferred into a new (labelled) scintillation or shell vial using a 10-mL pipette with a minimum ≥ 2 mm opening. The minimum 2 mm opening can be achieved by either purchase of a wide-bore pipette tip, or by cutting a tip to the required size. Thereafter, 1 mL of 0.5% glutaraldehyde is added to preserve the embryos and larvae for later examination.

[23] By requiring ≥ 70% normal larvae at presumptive end a safeguard is provided to ensure that the validity criteria of ≥ 60% normal larvae will be met when scoring the "water-only" controls.

Experience has shown that sediment carry-over can pose a problem when counting and scoring preserved organisms.[24] Therefore, during this transfer, care is taken to minimize the disturbance to the sediment in the vial (and sediment carry-over to the new vial), while ensuring that all test organisms within the overlying water are transferred.[25] The "water-only" controls paired with the sediment samples must also be transferred to new vials (as if it were a sediment sample) prior to preservation with glutaraldehyde.

For all replicates and treatments in the reference toxicity test (see Section 5), a 1-mL aliquot of 0.5% glutaraldehyde is added directly to each test vial at the end of the test. The "water-only" controls paired with the reference toxicant are also preserved without transfer to a new vial.

Thereafter, all vials containing test organisms and preservative are capped and stored at ambient room temperature until their contents are examined and larval development is evaluated. This examination should take place as soon as possible, and must be completed within 4 weeks following the end of the test.

4.7.2 Counting and Scoring Embryos and Larvae

Each laboratory technician responsible for counting and scoring echinoid embryos and larvae must be trained and experienced in how to do so. The photomicrographs showing normal and abnormal pluteus larvae and earlier life stages are provided in Figures 2 and 3 are useful in this respect.[26] Additional photomicrographs of developing echinoid larvae can be found in Lesser and Barry (2003). For each of the candidate echinoid species for use in this reference method, a clear distinction between fertilized and unfertilized eggs must be consistently recognized. Investigators must also be able to distinguish "normal" larvae (pluteus and prism) from abnormal ones (i.e., those with developmental anomalies such as asymmetrical or missing arms, and/or retarded development). Prism larvae are typically triangular (pyramid) in shape without arm-like extensions. Normal pluteus larvae will typically have a pyramid shape supported by a framework of skeletal rods, an internal gut that is attached to the body wall at both ends and consists of three distinctive regions, and at least one pair of post-oral arms. Arm length varies with species (APHA *et al*., 2005). Each laboratory using a different species should carefully compare well-developed embryos from controls with gradations of abnormal development in a toxicant to consistently identify normal and abnormal for their given species.

[24] Overlying water should not be poured into the preservation vials as this may increase sediment transfer and obscure organisms making it difficult to evaluate life stage.

[25] Pluteus larvae can often be observed in the overlying water when the test vial is held against a light.

[26] Figure 2 is reprinted with permission from ASTM (2004). A copy of the complete standard may be obtained from ASTM International, www.astm.org.

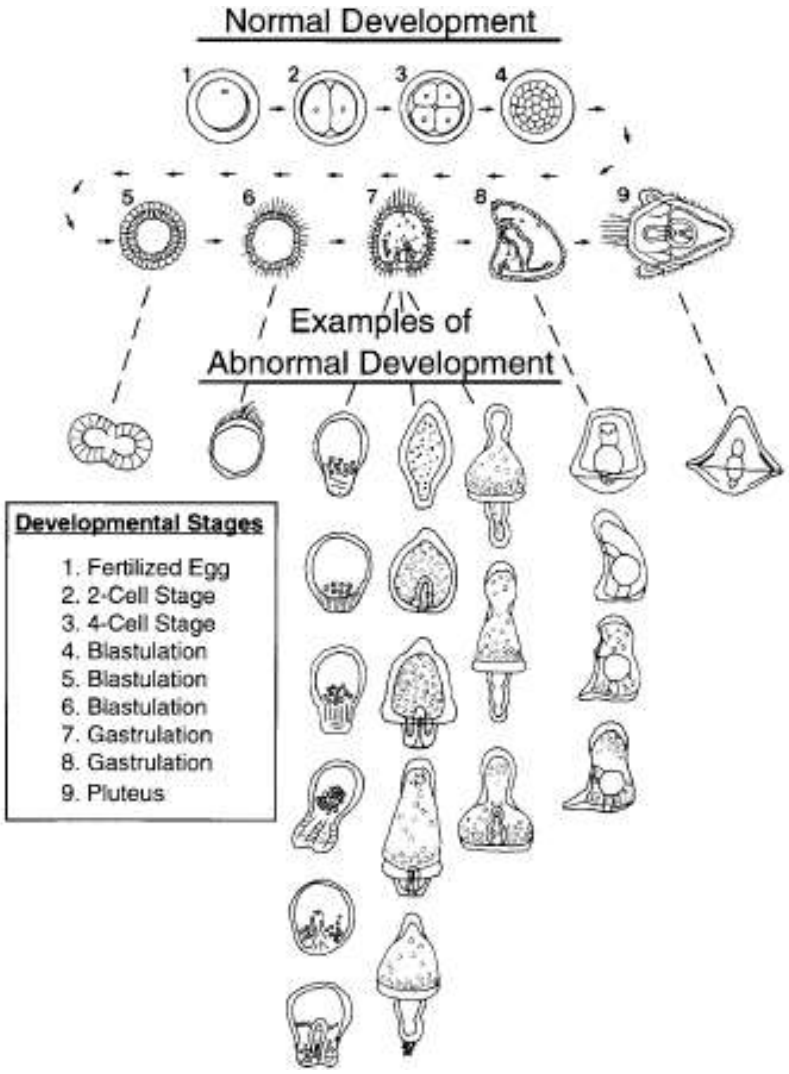

Figure 2 Drawings exemplifying key developmental stages of normal echinoid larvae occurring during the first 48 to 96 hours of development and examples of abnormal or arrested development. Reprinted with permission from ASTM 2004. A copy of the complete standard may be obtained from ASTM International, www.astm.org. Examples of normal and abnormal echinoid embryo development are from Kinae *et al.* 1981; Rulon 1956; Timourian 1969.

Examples of normal and abnormal echinoid embryo development are from: Kinae N, Hashizume T, Makita T, Tomita I, and Kimura I. "Kraft Pulp Mill Effluent and Sediment can Retard Development and Lyse Sea Urchin Eggs," *Bulletin of Environmental Contamination Toxicology,* 1981, Vol 27, pp. 616–623; Rulon, O., "Effects of Cobaltous Chloride on Development in the Sand Dollar," *Physiological Zoology,* 1956, Vol 29, pp. 51–63; and Timourian H, "The Effect of Zinc on Sea Urchin Morphogenesis," *Journal of Experimental Zoology,* 1969, Vol 169, pp. 121–132.

