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Biological Test Method: Fertilization Assay Using Echinoids (Sea Urchins and Sand Dollars)

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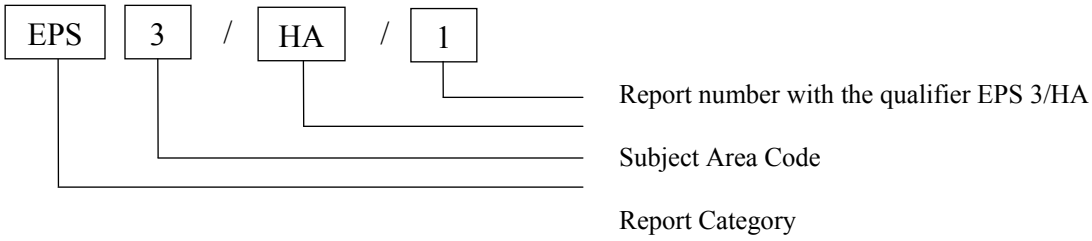

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Abstract

Methods recommended by Environment Canada for performing a sublethal marine toxicity test using gametes obtained from sea urchins or sand dollars are described in this report. This second edition of EPS 1/RM/27, published in 2011 supersedes the first edition that was published in 1992, and amended in 1997. It includes numerous procedural modifications as well as updated guidance and instructions to assist in performing the biological test method.

In the test, sperm are exposed to the substance or material being tested. Eggs are then added, and the success of fertilization under continued exposure to the same concentration of test substance or material is measured. The endpoint is decreased success of fertilization, described in terms of the concentration estimated to cause a specified percent inhibition (ICp). The test is quick and is among the most sensitive of marine sublethal toxicity tests. Because the gametes and the success of fertilization usually represent a sensitive part of the life cycle, this assay should be considered as a powerful and meaningful sublethal test. The test may be run with a minimum of seven concentrations of test substance or material to determine the threshold of effect, or with one concentration as a regulatory or pass/fail test.

*Recommended species for use in this test are the green sea urchin (*Strongylocentrotus droebachiensis*) found on the Atlantic, Pacific and Arctic coasts of Canada, the Pacific purple sea urchin (*Strongylocentrotus purpuratus*), the eccentric sand dollar (*Dendraster excentricus*) found in the Pacific, the Atlantic purple sea urchin commonly called Arbacia (*Arbacia punctulata*), and the white sea urchin from California (*Lytechinus pictus*).*

Procedures are given 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 3 days of arrival at the laboratory), and for obtaining sperm and eggs for a test. General or universal conditions and procedures are outlined for testing a variety of materials or substances for their effect on echinoid fertilization. Additional conditions and procedures are specific for testing sample(s) of chemical, effluent, receiving water, leachate, elutriate, or liquid derived from sediment or similar solid material. Instructions are included for test facilities, handling and storing samples, preparing test solutions and initiating tests, specific test conditions, appropriate observations and measurements, endpoints and methods of calculation, validation of the test, and the use of reference toxicants.

Résumé

Le présent document décrit les méthodes recommandées par Environnement Canada pour l'exécution d'un essai de toxicité sublétale en milieu marin avec des gamètes d'oursins globuleux ou d'oursins plats. Cette deuxième édition de la méthode SPE 1/RM27, publiée en 2011, remplace la première édition publiée en 1992, puis modifiée en 1997. Elle comporte de nombreuses modifications procédurales, de même que des conseils et des instructions à jour concernant la conduite de la méthode d'essai biologique.

Au cours de cet essai, on expose d'abord le sperme d'échinides à la substance ou à la matière d'essai. On ajoute ensuite des œufs d'échinides et on mesure le succès de la fécondation tout en maintenant l'exposition à une concentration constante de la substance ou de la matière d'essai. Le paramètre à mesurer est la diminution du succès de la fécondation, exprimée sous forme de concentration estimative causant un pourcentage précis d'inhibition (CI_p). L'essai demande peu de temps et compte parmi les essais de toxicité sublétale en milieu marin les plus sensibles. La survie des gamètes et le succès de la fécondation étant des éléments essentiels du cycle biologique, l'essai décrit ici constitue un instrument de mesure puissant et probant de la sublétalité. On peut utiliser, pour cet essai, au moins sept concentrations de la substance ou de la matière d'essai afin de déterminer le seuil à partir duquel s'exerce un effet, ou une seule concentration s'il s'agit d'un essai réglementaire à résultat unique (satisfaisant ou non satisfaisant).

*On recommande d'utiliser les espèces suivantes : l'oursin vert (*Strongylocentrotus droebachiensis*), qu'on trouve sur les côtes atlantique, pacifique et arctique du Canada, l'oursin violet du Pacifique (*S. purpuratus*) ou le clypéastre excentrique (*Dendraster excentricus*), qui vivent dans le Pacifique, l'oursin violet de l'Atlantique (*Arbacia punctulata*) et l'oursin blanc de Californie (*Lytechnius pictus*).*

Ce rapport présente les méthodes d'acclimatation et de maintien 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), de même que les procédures de collecte du sperme et des œufs nécessaires à l'essai. On y indique également les conditions et méthodes générales ou universelles à mettre en œuvre pour réaliser des essais visant à mesurer les effets d'un large éventail de substances ou de matières sur la fécondation chez les échinides. D'autres conditions et méthodes sont propres aux essais sur un ou plusieurs échantillons de substance chimique, d'effluent, d'eau réceptrice, d'élutriat, de lixiviat ou de liquide extrait de sédiments et de matières solides semblables. On a également inclus des directives concernant les installations d'essai, la manipulation et l'entreposage des échantillons, la préparation des solutions d'essai et la mise en route des essais, les conditions d'essais particulières, les observations et mesures appropriées, les paramètres des essais, les méthodes de calcul, la validation de l'essai et l'utilisation de toxiques de référence.

Foreword

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

- *for use in EC environmental toxicity laboratories;*
- *for testing that is contracted out by Environment Canada or requested from outside agencies or industry;*
- *in the absence of more specific instructions, such as are contained in regulations; and*
- *as a foundation for the provision of very explicit instructions as might be required in a regulatory program or standard reference method.*

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

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

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List of Abbreviations and Chemical Formulae

A	ampere
ANOVA	analysis of variance
°C	degree(s) Celsius
cm	centimetre(s)
CV	coefficient of variation
d	day(s)
DO	dissolved oxygen (concentration)
g	gram(s)
g/kg	grams per kilogram
h	hour(s)
HCl	hydrochloric acid
HSB	hypersaline brine
ICp	inhibiting concentration for a (specific) percent effect
KCl	potassium chloride
L	litre(s)
LOEC	lowest-observed-effect concentration
M	molarity (concentration)
m	metre(s)
mg	milligram(s)
min	minute(s)
mL	millilitre(s)
mm	millimetre(s)
mS	millisiemen(s)
N	Normal
NaOH	sodium hydroxide
nm	nanometre(s)
NOEC	no-observed-effect concentration
SD	standard deviation
SI	Système internationale d'unités
sp	species
TIE	toxicity identification evaluation
TM (TM)	Trade Mark
µg	microgram(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: All definitions are given in the context of the procedures in this report, and might not be appropriate in another context.

Grammatical Terms

Must is used to express an absolute requirement.

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

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

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

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

Technical Terms

Acclimation is 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"(‰).

Dispersant is a chemical substance which reduces the surface tension between water and a hydrophobic substance (e.g., oil), thereby facilitating the dispersal of the hydrophobic substance or material throughout the water as an emulsion.

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

Embryo means the undeveloped young animal, before it hatches from the egg.

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

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

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

Larva (plural, larvae) is a recently hatched organism which has physical characteristics other than those seen in the adult of the species.

Lux is a unit of illumination based on units per square metre. One lux = 0.0929 foot-candles and one foot-candle = 10.76 lux. For conversion of lux to quantal flux [μmol/(m^2 · s)], the spectral quality of the light source must be known. Light conditions or irradiance are properly described in terms of quantal flux (photon fluence rate) in the photosynthetically effective wavelength range of approximately 400 to 700 nm. The relationship between quantal flux and lux or foot-candles is highly variable and depends on the light source, the light meter used, the geometrical arrangement, and the possibilities of reflections (see ASTM, 1999). An approximate conversion between quantal flux and lux, for full-spectrum fluorescent light (e.g., Vita-Lite® by Duro-Test®), is as follows: one lux is approximately equal to 0.016 μmol/(m^2 · s) (Deitzer, 1994; Sager and McFarlane, 1997).

Monitoring is the routine (e.g., daily, weekly, monthly, quarterly) checking of quality, or collection and reporting of information. In the context of this report, it means either the periodic (routine) checking and measurement of certain biological or water-quality variables, or the collection and testing of samples of effluent, leachate, elutriate, *marine*/*estuarine* receiving water, or pore water for toxicity.

Percentage (%) is a concentration expressed in parts per hundred parts. One percent represents one unit or part of material or substance (e.g., chemical, effluent, leachate, elutriate, receiving water, or pore water) diluted with water to a total of 100 parts. Concentrations can be prepared on a volume-to-volume or weight-to-weight basis, and are expressed as the percentage of test substance or material in the final solution.

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

Photoperiod is the duration of illumination and darkness within a 24-h day.

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

Pre-treatment is, in this report, *treatment* of a sample or dilution thereof, before exposure of *gametes*.

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

Reference method refers to a specific *protocol* for performing a toxicity test, i.e., a biological test method with an explicit set of test procedures and conditions, formally agreed upon by the parties involved and described precisely in a written document. Unlike other multi-purpose (generic)

biological test methods published by Environment Canada, the use of a reference method is frequently restricted to testing requirements associated with specific regulations.

Salinity is the total amount of solid 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 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.

Turbidity is the extent to which the clarity of water has been reduced by the presence of suspended or other matter that causes light to be scattered and absorbed rather than transmitted in straight lines through the sample. It is generally expressed in terms of Nephelometric Turbidity Units.

Terms for Test Materials or Substances

Chemical is, in this report, any element, compound, formulation or mixture of a chemical substance that might enter the aquatic environment through spillage, application, or discharge. Examples of chemicals that are applied to the environment are insecticides, herbicides, fungicides, sea lamprey larvicides, and agents for treating oil spills.

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

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

Control sediment means uncontaminated (*clean*) sediment which does not contain concentrations of one or more contaminants that could affect the fertilization of echinoid eggs. In this report, control sediment is natural, field-collected sediment from an uncontaminated site, with pore water that is known to enable an acceptable egg fertilization rate. This sediment must contain no added test material or substance, and may provide a basis for interpreting data derived from toxicity tests using test sediment(s).

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

Deionized water is water that has been purified to remove ions from solution by passing it through resin columns or a reverse osmosis system.

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.

Distilled water is water that has been passed through a distillation apparatus of borosilicate glass or other material, to remove impurities.

Effluent is any liquid waste (e.g., industrial, municipal) discharged to the aquatic environment.

Elutriate is an aqueous solution obtained after adding water to a solid material (e.g., sediment, tailings, drilling mud, dredge spoil), shaking the mixture, then centrifuging or filtering it or decanting the supernatant.

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

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. It 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.

Interstitial water - see *pore water.*

Leachate is water or wastewater that has percolated through a column of soil or solid waste within the environment.

Marine water is seawater from or within the ocean, sea, or an inshore location where there is no appreciable dilution of water by natural fresh water derived from land drainage.

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

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.

Produced water is mainly salty water brought up along with oil and gas during its production. Produced water originates from water contained in oil and gas reservoirs that is produced along with the oil and gas. Produced water might be problematic in the environment due to its highly saline nature.

Receiving water is a natural seawater (e.g., in a *marine* or *estuarine* waterbody) that has received a discharged waste, or else is about to receive such a waste (e.g., it is just "upstream" or up-current from the discharge point). Further descriptive information must be provided to indicate which meaning is intended.

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

Sediment is a natural particulate material, which has been transported and deposited in water and then deposited on the sea floor. The term can also describe a material that has been experimentally prepared (formulated) using selected particulate material (e.g., sand of a particular grain size, bentonite clay, etc.) for experimental purposes.

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

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

Upstream water is natural seawater (e.g., in a *marine* or *estuarine* waterbody) that is not influenced by the effluent (or other test material or substance), by virtue of being removed from it in a direction against the current or sufficiently far across the current.

Wastewater is a general term that includes effluents, leachates, and elutriates.

Statistical and Toxicological Terms

Acute means within a short period of exposure in relation to the life span of the organism, and 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.

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

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

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

Endpoint means the measurement(s) or value(s) that characterize the results of the test (e.g., IC25). It also means the response of the test organisms that is measured (e.g., fertilization).

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

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

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

ICp is the inhibiting concentration for a (specified) percent effect. It represents a point estimate of the concentration of test substance or material that causes a designated percent impairment in a *quantitative* biological function such as a growth rate, or number of young per brood, compared to the control. For example, an IC25 could be the concentration estimated to cause a 25% reduction in growth rate, 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 the present echinoid test, unmeasured effects on sperm, on eggs, and on the fertilization process are given an overall assessment by the percent inhibition of fertilization. The term *effective concentration* for a specified percent effect (ECp), such as the median effective concentration (EC50) or an EC25, is not appropriate in tests of this kind because it is limited to *quantal* measurements, e.g., an estimate that 25% of the individual organisms exposed to that concentration would show a particular effect. The echinoid fertilization assay does not completely conform with the requirements for an ECp (such as the EC50), because the number of quantal observations is large (i.e., ≥100). As such, the change in percent effect caused by one individual reacting would be low enough that the data can be treated as if they represent a continuous distribution. Environment Canada (2005), therefore recommends estimating the ICp, a quantitative endpoint, for the echinoid fertilization test.

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

LOEC is the lowest-observed-effect concentration. This is the lowest concentration of a test material or substance to which organisms are exposed, that causes adverse effects on the organism, effects which are detected by the observer and are statistically significant. For example, the LOEC might be the lowest concentration at which fertilization success differed significantly from that in the control.

NOEC is the no-observed-effect concentration. This is the highest concentration of a test material or substance to which organisms are exposed, that does not cause any observed and statistically significant adverse effect on the organism. For example, the NOEC might be the highest tested concentration at which an observed variable such as fertilization success did not differ significantly from that in the control. NOEC customarily refers to sublethal effects, and to the most sensitive effect unless otherwise specified.

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

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

Quantal is an adjective, as in quantal data, quantal test, etc. A quantal effect is one for which each test organism either shows the effect of interest or does not show it. For example, an animal might either live or die, or an egg might be fertilized or not fertilized. Quantal effects are typically expressed as numerical counts or percentages thereof. *Quantitative* estimates are performed on quantal data if the number of quantal observations is large (i.e., counting 100 - 200 eggs per container) (EC, 2005).

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

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

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

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

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

Toxicant is a *toxic* substance or material.

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

Toxicity Identification Evaluation (TIE) describes a systematic sample *pre-treatment* (e.g., *pH* adjustment, filtration, aeration) followed by tests for toxicity. This evaluation is used to identify the agent that is primarily responsible for toxicity in a complex mixture. The toxicity test can be lethal or sublethal.

Toxicity test is a determination of the effect of a substance or material on a group of selected organisms, 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 concentrations of chemical, effluent, receiving water, leachate, elutriate, or interstitial water derived from sediment or similar solid material.

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

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 effluent, elutriate, leachate, receiving water, or control water).

Warning chart is a graph used to follow changes over time in the endpoints for a reference toxicant. The date of the test is on the horizontal axis and the 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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Revisions to the sediment testing procedures provided in this 2nd edition test method document are predominantly based on the results of an inter-laboratory study carried out in 2008 designed to investigate the improvement of the porewater-testing component of EPS 1/RM/27. Participants of this inter-laboratory study are gratefully acknowledged for their participation and their input into the report, and include: Ken Doe and Paula Jackman (Atlantic Laboratory for Environmental Testing, Environment Canada, Moncton, NB); Craig Buday (Pacific and Yukon Laboratory for Environmental Testing, North Vancouver, BC); and Scott Carr and Jim Biedenbach (Columbia Environmental Research Centre, TAMU-CC, Centre for Coastal Studies, Corpus Christi, TX, USA). Special thanks to Rick Scroggins and Lisa Taylor (Biological Methods Section, Environment Canada, Ottawa, ON) who acted as Scientific Authorities and provided technical input and guidance throughout the study.