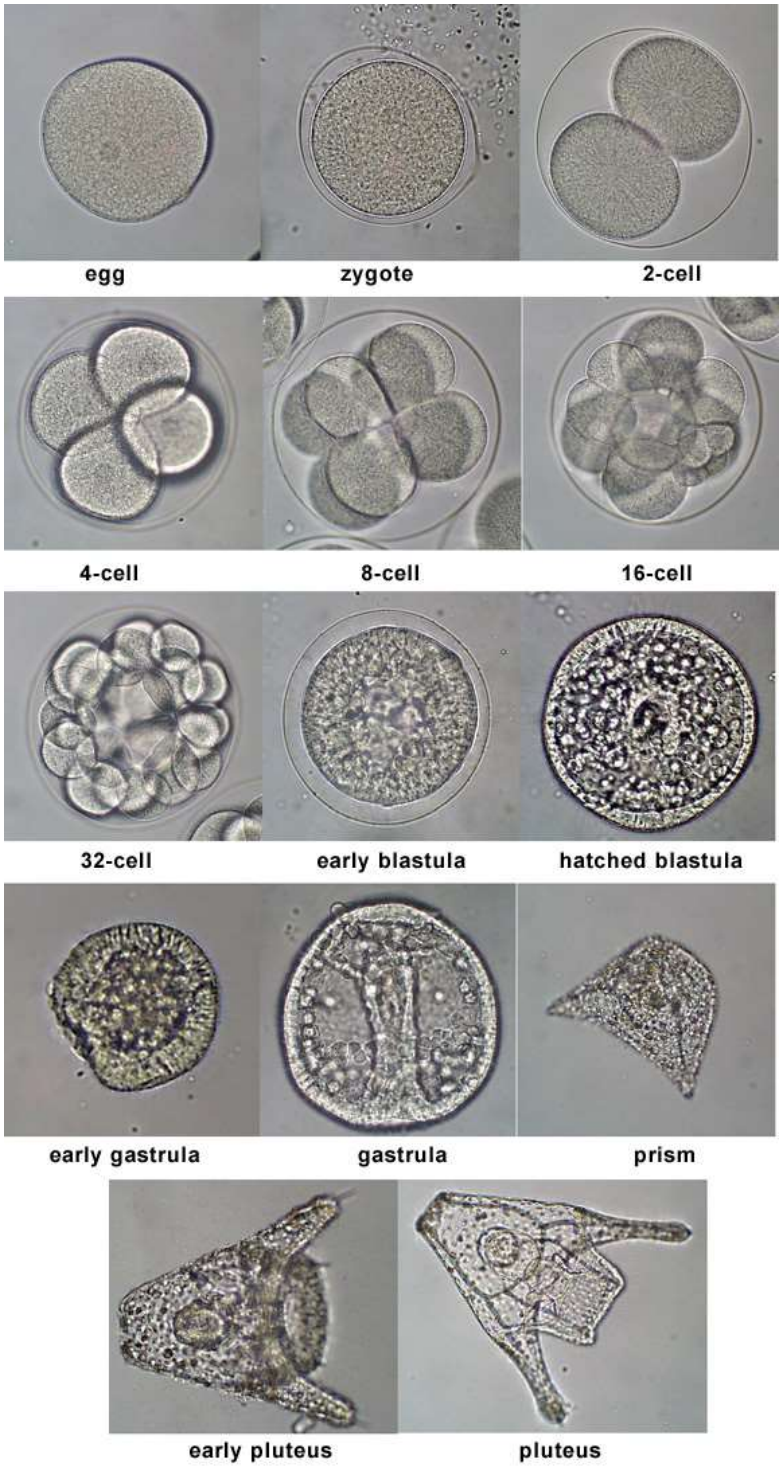

Figure 3 Normal developmental stages of echinoid embryos (*Lytechinus*). Photo reprinted with permission from Dr. Judy Cebra-Thomas, Millersville University, Millersville, PA (www.millersville.edu/~jcebrathomas/cebra_thomas/DB_lab/Urchin/urchin_stage.html).

Using a "total count" approach, for each replicate, all embryos and larvae recovered from each replicate at the end of the test must be counted and scored. The number counted should not exceed the number of eggs (fertilized and unfertilized) placed in each vial at the start of the test (~200; see Section 4.5.4), but in some instances could be slightly more, or could also be appreciably less (e.g., due to mixing or pipette accuracy).[27]

Two approaches can be used to count and score the preserved larvae in each test vial: scoring in-vial using an inverted-microscope or use of a Sedgwick-Rafter cell. [28]

Using an inverted microscope allows all organisms to be evaluated in a single vial without the need for multiple transfers on to a separate cell or counting chamber. However, this approach would also necessitate using shell vials for testing (rather than scintillation vials).

If a Sedgwick-Rafter cell (or other similar chamber) is used, the contents of each vial containing these organisms should be transferred to the chamber or cell for counting. Since the volume of the cell is only 1 to 2 mL, it might be necessary to prepare and count more than one slide to enumerate all embryos and larvae retrieved from each vial. Since embryos and larvae normally sink after preservation, much of the excess water in the vial can usually be carefully decanted or otherwise removed, to reduce the volume of residual water to be examined under the microscope. Care must be taken when discarding the extra water, to prevent the inadvertent loss of embryos or larvae to be counted and scored.

When using a Sedgwick-Rafter cell, a practical approach involves the use of a 10-mL pipette set at 8.5 mL to extract (and discard) most of the volume of seawater that does not contain the preserved organisms. Thereafter, the remaining 1.5-mL volume in the vial is gently swirled by hand to re-suspend the embryos and larvae. This smaller volume is then recovered using a transfer pipette, and placed in the counting chamber. A 0.5-mL volume of filtered seawater is then added to the vial that previously contained the preserved specimens, and the vial is gently swirled by hand to ensure that any remaining embryos and larvae that might have stuck to the sides of the vial are detached and recovered for scoring. While this may not be possible for some sediment samples, vigorous shaking and mixing must be avoided as this could damage the organisms (AquaTox, 2013). Each vial should be held up to the light while swirling it, to see if any embryos or larvae remain adhered to the sides of the vial and, if so, ensure their recovery.[29]

The contents of each vial (if using an inverted microscope) or Sedgwick-Rafter cell containing organisms from a single test vial should be examined under a compound microscope at a suitable resolution (e.g., 100× magnification). For each test replicate, the number of i) normal larva (prism or pluteus), and ii) abnormal larva are counted and documented. Any unfertilized eggs observed must not be counted or scored.[30]

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[27] Some normal or abnormal larvae and embryos might be mixed with the sediment in the test vials and left behind during the transfer step. "Missing" larvae in the test treatments that exceed the "missing" counts from the control or reference sediment are considered to have died or been so abnormal as to have been trapped in the sediment. Accordingly, "missing organisms" are considered to be non-survivors for the purpose of this reference method (as per Section A1.5 in ASTM, 2007).

[28] A Sedgwick-Rafter cell is an economical method for scoring, but a cell typically only holds 1 to 2 mL of solution and multiple transfers are needed to score all organisms. In comparison, using an inverted microscope all organisms can be evaluated in a single (shell) vial without the need for multiple transfers. However, an inverted microscope could be a more costly equipment option for many laboratories (cost will vary, but can range from $4000 to $6000).

[29] Method development work has shown average organism loss during transfers (i.e., from exposure vessel to preservation vial and to counting cell) will be ~10%.

[30] Depending on the study objectives, investigators might also wish to score embryos according to more detailed life stages (e.g., gastrula, blastula, or uncleaved fertilized egg). In addition, larvae observed might be developed to the pluteus stage, or might be only developed to the earlier "prism" stage. For purposes of this reference method, distinction as to these two developmental stages is somewhat difficult and unnecessary. Rather, any larvae observed should simply be counted and scored as "normal larvae" or "abnormal larvae" (McLeay, 2007).

Typical time for scoring 1 vial of a "water-only" sample (using Sedgwick-Rafter cell) was ~15 minutes, compared to 30+ minutes for 1 vial of organisms recovered from a sediment sample.

The mean recovery success rate for each treatment must be calculated and reported alongside the associated summary data for the test endpoint, once counting and scoring for the treatments has been completed.[31] This value (i.e., the recovery success rate) is calculated by dividing the mean for the total number of all life stages recovered from each replicate of the treatment, by the mean for the total number of all life stages recovered from the "water-only" controls and multiplying by 100.

4.8 Test Endpoints and Calculations

The endpoint for this reference method is based on the number of normal larvae produced in each replicate by the end of the test, and the associated % normal larvae calculated for each treatment, using the test procedures and conditions defined herein.

When determining "% normal larvae" for each treatment, all deformed and delayed larvae, embryos (deformed or normal), and fertilized eggs that were counted and scored (Section 4.7) must be considered as "abnormal" (APHA *et al*., 2005), with the remaining number(s) (i.e., the number of normal larvae found in each replicate) used in the determination.

Once the counting and scoring of all organisms recovered has been completed (see Section 4.7), the number of normal larvae (prism and pluteus) in each replicate must be determined and recorded. In addition, the mean value representing "% normal larvae" must be calculated and recorded for all replicates from the same treatment (n = 6).

As per APHA *et al*. (2005), the percentage normal larvae (i.e., P_n) is calculated for each treatment as follows:

$$P_n = 100(L_n/E_n)$$

where: L_n = mean number of normal larvae in 6 replicates at test end, and

E_n = mean number of embryos in 6 replicates at start of test in "water-only" controls

In the case of a reference toxicant test conducted simultaneously with sediment samples, Ln will be based on 3 replicates and and En will be based on 6 replicates. In the case of a reference toxicant test conducted alone, both En and Ln will be based on 3 replicates.

The mean number of embryos at the start of the test (E_n) to be used in this calculation is the mean number of newly fertilized eggs determined for the six (or more, depending on test design) replicate "water-only" controls, at the start of the test (see Section 4.6.1). The value for L_n is represented by the mean number of normal larvae determined for each of the six (or more) replicates in that treatment.

4.9 Test Validity Criteria

For this reference method, the criteria used to judge whether the test results are valid or not is based on the quality of embryo development in the replicates representing the "water-only" control and in the control sediment replicates.

For the findings of a test to be considered as valid, an average of ≥ 60% of the embryos must be judged to be normally developed larvae at the end of the test in the: 1) "water-only" control, and 2) laboratory control sediment. The validity criteria must be met in the "water-only" controls paired and scored in conjunction with the reference toxicant as well as the sediment samples.

[31] A series of comparative tests were conducted to determine the recovery success rates for each of the candidate test species included in this reference method. These tests measured and compared recovery rates at test end for embryos and larvae held in vials containing control sediment, reference sediment, or contaminated sediment, with those recovery rates for the "water-only" controls included in the same test. The values for % recovery success in each treatment proved useful when interpreting the findings (i.e., % normal larvae) for each treatment. High recovery rates were achieved for "clean" sediments including those with a high percentage of fines. The results for recovery success rates also assisted in choosing the control sediment and reference sediment(s) to be used in a particular series of tests (McLeay, 2010).

The value for "% normal larvae" (P_n) achieved in the "water-only" and sediment controls at the end of the test is calculated using the same equation described in Section 4.8, except that "L_n" represents the mean number of normal larvae found in the six (or more) replicates of the "water-only" control groups (or sediment control groups) at the end of the test.

Procedure for Testing a Reference Toxicant

The routine use of a reference toxicant is required to assess the relative sensitivity of the batches of gametes that are used, under standardized test conditions, and the precision and reliability of data produced by the laboratory for the selected reference toxicant(s), as well as the technical proficiency of the lab staff conducting the test (Environment Canada, 1990).

A multi-concentration "water-only" reference toxicity test must be performed in conjunction with each batch of organisms used in one or more sediment toxicity tests conducted per day according to this reference method. This multi-concentration test must be undertaken at the same time as the definitive test, using replicate groups of newly fertilized eggs from the same batch as those used to conduct the definitive test.[32]

Criteria considered in recommending appropriate reference toxicants for this test include:

- chemical readily available in pure form;
- stable (long) shelf life of chemical;
- highly soluble in water;
- stable in aqueous solution;
- minimal hazard posed to user;
- easily analyzed with precision;
- good dose-response curve for echinoid gametes;
- known influence of pH on toxicity of the chemical, in this test; and
- known influence of salinity on toxicity of the chemical, in this test.

Copper (as copper sulphate or copper chloride) is recommended for use as the reference toxicant for this test. Copper sulphate or copper chloride should be used for preparing stock solutions, which should be acidic (pH 3 to 4), and may be used when prepared, or stored in the dark at 4 ± 2°C for several weeks before use. Concentration of copper should be expressed as µg Cu/L.

The reference toxicity test must be performed as a multi-concentration test in which an IC50 is derived, based on % normal larvae (see Section 4.8). Results must be calculated and reported as µg Cu/L.[33] Regression analysis (with binomial weighting) is the principal technique used to derive the IC50. If regression analysis is not suitable, ICPIN may be used. A minimum of three replicates per treatment (concentration) and a minimum of five concentrations (plus the "water-only" control) are required.[34] Guidance in Section 4.5.2 of Environment Canada (2011) and in Section 6 of Environment Canada (2005) with regard to determining an *ICp* using regression (or other) analyses should be considered and followed.

To provide a high degree of standardization for this reference toxicity test, and in agreement with other treatments in this reference method, salinity of the control/dilution water used for reference toxicant testing must be within the range of 30 ± 2 g/kg.

Concentrations of reference toxicant in all stock solutions should be measured chemically by appropriate methods (e.g., APHA *et al*., 1989, 2005). Upon preparation of the test solutions, aliquots should be taken from at least the control, low, middle, and high concentrations, and analyzed directly or stored for future analysis, in case the ICp is outside warning limits. If stored, sample aliquots must be held in the dark at 4 ± 2°C. Copper solutions should be

[32] This requirement is consistent with Environment Canada's two existing Reference Methods for measuring the toxicity of sediment samples using other test organisms and means (Environment Canada, 2002; 1998).

[33] Based on results generated during method development and round robin testing, mean IC50s for *L. pictus*, *S. purpuratus*, and *D. excentricus* were 73.6 µg Cu/L (CV = 23.9%; n = 20), 21.7 µg Cu/L (CV=33.1%; n = 18) and 14.3 µg Cu/L (CV = 10.7%; n = 6), respectively.

[34] For laboratories new to this method, the use of seven exposure concentrations is recommended. The dilution series should be structured to capture 2 partial effects.

preserved before storage (APHA *et al*., 1989, 2005). Stored aliquots requiring chemical measurement should be analyzed promptly upon completion of the toxicity test. Calculations of ICp 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., five data points) are available (Environment Canada, 1990), a *warning chart* must be prepared and updated for each reference toxicant used. Successive ICps are plotted on this chart and examined to determine whether the results are within ± 2 SD of values obtained in previous tests. The geometric mean ICp together with its upper and lower warning limits (± 2 SD calculated on a logarithmic basis)[35] are recalculated with each successive ICp until the statistics stabilize (USEPA, 1989; Environment Canada, 1990).

A separate warning chart is required for each echinoid species used in the definitive test for sediment toxicity performed according to this reference method. Data plotted on this warning chart are to be derived using the same reference toxicant (i.e., copper sulphate) and identical test procedures and conditions consistent with those described herein for this reference method.

If a particular IC50 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 batch of gametes or the precision of the toxicity data produced by the laboratory are in question. Rather, it provides a warning that this might be the case. A thorough check of all holding and test conditions is required at this time.

Test results that usually fall within warning limits do not necessarily indicate that a laboratory is generating consistent results. A laboratory that produced extremely variable data for a reference toxicant would have wide warning limits; a new datum-point could be within the warning limits but still represent undesirable variation in results obtained in the test. For guidance on reasonable variation among reference toxicant data (i.e., warning limits for a warning chart), please refer to Section 2.8.1 and Appendix F in Environment Canada, 2005.

If an IC50 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.

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

Data Analysis and Interpretation

6.1 Data Analysis

The objective of the data analysis is to quantify contaminant effects on replicate groups of test organisms exposed to various treatments of concern, and to determine if these effects are statistically different from those occurring in a reference or control sediment.

Initially, endpoints [i.e., "% normal larvae" (P_n); see Section 4.8] are calculated for the *replicate samples* representing each treatment (including those representing the reference and control treatments).

Each study consists of a minimum of eight vials containing control sediment, eight vials containing each test sediment under investigation, and, if available and included as part of the field sampling program, a minimum of eight vials representing a suitable reference sediment.[36] A test sediment might be represented by replicate samples of dredged material from a particular depth or locale (sampling station) of interest, or replicate samples of field-collected sediment from a particular station within or adjacent to an ocean disposal site. A test treatment will be represented by six or more subsamples (i.e., laboratory replicates) of a single (non-replicated) sample of sediment from a particular sampling station or site-specific depth. In each case, the test treatment is represented by ≥ 6 replicates used to evaluate organism development.

Power analysis was performed using the data generated during the round robin evaluation to achieve a minimum of 80% power in detecting a 30% decrease in Pn (i.e., critical effect size; AquaTox, 2013). The analysis was consistent with recommendations from Bosker *et al.* (2013).

Statistical comparisons of biological data for the replicates representing each test treatment (i.e., potentially contaminated sediment from a single sampling station and depth) with that for replicate samples of reference sediment, must be applied whenever possible or appropriate. To this end, a reference sediment should be uncontaminated (e.g., measured substances should be below sediment quality guidelines) and similar to test sediments in grain size and TOC. In addition, the reference sediment should give an acceptable biological response (i.e., % normal larvae in a reference sediment should not be more than 10% lower than that in control sediment). Such comparisons provide a site-specific basis for evaluating toxicity. Statistical comparisons of biological data for test sediment(s) with that for the control sediment(s) should be made if the samples of reference sediment prove unsuitable for comparison with samples from other sites (e.g., due to physicochemical characteristics that are atypical of test sediments, or other confounding factors).

Organism response in one or more test sediments (e.g., from multiple sites) is compared with the control sediment and reference sediment response. Echinoid larval data presents a unique case in toxicity data analysis. The data often meet the assumption of normality (even though the data are by nature, binomial), as a result of the high number of replicates (~200).[37] Accordingly, the recommendations made here emphasize techniques for quantitative (continuous) data (Environment Canada, 2011).

This reference method is designed to determine if organism response (% normal larvae) in one test sediment is less than that in a reference sediment or control sediment. The required statistical test is the Welch's *t*-test, performed as a one-sided test.[38, 39]

[36] Test organisms are added to all eight vials, six of which are used to evaluate organism development, while the remaining two vials are used for water quality measurements at test initiation and termination.

[37] For proportions between 0.20 and 0.80, about 80% of simulated data sets were found to be normally distributed (Zajdlik & Associates Inc., 2010).

[38] Welch's *t*-test is used to compare two samples which may have unequal variance. Recent work (Zheng *et al*., 2013) has shown that Welch's *t*-test performs well even

A Shapiro-Wilk test must also performed, to determine if the assumption of normality is met. If (and only if) the Shapiro-Wilk $P < 0.05$, then a Mann-Whitney U-test (one-sided) must also performed.[40, 41] Results of all tests must be reported.[42]

Correction of fertilization using Abbott's formula I not necessary.[43]

For each test sediment, including the control and reference sediment(s), the mean (± SD) % normal larvae as determined at the end of the test, must be reported.

6.2 Interpretation of Results

Interpretation of results is not necessarily the sole responsibility of the laboratory personnel undertaking the test; this might be a shared task which includes an environmental consultant or other qualified persons responsible for reviewing and interpreting the findings.

Environment Canada (1999) provides useful advice for interpreting and applying the results of toxicity tests with environmental samples, and should be referred to for guidance in these respects. Initially, the investigator should examine the results and determine if they are valid. In this regard, the criteria for a valid test (see Section 4.9) must be met.

The findings of the reference toxicity test that was initiated with the same batch of organisms as those used in the sediment toxicity test (see Section 5) must be considered during the interpretive phase of the investigation. These results, when compared with historic test results derived by the testing facility using the same reference toxicant, test organism, and test procedure (i.e., by comparison against the laboratory's warning chart for this reference toxicity test), will provide insight into the sensitivity of the test organisms as well as the laboratory's testing precision and performance at the time that the sediment toxicity test was conducted.

The known physicochemical characteristics of each sample of test material (including that for control and reference sediment) must be reviewed and considered when interpreting the results. The analytical data determined for whole sediment should be compared with the known tolerance limits for the echinoid species used in the test. Values which approach (but do not exceed) the known tolerance limits (e.g., for ammonia) for each species could reduce their tolerance to contaminants within the sample, and thus have influenced the test results.