Introduction

1.1 Background

Aquatic *toxicity tests* are used within Canada and elsewhere to measure, predict, and control the discharge of *substances* or *materials* that *might* be harmful to aquatic life in the environment. Recognizing two decades ago that no single test method or test organism can be expected to satisfy a comprehensive approach to environmental conservation and protection, the Inter-Governmental Ecotoxicological Testing Group (see Appendix B) proposed the development and standardization of a set of single-species aquatic toxicity tests which would be broadly acceptable, and would measure different *toxic* effects using organisms representing different trophic levels and taxonomic groups (Sergy, 1987). A test based on fertilization success using *gametes* of sea urchins or sand dollars was one of several "core" aquatic toxicity tests which was then selected to help meet Environment Canada's testing requirements.

The first edition of this biological test method was published by Environment Canada in December 1992 as Report EPS 1/RM/27 (EC, 1992c), and amended in November 1997. After 15 years of application by private and public sector testing laboratories, Environment Canada recognized that specific aspects of the test method needed to be changed. This revision began with the preparation and circulation of a questionnaire to Canadian and US *toxicology* testing laboratories with experience in conducting echinoid fertilization assays. The purpose of the questionnaire was to collect details on the echinoid species used in testing, the conditions for holding and acclimating organisms, and the spawning and fertilization techniques employed by the various laboratories using the method. Guidance derived from the feedback provided by the laboratories responding to the questionnaire has been included herein, where applicable. Revisions to sediment (*pore water*) testing procedures are largely based on the results of an inter-laboratory study which investigated the improvement (i.e., improved test sensitivity and reduced influence of confounding factors on test results) of the porewater testing component of this echinoid fertilization assay (Miller, 2008).

The current (second) edition includes numerous procedural improvements, updated and more explicit guidance, as well as instructions for the use of revised statistics (i.e., regression analyses) when calculating the test *endpoint* for fertilization inhibition.

Universal procedures for conducting a fertilization assay with echinoid *gametes* are described in this second edition of Environment Canada's Report EPS 1/RM/27. Also presented herein are specific sets of test conditions and procedures, required or recommended when using the test to evaluate different types of substances or materials (e.g., samples of one or more *chemicals*, *effluents*, *receiving waters*, *leachates*, *elutriates*, or *interstitial waters* [*pore waters*] derived from *sediment* or similar solid material; see Figure 1). Those procedures and conditions relevant to the conduct of the test are delineated and, as appropriate, discussed in explanatory footnotes.

In formulating these test conditions and procedures, an attempt was made to balance scientific, practical, and financial considerations, and to ensure that the results will be accurate and precise enough for the majority of situations in which they would be applied. It is assumed that the user has a certain degree of familiarity with aquatic toxicity tests. Guidance regarding test options and applications is provided here. Explicit instructions that *might* be required in a regulatory *protocol* or *reference method* are not provided, although the report is intended to serve as a guidance document useful for that and other applications.

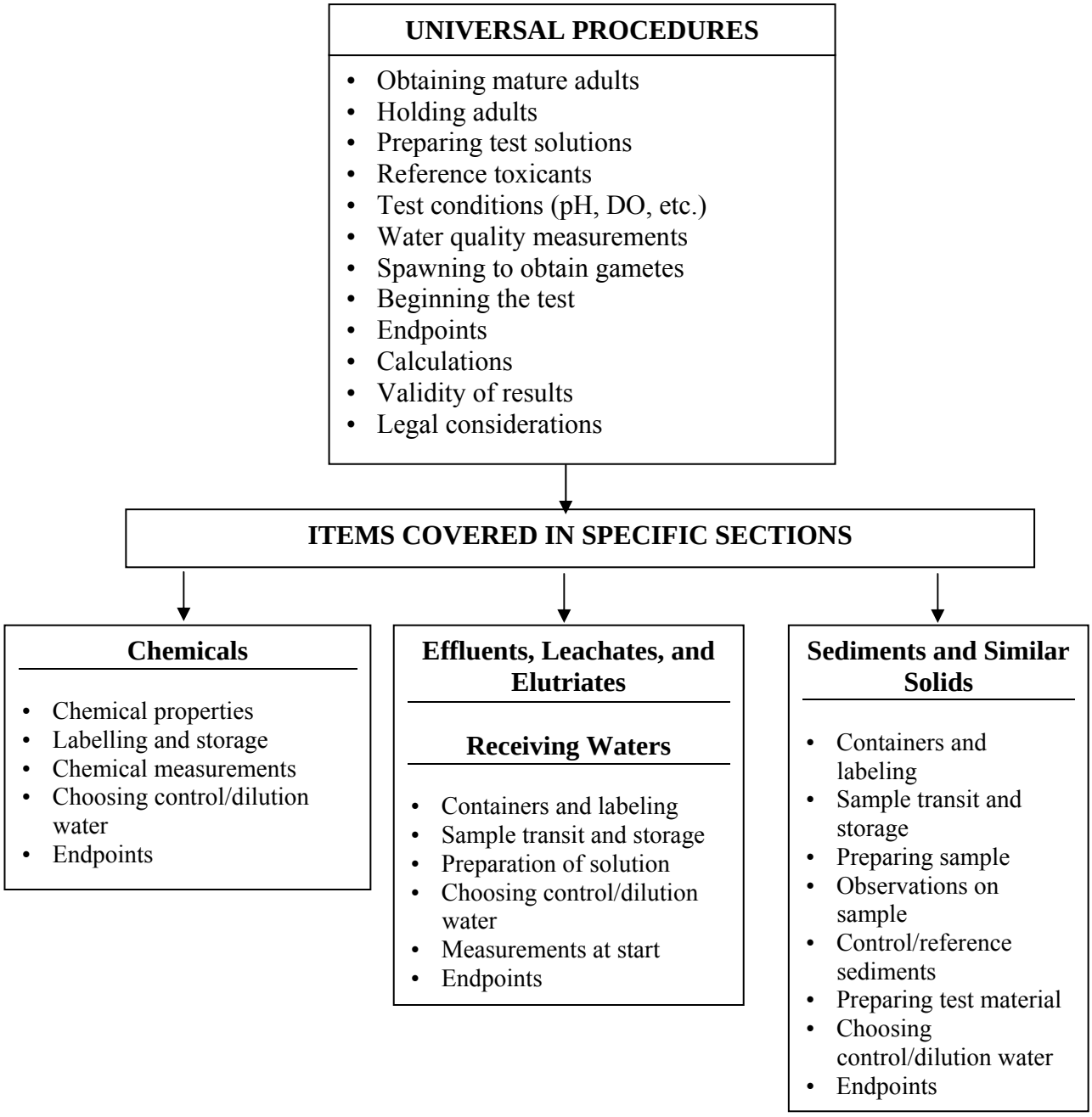

Figure 1 Diagram of Approach Taken in Delineating Test Conditions and Procedures Appropriate for Various Types of Materials or Substances

For guidance on the implementation of this and other biological test methods and on the interpretation and application of the endpoint data, consult Report EPS 1/RM/34 (EC, 1999).

1.2 General Aspects of Echinoids and Their Use in Tests

Sea urchins and sand dollars belong to the Phylum Echinodermata, Sub-phylum Echinozoa, and Class Echinoidea, and, therefore can collectively be called "echinoids". Other members of the phylum, not included in this test method, are the sea stars ("starfish"), brittle and basket stars, sea cucumbers, and crinoids or sea lilies and feather stars. The phylum has worldwide marine distribution and about 6000 living species are known. Seven species of sea urchins and three species of sand dollars are commonly found in the coastal *marine waters* of Canada.

Echinoids and other members of the phylum are considered to be structurally advanced and complex invertebrates. They have many sophisticated features and many similarities to chordate animals including the basic pattern of embryonic development and some biochemical processes. The apparent radial arrangement of the body in five parts around a central axis is superimposed on a primary bilateral organization. There is a true internal skeleton covered by a thin epidermis. The skeleton is of small jointed calcareous plates, which in sea urchins and sand dollars are fused together into a solid *test*, or "shell", the latter term being used in this report for convenience. There is a well-developed coelom or internal body cavity, most of which surrounds the internal organs (Figure 2). Another part of the coelom is a tube-like *water vascular* ("hydraulic") system running to all parts of the body, used to manipulate small tube feet for locomotion, and to perform other functions.

Sea urchins are spherical and covered in spines, while sand dollars are flattened on the oral-aboral axis and generally disk-shaped (Figure 2). The oral surface is oriented downwards. A *peristomial membrane* surrounds the mouth in sea urchins, and injection of a chemical solution through that membrane and into the coelom is part of the procedure in these tests. For sand dollars, injection has to be through the mouth opening. The anus of sea urchins is on the aboral (upper) surface, but in sand dollars it is on the same surface as the mouth.

The sexes are separate but cannot be distinguished externally. There are large internal gonads (Figure 2) with outlets on the aboral surface, as five genital pores in urchins and four in sand dollars. One of the pores of urchins is in the *madreporite*, an obvious large plate of the shell, which is a terminus of the animal's water vascular system.

The *gametes* (sperm and eggs) are simply passed through the pores to the sea for fertilization.

The early development of sea urchins from egg to late larval stage ("pluteus" stage) is of great embryological interest, and more than 5000 papers were published on the topic by 1980 (NRC, 1981). This background has led to the use of young stages of urchins in toxicity tests over many decades (Lillie, 1921; Drzewina and Bohn, 1926; Bougis, 1959), with a particularly thorough study of metal *toxicity* using fertilization in a sea urchin completed in the first quarter of the last century (Hoadley, 1923). Both sea urchins and sand dollars are now frequently used as standard organisms in toxicity testing (reviewed in Dinnel *et al*., 1987, 1988), and an extensive background of toxicological data has accumulated (Kobayashi, 1984).

The echinoid fertilization assay is sensitive. A major effect on egg fertilization, for example, was caused by municipal effluents at concentrations which were one-tenth of those causing 50% mortality of fathead minnows in a four-day test (Oshida *et al*., 1981). It was the second to third most sensitive among six *sublethal* tests (marine and freshwater) used in an inter-laboratory survey of effluent toxicity in California (Anderson *et al*., 1991). The 80-min echinoid fertilization assay

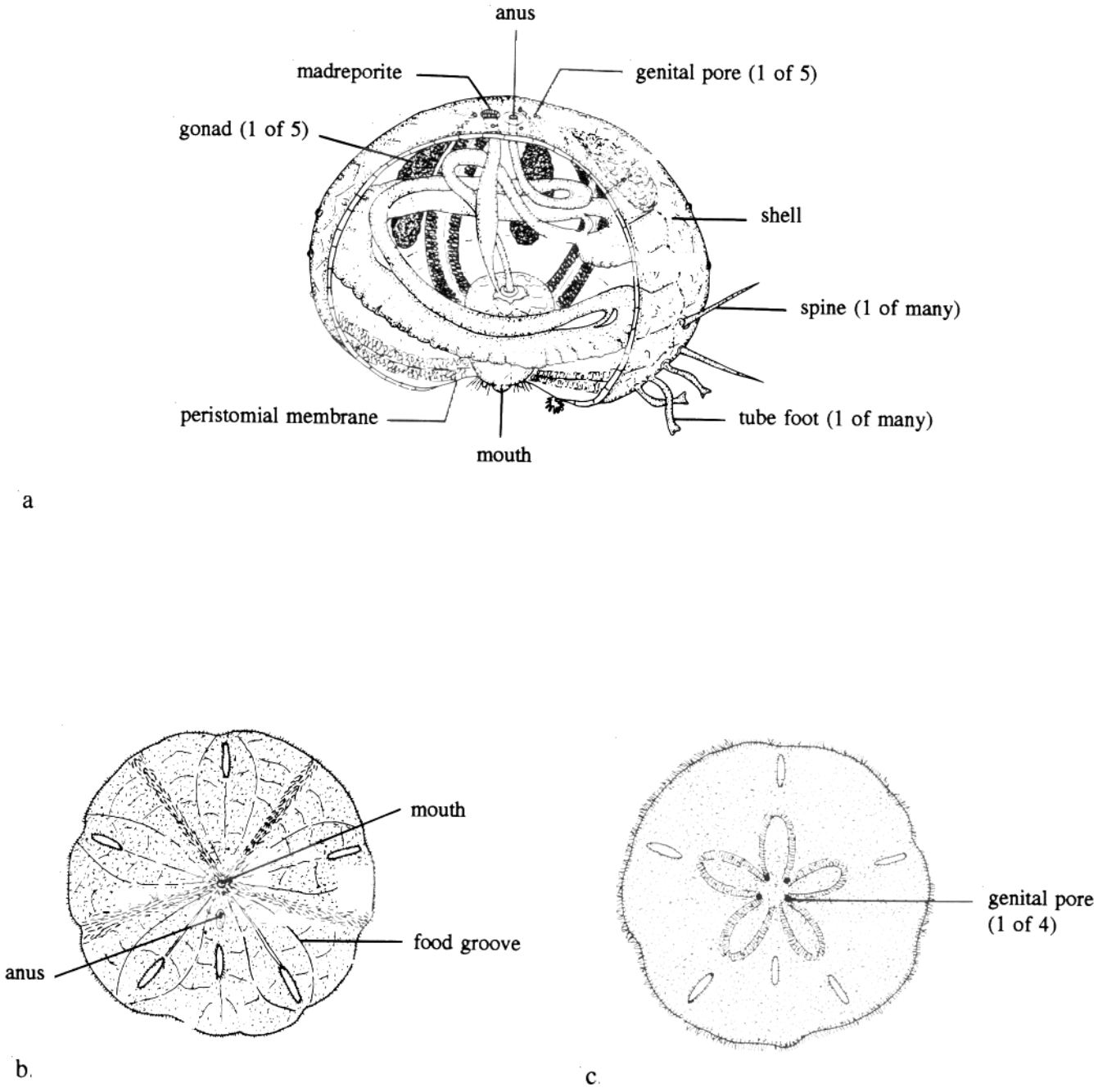

Figure 2 General Appearance of Echinoids

a. Cut-away view of a typical sea urchin, Arbacia, showing location of genital pores on the aboral (upper) side. Only two or three of the numerous spines and tube feet are indicated. b. Oral side (normally down) of a typical sand dollar. c. Aboral side of a sand dollar showing location of the genital pores. [Drawings by M.A. White, after Storer *et al.* (1979) and Barnes (1974)].

was more sensitive to the effluent from a municipal wastewater treatment plant than were 48-hour tests with oyster and crab embryos and larvae (Dinnel and Stober, 1987). Variable results were obtained in a comparison of the toxicities of metal and organic compounds using the fertilization assay, a bacterial luminescence assay, and *acute* lethality tests with fish and crustaceans. Sometimes the echinoid test was one, two, or three orders of magnitude more sensitive, and sometimes an order of magnitude less sensitive (Nacci *et al*., 1986). Results from echinoid fertilization assays were similar in sensitivity to those from embryo-larval tests with crab, squid, and fish, and were quite sensitive to metals, but much less so to pesticides than were tests of acute lethality using marine fish (Dinnel *et al*.,1989). For pulp mill wastes, NCASI (1992) cites work of Johnson *et al*. (1990) that embryo-larval tests with oysters were approximately an order of magnitude more sensitive than the echinoid fertilization assay. In turn, the echinoid assay was about as sensitive as a reproductive test using red alga, and was more sensitive, often by an order of magnitude, than other sublethal marine tests on growth and development of larval fish (silversides minnows and sheepshead minnows) or juvenile mysid shrimps (Schimmel *et al*., 1989).

The fertilization assay is a sensitive *sublethal* test. Gametes of echinoids are either the most sensitive of the developmental stages, or are among the most vulnerable stages of the entire life cycle, when tested using various *toxicants* (Kobayashi, 1980, 1984). The fertilization assay is not a *chronic* test, however, because of its very short duration relative to the life spans of the species (some years). The fertilization assay described in this report is not intended to replace *chronic toxicity* tests using echinoids, because it might not estimate the effects of longer exposures. However, this test can be expected to yield results closer to such chronic tests than would conventional lethality tests with marine or freshwater species (e.g., EC, 1990a, 1990b, 1990c).

Precision of the test appears to be satisfactory. The USEPA (2002) determined that within-laboratory *coefficients of variation* (CV) using *reference toxicants* and one species of sea urchin (*A. punctulata*) for IC50s were 23% to 48%, and for IC25s were 29% to 55%. A CV of 74% was found for IC50s of copper tested by six laboratories using four species of echinoids in an effluent testing program, compared to CVs of 29% to 38% obtained with sublethal tests on single species (*Ceriodaphnia* reproduction, and early life stages of fathead minnows and oysters, Anderson and Norberg-King, 1991). In five single-species comparisons among Canadian laboratories, the CVs were 62%, 65%, 75%, 82% and 110% for IC25s of copper (tests involved three species of sea urchins with total exposure times of 20 minutes). IC50s from the same tests showed lower CVs, with values 23%, 48%, 57%, 80% and 94% (Miller *et al*., 1992). These interlaboratory CVs, averaging 79% for IC25s and 63% for IC50s, are similar to the precision for chemical analyses, e.g., an average CV of 30 to 60% found in an interlaboratory comparison of chemical analyses of priority pollutants (Rue *et al*., 1988; Gossett *et al*., 2003). Unpublished results for an interlaboratory round-robin sponsored by the USEPA are apparently similar, with CVs of 57% for 40-min fertilization assays and 86% for 80-min assays (NCASI, 1992).