All physicochemical data determined for the overlying water during the sediment toxicity test (see Sections 4.6.3 and 4.6.6) should also be reviewed and considered when interpreting the findings. If, for example, records indicate that the dissolved oxygen concentration in the overlying water within one or more test chambers fell to levels below 40%

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when variances are equal. As a result, a separate *t*-test to accommodate the case of equal variance is redundant and not needed.

[39] Advice for performing this test in CETIS is available. Requests can be emailed to methods@ec.gc.ca

[40] A cut-off value of 0.05 is used to assess normality, following advice given in Table 16.1 of USEPA (2000).

[41] The Mann-Whitney U-test is a non-parametric analog to the *t*-test. As a non-parametric test, it does not require the assumptions of normality and equality of variance to be met.

[42] This reference method uses both effect size and statistical significance to determine if sediments "pass" or "fail." Advice from a statistician, environmental consultant or other qualified persons should be obtained if the target effect size is exceeded, both the Welch's *t*-test and the Mann-Whitney U-test were performed, and the two statistical tests yield different outcomes (e.g., Welch's *t*-test $P < 0.05$ and Mann-Whitney U-test $P \geq 0.05$).

[43] Abbott's formula would only be recommended if: (i) the difference in % normal larvae among sites is due to an effect other than a site effect (e.g., culture health, poor exposure conditions); or (ii) the absolute values of % normal larvae for each treatment are required (instead of the difference from controls) (Zajdlik & Associates Inc., 2010). If laboratories meet all requirements for culture health and the conditions of this standardized test, then (i) would not be expected to occur. It is assumed the purpose of the investigation is focussed on the difference in % normal larvae between sites, and accordingly, (ii) would not be expected to occur.

of saturation, this oxygen depression might have contributed to any toxic responses observed therein. Measurements of ammonia in overlying water at the start and end of the test (Section 4.6.3 and 4.6.6) must also be converted to the respective values for un-ionized ammonia (based on the concurrent measurements of pH and temperature for the overlying water). These values should be considered together with the known species' tolerance to ammonia, when interpreting the test results (Appendix F).

To assess sediments intended for disposal at sea, biological tests are required in the permit application phase by the *Canadian Environmental Protection Act, 1999* (CEPA 1999). These biological methods are used to confirm predictions of no significant biological impacts arising from disposal at sea. No single test should be used to assess environment impact, and therefore, when combined with chemical analysis, a battery of biological tests provides a more comprehensive evaluation of potential impacts on the marine environment. For this reason, four Environment Canada toxicity test methods (i.e., using amphipod, echinoid, photoluminescent bacteria, and polychaete) and a bioaccumulation test [i.e., USEPA (1993) test method using marine worms or bivalves], are in the current battery for disposal at sea assessments in Canada. However, during surveys on the highly contaminated sediments in Sydney Harbour, one of these tests (the echinoid fertilization test, EPS 1/RM/27; Environment Canada, 2011) on sediment pore water produced unexplainable results (Zajdlik *et al.*, 2000; Tay, 2000), likely due to background ammonia. As a result, during "The Contaminated Dredged Materials Management Decisions Workshop" (Agius and Porebski, 2008), it was recommended that Environment Canada standardize a new echinoid test that would eliminate the confounding effects of ammonia.

The outcome is the present echinoid embryo/larval sediment-contact test, which is intended to be used within the aforementioned test battery, and replaces the fertilization assay using echinoids (EPS 1/RM/27; Environment Canada, 2011). The present method can be used to determine if one or more test sediments are toxic to the test organisms. The method does not provide instructions on conducting multi-concentration or dilutions of contaminated sediment and thereby, the test does not provide information on the degree, magnitude, or cause of toxicity.

Various criteria have been used by regulators or permittees to judge if samples of test sediment pass or fail a sediment toxicity test (Environment Canada, 1998; 2002; WSDOE, 2008). For instance, some investigators have employed two conditions for the determination of an environmentally significant response, those being, the response in the test sediment under evaluation must be greater than 20% different from the control response; and, a comparison between mean test sediment and mean reference responses be statistically significant (WSDOE, 2008).

In keeping with other Environment Canada (1998) interpretations, the following two-part guidance is used when judging if samples of test sediment "pass" or "fail" a test for sediment toxicity, using this reference method:

1. Test sediment from a particular sampling station and depth is judged to have failed this sediment toxicity test if the % normal larvae development for the replicate groups of test organisms exposed to this sediment is more than 20% lower than that in the reference sediment and is significantly different ($P < 0.05$).[44]

2. In the absence of an acceptable reference sediment, the test sediment is judged to have failed this sediment toxicity test if the % normal larval development for the replicate groups of test organisms exposed to this sediment is more than 30% lower than that in the control sediment and is significantly different ($P < 0.05$).

As part of the Ocean Disposal permitting program the pass/fail criterion can designate when dredged material is considered acceptable for ocean disposal. A failure (of this test alone or in combination with other acute, sublethal, or bioaccumulation tests) can lead proponents to investigate remediation or dredged material management options other than open-water disposal.

[44] For example, if Pn for a reference sediment was 80%, the effect "cut off" would be 64%.

Reporting Requirements

Each test-specific report must indicate if there has been any deviation from any of the "must" requirements delineated in Sections 2 to 6 of this *reference method*, and, if so, provide details of the deviation. The reader must be able to establish from the test-specific report whether the conditions and procedures preceding and during the test rendered the results valid and acceptable for the use intended. Section 7.1 provides a list of the items that must be included in each test-specific report. A list of items that must either be included in the test-specific report, provided separately in a general report, or held on file for a minimum of five years, is found in Section 7.2. Specific monitoring programs or regulations might require selected test-specific items listed in Section 7.2 (e.g., details regarding the test material and/or explicit procedures and conditions during sample collection, handling, transport, and storage) to be included in the test-specific report, or might relegate certain test-specific information as data to be held on file.

Procedures and conditions that are common to a series of ongoing tests (e.g., routine toxicity tests for monitoring or *compliance* purposes) and consistent with specifications in this document, may be referred to by citation or by attachment of a general report which outlines standard laboratory practice.

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

- a record of the chain-of-continuity for samples tested for regulatory or monitoring purposes;
- a copy of the record of acquisition for the sample(s);
- certain chemical analytical data on the sample(s);
- bench sheets for the observations and measurements recorded during the test;
- bench sheets and warning chart(s) for the reference toxicity tests;
- detailed records of the source and health of the adult echinoids used to provide gametes 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.

7.1 Minimum Requirements for Test-Specific Report

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

7.1.1 Test Substance or Material

- brief description of sample type (e.g., dredged material, reference sediment, contaminated or potentially contaminated field-collected sediment or control sediment) or coding, as provided to the laboratory personnel;
- information on labelling or coding for each sample; and
- date of sample collection; name of person(s) collecting sample; date and time sample received at test facility.

7.1.2 Test Organisms

- species, source, and date of collection;
- brief description of holding time and conditions, for adults;
- percentage of mortalities among adults shipped and held ≤ 3 days before spawning (i.e., 7 days prior to shipping mortality rate information

provided by the supplier and/or daily mortality rates of adults once received and within 3 days prior to spawning);

- the average daily and cumulative 7-day percentage of mortalities among the adults being acclimated and held for longer periods (i.e., > 3 d) at the laboratory; and

- any unusual appearance, behaviour, or treatment of adults or gametes, before the test is started.

7.1.3 Test Facilities and Apparatus

- name and address of test laboratory;

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

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

7.1.4 Control Sediment and Control/Dilution Water

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

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

- source(s) of sediment used as control sediment.

7.1.5 Test Method

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

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

- name and citation of programs and methods used for calculating statistical endpoints.

7.1.6 Test Conditions and Procedures

- design and description if any deviation from, or exclusion of, any of the procedures and conditions specified in this document;

- number of discrete samples per treatment; number of replicate test chambers for each treatment; number and description of treatments in each test including the control(s);

- volume of sediment and overlying water in each test chamber;

- number of males and females used to pool sperm and eggs;

- brief statement indicating whether a gamete viability check and pre-test was performed;

- sperm:egg ratio used in testing, including estimated initial sperm stock density;

- number of eggs per test chamber and egg density;

- pre-test fertilization period (minutes);

- mean fertilization (%) at test start;

- time between fertilization and test initiation;

- period of time test vessels (e.g., larvae) are stored prior to enumerating results;

- date when test was started and ended; statement of test duration;

- date when each test chamber was scored;

- each sediment sample-percent coarse-grained sediment (e.g., particles > 2.0 mm), percent sand (e.g., particles > 0.063 to ≤ 2.0 mm), percent silt (e.g., particles > 0.002 to ≤ 0.063 mm), percent clay (e.g., particles ≤ 0.002 mm), percent water content (moisture), total organic carbon content, total ammonia, sulphide, and pH;

- indicate if any samples of test sediment (including reference sediment) were press-sieved to remove large particles and/or detritus or indigenous organisms, including the procedure and mesh size used if applied;

- at the start and end of the test, temperature, salinity, dissolved oxygen content, and pH in the overlying water in the two replicates dedicated for this purpose for each sample under evaluation;

- at the start and end of the test, total and un-ionized ammonia in all sediment exposures, including in the corresponding "water-only" control tested in conjunction with the sediment samples;

- for the reference toxicant test, at the start and end of the test, total and un-ionized ammonia in the corresponding "water-only" control tested in conjunction with the reference toxicant; and

- date when the reference toxicity test was performed and brief statement indicating whether it was performed under the same experimental conditions as those used with the test sample(s); and description of any deviation from or exclusion(s) of any of the procedures and conditions specified for the reference toxicity test in this document.