Prior to 1992, the echinoid fertilization assay had been used in several Canadian aquatic toxicity laboratories, both governmental and industrial. Standard test methods had been described in British Columbia (B.C. MOE, 1990; van Aggelen, 1988), and by consulting companies (Beak, 1988; EVS, 1989). At the national level, a trial of methods had been carried out by certain Environment Canada laboratories (see Appendix C), under the sponsorship of a federal-provincial body (IGATG, 1991). Additional interlaboratory trials, involving federal, provincial (B.C. Ministry of Environment), and private testing facilities, were done the following year (Miller *et al*., 1992). Echinoid tests were reviewed and recommended by an Environment Canada scientist (Wells, 1982, 1984), but prior to 1992,

no standard method had been published by a Canadian federal government agency.

Since it's publication in 1992, and formal amendment in 1997, Environment Canada's *Fertilization Assay Using Echinoids (Sea Urchins and Sand Dollars)* (EPS 1/RM/27) has been used extensively in two important programs falling under the authority of the Canadian Environmental Protection Act (CEPA), and has also been applied under specific regulations of the Canadian Fisheries Act. Under the Disposal at Sea Program, this sublethal test uses echinoid fertilization on sediment pore water to help evaluate the suitability of dredged material for disposal at sea (CEPA, 1999; Government of Canada, 2001). Under the Environmental Effects Monitoring Program, the sublethal toxicity of pulp and paper, and mining effluent discharged to the marine environment is assessed using echinoid fertilization (DFO, 1992, 2002).

In the United States, several groups have provided methods for conducting sublethal toxicity tests using echinoids. The United States Environmental Protection Agency has developed authoritative procedures for species of echinoids indigenous to their Atlantic (USEPA, 1988, 1994, 2002) and Pacific (Chapman, 1991, 1992a; USEPA, 1995) coasts. Fertilization assays were also being developed by the American Society for Testing and Materials (ASTM, 1990), however, only the echinoid *embryo* test was formalized and published (ASTM, 2002). A critique of methodology was provided by NCASI (1992), with special relevance to pulp and paper effluents. In addition, a number of consulting companies and other marine laboratories have written procedures for their own organizations (see Appendix D).

Numerous papers have been published by various authors and groups of authors who use standard techniques. Notable among these papers are those of Kobayashi, Dinnel and co-authors, and Pagano and fellow-workers. Some of their papers are in the reference list, and many others are in the bibliography (Appendix E).

There are several reasons for choosing an echinoid fertilization test as a method of assessing sublethal toxicity in Canadian marine locations. In general, the test is quick, sensitive, and relatively simple. Some advantages are:

- Much of the biology and life history of major species are documented.
- The organisms are commonly and widely distributed on the three Canadian coasts.
- Adult sea urchins and sand dollars are easily collected in shallow waters.
- Adults are readily held in the laboratory and conditions can be manipulated to lengthen their spawning season.
- Gametes of consistent quality and sensitivity can be obtained.
- Success of fertilization is a sensitive and fundamental sublethal effect to measure.
- The fertilization assay is rapid and economical because it is small-scale, easy to do, and uses ordinary facilities and supplies.
- Echinoid eggs are already haploid when released, unlike those of most animals, and so the need for a mandatory waiting period before use is avoided.
- The test has a relatively simple and objective endpoint.
- Echinoids are available worldwide, and are frequently used as standard marine species for regulatory and research purposes. They can be easily shipped, and used at inland laboratories.

(NRC, 1981; Dinnel and Stober, 1985; Esposito *et al*., 1986; Dinnel *et al*., 1987).

In addition to general toxicity testing in a marine venue, the echinoid fertilization test would seem suitable for identifying the sublethally toxic components of complex effluents, using the "*Toxicity Identification Evaluation*" or TIE

procedures described by the USEPA (1991a, 1991b).

The purpose of this "generic" report is to provide standardized Canadian methods for testing the sublethal *toxicity* of various substances or materials using echinoid *gametes*. Preferred choices are given among the alternatives available within a standard framework, for choice of species, exposure times, single-concentration (pass-fail) test versus multi-concentration test, test volumes, and type of water used for dilution and the controls. The echinoid test procedures in existing documents vary in their coverage of endpoints, and also differ in issues such as *pH* adjustment, alternative procedures for various objectives, selection of *control/dilution water*, and how to deal with samples that contain appreciable solids or floating material. This report is intended for evaluation of sublethal toxicity in samples of chemical, effluent, leachate, elutriate, receiving water, and liquid derived from sediment and similar solid materials. The rationale for selecting certain approaches is given.

The method is meant for use with seawater-acclimated animals and seawater as the dilution and *control* water. Depending on the test objectives, this seawater may be reconstituted or natural, but should approach the *salinity* of full-strength seawater. Other tests, using freshwater-acclimated fish or other sensitive freshwater organisms, are available for evaluating the *lethal* and sublethal toxicity of chemicals or *wastewaters* that are destined for, discharged to, or within the freshwater environment (See Appendix A).

Test Organisms

2.1 Species

The test *must* be carried out using one of the species listed below.

Strongylocentrotus droebachiensis (O.F. Müller), the **green sea urchin**, a circumpolar species found on the Canadian Atlantic and Pacific coasts and across the Arctic Ocean to 80 °N.

Strongylocentrotus purpuratus (Stimpson), called in this report the **Pacific purple sea urchin** (and commonly called the purple sea urchin), found on the Pacific coast of Canada and southwards to Baja California (Meinkoth, 1981).

Dendraster excentricus (Eschscholtz), a sand dollar of the Pacific coast of Canada and southwards, called in this report the **eccentric sand dollar**, a standard common name (Meinkoth, 1981).

Arbacia punctulata (Lamarck), called in this report **Arbacia**, although the common name of "Atlantic purple sea urchin" is sometimes used (Meinkoth, 1981). Found on the Atlantic coast of the United States from Cape Cod southerly into the Caribbean and Gulf of Mexico.

Lytechinus pictus (Verrill), the **white sea urchin**, found from southern California to Panama.

The first four species can be collected on one or more Canadian coasts. All five species can be purchased from biological supply houses and shipped to the test laboratory.

All of these species have been listed as echinoids commonly used in the laboratory (NRC, 1981). Most of the species have been used frequently in toxicity tests (Appendix D). According to feedback provided by Canadian and US laboratories in response to a questionnaire circulated prior to the preparation of this second edition (see Section 1.1), the green sea urchin (*S. droebachiensis*) has been used the least of the five candidate test species included in the first edition of EPS 1/RM/27. This species is desirable from a Canadian perspective, however, since it is the only echinoid sp., listed herein, that is found in Pacific, Atlantic, and Arctic waters. In recent testing, it was confirmed that green sea urchin eggs are relatively large (i.e., 3 to 4 times larger than the other species listed herein), have highly visible fertilization membranes, and show good fertilization rates in uncontaminated seawater (Jackman, pers. comm., 2008) . These factors along with their presence on all three Canadian coasts have resulted in the green sea urchin being retained in this second edition of EPS 1/RM/27.

In general, toxicity results from fertilization assays using echinoids appear to be similar among species (Kobayashi, 1984; Nacci *et al*., 1986). There might, however, be differences in species sensitivity, depending on the toxicant being tested. For example, the eccentric sand dollar appears to be about 1.4 times more sensitive to sodium dodecyl sulphate than the Pacific purple sea urchin, and 1.7 times more sensitive to bleached sulphite mill effluent than the green sea urchin (NCASI, 1992). In a more recent study involving 3 species of sea urchins (Pacific purple, Arbacia, and white) and 1 species of sand dollar (eccentric), the white sea urchin and the eccentric sand dollar were found to be the most sensitive to specific samples of sediment pore water from Vancouver and Halifax Harbours, whereas Arbacia had the greatest sensitivity to ammonia. In the same study, the Pacific purple sea urchin and the eccentric sand dollar had the greatest sensitivity to copper (Jackman and Doe, 2004).

The common sand dollar, *Echinarachnius parma* (Lamarck), has not been used frequently in

toxicity tests, and performed poorly in a multi-species interlaboratory evaluation of this echinoid fertilization assay (Miller *et al*., 1992). Accordingly, the common sand dollar is not presently recommended for the test until research proves suitable. The circumpolar distribution of the common sand dollar, including its frequent occurrence along the Atlantic coast of Canada southwards to Maryland (U.S.A.), support further research with this species. Adult common sand dollars were successfully used for month-long toxicity tests in Newfoundland by Osborne and Leeder (1989). The effect of growth-inhibiting chemicals and sediment contaminants on the early life stages of this sand dollar have been studied (Karnofsky and Simmel, 1963; Meador *et al*., 1990).

2.2 Life Stage, Size, and Source

Mature and gravid echinoids should be obtained to provide the *gametes*. Adult sizes range from about 3-cm diameter upwards for the various species (Table 1); a common size-range for specimens in the laboratory is 5 to 6 cm.

All adults used to provide gametes for a test should be derived from the same batch and source. The native species can be collected from clean-water coastal marine locations, some in shallow water at low tide, or by diving. All species can also be purchased from biological supply houses. Adult echinoids must be positively identified to species. 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. Records accompanying each *batch* of test organisms 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. To ensure that the echinoids' health is maintained during transit, appropriate temperature, DO, and *salinity* conditions should be maintained as much as possible. Shipping containers should be insulated to minimize changes in temperature during transit. If the organisms cannot be delivered on the same day that they are shipped, the transport containers should be stored in such a way that the temperature of the echinoids is held as constant as possible. The temperature of the test organisms should be recorded upon departure from the supplier's facility and upon arrival at the testing laboratory.

The spawning seasons listed in Table 1 show that in a given location, tests could be carried out for much of the year by collecting sea urchins and sand dollars at appropriate times.

The testing season could be lengthened by maintaining the adults at warm or cool temperatures to encourage early or late spawning. The green sea urchin is in spawning condition for only a few months in the spring (see Table 1), however Canadian laboratories might be able to obtain gametes of the green sea urchin over most or all of the year by such changes in holding conditions (Wells, 1982, 1984). The other alternative would be to purchase species that had a suitable spawning time, from another location. It should be realized that animals from different sources and climatic conditions might show variations in timing and length of spawning season, or in the optimum temperature for bringing about spawning. Sea urchins that are spawned early in the season can sometimes provide gametes again in a month or six weeks if fed a proper diet (Dinnel and Stober, 1985). These sea urchins should be held in a separate tank after the first spawning.

Table 1 General Features and Conditions for Spawning Echinoids to be Used as a Source of Gametes

Species	Spawning Season*	Maximum Diameter of Adult (cm)**	Holding Temperature in Laboratory (°C)***
Green Sea Urchin	- generally April, but March to May at specific Canadian locations; a later cycle to June in the St. Lawrence estuary (January, June +)	8.3	**12 ± 2**, ≤15
Pacific Purple Sea Urchin	- generally January to May, optimally January to March for feral animals; late October to April on California coast (December, June)	10	**10 ± 2**, ≤17
Eccentric Sand Dollar	- May through summer to October (February to December)	9	**13 ± 2**, ≤17
Arbacia	- June to August on Atlantic Coast; January to April on Gulf Coast********	5.1	**17 ± 2**, ≤22
White Sea Urchin	- March through summer to November	♂2.8 ♀3.2	**13 ± 2,** ≥8 & ≤17

* Months in parentheses indicate possible extended spawning in the laboratory by holding at warm or cool temperatures. Information is taken from references used for Appendix D; from Meinkoth, 1981; NRC, 1981; Strathmann, 1987; Starr, 1990; and from information provided by reviewers listed in the Acknowledgements.

** The indicated sizes are the largest to be expected. Specimens held in the laboratory are often 5- to 6-cm diameter for most of the species.

*** Temperatures in bold type are the optimum ranges identified to obtain gametes in normal fashion, as derived from Appendix D, NRC (1981), as well as feedback provided by Canadian and US laboratories in response to a questionnaire circulated prior to the preparation of this second edition (see Section 1.1). The "≤" (and "≥" in the case of the white sea urchin) value(s) listed for each species is a temperature that should not be exceeded in order to avoid spontaneous spawning and/or increased mortality (see Section 2.3.5). Other temperatures could be used to speed or slow the maturation process, or the seasonal temperature of incoming natural seawater could be accepted.

**** Two different populations become fertile at different times of the year (i.e., Arbacia from Atlantic Coast are fertile in the summer months, and organisms from Gulf Coast are fertile in the winter months), providing a great amount of flexibility as far as obtaining fresh test organisms (Biedenbach and Carr, pers. comm., 2008).

Maturation should be checked before attempting to carry out a toxicity test with the gametes. Sperm and eggs obtained outside the main period of maturation can give poor fertilization rates and poor test results. Inspections for state of maturity require some experience on the part of the investigator, but can be assessed by spawning a sample of echinoids and examining the gametes (Section 4.2.1). Mature sperm are minute and quickly become very active in seawater. Mature eggs rapidly become spherical in seawater. Immature eggs have a clear spot in the cytoplasm. Some adults could be sacrificed to examine the gonads, and to obtain gametes directly instead of by forced spawning. In sea urchins, mature ovaries are coloured yellow to red depending on species, and testes are white.

Shipping extremely ripe or gravid individuals under stressful conditions (e.g., extreme temperature changes) might cause spawning or mortality during shipment or upon receipt. This can be avoided by having the animals acclimated to laboratory conditions, as much as possible, prior to being shipped. Adults should 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. Shipping these organisms submerged in water might lead to oxygen depletion. The eccentric sand dollar, on the other hand, should be shipped in a small amount of chilled seawater. If adult echinoids spawn prematurely (i.e., during shipment or upon receipt in the laboratory), they can be separated by sex, and from those that have not spawned, and then housed in separate holding tanks.

Moving animals from one location to another marine location raises serious questions of introducing non-native species or transporting diseases and parasites. Any proposed procurement, shipment, or transfer of echinoids should be submitted for the approval of federal, provincial, or regional authorities. 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 (Appendix C). Application for a permit to bring in animals must be made to the above-mentioned committee, to the appropriate provincial agency, or to the Regional Director-General of the Department of Fisheries and Oceans (DFO), depending on procedures in place locally.

Testing laboratories might be required to establish and use a quarantine section within their facilities, where imported organisms or gametes 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. Standard operating procedures detailing quarantine operations and procedures might also be required by provincial agencies or DFO.

2.3 Holding and Acclimating Adults in the Laboratory

2.3.1 General

Groups of male and female echinoids are held in tanks and used to provide gametes when required for a test. There is no particular limitation on time that the adults may, or must be kept in the laboratory before providing gametes. All five test species have been successfully maintained in spawning condition in the laboratory for extended periods of time (i.e., 3 months to 1 year), although some species (i.e., Arbacia and the white sea urchin) are reportedly more easily maintained than others. 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*). Problems associated with holding these test species for extended periods of time include: adults

spontaneously spawn prior to testing; adults spawn for only a short period of time after arrival at the laboratory; and difficulty keeping them healthy or alive for extended periods of time (>3 months). As a result, this second edition of EPS 1/RM/27 includes an option for "holding adults for immediate use", where gametes may be collected within a short period of time (≤ 3 days) after the adults are received at the laboratory.[1]

For adults that are to be spawned and gametes tested within a 3-day period after the adults arrive at the testing laboratory, confirmation should be obtained from the supplier that the 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 if any time for acclimation upon arrival at the testing laboratory. Even with "holding for immediate use", the adults should be moved to laboratory holding conditions as gradually as possible, so that the stress of the rapid changes in holding conditions for the sexually-mature adults does not influence the test results for their gametes (i.e., test validity criteria are met, see Section 4.5.1). 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. This gradual exposure should minimize any stress on the animals caused by different water quality characteristics. For adults that are to be spawned for testing on the same day that they arrive at the laboratory, a minimum holding period of three hours 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. For adults shipped in water, a useful procedure for moving adults from their shipping water to control/dilution water prior to spawning is to hold them for 1-2 h in a 50:50 mixture of shipping water:control/dilution water, then for 1-2 h in a 25:75 mixture of shipping water:control/dilution water, followed by a final 1-2 h in 100% control/dilution water before they are spawned. Another useful procedure is to siphon off 20 to 30% of the shipping water every 1 to 2 hours and replace it with laboratory control/dilution water until at least three exchanges have been made. 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.

Some Canadian and US laboratories have had good success in holding both the eccentric sand dollar and the Pacific purple sea urchin in the laboratory for extended periods of time. Success in holding both of these species might be due to the use of fairly simple systems with lots of clean natural seawater flowing continuously through the holding tanks.[2] For the Pacific purple sea urchin, these conditions might include temperatures below 15 °C, high DO, pH > 8, good water flow, prompt removal of fecal

[1] Responses to a questionnaire provided by Canadian and US laboratories experienced in echinoid fertilization testing (see Section 1.1) showed that several laboratories had difficulty in maintaining the Pacific purple sea urchin and the eccentric sand dollar in captivity for extended periods of time, and therefore resorted to spawning them on the day of, or within a few days after shipping (i.e., without acclimation). Adult echinoids do not necessarily need to be acclimated for several days to test conditions prior to spawning since it is not them, but rather their sperm and eggs, that are used in the fertilization test. As long as the test organisms can deliver viable gametes that meet the criterion for test validity (see Section 4.5.1), gametes may be taken on the same day or within a few days after the spawning adults are received in the laboratory.

[2] Laboratories with flow-through systems had more success (i.e., less mortality) in holding groups of Pacific purple sea urchins for extended periods of time than those with static tanks, in a recent survey of US and Canadian laboratories (See Section 1.1).

material, and organisms being held in the dark (Bay and Greenstein, pers. comm., 2008; and Buday, pers. comm., 2008). Problems experienced by other laboratories could be due to a combination of adverse conditions during transport, poor exchange rate of seawater in the holding tanks, and prolonged exposure to artificial sea salts of a source and mixture that is foreign to the echinoids.

Both Arbacia and the white sea urchin have been successfully and easily held in the laboratory for extended periods of time (> 1 year), and spawned repeatedly (every 4 to 6 weeks) throughout the year when maintained under the right conditions (Doe and Jackman, pers. comm., 2008; Jonczyk and Holtze, pers. comm., 2008; and Carr, Nipper, and Biedenbach, pers. comm., 2008). They can be sexed and housed in separate aquaria to facilitate the quick selection of the appropriate numbers of males and females required for testing. Most laboratories report very little mortality with either of these two species during acclimation and holding. Both Arbacia and the white sea urchin are easily acclimated and maintained in closed, recirculating, temperature-controlled aquariums.

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 and a minimum holding time of 3 or 4 days, 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 (see Section 4.5.1) of the test.

Echinoids must be handled with care and should not be subjected to sudden shocks or changes in holding conditions. In particular, large changes in temperature or hydrostatic pressure might stimulate spawning at a time that is not desired by the investigator (Dinnel and Stober, 1985). Some laboratories that use natural seawater without fine filtration have noticed mass spawning of sea urchins occurring at times of plankton blooms, and the phenomenon has been observed in Canadian waters (Starr, 1990; Starr *et al*., 1990). In addition, spawning by individual animals might induce others to spawn, so such animals should be isolated immediately upon detection, to prevent mass spawning.

For adult echinoids that are going to be maintained in the laboratory for an extended period of time (i.e., > 3 days), the recommended conditions for holding echinoids, outlined in the following Sections 2.3.2 to 2.3.10 and summarized in Tables 1 and 2, are intended to allow some degree of flexibility within a laboratory, while at the same time standardizing those elements which, if uncontrolled, might affect the health of animals or viability of their gametes. Where applicable, guidance on "holding for immediate use" (i.e., spawning of adults within 3 days of arrival at the laboratory) is also provided. Recommended conditions have been drawn, in general, from Appendix D, and guidance derived from the feedback provided by US and Canadian laboratories responding to a questionnaire (see Section 1.1). Further details and rationale are given in some of the publications included in Appendix D, and in the References, particularly ASTM (1990), USEPA (1988, 1994, 1995, 2002), NCASI (1991), and papers of Dinnel and colleagues listed in the References and in the Bibliography of Appendix E. In addition, the following website, developed at Stanford University, might also provide useful details related to sea urchin care and embryology: http://www.stanford.edu/group/Urchin/.

2.3.2 Holding Containers

Adults may be held in aquaria, troughs, or tanks made of nontoxic materials such as glass, stainless steel, porcelain, fibreglass-reinforced polyester, perfluorocarbon plastics (Teflon ™), acrylic, polyethylene, or polypropylene. Tanks containing about 50 to 150 L of water, and fitted with a standpipe drain, are most commonly used. The holding tanks should be located away from

any major physical disturbances and preferably in a location separate from that used for testing. To help avoid undesired mass spawning, adults should be held in groups of 20 or fewer animals.

For sea urchins, the water depth should be ≥20 cm. For sand dollars, trays are frequently used, for example, 1 × 2 m with a water depth of 10 cm. There should be 2 to 3 cm of sediment or sand, rich in detritus including settled algal cells, on the bottom of containers used for sand dollars.

2.3.3 Lighting

The lighting conditions for holding echinoids currently used by various Canadian and US laboratories are quite varied and include: ambient laboratory light levels (100 - 500 *lux*), with a 16-h light : 8-h dark *photoperiod*; natural outside light and seasonal photoperiod (i.e., tanks are outside); or complete darkness. For sea urchins, the strength of lighting and photoperiod do not seem to be of major importance, and a low intensity of normal laboratory lighting is most common. 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.

For adult echinoids that are to be held for a prolonged period (i.e., > 3 days) prior to collecting gametes for use in a test, ambient laboratory lighting (100 - 500 *lux*) and a 16-h light : 8-h dark photoperiod are recommended. For tanks that are maintained outside, normal daylight and a seasonal photoperiod are recommended.

For the Pacific purple sea urchin, some laboratories have reported that cultures have been maintained successfully in complete darkness for extended periods of time. Constant darkness might disrupt some of the seasonal patterns of the animals that provide them a cue to spawn, so that they will maintain ripe gonads for a longer period of time (Bay, pers. comm., 2008). In instances where adults are transferred to a testing facility for "same-day" collection of gametes or collection of gametes within an ensuing period of 3 days or less, lighting conditions representing those to be used in the test are recommended.

2.3.4 Water

The water in containers holding adults should be renewed continuously or periodically to prevent a buildup of metabolic wastes. The water 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 (EC, 2001). Any commercially-available sea salts (e.g., Instant Ocean™, Ocean Pure Sea Salt™, Red Sea Salt™) or appropriate mixture of reagent-grade salts (e.g., modified GP2; see Bidwell and Spotte, 1985 or Table 2 in USEPA, 1994 or USEPA, 1995), 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. Assessment of other variables such as total dissolved gases, ammonia, nitrogen, nitrite, metals, pesticides, suspended solids, and total organic carbon, should be performed as frequently as necessary to document water quality.

As a general guideline for the optimal maintenance of high-quality water, the flow rate of seawater in "once-through" systems should provide 5 to 10 L/d or more for each organism held, and have a flow that equals the tank volume in 6 to 12 h. For static holding tanks, a similar and acceptable exchange rate would be replacement of most of the water on a daily basis. There is no apparent consensus for optimal

Table 2 Checklist of Recommended Conditions and Procedures for Holding and Acclimating Echinoid Adults

Source of Adults	–	collected from clean-water areas or purchased from supply houses
Water	–	uncontaminated natural seawater or *reconstituted* (artificial) *seawater*; flow-through or semi-static (e.g., once every 24 h) replacement; average salinity from 28 to 34 g/kg, and individual measurements not outside 25 to 36 g/kg; rate of salinity change ≤5 g/kg/d 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
Temperature	–	from 10 to 17 °C depending on species, somewhat lower or higher to delay or speed spawning, see Table 1; rate of temperature change ≤5 °C/d for adults to be held for >3 d
Oxygen/aeration	–	dissolved oxygen 80 to 100% saturation; maintained by aeration with filtered, oil-free air if necessary
pH	–	within the range 7.5 to 8.5, in normal circumstances 8.0 ± 0.2
Water quality	–	monitor temperature, salinity, dissolved oxygen, pH, and flow to each holding tank, preferably daily
Lighting	–	normal laboratory lighting at low intensity, 16-h light : 8-h dark; normal daylight, seasonal *photoperiod*; or complete dark. Lighting conditions not considered critical
Feeding	–	for sea urchins; kelp, other macroalga, or romaine lettuce, spinach and carrots; for sand dollars; provide sediment with detritus and alga, use lighting to encourage growth of algae, and if necessary add cultured alga or algal paste
Cleaning	–	removal of old alga, fecal material, and debris, daily or as required, unless intended as food
Disease/mortality	–	monitor mortality daily; for adults held >3 d, mortality should be ≤2%/d averaged over 7 d preceding collection of gametes, and cumulative mortality over the same 7-d 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

amounts of water and exchange times in the existing methods (Appendix D), nor among Canadian and US laboratories responding to a questionnaire in a recent survey (see Section 1.1).[3] Most methods do not specify the flow, and the few that do, range from a high rate of hundreds of litres per animal per day, with an inflow equalling the tank volume in a few minutes, to lower rates which equal the tank volume in about 5 h. NCASI (1991, 1992) uses seawater flows similar to those recommended here, with 7 to 14 L/d per sand dollar and flow that equals the tank volume in 1.3 to 2.7 hours.

"Less-than-optimal" exchange rates and loading densities may be used provided that the criterion for survival in holding tanks (see Section 2.3.10) as well as those for test validity (see Section 4.5.1) are achieved. The regular *monitoring* and documentation of water quality variables in holding tanks (ammonia and nitrite in particular) is highly recommended, and values should be compared to the recommended target values to ensure that metabolic wastes do not reach harmful levels in the holding tanks. Target values, recommended for the protection of aquatic organisms, are ≤0.02 mg/L of un-ionized ammonia and ≤0.06 mg/L of nitrite (CCREM, 1987).[4]

The average *salinity* of the holding water should be 28 to 34 g/kg, preferably 30 to 32 g/kg. Extreme salinity values must not be less than 25 or should not be more than 36 g/kg during holding of echinoids.[5] 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/day and must be ≤5 g/kg/day. Certain situations (e.g., adults spawned for testing on the same day they arrive in the laboratory), however, might require a daily shift of more than 5 g/kg/day. Many laboratories have reported that for tests initiated on the same day or the day after adults arrive in the laboratory, rapid changes in salinity of 6 - 8 g/kg have no effect on the gametes of the test organisms. Therefore, 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 (see Section 4.5.1), and the sensitivity of the gametes in *reference toxicant* tests is not affected (see Section 4.6).

There are reportedly some species-specific differences in salinity tolerance among the echinoids listed herein. During a recent survey (see Section 1.1), some Canadian and US laboratories reported that the Pacific purple sea urchin and the white sea urchin thrive better at

[3] Less-than-optimal exchange rates are currently being practiced by some Canadian and US laboratories recently surveyed. Laboratories with static holding systems reported tank sizes ranging from 10 L to almost 600 L, and exchange rates ranging from 50% daily to 100% every 1 to 2 weeks. Laboratories with larger holding tanks reported using natural seawater and recirculating systems with large-capacity biofilters. Loading densities in static systems ranged from 1 organism/L to 1 organism/10 litres, with the majority reporting loading densities of 1 to 4 organisms/L. Those laboratories reporting higher loading densities also reported more frequent exchange rates. Laboratories with flow-through systems reported tank sizes ranging from 100 to 150 L, and flows of ~5 L/minute.

[4] The recommended target values are criteria for fresh water; however, they should also be protective for marine animals. There are no well established criteria for ammonia in seawater although the freshwater objectives are very well documented. The recommended limit for nitrite is not likely to be reached in marine waters under usual circumstances.

[5] The average *salinity* of world oceans is 34.7 g/kg, and varies from 32 to 37 g/kg except in the Arctic and nearshore areas where salinity can be less than 30 g/kg, or in hot areas of high evaporation rate, where salinity can be over 40 g/kg (Thurman, 1975; McCormick and Thiruvathukal, 1976). Echinoderms are well known to be osmo-conformers with narrow salinity tolerances. Himmelman *et al*. (1984) showed that at 24 to 25 g/kg compared to 30 g/kg, the green sea urchin had a decreased ability to right itself after being inverted, and that ability was a meaningful indicator of general health and physiological state of the animal. Of the existing methods for holding echinoids, those that recommend salinities do not specify values typical of the open ocean, but lower ones, mostly 27 to 30 g/kg with extremes of 25 to 35 g/kg (Appendix D). The normal coastal ocean salinity and guidance from past success in holding echinoids has been used in the present report, particularly with regard to lower limits of salinity (see Section 4.3.2).

higher salinities (34-35 g/kg), however other laboratories reported being able to maintain these species at salinities of 28 to 30 g/kg without problems. For the white sea urchin, salinities <28 g/kg might result in stressed animals and high mortality. Some laboratories reported that the eccentric sand dollar has been found to be slightly more sensitive to large salinity changes, and to salinities lower than 32 g/kg.

Water entering the containers should not be supersaturated with gases, as might occur if the water were warmed. If that is a valid concern, total gas pressure in the water should be checked frequently (Bouck, 1982). Remedial measures must be taken (e.g., use of aeration columns or vigorous aeration in an open reservoir) if dissolved gases exceed 100% saturation.

If reconstituted (artificial) seawater is to be used as dilution and control water (see Section 4.1.1 and Section 5.3), and if adults are going to be held for >3 days after arrival in the laboratory, adults should be acclimated to that water for at least three days immediately before they are forced to spawn. Holding in *reconstituted seawater* or in limited seawater supply might require filtration and recirculation of water, or its periodic renewal in static systems; ammonia and nitrite should then be measured frequently to check that they do not reach harmful levels.

If reconstituted (artificial) seawater is to be used, 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 a suitable fresh water (see EC, 2001 for guidance). The HSB should have a salinity of 90 ± 1 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 (EC, 2001), however, longer periods of aging (i.e., ≥3 days) with aeration are recommended.[6]

The use of HSB derived from an uncontaminated, source of high quality (and preferably high salinity) natural seawater is recommended (EC, 2001), however, artificial hypersaline brine may also be prepared using commercially available dry ocean salts (e.g., Instant Ocean™) or reagent-grade salts (i.e., "modified GP2;" see Bidwell and Spotte, 1985 or Table 2 in USEPA, 1994 or USEPA, 1995). Any artificial HSB which is prepared using commercial sea salt or reagent-grade salts must be filtered (≤1 µm), and then aerated vigorously for a minimum of 24 h before use, however longer periods of aging (i.e., ≥3 days) with aeration are recommended.[6] HSB derived from natural seawater should be filtered (≤1 µm) and may be used immediately for salinity adjustment. Unused portions of prepared natural or artificial HSB should be capped and stored in the dark at 4 ± 2 °C until used (EC, 2001). Additionally, testing laboratories should obtain the "best quality" of commercial sea salts (e.g., Forty Fathoms™ Toxicity Test Grade) available from the supplier. The suitability and consistency of any new products or batches

[6] Research has shown that the type (i.e., brand) of commercially-available sea salts and the way in which it is prepared might significantly influence toxicity test results. In this study the fertilization success of the Pacific purple sea urchin was compared among treatments of different types of artificial sea salt and the preparation techniques used. Six commercial brands (Instant Ocean™, Crystal Sea Marine Mix™, Kent Sea Salt™, Sea Chem Reef Salt™, Ocean Pure Sea Salt™, and Red Sea Salt™) were prepared with different treatments (i.e., *deionized water* vs. *dechlorinated water*, aeration vs. no aeration, and aging for 24 vs. 48 hrs). The results indicated that Ocean Pure Sea Salt™ and Red Sea Salt™ preparations, aged for a minimum of 48 hrs with aeration produced the highest rate of fertilization (similar to fertilization rates observed in natural seawater that had been filtered and sterilized) (Pickard *et al*., 2008). Also, responses to a questionnaire provided by Canadian and US laboratories experienced in echinoid fertilization testing (see Section 1.1) indicate that reconstituted water or HSB prepared by the direct addition of dry salts should be aged (and aerated) for a minimum of 48 hours and longer ≥3 days up to 1-2 weeks would be preferable. Respondents indicated that without aging, the fertilization of eggs is reduced.

should be evaluated for their ability to meet the test-specific validity criteria (see Section 4.5.1), before artificial seawater is used to prepare HSB or control/dilution water (EC, 2001), since some investigators feel that specific types and/or batches of sea salt might produce low fertilization rates in controls, produce unwanted toxic effects, and/or sequester test substances. If ocean salts are used to prepare HSB or control/dilution water, the suitability and consistency of these salts should also be verified by testing.