7.1.7 Test Results

- at start of test, the number (and percentage) of fertilized and unfertilized eggs in each of six replicate "water-only" vials, including the mean (± SD) (mean represents the "En" value used when calculating the test endpoint for each treatment);

- at test end:

  - % normal larvae (P_n) (± SD) in "water-only controls";

  - for all treatments and "water-only" control, number of (i) normal larvae (prism or pluteus) including mean (± SD), and (ii) abnormal larvae; in each replicate, including mean (± SD);

  - % normal larvae (P_n) (± SD) in all treatments and controls;

- mean recovery success rate for each treatment;

- any outliers, and the justification for their removal;

- type and results from all statistical analysis and comparisons of the data;

- the duration and results of any toxicity tests with the reference toxicant(s) performed at the same time of the test, together with the geometric mean value (± 2 SD) for the same reference toxicant(s) as derived at the test facility in previous tests with the same species; and

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

7.2 Additional Reporting Requirements

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

7.2.1 Test Substance or Material

- records of sample chain-of-continuity and log-entry sheets; and

- conditions (e.g., temperature, in darkness, in sealed container) of sample upon receipt and during storage.

7.2.2 Test Organisms

- records of taxonomic confirmation of species; all supplier's records provided with each shipment, including number of test organisms shipped, as well as date and time of shipment; temperature, dissolved oxygen concentration, and pH of any water in shipment container(s) [or of shipment container(s) if adults are shipped dry] when shipped and upon arrival;

- detailed description of holding conditions and procedures for adults, including: facilities and apparatus; lighting; water source and quality; water pre-treatment; water exchange rate and procedure for replacement; density of adults in tanks; temperature in those tanks;

- type and source of food for adults in tanks; procedures for preparation and storage of food; feeding procedures, frequency, and ration;

- incidence of diseased adults; details regarding any treatment of adults for disease;

- records of checks and findings for spawning success and time, and fertilization success rates before test; and

- procedures and conditions for inducing spawning and collecting gametes, and or adding them to test vessels.

7.2.3 Test Facilities and Apparatus

- description of systems for providing lighting and regulating temperature during the incubation of test vessels;

- description of pipettes and disposable tips used to prepare test concentrations and transfer test organisms; and

- description and calibration record of balance used for weighing sediment.

7.2.4 Control Sediment and Control/Dilution Water

- procedures for pre-treatment of control sediment (e.g., sieving, settling of sieved fines);

- any water pre-treatment (i.e., procedures and conditions for salinity adjustment, filtration, sterilization, temperature adjustment, de-gassing, aeration);

- sediment and water storage conditions and duration before use; and

- measured water quality variables before or at time of starting the test.

7.2.5 Test Method

- description of laboratory's previous experience with this reference method and training records of technicians qualified to conduct the test;

- procedures used in preparing and storing stock and/or test solutions of chemicals; and

- methods used (with citations) for chemical analyses of test material (sediment and overlying water); details concerning sampling, sample/solution preparation and storage, before chemical analyses.

7.2.6 Test Conditions and Procedures

- photoperiod, light source, and intensity adjacent to surface of overlying water in test vials;

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

- holding conditions for preserved vials (i.e., before and during scoring);

- any other chemical measurements (e.g., contaminant concentrations, acid volatile sulphides, biochemical oxygen demand, chemical oxygen demand, total inorganic carbon, cation exchange capacity, redox potential, pore water hydrogen sulphide) made before the test on the test sediment (including control and reference sediment);

- any other observations or analyses made on the test sediment (including samples of control or reference sediment; e.g., qualitative or quantitative data regarding indigenous macrofauna or detritus, geochemical analyses); and

- appearance of each sediment sample (or mixture thereof) and of the overlying water in the test vials; changes in appearance noted during the test.

7.2.7 Test Results

- results of the gamete viability check and pre-test;

- values from presumptive test end;

- graphical presentation of data;

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

- any other observed effects; and

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

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

Title of Biological Test Method or Guidance Document	Report Number	Publication Date	Applicable Amendments
A. Generic (Universal) Biological Test Methods			
Acute Lethality Test Using Rainbow Trout	EPS 1/RM/9	July 1990	May 1996 and May 2007
Acute Lethality Test Using Threespine Stickleback *(Gasterosteus aculeatus)*	EPS 1/RM/10	July 1990	March 2000
Acute Lethality Test Using *Daphnia* spp.	EPS 1/RM/11	July 1990	May 1996
Test of Reproduction and Survival Using the Cladoceran *Ceriodaphnia dubia*	EPS 1/RM/21 2nd Edition	February 2007	–
Test of Larval Growth and Survival Using Fathead Minnows	EPS 1/RM/22 2nd Edition	February 2011	–
Toxicity Test Using Luminescent Bacteria *(Photobacterium phosphoreum)*	EPS 1/RM/24	November 1992	–
Growth Inhibition Test Using a Freshwater Algae	EPS 1/RM/25 2nd Edition	March 2007	–
Acute Test for Sediment Toxicity Using Marine or Estuarine Amphipods	EPS 1/RM/26	December 1992	October 1998
Fertilization Assay Using Echinoids (Sea Urchins and Sand Dollars)	EPS 1/RM/27 2nd Edition	February 2011	–
Toxicity Tests Using Early Life Stages of Salmonid Fish (Rainbow Trout)	EPS 1/RM/28 2nd Edition	July 1998	–
Test for Survival and Growth in Sediment Using the Larvae of Freshwater Midges *(Chironomus tentans* or *Chironomus riparius)*	EPS 1/RM/32	December 1997	–

[a] These documents are available for purchase from Communications Services, Environment Canada, Ottawa, Ontario, K1A 0H3, Canada. Printed copies can also be requested by email at: epspubs@ec.gc.ca. These documents are freely available in PDF at the following website: www.ec.gc.ca/faunescience-wildlifescience/default.asp?lang=En&n=0BB80E7B-1. For further information or comments, contact the Chief, Biological Assessment and Standardization Section, Environment Canada, Ottawa, Ontario K1A 0H3.

Title of Biological Test Method or Guidance Document	Report Number	Publication Date	Applicable Amendments
A. Generic (Universal) Biological Test Methods (continued)			
Test for Survival and Growth in Sediment and Water Using the Freshwater Amphipod *Hyalella azteca*	EPS 1/RM/33 2nd Edition	June 2012	–
Test for Measuring the Inhibition of Growth Using the Freshwater Macrophyte, *Lemna minor*	EPS 1/RM/37 2nd Edition	January 2007	–
Test for Survival and Growth in Sediment Using Spionid Polychaete Worms *(Polydora cornuta)*	EPS 1/RM/41	December 2001	–
Tests for Toxicity of Contaminated Soil to Earthworms *(Eisenia andrei, Eisenia fetida,* or *Lumbricus terrestris)*	EPS 1/RM/43	June 2004	June 2007
Tests for Measuring Emergence and Growth of Terrestrial Plants Exposed to Contaminants in Soil	EPS 1/RM/45	February 2005	June 2007
Test for Measuring Survival and Reproduction of Springtails Exposed to Contaminants in Soil	EPS 1/RM/47 2nd Edition	February 2014	–
Test for Growth in Contaminated Soil Using Terrestrial Plants Native to the Boreal Region	EPS 1/RM/56	August 2013	–
B. Reference Methods[b]			
Reference Method for Determining Acute Lethality of Effluents to Rainbow Trout	EPS 1/RM/13 2nd Edition	December 2000	May 2007
Reference Method for Determining Acute Lethality of Effluents to *Daphnia magna*	EPS 1/RM/14 2nd Edition	December 2000	–
Reference Method for Determining Acute Lethality of Sediment to Marine or Estuarine Amphipods	EPS 1/RM/35	December 1998	–
Reference Method for Determining the Toxicity of Sediment Using Luminescent Bacteria in a Solid-Phase Test	EPS 1/RM/42	April 2002	–

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

Title of Biological Test Method or Guidance Document	Report Number	Publication Date	Applicable Amendments
C. Supporting Guidance Documents			
Guidance Document on Control of Toxicity Test Precision Using Reference Toxicants	EPS 1/RM/12	August 1990	–
Guidance Document on Collection and Preparation of Sediment for Physicochemical Characterization and Biological Testing	EPS 1/RM/29	December 1994	–
Guidance Document on Measurement of Toxicity Test Precision Using Control Sediments Spiked with a Reference Toxicant	EPS 1/RM/30	September 1995	–
Guidance Document on Application and Interpretation of Single-Species Tests in Environmental Toxicology	EPS 1/RM/34	December 1999	–
Guidance Document for Testing the Pathogenicity and Toxicity of New Microbial Substances to Aquatic and Terrestrial Organisms	EPS 1/RM/44	March 2004	–
Guidance Document on Statistical Methods for Environmental Toxicity Tests	EPS 1/RM/46	March 2005	June 2007
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	–

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Procedural Variations for Echinoid Embryo/Larval Toxicity Tests, as Described in Canadian and United States Methodology Documents[a]

Source documents are listed here chronologically by originating agency rather than by author(s).

USEPA and PSWQA. 1995. represents USEPA and PSWQA, Echinoderm Embryo Sediment Bioassay, p. 40–48, In: *Recommended Guidelines for Conducting Laboratory Bioassays on Puget Sound Sediments*, United States Environmental Protection Agency (Seattle, Washington) and Puget Sound Water Quality Authority (Olympia, Washington), July 1995.

APHA *et al.* 2005. represents APHA, AWWA, and WEF (American Public Health Association, American Water Works Association, and Water Environment Federation), Echinoderm Embryo Development Test, 8810 D, p. 8-143 – 8-145, In: *Standard Methods for the Examination of Water & Wastewater*, 21st ed., Washington, DC.

ASTM. 2007. represents ASTM (American Society for Testing and Materials), Standard Guide for Conducting Static Acute Toxicity Tests with Echinoid Embryo, E 1563-98 (Reapproved 2004), p. 720–741. In: *2007 Annual Book of ASTM Standards*,, Vol. 11.06, ASTM, West Conshohocken, Pennsylvania, USA.

SCCWRP. 2008. represents SCCWRP, Standard Operating Procedure T04: Sea Urchin, *Strongylocentrotus purpuratus*, Embryo Development Test, Southern California Coastal Water Research Project, Costa Mesa, CA, 2004 SOP revised October 10, 2008.

EC. 2013. represents the current EC (Environment Canada) method.

[a] Based on documents available as of June 2013.

1. Test Species, and Test Material or Substance

Document	Recommended Test Species[a]	Test Material or Substance
USEPA and PSWQA 1995	AP, SD, SP, DE	sediment
APHA *et al.* 2005	AP, SD, SP, DE	sediment, effluent, receiving water, chemical
ASTM 2007	AP, SD, SP, DE	sediment (Annex A1), effluent, leachate, surface water, particulate matter, chemical
SCCWRP 2008	SP	effluent in marine waters
EC 2013	SP, LP, DE	sediment

[a] AP—*Arbacia punctulata* (Atlantic purple sea urchin); SD—*Strongylocentrotus droebachiensis* (green sea urchin; circumpolar species); SP—*Strongylocentrotus purpuratus* (Pacific purple sea urchin); LP—*Lytechinus pictus* (white sea urchin, found from southern California to Panama); DE—*Dendraster excentricus* (eccentric sand dollar; Pacific species)

2. Source of Adults

Document	Source of Adults
USEPA and PSWQA 1995	adults collected from uncontaminated sites; if from a commercial harvester, original collection area to be identified
APHA *et al.* 2005	collect from the field during natural spawning season; organisms from a commercial supplier may be used
ASTM 2007	adults are brought into the laboratory and identified to species
SCCWRP 2008	adults purchased through commercial suppliers or collected from uncontaminated, rocky intertidal areas
EC 2013	adults may be obtained from commercial suppliers or collected by laboratory personnel; marine collection site from which adults are obtained should be uncontaminated

3. Transportation, Holding, and Acclimation of Adults

Document	Guidance on Transportation, Holding, and Acclimation of Adults
USEPA and PSWQA 1995	transport within 24 h of collection or purchase; keep cool while in transit; place in flowing seawater upon receipt; temperature-controlled, aerated seawater is needed; flow rates typically > 28 L/h per individual; sand dollars held on a bed of sand; feed a natural or cultivated alga; clean holding tanks several times per week; remove dead specimens immediately
APHA *et al.* 2005	avoid sudden or extreme variations in temperature and salinity during transport; hold using either a flow-through seawater supply or a recirculating filter system; feed sea urchins *ad libitum* brown macroalgae and substitute romaine lettuce or commercial fish food if fresh seaweed is unavailable; holding temperature varies with species and should be similar to that at the collection site; hold at 28 to 34 g/kg salinity
ASTM 2007	acclimate animals to laboratory water over a period of two or more days; change temperature at a rate of ≤ 2°C/day and salinity at a rate ≤ 1 g/kg per day; observe daily; remove dead or stressed animals daily; if gonads are not ripe, adults should be held and fed until they reach a suitable reproductive state
SCCWRP 2008	animals best transported "dry" (surround with moist seawater or damp paper towels); hold at collection or culture temperature; maintain in complete darkness at 12°C to 15°C and at a salinity of 32 ± 2 g/kg; best to provide flowing seawater at ~5 L/min; preferred food is *Macrocystis*; decaying food and fecal material should be removed
EC 2013	follow guidance for holding and acclimation in second edition of Report EPS 1/RM/27; all adults used to provide gametes for a test must be derived from the same population and source, and must be obtained from sexually mature adults; inspect daily and remove dead or diseased animals upon observation; adjust temperature at a rate ≤ 3°C/day upon receipt of animals in the laboratory; holding temperature is species-specific; recommend acclimating adults to test temperature for a minimum of 3 days preceding gamete collection; average salinity of the holding water should be 28 to 34 g/kg, but preferably 30 to 32 g/kg; adjust salinity gradually (≤ 1 g/kg per day) upon receipt of adults; organisms to be held in the laboratory for extended periods of time, the rate of any salinity adjustment should be ≤ 3 g/kg per day and must be ≤ 5 g/kg per day; normal laboratory lighting at low intensity is acceptable

4. Spawning and Fertilization

Document	Guidance on Spawning and Fertilization
USEPA and PSWQA 1995	induce spawning by injecting KCl; initiate fertilization within 1 h of spawning, using a sperm:egg ratio of ≤ 2000:1; adjust density of fertilized eggs to 20 000 to 30 000 per mL when > 90% of eggs show membrane formation within 10 to 15 minutes
APHA *et al.* 2005	induce spawning by injecting KCl or, for suitable species (primarily *A. punctulata),* using electrical stimulation; collect sea urchin sperm in the "dry" condition; activate sperm by dilution in seawater and use within 30 min; use a sperm:egg ratio of 200:1 to 1,000:1; assess fertilization rate after 10 min and add more sperm if < 90% fertilized; should restart using different gametes if fertilization is < 90% at that time
ASTM 2007	induce spawning by injecting KCl or using electrical stimulation; collect sperm by wet or dry spawning, and eggs by wet spawning; adjust egg density to 20 to 50 eggs/mL before adding sperm; use 10^5 to 10^7 sperm/mL in the final solution
SCCWRP 2008	induce spawning by injecting KCl; only collect gametes for the first 15 min after each animal starts releasing; use a sperm:egg ratio of 500:1; adjust density of fertilized eggs to 2500 eggs/mL; if fertilization is not ≥ 90% after 10 min, add additional sperm and recheck after another 10 min; if fertilization is still not ≥ 90%, restart using different gametes
EC 2013	follow guidance for spawning and fertilization found in second edition of Report EPS 1/RM/27; fertilization rate must be ≥ 90% for test to proceed; sperm:egg ratio is species-dependent; a target embryo (i.e., newly fertilized eggs) density of ~200 eggs per 200 µL aliquot (in 10 mL exposure volumes) is required (equivalent to a density of 20 eggs per 20 µL or 100 000 eggs per 100 mL); this represents the addition of ~200 eggs (≥ 90% fertilized and < 10% unfertilized) to each 10-mL volume

5. Test Chambers and Initial Contents

Document	Test Chamber	Amount of Sediment	Volume of Seawater
USEPA and PSWQA 1995	1-L jar or beaker[a]	18 g (wet wt)	900 mL
APHA *et al.* 2005	glass, various[b]	not indicated	variable[f]
ASTM 2007	1-L jar or beaker[c]	18 g (wet wt)	900 mL
SCCWRP 2008	20-mL glass vial[d]	not applicable	9.9 mL
EC 2013	20-mL glass vial[e]	0.5 g (wet wt)	10 mL

[a] Use a standard 1-L glass jar or beaker (10-cm internal diameter); cover with an 11.4-cm-diameter watchglass.

[b] Use glass chambers of 10-mL to 1-L capacity; cover loosely or seal; disposable scintillation vials are suitable.

[c] Use either 1-L glass beakers or ~950-mL glass canning jars; loosely cover with watchglass of non-toxic plastic (if beaker), or a lid with a Teflon liner (if a canning jar); use of smaller chambers (and equivalent volumes of sediment and overlying water) might be satisfactory.

[d] Use 20-mL glass scintillation vials with polypropylene caps.

[e] Use 20-mL glass scintillation or shell vials; loosely cover with plastic film or sheet of transparent Plexiglass; tightly seal (e.g., using Saran wrap) if test sediment contains appreciable volatiles.

[f] Depends on volume of test chamber; volume used should provide an initial density of ~25 embryos/mL.