Hypersaline brine derived from natural seawater may be prepared by concentrating seawater (natural or, less desirably, artificial) by freezing or evaporation. The seawater should be filtered to at least 10 µm before placing it into the freezer or the evaporation chamber. Once prepared, its salinity should be 90 ± 1 g/kg (EC, 2001). If prepared by freezing, freeze at -10 to -20 °C for ≥6 h, and collect the HSB under the ice when it reaches a salinity of 90 ± 1 g/kg. If prepared by evaporation, heat the seawater in a non-corrosive, non-toxic container at ≤40 °C while aerating it, until the desired salinity (i.e., 90 ± 1 g/kg) is achieved (USEPA, 1994, 1995; EC, 2001). Regardless of which technique is used (i.e., freezing or evaporation), the salinity of the brine should be monitored during its preparation, and must not exceed 100 g/kg. HSB may be added to natural seawater, fresh water, *distilled water*, *deionized water*, or test samples, to increase the salinity to the level desired for testing. Guidance in Environment Canada (2001) should be followed when preparing, aging, and storing HSB. If HSB with a salinity of 90 g/kg is used to prepare control/dilution water with a salinity of 30 g/kg (see Sections 3.4 and 4.1.1), the maximum concentration of effluent (or other freshwater sample) that could be tested would be 67%.[7] If, however sample salinity is adjusted by the direct addition of dry salt to the freshwater sample, toxicity can be determined at 100% test concentration.

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. If municipal or natural freshwater sources are used, this water should also be chemically assessed as appropriate to document its quality, for example the items listed at the beginning of this Section (2.3.4).

If municipal drinking water is to be used for preparing reconstituted seawater, effective dechlorination must rid the water of any harmful concentration of chlorine. The target value for total residual chlorine in water used for holding, control tests or dilution, is ≤0.002 mg/L (CCREM, 1987). Available chlorine as low as 0.05 mg/L is a potent spermicide for echinoids (Muchmore and Epel, 1973). Vigorous aeration of the water might strip out volatile chlorine gas. The use of activated carbon (bone charcoal) filters and subsequent ultraviolet radiation (Armstrong and Scott, 1974) is recommended for removing residual chloramine and other chlorinated organic compounds.[8]

2.3.5 Temperature

Echinoids may be held at the optimum temperature ranges identified herein (Table 1), or, if desired, at normal seasonal temperatures (i.e., using the temperature of the incoming natural seawater supplied to the laboratory). A pre-spawning optimum temperature of 12 ± 2°C for the green sea urchin, 10 ± 2°C for the Pacific purple sea urchin, 13 ± 2°C for the eccentric sand dollar, 17 ± 2°C for Arbacia, and 13 ± 2°C for the white sea urchin, should be maintained.[9]

[7] About 33% of the test solution would have to be brine, in order to attain the desired salinity.

[8] Thiosulphate or other chemicals effective in removing residual chlorine from water should not be added to reconstituted seawater that will be used as control/dilution water in toxicity tests. Such chemical(s) could alter sample toxicity.

[9] At the beginning of the spawning season, or just before that season, it might be desirable to keep organisms at somewhat lower temperatures than indicated, to prevent spawning. Advice should be sought from the people collecting at a particular site.

Groups of adults may be held for delayed spawning at temperatures that are lower than the seasonal norm for their habitat, the exact values varying with the species and the desired degree of delay. Laboratories responding to a questionnaire, however, indicated that they had little experience with holding adults at lower temperatures since it is typically not necessary to manipulate test organisms at lower temperatures to delay spawning. Arbacia and white urchins can be held in the laboratory for extended periods of time and therefore it is unnecessary to hold them at lower temperatures to delay spawning, however Arbacia has been reported to feed and regenerate faster if they are held at 15°C after spawning. Similarly, temperature may be raised to encourage early development of gametes. Excessively high temperatures should be avoided in order to prevent spontaneous spawning and stress. Recommended upper limits are 15 °C for green sea urchins, 17 °C for Pacific purple urchins and eccentric sand dollars, 22 °C for Arbacia, and 17 °C for white sea urchins, a species which should not be held at less than 8 °C.

Gradual *acclimation* to test temperature before gamete collection is advised, even if the gametes are to be collected on the day of, or the day after, the spawning adults are received in the laboratory. Water temperatures should be changed to the desired value at a rate of ≤5 °C per day. Certain situations (e.g., adults spawned for testing on the same day they arrive in the laboratory), however might require a daily shift of more than 5 °C. For rapid temperature adjustment of adults held ≤ 3 days prior to spawning, the procedure described in Section 2.3.1 for mixing culture water (or shipping water) and control/dilution water should be used.[10]

[10] Adults are held for 1-2 h in a 50:50 mixture of shipping water:control/dilution water, then for 1-2 h in a 25:75 mixture of shipping water:control/dilution water, followed by a final 1-2 h in 100% control/dilution water before they are spawned. Another useful procedure is to siphon off 20 to 30% of the shipping water every 1 to 2 hours and replace it with laboratory control/dilution water (minimum of three exchanges).

2.3.6 Dissolved Oxygen

The dissolved oxygen (DO) content of the water within holding containers should be 80 to 100% of air saturation. If necessary to achieve that, mild aeration of the water should be carried out using filtered, oil-free compressed air. Such aeration through a commercial aquarium airstone also assists in mixing the water. Overly vigorous aeration should be avoided.

2.3.7 pH

The *pH* of water used for holding adults should normally be in the range 8.0 ± 0.2, and must be within limits of 7.5 to 8.5.[11] The average pH of ocean waters is 8.1 (Thurman, 1975) and seawater has a strong buffering capacity. Coastal waters have a lower salinity than the open ocean, however, and some variation occurs from runoff of fresh water. Uncontaminated seawater is normally within the range of 7.5 to 8.5, whether it is brackish or full-strength, although the extremes of that range would be unusual. Existing methods for toxicity tests with echinoids do not give recommendations for the pH of water used to hold adults (Appendix D). Most laboratories surveyed (see Section 1.1) indicated that the pH range defined herein was not problematic for holding, acclimating, or spawning the echinoid species included in this biological test method. It has been reported, however, that the Pacific purple sea urchin has difficulty acclimating to waters with pH > 8.1 and that high mortality might be experienced if adults are to be held at a pH of >8.1 for longer than a few days (Carr, Nipper, and Biedenbach, pers. comm., 2008).

2.3.8 Feeding

Adult echinoids that are spawned for testing within 3 days of arrival at the laboratory do not require feeding.

Sea urchins that are held in the laboratory for an extended period of time (i.e., > 3 days) should be fed with kelp or macroalga (*Laminaria,*

[11] Aquaculture information suggests that pH below 7.9 might be stressful for marine species in general (Bay and Greenstein, pers. comm., 2008).

Nereocystis, Macrocystis, Egregia, Hedophyllum) or, alternatively, with romaine lettuce. During a recent survey (see Section 1.1), Canadian and US laboratories provided feedback on their experience with the relevance of diet on adult survival, health and spawning success. Most laboratories feed urchins leafy greens (romaine lettuce, spinach, macroalgae), supplemented with carrots and/or algal pellets. One laboratory reported that the nutritional state of the adult sea urchins can affect the sensitivity of their gametes to contaminants and that romaine lettuce alone did not provide adequate nutrition. Researchers at that laboratory found that romaine lettuce supplemented with leaf spinach and carrots offered a better response (Carr, Nipper, and Biedenbach, pers. comm., 2008). Another response to the survey indicated that carrots were an essential component of food provided and enabled the long-term holding of adults with viable gametes (Agius, pers. comm., 2008). Food should be added frequently enough (weekly, daily) that it is always available to the urchins (i.e., *ad libitum*), and old or decomposing food should be removed. Restricted food supply would likely limit success in holding animals in good spawning condition (Bay and Greenstein, pers. comm., 2008). Sea urchins have been held in the laboratory for years using macroalgae. The brown alga *Fucus* has been recommended as food (EVS, 1989) and also recommended against use (Dinnel *et al*., 1987). The green sea urchin in Newfoundland eats *Fucus* and other brown alga such as *Alaria esculenta* as a major component of diet (Himmelman and Steele, 1971). The apparent feeding preference of the sea urchins being held should guide the investigator on use of *Fucus* and other potential food.

Sand dollars normally ingest particles selectively from the bottom and make use of the organic detritus available to them, including microalgae. For this reason, the natural and uncontaminated sediment used on the bottom of containers holding sand dollars should contain such detritus, and especially, settled plankton. Sand dollars have been said to require microalgae such as diatoms on the surfaces of sediment particles, and sufficient lighting can encourage growth of such algae on the sediment, increasing the success of long-term holding of the animals. Algae from a culture, or as an algal paste should be added to the sediment, if necessary (ASTM, 1990).

There are alternatives for feeding sand dollars which might sometimes be useful. Shredded eel grass (*Zostera* sp.) or even spinach could be added weekly, so that the animals can feed on the detritus (EVS, 1989). Flaked fish food may be used as a supplement (NCASI, 1991). However, any decomposing food in the tanks should be removed.

2.3.9 Cleaning the Holding Containers

Holding containers should be cleaned by scrubbing and rinsing before introducing a new *batch* of adults. Disinfectants may be used if it is desired to minimize the transmission of disease. Suitable disinfectants include those containing chlorinated or iodophore compounds or n-alkyl dimethyl benzyl ammonium chloride (e.g., Comet™, Ovidine™, Argentyne™, Roccal™). Disinfectants are toxic to aquatic animals, and traces could carry over on the tanks and affect the echinoids. If disinfection is used, each container must be thoroughly rinsed with the water used for holding.

When holding adults, the containers should be kept reasonably clean. Old macroalga should be removed from urchin tanks, daily or as required. Periodic siphon-cleaning can be used in containers holding sea urchins, and also in sand dollar containers for removing light detritus, fecal pellets, or replacing the sediment. Shell fragments could be left in tanks with sea urchins, since healthy urchins commonly cover themselves with such fragments.

2.3.10 Disease and Mortality

Adult mortality should be low if organisms are acclimated properly to laboratory conditions. Occasionally, laboratories experience some adult mortality in the first week or two after the organisms arrive at the laboratory, or when adults are shipped with very ripe gonads and there is spontaneous spawning. Laboratories have also reported increased mortality in organisms

collected and spawned late in their spawning season (i.e., September or later for the eccentric sand dollar and April or later for the Pacific purple sea urchin). Additionally, some species and/or batches demonstrate a high rate of mortality after spawning.

Adults should be inspected upon arrival at the laboratory and thereafter, daily, for signs of disease. Dead individuals should be removed immediately. 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 seven days preceding 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 to be used for testing again.

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 should be obtained for the batch of organisms shipped from the supplier, and should not exceed 20%. No adults are to be used for same-day gamete collection (i.e., testing on the day that adults arrive at the laboratory), if their cumulative mortality rate exceeds 20% upon their receipt at the laboratory. This same criterion for mortality applies in instances where adults are held briefly (i.e., up to 3 days) before spawning (i.e., the cumulative mortality rate upon receipt and for the ≤3 days before spawning must be <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 discarded or held for an extended period until the mortality rate is acceptably low. Discard also, any moribund animals, sea urchins with significant loss of spines, and sand dollars with patches of fungus. Moribund sea urchins can usually be distinguished by lack of activity of the tube feet, inability to right themselves when turned over, and in particular by lack of adhesion to the substrate. Moribund sand dollars are usually distinguished by external appearance and activity. Such individuals often show patchy or overall pale colour as the epidermis degenerates, and do not rebury themselves. There is only weak activity of tube feet upon close inspection (magnifying glass or dissecting microscope), coupled with limpness of spines and pedicellaria (small pincer-bearing appendages among the tube feet). Dead sand dollars develop a coating of slime and often turn black.

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 discarded.

Test System

3.1 Facilities and Apparatus

Tests are to be conducted in a facility isolated from general laboratory disturbances. If a separate room is unavailable, the test area should be subject to minimal dust and fumes.

Construction materials and any equipment that might contact the test solutions or control/dilution water should not contain any substances that can be leached into the solutions or increase sorption of test substance or material (see Section 2.3.2). The laboratory must have the instruments to measure the basic variables of water quality (temperature, *salinity*/*conductivity*, dissolved oxygen, *pH*), and must be prepared to undertake prompt and accurate analysis of other variables such as ammonia.

All test solutions should be maintained within ± 1 °C of the desired temperature. This can be achieved using various types of equipment such as a temperature-controlled water bath in which test vessels are immersed.

3.2 Lighting

Normal laboratory lighting is satisfactory for the test.

3.3 Test Vessels

The three options for initial volume of test solution are 10.0 mL, 5.0 mL, and 2.0 mL; vessels must be suitable for containing the selected volume. These volumes span the usual range used in other written methods (Appendix D). The 10-mL volume is normally standard, and the smaller volumes are used for special purposes (see introduction to Section 4).

Borosilicate glass vials or tubes are to be used as test vessels. A capacity of about 20 mL is recommended for use with 10 mL of test solution, as is common practice (Appendix D, item 8). For smaller volumes of test solution, size of the vessels should be scaled down, to about double the volume of solution or somewhat more, e.g., vessels of 5 mL capacity for 2 mL of test fluid. However, larger vials may be used if desired, and vials up to 13 mL are sometimes used for 2 mL of test solution (Appendix D). A standard size should be selected and used within a laboratory.[12]

The test vessels should have caps or some other seal, to avoid potential contamination from the air and loss of volatile components. The seal could be a sheet of plastic film which covers all the vessels in a test. The vessels should normally be of the disposable type, new and unwashed before use. An option is to reuse tubes after thorough washing and rinsing, but that technique has been known to result in measurable toxicity,[13] and is not recommended.

Considerable latitude is allowed in the design and shape of test vessels. For a given test, however, every *treatment* must use containers of identical type, size, and shape. Plastic vessels are not to be

[12] Variations in size of test vessel might affect the results of the test through changes in relative depths, relative surface area of the fluid, and other variables, in ways that are as yet unrecognized. General agreement on exact sizes of test vessels does not appear to have occurred at the time of publication of this document. If such agreement develops in the future, investigators should harmonize with the trend, in order to minimize any potential effect of vessel size or proportion.

[13] Dinnel *et al*., (1987) tested different methods of preparing various kinds of vials. Fertilization was excellent in unwashed borosilicate tubes, and absent in acid-washed and rinsed glass tubes (i.e., very toxic). Fertilization was very poor in unwashed polystyrene and polyethylene tubes, and somewhat less toxic in washed plastic tubes, despite successful use of plastic tubes for other purposes such as tissue culture. Some laboratories have encountered sporadic toxicity in certain batches of new unwashed tubes, while most batches did not show toxicity.

used since there is evidence of deleterious effects on fertilization success (Dinnel *et al*., 1987). In descriptions of existing procedures (Appendix D) the vessels are mostly disposable tubes of one kind or another, with caps, and made of borosilicate glass (such as Pyrex™). They are variously described as *scintillation vials, culture tubes, test tubes*, or simply as *tubes* or *vials*, and vessels of those designs would seem satisfactory, if of the appropriate size.

3.4 Control/Dilution Water

Depending on the test material or substance and intent (Sections 5 to 8), the control/dilution water may be uncontaminated natural seawater, reconstituted (artificial) seawater, or a sample of receiving water collected beyond the influence of the zone of contamination. Artificial seawater can be made up to the test salinity by adding the appropriate amount of commercially-available dry ocean salts (e.g., Instant Ocean™, Red Sea Salt™) or reagent-grade salts (e.g., modified GP2; see Bidwell and Spotte, 1985 or Table 2 in USEPA, 1994 or USEPA, 1995) to *deionized water*, or by adding appropriate quantities of natural or artificial hypersaline brine to deionized water (following guidance in EC, 2001 and Section 2.3.4). A supply of uncontaminated natural seawater with a lower (i.e., <28 g/kg) or higher (i.e., >32 g/kg) salinity can be mixed with the appropriate amount of dry ocean salts, reagent-grade salts, natural or artificial HSB, or deionized water sufficient to adjust its salinity to within the test range. During prolonged storage (>1 day), natural or artificial seawater prepared for use as *dilution water* should be refrigerated (4 ± 2°C) to minimize microbial growth (EC, 2001). If receiving water is used, conditions for collection, transport, and storage should be as described in Section 6.1. Control/dilution water used in any given test must be from the same source (if natural seawater) or the same batch (if reconstituted water), and artificial seawater should not be used after 14 days following its preparation (EC, 2001). All marine waters used as a source of control/dilution water, including the laboratory supply of natural seawater, should be passed through a filter with a pore size of approximately 60 µm (USEPA, 1994).