6. Pre-Test Manipulation and Conditions for Contents of Test Chambers

Document	Initial Agitation	Pre-Test Incubation Conditions
USEPA and PSWQA 1995	shake vigorously for 10 seconds	allow to settle for 4 h before adding embryos
APHA *et al.* 2005	not indicated[a]	not indicated[a]
ASTM 2007	stir or shake vigorously for 10 seconds	allow to settle for 4 h before adding embryos
SCCWRP 2008	not applicable [b]	not applicable [b]
EC 2013	mix by agitating individual vials on a vortex mechanical shaker at a rate of 1800 rpm for 10 seconds	incubate overnight

[a] The test method is designed for various test materials (included sediment) or substances, and does not address pre-test treatment and conditions when testing samples of sediment or other solid material.

[b] The test is designed for effluent in marine waters, and is not intended for measuring the toxicity of samples of sediment.

7. Test Treatments and their Replication

Document	Test Sediment (Contaminated)	Reference Sediment	Control Sediment	"Water-Only" Controls
USEPA and PSWQA 1995	5 + 1[a]	5 + 1[a,d]	yes[h]	5 + 5[j]
APHA *et al.* 2005	5 + 1[a]	NI[e]	NI	5 + 5[k]
ASTM 2007	5 + 1[a]	5 + 1[a,f]	5 + 1[a,i]	6 + 5[l]
SCCWRP 2008	NA[b]	NA	NA	5 + 1[n]
EC 2013	6 + 2[c]	6 + 2[c,g]	6 + 2[c,i]	6 + 2 + 6 + 9[m]

[a] Besides the five replicates for each sample of sediment that are used for biological data, an additional replicate of each sample is used for monitoring the quality of the overlying seawater in the test chambers.

[b] Not applicable.

[c] The seventh and eighth (or more, depending on analytical requirements) replicates are used for monitoring the chemistry of the overlying water at the beginning and end of the test.

[d] The design of field surveys might include a reference sediment from an area known to be free of chemical contamination. This provides a basis for comparison of potentially toxic and nontoxic conditions (no guidance is offered in this respect).

[e] Not indicated.

[f] If field-collected sediments are being tested, reference sediments should be tested in addition to control sediments, or reference sediments can be considered the control sediments.

[g] If available and included as part of the field sampling program, a minimum of eight replicates representing one or more samples of reference sediment should be incorporated as part of the test design.

[h] Brief mention is made of the use of control sediment, although no guidance is provided on the number of replicates or the application of data for this sediment when judging sample toxicity.

[i] The test design requires (a "must") the use of control sediment, as well as "water-only" controls.

[j] Two "water-only" control series are prepared. One set is used as a "sacrificial control" to monitor embryo development.

[k] Besides the five (or more) replicate controls that are carried forward until test end, at least five additional controls are required when using the "complete count method" in order to estimate the actual number of embryos in each test chamber at the start of the test.

[l] Six "water-only" controls are used for the biological data and (in the sixth test chamber) water-quality data. An additional five "water-only" controls are required for an estimate of the number of embryos in each test chamber at the start of the test, and to monitor development.

[m] Twenty-three (or more) "water-only" vials are used as follows: six vials are used to provide an estimate of the number of fertilized eggs (embryos) in each treatment at the start of the test (En), which is used when calculating % normal larvae for each treatment at the end of the test. Two vials are used for monitoring the quality of water representing the "water-only" control, at the beginning and end of the test. Six "monitoring" vials are used to determine the % normal larvae in the seawater used as overlying water for the "water-only control," during 1-h hour immediately preceding the presumptive end of the test. The "final" nine "water-only" vials are used to confirm the test met the validity criterion. One set of vials (6 final "water-only" controls) is paired with the sediment samples and 3 "water-only" controls must be paired with the reference toxicant.

[n] Besides five replicate control solutions, a sixth replicate control should be set up for physical/chemical measurements.

8. Starting the Test

Document	Age of Organisms at Start of Test	Aliquot of Eggs[a] Transferred	Number of Eggs[a] per Test Chamber	Initial Density of Eggs[a] in Test Chamber
USEPA and PSWQA 1995	within 2 h of fertilization	1.0 mL	~25 000	~28 per mL seawater
APHA *et al.* 2005	2 to 4 h after fertilization	varies[b]	varies[b]	~25 per mL seawater
ASTM 2007	within 4 h of fertilization	NI[c]	20 000–40 000	22 to 44 per mL seawater
SCCWRP 2008	within 1 h of fertilization	0.1 mL	~250	~25 per mL seawater
EC 2013	2 to 4 h after fertilization	200 µL	~200	~20 per mL seawater

[a] Including unfertilized eggs as well as fertilized ones.

[b] Depends on size of test chamber (10-mL to 1-L capacity).

[c] Not indicated. Volume of aliquot to be < 1% of the total volume of liquid in the test chamber.

9. Conditions During the Test

Document	Sediment:Water Ratio	Temperature[a]	Salinity	Aeration	Lighting
USEPA and PSWQA 1995	0.2 g/10 mL	15 ± 1°C	28 ± 1 g/kg	normally no[g]	14-h L:10-h D
APHA *et al.* 2005	NI[b]	20°C for AP 15°C for SP & DE 12°C for SD	28 to 34 g/kg	NI	ambient[h]
ASTM 2007	0.2 g/10 mL	20°C for AP 15°C for DE 12 or 14°C for SP 12°C for SD[d]	27 to 36 g/kg[e]	normally no[h]	NI
SCCWRP 2008	NA[c]	15 ± 1°C	34 to 2 g/kg	no	12-h L:12-h D
EC 2013	0.5 g/10 mL	15 ± 1°C for SP or DE 20 ± 1°C for LP[d]	30 to 2 g/kg[f]	no	16-h L:8-h D[i]

[a] AP—*Arbacia punctulata*; SP—*Strongylocentrotus purpuratus*; SD—*Strongylocentrotus droebachiensis*; LP—*Lytechinus pictus*; DE—*Dendraster excentricus*.

[b] Not indicated.

[c] Not applicable; test is designed for aqueous substances (i.e., chemicals).

[d] In addition, the instantaneous temperature must be within 3°C of the daily mean temperature at all times.

[e] In addition, the salinity should not vary by more than 1 g/kg among treatments or any renewals during a test.

[f] The salinity of the overlying water must not be adjusted at the start of the test nor at any time thereafter.

[g] If dissolved oxygen in any test chamber declines below 60% of saturation, gently aerate all test chambers until test end. To aerate, use a pipette inserted mid-depth in the water column, with an air flow of ~100 bubbles/minute.

[h] Ambient laboratory light levels and photoperiods are adequate for all species.

[i] An intensity of 500 to 1000 lux, adjacent to the surface of the overlying water in each test chamber, is recommended.

10. Test Duration

Document	Guidance on Test Duration
USEPA and PSWQA1995	end test at 48 h or when > 90% of embryos in the seawater control have reached the 4-armed pluteus stage (whichever is later, and within 48 to 96 h)
APHA *et al.* 2005	*A. punctulata*, 48 h at 20°C; *D. excentricus*, 72 h at 15°C; *S. purpuratus*, 72 h at 15°C or 96 h at 12°C; *S. droebachiensis*, 96 h at 12°C [a]
ASTM 2007	test duration depends on species and temperature, and should be either 48 h, 72 h, or 96 h[b]
SCCWRP 2008	end the test at 72 h
EC 2013	presumptive test end is 48 h if *L. pictus*, and 72 h if *D. excentricus* and 96-h if *S. purpuratus*[c]

[a] These are target times; a few extra hours might be allowed to help assure that most (> 90%) control larvae have attained the normal pluteus stage.

[b] Test duration is based on the time for ≥ 70% of the embryos in the control solutions to develop to the pluteus stage. Continue the test beyond the usual time (for that species and temperature) if necessary, but record this time extension as a test deviation.

[c] During the one hour preceding the species-specific times, determine % normal larvae in six of the "water-only" controls; if normal larvae is ≥ 70%, the test must be terminated; if at presumptive test end, % normal is < 70%, extend the test for an additional 24 h in order to ensure the test validity criteria will be met (i.e., end test at 72 h if *L. pictus*; at 96 h if *D. excentricus*; and at 120 h if *S. purpuratus*).

11. Monitoring Quality of Overlying Seawater During Test

Document	Variables Monitored and Frequency
USEPA and PSWQA 1995	daily measurements of temperature, pH, salinity, and dissolved oxygen, in replicate of each treatment dedicated for this purpose; measure sulphides and ammonia at start and end of test
APHA *et al.* 2005	measure initial and final water quality (variables not specified) for each treatment
ASTM 2007	daily measurements of pH, salinity, dissolved oxygen, and temperature; in at least one test chamber, measure temperature several times per day or use a maximum-minimum thermometer; recommend measuring ammonia and sulphide at start and end of test (must measure if sediment with high organic content)
SCCWRP 2008	measure pH, salinity, and dissolved oxygen at start and end of test; measure temperature daily
EC 2013	must measure temperature, pH, salinity, dissolved oxygen, and ammonia (total and, by calculation, un-ionized) at start and end of test

12. Ending the Test

Document	Sample Preserved	Preservative	Storage Time	Counting and Scoring
USEPA and PSWQA 1995	3 aliquots[a]	5% buffered formalin	archive 2 aliquots[f]	use Sedgewick-Rafter cells; % survival, normal/abnormal larvae[i]
APHA *et al.* 2005	aliquot or vial contents[b]	5% buffered formalin	NI	use Sedgewick-Rafter cells or count directly (inverted microscope)[j]
ASTM 2007	aliquot(s)[c]	5% buffered formalin	NI	use Sedgewick-Rafter cells[k]
SCCWRP 2008	vial contents[d]	4% buffered formalin or 1% glutaraldehyde	NI[g]	use Sedgewick-Rafter cells or count directly (inverted microscope)[l]
EC 2013	transfer via pipette[e]	0.5% glutaraldehyde	4 weeks[h]	use Sedgewick-Rafter cell or count directly (in-vial with an inverted microscope)[m]

[a] Water and organisms in each 1-L test chamber are carefully and gently stirred to suspend them, liquid is decanted, and three 10-mL aliquots are removed by pipette and placed in 10-mL screw-cap vials followed by the addition of preservative.

[b] Preservative is added directly to the test chamber if vials or culture tubes are used. Otherwise, mix chamber contents, transfer a 10-mL aliquot to a vial, and add preservative.

[c] Water and organisms in each 1-L test chamber are carefully decanted, mixed to re-suspend organisms, and one or more 10-mL aliquots removed by pipette and placed in 10-mL screw-cap vials followed by the addition of preservative.

[d] The preservative is added directly to each test vial, at the end of the test.

[e] Water overlying sediment in each test vial is carefully transferred into a new scintillation or shell vial using a 10-mL pipette with a minimum $\geq$ 2 mm opening. Thereafter, 1 mL of 0.5% glutaraldehyde is added to preserve the embryos and larvae for later examination.

[f] The contents of one of the three aliquots are counted and scored, while the other two are archived. Storage time is not indicated.

[g] Not indicated.

[h] Preserved organisms must be counted and scored within 4 weeks of test end.

[i] Normal and abnormal larvae are enumerated separately. Percent survival is based on number of surviving relative to initial count.

[j] Either count all organisms (complete count method) or do a relative count of at least 100 organisms (embryos and larvae); score as normal pluteus larvae, abnormal larvae, deformed embryos, normal-appearing embryos, and uncleaved fertilized eggs.

[k] Count all embryos and larvae found in each preserved test vial; score as normal (i.e., normally developed pluteus larvae) and abnormal (i.e., grossly deformed pluteus larvae or embryos that failed to develop into pluteus larvae).

[l] Count the first 100 embryos (and larvae) encountered using a multi-unit hand counter; do not count unfertilized eggs; score as normal larvae, abnormal larvae, and earlier life stages (i.e., fertilized eggs and embryos at the blastula or gastrula stage).

[m] Count the number of normal larva (prism or pluteus) and abnormal larva. Unfertilized eggs observed must not be counted or scored.

13. Biological Endpoints

Document	Normal Larvae (%)	Survival (%)	Abnormal Survivors (%)	Multi-Concentration Test
USEPA and PSWQA 1995	yes[a]	yes[e]	yes[f]	NI[g]
APHA *et al.* 2005	yes[b]	no	no	NI
ASTM 2007	yes[c]	no	no	EC50[h]
SCCWRP 2008	yes[d]	no	no	NOEC[i]
EC 2013	yes[b]	no	no	NI[g]

[a] The primary biological endpoint for the test is % normal larvae. This endpoint is calculated by dividing the number of normal larvae found in a treatment at test end by the number of embryos representing that treatment at the start of the test, and multiplying by 100. The endpoint represents the combined effects of the contaminant(s) on survival and (normal) development.

[b] The biological endpoint for this test is % normal larvae. Using the "complete count method", the % normal larvae is calculated by dividing the number of normal larvae found in a treatment at test end by the number of embryos representing that treatment at the start of the test, and multiplying by 100.

[c] ASTM (2007) calculates and expresses the results for the test as the percentage of embryos in each replicate of a treatment that did not result in normal pluteus larvae. This calculation is essentially the same as the one for *% normal larvae*, as it is based on the combined effects of the contaminant(s) on survival and development, and it uses the data for the number of embryos at the start of the test and the number of normal larvae at the end of the test.

[d] The biological endpoint for the test is based on % normal larvae. It is determined using the counts of 100 larvae, blastulae, gastrulae, and fertilized eggs in each replicate; any unfertilized eggs observed are not counted.

[e] A secondary biological endpoint for the test is % survival. According to USEPA and PSWQA (1995), this endpoint is calculated by dividing the number of surviving test larvae by the number of control larvae, and multiplying by 100.

[f] Another secondary biological endpoint for the test is abnormal survivors (%). According to USEPA and PSWQA (1995), this endpoint is calculated by dividing the number of (surviving) abnormal larvae by the number of normal and abnormal survivors, and multiplying by 100.

[g] Not indicated (except for a reference toxicity test).

[h] For a multi-concentration test, the EC50 (Median Effective Concentration) is calculated using the mean values for % normal larvae determined for each concentration.

[i] For a multi-concentration test, the NOEC (No Observed Effect Concentration) is calculated using the mean values for % normal larvae determined for each concentration.

14. Reference Toxicity Test

Document	Chemical(s)	Required?	Biological Endpoint	Statistical Endpoint
USEPA and PSWQA 1995	cadmium chloride sodium dodecyl sulphate	yes[b,c]	% normal larvae	EC50[f]
APHA *et al.* 2005	NI[a]	NI	NI	NI
ASTM 2007	NI	no[d]	NI	NI
SCCWRP 2008	Cupric chloride	yes[e]	% normal larvae	NOEC[g]
EC 2013	Copper (as copper sulphate or copper chloride)	yes[b,c]	% normal larvae	IC50[h]

[a] Not indicated.

[b] Must be performed in conjunction with the definitive sediment toxicity test.

[c] The test is performed without sediment. Otherwise, test procedures and conditions are identical to those used in the definitive embryo/larval sediment-contact test.

[d] A test using a reference toxicant might be useful for assessing the quality of embryos and larvae; such assessment can only be conducted simultaneously with the definitive toxicity test.

[e] Must be conducted concurrently with every effluent test.

[f] Median Effective Concentration.

[g] No Observed Effect Concentration.

[h] Inhibiting Concentration for a specified (50%) effect.

15. Requirement(s) for a Valid Test Result

Document	Test Requirement(s)
USEPA and PSWQA 1995	"at least 70 percent of the larvae [in 5 replicates of the seawater control] must achieve a normal pluteus stage"; "the recommended biological criterion of acceptability is that the larvae...must not incur more than 30-percent combined mortality/abnormality during 48-96 hours of exposure to the bioassay seawater"
APHA *et al.* 2005	"dilution water quality should be sufficient to produce ≥ 70% normal development (relative to initial number of embryos) in control samples"
ASTM 2007	"dilution water quality should be sufficient to produce ≥ 70% normal development (relative to initial number of embryos) in control samples"; "for a toxicity test with echinoid embryos to be acceptable, an average of ≥ 70% of the embryos maintained in the dilution water control chambers must be judged to be normally developed pluteus larvae"
SCCWRP 2008	"the percentage of abnormal embryos in the dilution water control cannot exceed 20% in order for the test to be considered acceptable" [a]
EC 2013	"for the findings of a test to be considered as valid, an average of ≥ 60% of the embryos must be judged to be normally developed larvae at the end of the test in the: 1. 'water-only' control, and 2. laboratory control sediment. The validity criteria must be met in the 'water-only' controls paired and scored in conjunction with the reference toxicant as well as the sediment samples" [b]

[a] SCCWRP (2008) also states that, if within 10 minutes of fertilization the fertilization success rate is not ≥ 90%, an additional volume of sperm is added. After waiting 10 minutes; the fertilization success rate is rechecked. If fertilization is still not 90%, the test must be restarted with different gametes.

[b] At the start of the test, the fertilization success rate must average ≥ 90% for the test to proceed.

Comparison of Species Sensitivity to Ammonia[a]

Species	Endpoint	Result[b]	Type
Dendraster excentricus	72-h EC50	2.39 mg/L (2.24–2.52)	Total[c]
	72-h EC50	51.8 µg/L (47.5–55.7)	Un-ionized (NH_3-N)[d]
	72-h EC50	3.59 mg/L (3.33–3.77)	Total
	72-h EC50	76.1 µg/L (66.6–83.1)	Un-ionized (NH_3-N)
Strongylocentrotus purpuratus	96-h EC50	3.31 mg/L (2.99–3.53)	Total
	96-h EC50	52.5 µg/L (47.0–56.2)	Un-ionized (NH_3-N)
Lytechinus pictus	48-h EC50	1.91 mg/L (1.53–2.15)	Total
	48-h EC50	78.6 µg/L (52.1–96.1)	Un-ionized (NH_3-N)

[a] Based on method development research conducted by Environment Canada's Atlantic Laboratory for Environmental Testing (ALET) and Pacific and Yukon Laboratory for Environmental Testing (PYLET).

[b] 95% confidence intervals in parentheses

[c] Endpoint was calculated using the average measured total ammonia at test start and test end per test exposure concentration.

[d] Un-ionized ammonia was calculated from the measured total ammonia at test start and test end and averaged per test exposure concentration.

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