Salinity of control/dilution water should be 30 g/kg and must be in the range 28 to 32 g/kg. Lower salinities should be adjusted upwards using aged hypersaline brine with a salinity of 90 ± 1 g/kg (see Section 2.3.4), and higher ones should be adjusted downwards with deionized water, distilled water, or uncontaminated fresh water.[14]

The pH of control/dilution water must be in the range 7.5 to 8.5, and should normally be 8.0 ± 0.2. Those values would usually be obtained because of the natural buffering capacity of seawater. If not, adjustment should be made with acid or base (Section 4.3.4).

Control/dilution water must be adjusted to the test temperature before use. It should not be supersaturated with excess gases (see Section 2.3.4), and must contain dissolved oxygen at 90 to 100% of the air-saturation value before use. If necessary, achieve that level by aerating vigorously with oil-free compressed air passed through air stones.

[14] Gametes of some or all of the test species would presumably tolerate salinities outside the recommended limits (see Section 4.3.2 and associated footnote), particularly higher salinities close to the oceanic average of nearly 35 g/kg. Salinities near 34 or 35 g/kg might, in fact, be less stressful than lower ones in the vicinity of 28 to 30 g/kg. The range of values recommended here considers the natural oceanic salinities and also the ranges used successfully in existing echinoid methods, i.e., salinities that are mostly near 30 g/kg (Appendix D).

Universal Test Procedures

Procedures described in this section apply to all the tests of particular chemicals, wastewaters, or receiving-water samples described in Sections 5, 6, and 7, and liquid samples derived from sediment (i.e., pore water) or similar solid materials, described in Section 8. All aspects of the test system described in the preceding Section 3 must be incorporated into these universal test procedures. The summary checklist of recommended conditions and procedures in Table 3 includes not only universal procedures but also those for specific types of test materials or substances.

There are some choices allowed within the general test procedures given in this report. Three options are available for duration of exposure. The shortest duration is a 10-min exposure of sperm, continued for an additional 10-min after eggs are added. That is the recommended standard exposure and it would minimize aging of *gametes* during a test or set of tests. The short exposure would also be most suitable for intensive programs involving many tests. For example, when attempting to identify toxic compounds in a complex effluent (TIE programs), successive manipulations of the effluent could be done before it aged appreciably.

A second option is a 20-min exposure of sperm plus 20 min of sperm plus eggs. That exposure might be used if it were desired to parallel certain existing methods or research results (Appendix D). The longest duration is 60 min of sperm plus 20 min of sperm plus eggs, an option that might be selected if maximum sensitivity were desired in the test. This longest exposure is also associated, however, with increased variation in results (see Section 4.2.4).

Three options are also available for the volume of test solution, which can be 10, 5, or 2 mL of each concentration of the sample. The 10-mL volume would be the usual standard choice and is preferred by Canadian investigators. The larger volume should be most convenient for manipulations by the operator and might improve the relative precision in handling small volumes. The smaller volumes require fewer adults to provide an assured supply of gametes, and can require less space in a water bath or constant temperature chamber. Small volume might be important for some investigations such as trials with pilot-plant outputs, perhaps as part of a TIE program.

4.1 Preparing Test Solutions

All test vessels, measurement devices, stirring equipment, and pails for transferring organisms must be thoroughly cleaned and rinsed in accordance with standard operational procedures. Control/dilution water should be the final rinse water for items which are to be used immediately in setting up the test; distilled or deionized water should be used as the final rinse for items which are to be stored after allowing them to dry.

4.1.1 Control/Dilution Water

The same control/dilution water must be used for preparing the control and all test concentrations. Each test solution must be well mixed with a glass rod, Teflon™ stir bar, or other device made of non-toxic material.

The temperature of the control/dilution water and the sample or each test solution must be adjusted as necessary to within ± 1 °C of the

Table 3 Checklist of Recommended and Required Test Conditions and Procedures

Universal		
Test type	–	*static*; standard sperm exposure of 10 min, continuing with 10-min exposure of both sperm and eggs to allow fertilization; alternative exposures 20 + 20 min, or 60 + 20 min
Control/dilution water	–	filtered (60 µm) uncontaminated laboratory seawater; reconstituted (artificial) seawater, filtered (60 µm) "upstream" receiving water to assess toxic impact at a specific location, with additional control of laboratory seawater; dissolved oxygen (DO) content 90 to 100% saturation at time of use; salinity 28 to 32 g/kg, preferably 30 g/kg, and pH 7.5 to 8.5, preferably 8.0± 0.2
Organisms	–	each *replicate* test vessel receives about 2000, 1000, or 400 eggs, depending on the selected volume of test solution; sperm:egg ratio is ascertained in a pre-test as that which targets an optimum of 80% fertilization under control conditions
Number of concentrations	–	minimum of 7, plus control(s); recommend more (i.e., ≥10), plus control(s)
Replicates	–	≥3 per *treatment* (recommend 5) for calculation of *ICp;* ≥4 per *treatment* if single-concentration (e.g. full strength) for hypothesis testing
Vessel/solution	–	standard volume of 10 mL test solution, with alternatives 5 or 2 mL; borosilicate glass vessels, capped or sealed
Temperature	–	for the native species 15 °C (green sea urchins, Pacific purple sea urchins, eccentric sand dollars), and 20 °C for Arbacia and white sea urchins; range for individual test vessels ± 1 °C of desired temperature
Salinity	–	standard test salinity 30 g/kg, limits 28 to 32 g/kg; each test solution in that range and also within 1 g/kg of the control; adjust salinity of sample or test solutions as necessary using hypersaline brine (HSB) with a salinity of 90 ± 1 g/kg, commercially-available dry ocean salts, reagent-grade salts or, if sample salinity >32 g/kg, deionized water; adjust salinity of control/dilution water as necessary using HSB at 90 ± 1 g/kg or dry salts; test requires second set of controls adjusted to 30 ± 2 g/kg and prepared by adding aged HSB (90 ± 1g/kg) or dry salts to deionized water, if HSB or dry salts added to sample/test solutions and if control/dilution water differs in any respect
Oxygen/aeration	–	no pre-aeration of aliquots of sample (e.g., effluent) or test solution unless DO is estimated to be <40% or >100% saturation in any concentration, in which case aerate an aliquot of the sample for ≤20 minutes through a plastic or glass tube with a small aperture (e.g., 0.5 mm ID) at a rate ≤100 bubbles/min, before making up concentrations and starting the test; no aeration during test
pH	–	regulatory or monitoring tests normally require no adjustment of pH of sample or solution; for other purposes, adjustment or a second (pH-adjusted) test might be required or appropriate; limits of pH 7.5 to 8.5, preferably 8.0 ± 0.2, apply for minimizing direct effects of pH on the gametes, and maximizing the potential for detecting toxic chemicals
Lighting	–	normal laboratory lighting or natural sunlight; variable photoperiod
Observations	–	percentage of fertilized eggs among 100 to 200 inspected microscopically for each test vessel
Measurements	–	temperature, salinity, pH, and DO at start of exposure, in aliquots of test solutions for high, middle, low concentrations and control
Endpoints	–	in multi-concentration tests, ICp for fertilization success; in single-concentration tests, percent fertilization and whether significantly lower than control; in porewater tests, percent fertilization and whether significantly lower than control or reference pore water at each treatment level (i.e., for each porewater dilution)
Reference toxicant	–	copper is recommended; determine ICp for fertilization success; perform within 14 days of the definitive test , or concurrently with definitive test for every new *batch* of adults if held ≤ 3d
Test validity	–	average success of fertilization in control must be ≥60% and <98%

Chemicals

Solvents	–	to be used only in special circumstances; maximum concentration 0.1 mL/L
Concentration	–	measurement at start is recommended, in aliquots of high, medium, and low strengths and control(s)
Control/dilution water	–	as specified and/or depends on intent; reconstituted seawater if high degree of standardization required; receiving water if concerned with local toxic impact; otherwise, uncontaminated laboratory seawater

Effluents, Leachates, and Elutriates

Sample requirement	–	2 L should be adequate for the assay and for routine chemical analyses
Transport, storage	–	if warm (>7 °C), must cool to 1 to 7 °C with regular ice (not dry ice) or frozen gel packs upon collection; transport in the dark at 1 to 7 °C (preferably 4 ± 2 °C) using frozen gel packs as necessary; sample must not freeze during transit or storage; store in the dark at 4 ± 2 °C; use in testing should begin within 1 day and must start within 3 days of sample collection or elutriate extraction; extraction from sediment should occur within 2 weeks and must occur no later than 6 weeks after sampling
Control/dilution water	–	as specified and/or depends on intent; laboratory seawater or "upstream" receiving water for *monitoring* and *compliance*
Suspended solids	–	normally do not filter; filter effluent or leachate through 60 µm sieve if sample contains debris or indigenous organisms that could be confused with or attack the gametes or fertilized eggs; centrifuge elutriate

Receiving Water

Sample requirement	–	as for effluents, leachates, and elutriates
Transport, storage	–	as for effluents, leachates, and elutriates
Control/dilution water	–	as specified and/or depends on intent; if studying local impact use "upstream" receiving water

Sediment and Similar Solids

Transport, storage	–	temperatures as for effluents and leachates; test should start within 2 weeks and must start within 6 weeks
Preparing/testing	–	aqueous samples derived from sediments should be treated as for effluents, leachates, and elutriates; solvent-based extracts should have balanced solvent concentrations; this is not a suitable assay for the solids themselves
Reference sediment	–	parallel test with clean sediment of similar physicochemical properties (uncontaminated sediment), if possible; otherwise use any clean (control) sediment
Control/dilution water	–	as for effluents, leachates, and elutriates

test temperature, before starting the test. Sample or test solutions must not be heated by immersion heaters, since this could alter chemical constituents and toxicity. It might be necessary to adjust the salinity or pH of the sample of test substance or the test solutions (see Sections 4.3.2 and 4.3.4), or to provide preliminary aeration (Section 4.3.3).

Control/dilution water may be the laboratory's online supply of uncontaminated natural seawater, *"upstream" water* (i.e., receiving water) from a specific location under investigation, or reconstituted (artificial) seawater (see Section 2.3.4 and 3.4). As necessary, quantities of dry ocean salts (e.g. Instant Ocean™, Red Sea Salt™), reagent-grade salts (e.g., modified GP2; see Bidwell and Spotte, 1985 or Table 2 in USEPA, 1994 or USEPA, 1995), natural hypersaline brine, artificial hypersaline brine, or deionized water should be added to seawater to adjust it to the test salinity (30 ± 2 g/kg). Any HSB, dry ocean salts, or reagent-grade salts used must be from the same source as that used to adjust the salinity of the test sample or test solutions (see Sections 5.2 and 6.2).

If any HSB is added to the test sample/solutions to adjust salinity, the toxicity test must include a set of controls (*HSB controls*) prepared using only this HSB and deionized water, adjusted to the test salinity (30 ± 2 g/kg). Likewise, if any commercially-available dry ocean salts or reagent-grade salts are added to the sample or test solutions, the toxicity test must include a set of controls (i.e., *salt controls*) which is prepared using the same source, batch, and concentration of dry salts as that added to the test sample. A second set of controls (i.e., *dilution-water controls*), comprised of 100% dilution water, is required if any water used to dilute the sample differs in any respect from the *HSB controls* or *salt controls* (e.g., natural seawater with or without HSB or dry salts added; natural fresh water with HSB or dry salts added, etc.) (see Section 4.1.4).

If natural seawater must be stored, it should be held at the test temperature or cooler, and should be used in three days or less.

Portions of seawater (i.e., control/dilution water or control/reference pore water) used for determining sperm density (see Section 4.2.2) and the appropriate sperm-to-egg ratio to be used in the test (see Section 4.2.3) should be filtered to remove solids that might interfere with sperm counts. Filtration is particularly important for natural seawater. A filter of pore size approximately 60 µm (USEPA, 1994) is recommended for this purpose. Filtered water should be used in three days or less.

Receiving water may be used as control/dilution water to simulate local situations such as effluent discharge, a spill of chemical, or pesticide spraying. If that is done, a second control solution must be prepared using the laboratory seawater in which adults were kept (see Section 4.1.4). "Upstream" receiving water cannot be used, however, if it is clearly toxic and produces an invalid result in the control according to the criteria of this fertilization assay.[15] In such a case, reconstituted seawater (Section 2.3.4) or the laboratory's natural seawater should be used as control/dilution water. The laboratory water

[15] Lower levels of contaminants, already in the receiving water, might not affect the controls by themselves, but could add toxicity to that contributed by the substance or material being tested. In such cases, uncontaminated dilution water (reconstituted or natural seawater) would give a more accurate estimate of the individual toxicity of the substance or material being tested, but would almost certainly underestimate the total impact at the site of interest.

If the intent of the test is to determine the effect of a specific substance or material on a specific receiving water, it does not matter if that receiving water modifies sample toxicity by the presence of additional toxicants, or conversely by the presence of substances or materials that reduce toxic effects, such as humic acids. In the case of toxicity being added by the receiving water, it would be appropriate to include in the test, as a minimum, a second control of laboratory seawater or reconstituted seawater and, as a maximum, another series of concentrations using such clean water as diluent.

could also be used if the collection and use of receiving water is impractical.[16]

4.1.2 Concentrations

For any test that is intended to estimate the *ICp* by regression analysis (see Section 4.5.2), at least seven test concentrations plus a control solution (100% control/dilution water) must be prepared, and more *treatments* (≥10 plus a control) are recommended. An appropriate geometric dilution series might be used, in which each successive concentration is about 0.5 of the previous one (e.g., 100, 50, 25, 12.5, 6.3, 3.1, 1.6, etc.). Test concentrations may also be selected from other appropriate dilution series (e.g., 100, 75, 56, 42, 32, 24, 18, 13, 10, 7.5; see column 7 in Appendix F). A dilution factor as low as 0.3 (e.g., concentrations 100, 30, 9, etc.) is not recommended for routine use because of poor *precision* of the estimate of toxicity; however, it might be used if there is considerable uncertainty about the range of concentrations likely to be toxic.

Each desired concentration is prepared and the standard volume selected (10.0 mL, 5 mL, or 2 mL) is added to the *replicate* test vessels. These nominal concentrations of the solutions (or measured concentrations, see Section 5.4) are adopted as the concentrations of the test. The slight decrease in concentration upon addition of the aliquot of sperm suspension is neglected.[17] The nominal concentration during the exposure of sperm is adopted as the concentration of the entire test. There is a concentration decrease of about 9% in the final part of the test, after the suspension of eggs is added, but for purposes of characterizing the test, the initial concentrations for sperm exposure are used.[18]

In cases of appreciable uncertainty about sample toxicity, it is beneficial to run a range-finding or screening test for the sole purpose of choosing concentrations for the definitive test. Conditions and procedures for running the test can be relaxed. A wide range of concentrations (e.g., ≥2 orders of magnitude) should assist in selection for the full test.

Single-concentration tests could be used for regulatory purposes (e.g., pass/fail). They would normally use full-strength effluent, leachate, receiving water, elutriate or other liquid (i.e., pore water) from a sediment or similar solid, or an arbitrary or prescribed concentration of chemical. Use of controls would follow the same rationale as multi-concentration tests. Single-concentration tests are not specifically described here, but procedures are evident, and all items apply except for testing only a single concentration and a control.

4.1.3 Replication

If a multi-concentration test is conducted and an ICp is determined, each treatment including the control(s) must include a minimum of three *replicate* test vessels, and more than three (i.e., five) are recommended. If a single-concentration test (or multiple full-strength solutions) is conducted and hypothesis testing is used[19] each

[16] An alternative that could partially simulate receiving water would be to adjust the salinity (and perhaps the pH) of the laboratory seawater or reconstituted seawater to that of the receiving water. Depending on the situation, the adjustment might be to some particular seasonal value or mean. Adjustments could be made by methods mentioned in Section 2.3.4.

[17] The actual concentrations to which the sperm are exposed would be 99% of the concentrations of the test solution, since 0.1 mL of sperm suspension is added to 10 mL of test solution, and proportional additions are made to tests of smaller volume. For example, if full-strength effluent were being tested in a vessel, the sperm would actually be exposed to $10/10.1 \times 100\% = 99.0\%$. No allowance is made for that slight difference.

[18] In some cases, at least, the sperm will be more sensitive than the eggs, and therefore the concentration during the initial sperm exposure can be the operative factor. For example, the toxic components of the effluent from bleached kraft pulp mills are reported to act primarily on the sperm rather than the eggs, in an echinoid fertilization assay (Cherr *et al.*,1987). Kobayashi (1984) reviews his own work and that of other authors and concludes that male gametes are frequently "the most sensitive link in the success of fertilization and subsequent embryonic development".

[19] Note that this hypothesis testing is not used to derive NOECs and LOECs, and this test design does not involve dilutions. Instead, it is used to determine if significant differences exist between full-strength test solutions.

treatment including the control(s) must include a minimum of four *replicate* vessels[20] and more than four is recommended.

4.1.4 Controls

A control exposure which employs the same control/dilution water (*dilution-water control)* that is used to make up the test concentrations is required for all tests. A separate set of controls comprised solely of hypersaline brine (HSB) or dry salts in deionized water at a salinity of 30 ± 2 g/kg (Sections 2.3.4 and 4.1.1) is required if HSB or dry salts are added to the test sample or test solutions (Section 4.3.2), and if the dilution water differs from this *HSB control* or *salt control* in any respect. Each control must have the same number of replicates (i.e., at least 3) as for each of the other test solutions. The results for each *dilution-water control, HSB control*, or *salt control* used in a toxicity test must be examined to determine if they independently meet the test-specific criterion or criteria for test validity (see Section 4.5.1). In instances where two sets of control solutions are used (i.e., *HSB controls* or *salt controls* as well as *dilution-water controls*), the results for the toxicity test are considered to be valid and acceptable only if each set of control solutions independently meets the respective validity requirements (see EC, 2001 and EC, 2005 as well as Section 4.5.1). If, and only if, both sets of controls have met the validity criteria of the test, and the results of the two sets of controls are not statistically different from one another, then the results of the two sets of control solutions may be pooled (if desired) before calculating any statistical endpoints of the findings for each set of test concentrations versus those for the control solutions. Pooling control data for these two sets of controls before determining if the test results are valid or not is not acceptable (EC, 2001). If the controls are statistically different from each other by *t*-test (EC, 2005), then the two sets of data must not be pooled and the most applicable of the controls are used to calculate any statistical endpoints.

A set of *salinity controls* should be run if test salinity is, for any reason, outside the required range of 28 to 32 g/kg. If samples which were essentially fresh water (salinity ≤5 g/kg) were tested without adjusting salinity, salinity controls should be prepared by adding deionized or distilled water to a series of test vessels, at the same concentrations as used for the test liquid. The salinity controls indicate the effect of low salinity acting alone, but do not indicate any increased effect caused by interaction of low salinity with toxic substances or materials in the sample (see Section 4.3.2).

If a solvent is used in testing a chemical that is sparingly soluble, then a "solvent control" must be run with replicates, and must contain the solvent at the highest concentration present in any test concentration.

If receiving water is used as the control/dilution water, a second set of controls must be run using the laboratory seawater (artificial or natural) that was used for holding the adults (see Section 2.3.4).

Additional kinds of controls are not required, but are recommended to improve the ability to judge quality of results. A "low-sperm" control would use only half the number of sperm in order to check for "over-sperming", which is a common imperfection in this assay. If the normal control achieved >90% fertilization and the low-sperm control was not 5% lower than the rate in the normal control, over-sperming is indicated, with associated poor sensitivity of the test. A "toxicant/egg control", or "egg blank" uses a high concentration of the toxicant, but no sperm; it can indicate whether the sample being tested causes false fertilization membranes. A "control blank" with eggs but no sperm can reveal accidental contamination of stocks of eggs with sperm (Chapman, 1991).

[20] The requirements given here for replication are generally in line with those in other methodology documents reviewed in Appendix D. Five of the methods recommended "three or more" replicates, some acknowledging that four replicates would be required for nonparametric statistics. Five methods do not specify number of replicates.

4.2 Beginning and Performing the Exposure

Semen containing sperm is collected from several echinoids by forced spawning. Semen from each individual can be pooled before use. Eggs are collected, and can be pooled in the same fashion. Sperm are exposed to the test substance or material in each test vessel for either 10, 20, or 60 min. Then an appropriate number of eggs is added to each vessel, and exposure continues for 10 to 20 min to allow fertilization. Preservative is added to each vessel to end the exposure.

4.2.1 Collecting Gametes for the Test

Ideally, the sperm 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 must be performed to ensure that only good-quality gametes are being selected for use in the test. If good-quality gametes from three adults of each sex cannot be obtained (see following paragraph), and/or if in addition to the gamete check a pre-test is carried out with individual gamete combinations of at least 2 males and 2 females (as described in Section 4.2.3), it is permissible to use gametes from only one adult from each sex (i.e., 1 male and 1 female whose gametes yield good fertilization success when combined in a gamete check and pre-test).

A gamete check is required to ensure that a subsample of gametes from each of the several 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, three to five females and at least 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 semen from each male are stored separately on ice. A small portion of each male's sperm is then diluted with control/dilution water on 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. Small aliquots of eggs from each female having "good-quality" eggs are then placed in several scintillation vials. 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 for each female (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 - 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 minutes each mixture of sperm and eggs in each vial are observed under a microscope. 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.

The number of eggs fertilized in each vial should also be examined. If the proportion of eggs fertilized is high (i.e., 95-100%) in a particular vial, and a pre-test (see Section 4.2.3) is carried out on the same batch (i.e., gametes from the individual male and female being stored on ice) of gametes to determine the optimal sperm:egg ratio, then the original batches of sperm from the male and the eggs from the female for which combined aliquots (subsamples) showed a high fertilization rate, can be used in the definitive test. If a laboratory chooses not to run a pre-test to determine the "optimal" sperm:egg ratio, then good quality gametes pooled from at least three males and three females, as determined in a

gamete check, must be used. Only good-quality gametes are pooled and then used in the test.[21] If good-quality gametes are not available from three males and three females, fewer adults may be used, however a pre-test must be carried out to determine the optimal sperm:egg ratio for a given batch of gametes, prior to the definitive test, thereby improving the likelihood of successful control fertilization. "It is more important to use high quality [gametes] than it is to use a pooled population of [gametes]" (Chapman, 1992a).

The adults are stimulated to spawn by injecting potassium chloride.[22] 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 (Figure 2).[23] 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. An alternate method, that appears to work only with Arbacia, is stimulation of the shell for 30 seconds by electrodes supplied with 12 volts D.C.[24]

The preferred and recommended technique for collecting semen from male sea urchins is called "dry spawning". Once sperm is wetted, it has limited viability (see footnote 25), so in order to complete both a gamete check and a pre-test (see Section 4.2.3), and still have viable sperm for use in the definitive test, 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, and with control/dilution water covering only the lower half of the test or 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. Similar techniques may be used for collecting eggs from females, if desired, but they should be washed and stored as indicated below.

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)[25]; however, they should be suspended over the water column. (Experience indicates that sand dollars won't spawn if placed in a seawater-rinsed petri dish with their aboral surface in direct contact with the bottom of the dish).

For the alternative "wet spawning" method, each sea urchin or sand dollar is placed aboral side

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[21] Eggs should be large and round, sperm should be active and fertilization should be high (i.e., 90-100%).

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

[23] The following website provides a good description and an animated diagram for injecting sea urchins to induce spawning: http://www.stanford.edu/group/Urchin/ani-plus.htm.

[24] An electro-stimulator can be constructed from an aluminum box containing a mini-transformer, which converts 115 V current to 12/24 V current at 2.4 A. The out-going current is directed to two ports to which electrical probes are plugged (similar to probes used in electrical current testers). When plugged in, these two probes are touched to the top of the urchin near the gonopores for short periods of time (10-30 seconds), which results in the urchin spawning. Typically the urchin will spawn for only a short time while the stimulation is taking place. If the urchins are very ripe, however, they will continue to spawn even if the stimulation is removed (Carr, Nipper, and Biedenbach, pers. comm., 2008).

[25] Gamete viability for sand dollars is greatly enhanced when organisms are spawned into seawater with a salinity of >30 g/kg (Pickard, pers. comm., 2008).

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, decant as much water as possible 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.

If there is no spawning in 5 or 10 minutes, a second injection may be used, however this might cause the organisms to extrude gametes that are immature and of poorer 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, and eggs will appear as somewhat granular material, usually with a pastel colour (pinkish in sand dollars). Coloured products 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 by means of a scalpel, to provide a bore diameter of approximately 1 mm and reduce damage to the eggs.

Semen collected "dry" may be held on ice[26] for 4 h before "activation" in seawater, then used in a test in the subsequent 30 to 120-min period.[27] If sperm are collected in beakers of seawater, they should be used to start the test in a period ≥0.5 h 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.

The collected eggs are washed three times by diluting with 100 mL of control/dilution water, mixing, settling for 10 minutes, and decanting. If pigmented substance is obtained with the eggs, it might be important to rinse them soon after collection, since the substance might be toxic to the Pacific purple sea urchin and perhaps with other species.[28] 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 aerated gently during holding.

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[26] 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, pers. comm., 2008).

[27] Sperm commence a series of metabolic changes when exposed to seawater, enabling them to carry out fertilization. Following that activation, there is a period of an hour or less of vigorous activity, followed by gradual loss of vitality in the following 6 to 9 hours, the loss becoming more rapid at higher temperatures (Dinnel *et al*., 1987). Some work shows a large increase in variability of test results if sperm are held in seawater before testing. Comparison of the toxicity of bleached kraft pulp mill effluent for eccentric sand dollar fertilization showed that "pre-activation" of sperm for 60 min in seawater, before the 80-min test, increased the CV to 91%, from the value of 38% for sperm used without the pre-activation. Sensitivity of the test increased by a factor of 1.6 for pre-activation, i.e., a smaller relative change than the increase in variation (NCASI, 1992).

Activation of sperm can be delayed, and the useful period before starting a test prolonged by keeping the semen cool (on ice), in "dry" conditions as released from the adult. The time limits for holding gametes, given in Section 4.2.1, are strongly recommended as a means of standardizing the test. Other times and techniques may, however, be used if it is demonstrated that fertilization rates of about 90% are normally obtained in the controls. Some investigators report that "dry" semen can be held satisfactorily on ice for longer periods, e.g., 8 hours for Arbacia (Carr and Chapman, 1992), but 4 hours is a more usual maximum (Chapman, 1992a).

[28] Excess handling might decrease fertilization success rate, therefore washing the pigment from the eggs might be unnecessary (Buday, pers. comm., 2008).

4.2.2 Preparing Standard Suspensions of Gametes

Semen from the male sea urchins or sand dollars, chosen following the gamete check (see Section 4.2.1), 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 delivering 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 cell under 400 × magnification.[29] Dilute a small sample (0.1 to 1 mL) of the mixed suspension 100-fold to 10 000-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 minutes. 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) × (hemocytometer conversion factor) × (conversion of mm^3 to mL) ÷ (the number of squares counted). For a standard hemocytometer (Neubauer), the formula becomes:

No. sperm/mL = 100 × (No. of sperm counted) × 4000 × 1000 ÷ 400

Adjust the initial suspension of sperm to the desired concentration in a "standard sperm suspension", using control/dilution water.[30] The concentration of this standard sperm suspension is determined by the sperm:egg ratio that is selected (Section 4.2.3).

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. The advantage is a saving of time, since the measurement takes only one minute compared to 20 to 30 minutes with a hemocytometer (NCASI, 1992). That in turn allows tests to start sooner after collection of gametes. The concentrated collection of sperm is mixed with control/dilution water in a 1-cm spectrophotometer tube, just before starting the test. Standard turbidity meters designed for analysis of water samples may be used. NCASI (1992) reports that a range of 2.0 to 4.0 Nephelometric Turbidity Units (NTU) usually yields the desired numbers of sperm. A count of 2.5 million sperm/mL would be associated with about 3.0 NTU for the eccentric sand dollar, and about 2.7 NTU for the Pacific purple sea urchin. The turbidimetric technique can have precision that is almost as good as that obtained by counting. NCASI (1992) found an average CV of about 9% for repeated hemocytometer counts of single dilutions of sperm, and a CV of 12% for repeated hemocytometer counts of dilution to 5.0 NTU of sperm from three males. No evaluations of the turbidimetric method are available from other laboratories at the time of writing. The final criterion of whether turbidimetric assessment of sperm density was satisfactory would be the fertilization rate achieved in the control, during the test, compared to the validity criterion of ≥60%, and <98% fertilization (Section 4.5.1).

There are three options for initial test volume, the standard of 10 mL and of 5 or 2 mL. The concentrations of the gamete suspensions are the same for each. The amount of gamete suspension to be added is scaled down proportionally for the smaller test volumes. In the largest test volume (10 mL), there is 0.1 mL of sperm suspension added, and 1.0 mL of egg suspension. (See Table 4 for summary of numbers of gametes and volumes of gamete suspensions for the three sizes of test).

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

[30] The instructions for 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.

Table 4 Summary of Sperm and Egg Additions to Each Test Vessel for the Three Test Volumes

The numbers of sperm in columns 4/5 and 7 are governed by the sperm:egg ratios of 200:1 and 2500:1 selected as examples.

Initial test volume (mL)	Number of eggs	Volume of egg suspension	Number of sperm (millions) at usual sperm:egg ratios of		Volume of sperm suspension added (mL)	Usual range of concentration in sperm suspension (millions/mL)
			200:1	2500:1		
10	2000	1.0	0.4	5	0.1	4 to 50
5	1000	0.5	0.2	2.5	0.05	4 to 50
2	400	0.2	0.08	1	0.02	4 to 50

The numbers of gametes and procedures are given here for a test with initial volumes of 10 mL. The required strength of the sperm suspension must be calculated first. About 2000 eggs are used in the 10-mL test, and the ratio of sperm to eggs is often in the range 50:1 to 2500:1 (Section 4.2.3), although it might sometimes be higher, to 20 000:1 or more. Within the range 50:1 to 2500:1, the required number of sperm would be from 100 000 to 5 million. Since 0.1 mL of the sperm suspension is added in the test, the concentration of sperm required in the standard suspension will usually be in the range of one million to 50 million per mL.[31]

Calculations of proper dilution are easily done by the following standard chemistry formula:

$C1 \times V1 = C2 \times V2$

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

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:

$125 \times V1 = 40 \times 5$
therefore, $V1 = 1.6$ mL

Determine the density of the mixed suspension of eggs by counting and adjust to 2000 eggs/mL.

Counting can be done by adding to a Sedgwick-Rafter cell, 1 mL or less of the mixed suspension as required, then observing at 20 to 100 × magnification. It is often useful to dilute an aliquot 10-fold, 100-fold, or, in some instances, 1000-fold, for the purpose of counting. With experience, the original suspension can be diluted according to its appearance, to a few hundred eggs/mL, then a count is made with 0.5 mL. Other techniques of counting may be used if they are effective. Adjust the suspension to 2000 eggs/mL by adding control/dilution water to reduce the density, or settling eggs and decanting water to increase the density.

[31] Other volumes of sperm solution might be used, with appropriate changes in concentration of the sperm, but larger volumes are not recommended. For example, some existing procedures add 0.5 mL of sperm solution. That would decrease the concentration of the test substance or material by about 5%, however, and such a change is large enough that it should be accounted for in the calculations of effect. The recommended low volume of 0.1 mL of sperm suspension causes only a small change in concentration that can be neglected for most purposes.

For a test with an initial volume of 5 mL, exactly the same procedures are followed except that smaller volumes of the gamete suspensions are added to the test vessels (Table 4). The volume of sperm suspension added would be 0.05 mL (usually containing 2 to 25 million sperm, depending on the sperm:egg ratio required), and the volume of egg suspension added would be 0.5 mL (containing 1000 eggs).

For a test with an initial volume of 2 mL, proportionally smaller volumes of gamete suspensions are used. The added volume of sperm suspension would be 0.02 mL (usually containing 0.8 to 10 million sperm), and the added volume of egg suspension would be 0.2 mL (containing about 400 eggs).

4.2.3 Ratio of Sperm to Eggs

The optimum sperm-to-egg ratio should be determined by pre-test in each laboratory, as that which targets an optimum rate of 80% fertilization under control conditions.[32] Very low fertilization rates in the control would mean that effects of a toxicant on fertilization might be difficult to distinguish from the generally poor and variable background performance. Rates that are high indicate an excess of sperm that might mask an effect by compensating for part of the toxicity, thus reducing the sensitivity of the test and raising the IC25.[33] Several options are available for determining a suitable sperm:egg ratio, since the final criterion of a satisfactory test will be the actual rate of fertilization achieved in the control, which must be between the control limits of ≥60% and <98% for a valid test (Section 4.5.1).

Ratios that have been reported in the literature to give satisfactory fertilization range from 50:1 to 2500:1 for the various test species (Appendix D). The following sperm-to-egg ratios have been reported by Canadian and US laboratories in a recent survey (see Section 1.1) to achieve a fertilization range of 70 to 90%: green sea urchin, 2000:1 up to 5000:1[34]; Pacific purple sea urchin, commonly 100 to 500:1 but as low as 2:1 and as high as 2000:1; eccentric sand dollar, often about 200:1 to 400:1 but also reliably reported in the range 50:1 to 6000:1; white sea urchin, typically 20,000:1; and Arbacia, 2500:1. Such general guidance cannot, however, be depended on to yield satisfactory test results in any given laboratory or season. Canadian interlaboratory tests, for example, found that some sperm:egg ratios had to be an order of magnitude higher than values mentioned above (Miller *et al*., 1992).

[32] The target of 90% fertilization success was originally selected since it helps avoid the problems of too many and too few sperm. For this second edition, however, the target fertilization has been changed to 80% due to concern of the loss of test sensitivity with higher rates of control fertilization (see next footnote). In addition, most Canadian and US laboratories responding to a recent survey (see Section 1.1) indicated that they target 80% fertilization in the controls since better (i.e., more sensitive) results are achieved when the controls have 80% fertilization. It is possible that other agencies might, in the future, adopt standardized tests which stipulate higher fertilization rates under control conditions, perhaps ≥95%. The objective of those other agencies would be to decrease variability between laboratories, at some sacrifice of sensitivity. Achieving 80% fertilization often requires a fairly delicate balance of the gamete ratio and other factors, and suitable procedures must be established within each laboratory.

[33] There is some evidence in the literature, of appreciable loss of sensitivity of this test at higher rates of fertilization in the controls, resulting from high sperm:egg ratios. NCASI (1992) found that each of seven paired assays of pulp mill effluent showed decreased sensitivity in the test which had the higher rate of control fertilization (based on IC50s for gametes of eccentric sand dollars). The seven tests that averaged 94% fertilization in controls were 2.2-fold less sensitive, on average, than seven parallel tests with 84% average control fertilization. Another set of eleven assays carried out by NCASI (1992) on a sample of kraft mill effluent, showed a geometric average IC25 of 2.9% effluent at a low sperming ratio, but an IC25 of 6.7% at a high sperming ratio, a 2.3-fold loss of sensitivity. Similarly, Dinnel *et al*. (1987) found that the IC50 of silver for eccentric sand dollars was 23 µg/L at a low sperm-to-egg ratio, but was 37 µg/L at a high sperming ratio, a decrease in sensitivity by a factor of 1.6.

[34] Testing carried out in May 2008 confirmed that for the green sea urchin, a sperm:egg ratio of 5000:1 will yield ~90% fertilization at 10°C or 15°C with a 10 + 10 minute exposure (Jackman, pers. comm., 2008).

Ideally, the appropriate sperm:egg ratio should be determined just before each test, and with the gametes to be used in that test. The pre-test may be shortened and simplified to use one or two sperm:egg ratios that are thought to be low. 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 real test.

An alternative pre-test procedure may be used to determine the sperm:egg ratio to be used in order to target 80% fertilization in the controls (Carr and Chapman, 1995).[35] 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., 5) 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). The pre-test is performed like a regular test, with the addition of an appropriate aliquot of sperm to each vial, and then eggs added after the appropriate exposure time. 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 80% 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 targeted optimum fertilization rate in the controls.

For porewater testing, this pre-test method is recommended, and should include two replicates of a control pore water (see Section 8.1.4), in addition to the two replicates of control/dilution water and one replicate of each of three concentrations of a reference toxicant, previously described.[36] This pre-test can be combined with an extended gamete check by performing the test on specific combinations of gametes from individual males and females (e.g., sperm from each of several males is tested separately with the eggs from each of several females to determine the best quality gamete combination; see Section 4.2.1). As such, the gametes from individual males and females, which, when combined at the right sperm:egg ratio, result in the ideal percent control fertilization, and the appropriate sensitivity to a reference toxicant (i.e., will yield results that fall within the warning limits of a control chart). These gametes can then be chosen for use in the definitive test and the appropriate sperm:egg ratio is known.

In practice, experience at a given laboratory can establish a "standard" ratio that usually gives the desired results for a particular species. However, the routine use of a "standard" ratio risks lowering the quality of testing. If the standard ratio yielded $< 60\%$ fertilization in the control, or $\geq 98\%$ fertilization (Section 4.5.1), the test would be invalid and would have to be repeated using a different ratio. Other tests might lose sensitivity because of "over-sperming". The sperm:egg ratio might require adjustments with season, and 10-fold changes in requirements due to season are not unknown (Chapman, pers. comm., 1992b).

Because of the normal variation in percent fertilization for controls, a pre-test is highly recommended. Investigators familiar with the echinoid fertilization assay find that the time spent in a pre-test for each definitive test has, in the long run, saved considerable time, money, and sometimes irreplaceable samples.

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[35] In Carr and Chapman (1995), sperm dilutions (expressed as a ratio of volume of "dry" sperm:volume of seawater) rather than sperm:egg ratios are used to achieve and express various sperm concentrations, however for this second edition test method document, sperm:egg ratio will continue to be used for this purpose, as per the first edition of EPS 1/RM/27.

[36] This pre-test was developed at the Columbia Environmental Research Centre's Marine Ecotoxicology Research Station for porewater testing (Carr, Nipper, and Biedenbach, pers. comm., 2008). It was used in an inter-laboratory investigation conducted in 2008 which was designed to investigate the improvement of the porewater testing component of EPS 1/RM/27 (Miller, 2008). It was found to be favorable by participating laboratories, since it provided more information on which to base the choice of sperm:egg ratio to be used in the definitive test.

An alternative approach to circumvent control pre-tests is to include replicates of two or three sperm:egg ratios for each concentration used in the test including controls. Results for the ratio that yielded a fertilization rate of 80% in the controls would be used in calculating the ICp (Section 4.5.2). NCASI (1992) points out that this actually requires less of the investigator's time than running a pre-test and then a test, and has a further advantage of avoiding any changes of sperm activity during the interval from pre-test to test.

If the sperm:egg ratio was determined by a pre-test, or arbitrarily selected, the strength of the suspension of sperm is also fixed (Section 4.2.2). For example, if a sperm:egg ratio of 2000:1 were required for the 2000 eggs to be added, then 4 million sperm would be needed in the 0.1 mL of added suspension, or 40 million sperm per mL in the suspension.

4.2.4 Exposure of Gametes

Individual vessels are positioned for the exposure in a test tube rack or other rack, held in the water bath or other temperature-control facility. Vessel positions in the rack must be either completely randomized, or randomized in "columns" of the rack, each column representing one replicate of each concentration and control.[37] Each vessel must be clearly labelled or positions coded so that concentrations and replicates can be identified.

The temperature, salinity, dissolved oxygen, and pH levels in representative (i.e., controls plus at least the high, medium, and low concentrations if a multi-concentration test) aliquots of the test solutions must be measured when they are prepared. If required or permitted, values must/should be adjusted to acceptable levels (Section 4.3) before adding the solutions to the test vessels.

The test has three options for duration of exposure, options which are otherwise identical in their procedures. Obviously, only one of these options can be used in a given test, and for comparative tests. The shortest option is the standard exposure for normal testing and *monitoring*. It is 10 min of sperm exposure, with the addition of eggs at that time and an exposure that continues for a further 10 min of sperm plus eggs, i.e., the 20-min test. For Arbacia, however, the time for development of a fertilization membrane is slower than that for the other four test species, and therefore longer exposures (i.e., 20 + 20 min or 60 + 20 min) are recommended for this species.

Either of two longer exposures might also be used for special purposes such as research or comparison with other data. The second option is 20 min of sperm exposure followed by 20 min sperm plus eggs, the 40-min test. The longest option is exposure of the sperm for 60 min, plus 20 min, an 80-min test.[38]

The three options for volume of test solution are independent of the options for duration (thus nine options for test procedure). The option for an

[37] The randomized locations may be selected in various ways, and most laboratories will have customary procedures. Some laboratories have computer programs tailored to the needs of the echinoid test, which make use of the random numbers available in standard computer software. For a guarantee of objectivity and assured validity of statistical tests, a "blind" test could be done by having all vessels including controls and reference toxicants, coded by a person other than the one who will make the count of fertilization.

[38] The longer exposure is usually acknowledged as a more sensitive test of toxicity. The improved detection of a toxic effect is less pronounced, however, because there is usually a drop in fertilization rate in the control, as the sperm are held for longer periods before the addition of eggs. The drop in control fertilization might be as great as from 90% for a 10-min exposure of sperm to 30% for a 60-min exposure (Pagano *et al*., 1983). The apparent margin between performance of the control and test concentrations might not improve greatly with the longer exposure of sperm (test of cadmium, Pagano *et al*., 1986). Investigators in British Columbia have noted such a decreased rate of control fertilization in eccentric sand dollars, comparing sperm exposures of 10 minutes and 30 minutes (van Aggelen, pers. comm., 1992).

The short exposure (10 + 10 min) was favoured as a standard test by Canadian investigators participating in an interlaboratory comparison (Miller *et al*., 1992). The short exposure might partly compensate for loss of sensitivity by reduced variability. For example, in seven paired tests of toxicity of bleached pulp mill effluent to eccentric sand dollars, the short test (20 min total) was less sensitive by a factor of 2.4 compared to the long test (80 min total), but had a smaller CV by a factor of 2.0 (NCASI, 1992).

initial test volume of 10 mL is the usual standard and is described here.[39] The procedures for the smaller test volumes of 5 and 2 mL would be identical except that proportionally smaller volumes of gamete suspensions would be added (Table 4).

The solution of sperm is mixed, and to start the test, 0.1 mL is added to each test vessel, which already contains 10.0 mL of test solution (Section 4.1.2). At the end of the sperm exposure, the egg preparation is mixed and 1.0 mL is added to each test vessel. Automatic dispensing micro-pipettes are needed to accomplish these steps within narrow time limits. Care must be taken when adding sperm and eggs to the vessels; all of the fluid delivered from a pipette must enter the test solution rather than striking the side of the vessel, and the pipette tip must not touch the test solution. The suspension of gametes should be mixed after every second or third vessel is filled. After sperm have been added to all vessels, and again after eggs have been added, all vessels should be thoroughly mixed by swirling, in-and-out pipetting, or brief use of a vortex mixer.

A timing procedure should be used for adding sperm to vessels in sequence, for example one vessel every 5 seconds. The eggs should be added to the vessels in the same sequence (order of vessels) and with the same timing interval as was used for sperm, in order to equalize exposure periods. Termination of the test should again be done in the same sequence with the same timing. Additions to test vessels should not be done according to magnitude of concentration, but by replicate, i.e., the first set of replicates, then the second, then the third (Chapman, 1992a).

At the end of the sperm-plus-eggs exposure, the test is terminated by adding either 2 mL or less of 1% glutaraldehyde, or 2 mL or less of 10% buffered formalin to each test vessel.[40] (The amounts of preservative are divided by 2 and by 5 for the two smaller-volume test options). Preserved eggs should be counted within three days of test completion. During storage, vessels containing eggs should be sealed (e.g., using plastic film).

4.3 Test Conditions

This a *static* test without aeration and without renewal of test solutions. The test is carried out at 15 °C for the four native species, and at 20 °C for the listed non-native species. Salinities in all test vessels are normally within 1 g/kg of the control, in the range 28 to 32 g/kg. An attempt is made, if necessary, to raise the dissolved oxygen of all test solutions above 40% saturation before the test is started.

4.3.1 Temperature

A test temperature of 15 °C should be used for green sea urchins, Pacific purple sea urchins, and eccentric sand dollars. The test temperature should be 20 °C for the non-native Arbacia and white sea urchins. Temperatures of all test solutions should be within 1 °C of the intended value as determined by measurements in aliquots or test vessels without gametes (dedicated to temperature monitoring). Temperatures must be measured in aliquots of the control(s), high,

[39] The 10-mL volume was favoured by Canadian investigators in an interlaboratory comparison, and was adopted as the standard method in the trials (Miller *et al.*, 1992). There was concern that the smaller volumes might decrease the precision in handling small volumes of fluid, and might increase the "edge effects" at surfaces contacting the air and test vessels.

[40] Formalin is a 37% to 40% solution of formaldehyde. To make 10% buffered formalin, add 100 mL of formalin, 4 g monobasic sodium phosphate, and 6.5 g anhydrous dibasic sodium phosphate to 900 mL of distilled water. Experience has shown that the volume of chemical needed to preserve the eggs may be reduced by as much as a factor of 10. Eggs should be fixed using the least amount of chemical necessary for adequate preservation (Buday, pers. comm., 2008). Formalin is a hazardous substance, must be handled in a fume hood, and counting of eggs must take place with similar positive ventilation. It has been reported that formalin might form a white precipitate in test vessels containing pore water, whereas glutaraldehyde usually does not (Buday, pers. comm., 2008). Work with glutaraldehyde should also take place in a fume hood or well-ventilated area. Both substances should be kept away from areas used to hold and test organisms. Some authors add chlorine bleach solution immediately before enumeration of eggs, to neutralize excess formalin.

medium, and low concentrations before beginning the test.

The test temperatures recommended here are 3 ° to 7 °C higher than the values recommended for holding the adults of the same species, but within the biokinetic ranges. These somewhat elevated temperatures should make the test more sensitive in detecting some toxicants.[41] Some of the recommended temperatures conform with those previously used in Canadian methods or U.S. standard methods, but they necessarily diverge from some other methods, because of the variety employed elsewhere (Appendix D).

4.3.2 Salinity

A standard test should be carried out at a salinity of 30 g/kg. All test solutions should be in the 28 to 32 g/kg range, and they should also be within 1 g/kg of the salinity of the control.[42]

If a chemical is being tested, it should be made up to the test concentration(s) using a control/dilution water which has a salinity in the required range (see Sections 4.1.1, 5.2, and 5.3). The salinity of aqueous samples (e.g., chemical products or formulations made up in water; effluents; leachates) or test solutions should be measured before the test and, if outside the range 28 to 32 g/kg, should be adjusted to within this range using one of two approaches: (1) sample salinity may be adjusted by the direct addition of dry salt to the effluent or other material (e.g., leachate or elutriate); or (2) sample salinity may be adjusted by the addition of hypersaline brine (following guidance in EC, 2001 and in Section 2.3.4). Deionized water can be used to reduce the salinity of test samples. The sample must not be warmed to the test temperature before this salinity adjustment, rather, the temperature during salinity adjustment should approximate either that of the sample when received, or in instances when the sample is stored overnight in a refrigerator at 4 ± 2°C, that of the sample when it is removed from the refrigerator (EC, 2001).

If the first approach is chosen, either a mixture of commercially-available dry ocean salts (e.g., Instant Ocean™ , Red Sea Salt™) or reagent-grade salts (e.g., modified GP2; see Bidwell and Spotte, 1985 or Table 2 in USEPA, 1994 or USEPA, 1995) may be added to the undiluted sample, in a quantity sufficient to raise sample salinity to 30 ± 2 g/kg. Any sample to which dry salts are added directly must be aged for a period of no more than 16 to 24 hours before its use in a toxicity test (EC, 2001). To age the sample, the required quantity of salt must be added while stirring the effluent; thereafter, the salinity-adjusted (30 ± 2 g/kg) sample must be held for

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[41] In fertilization assays at a favourable salinity of 28 g/kg, the IC50s for silver decreased appreciably over the temperature series 7 °, 12 °, 17 °C. For green sea urchins, the IC50s were 215, 110, and 38 µg/L, respectively, while for eccentric sand dollars a smaller change of IC50s was 120, 88, and 66 µg/L (Dinnel *et al*., 1982). The IC50s for the insecticide endosulfan did not appear to be affected in a predictable way by test temperature.

[42] Gametes and *larvae* of echinoids are considered to have a fairly narrow range of salinity tolerance, and low salinity can have major effects on the estimate of toxicity in a fertilization assay. For instance, the fertilization rate for the Pacific purple sea urchin decreased at a salinity of 28 g/kg compared to 30 g/kg, with no toxicant present (Oshida *et al*., 1981). Contrasting work showed no decrease in fertilization rate of Pacific purple sea urchins until salinity was below 20 g/kg (Dinnel *et al*., 1987), a finding that might be partly explained by a high ratio of sperm to eggs, since that can partly compensate for low salinity. Green sea urchins showed a decreased rate of fertilization at 24 g/kg and less (Dinnel *et al*., 1987). Eggs of the eccentric sand dollar obtained from organisms spawned at lower salinities have been reported to be deformed in shape and will either not fertilize or will remain deformed after fertilization (Pickard, pers. comm., 2008). Increased sensitivity to toxicants can be caused by lowered salinity (ASTM, 1990). For example, green urchins tested at 10°C showed IC50s for silver of 94 µg/L, 45 µg/L, and 34 µg/L at salinities of 30, 28, and 26 g/kg respectively, although IC50s for the insecticide endosulfan showed little or no change (Dinnel *et al*., 1987). Lowered salinity was particularly effective in causing increased toxicity of silver, with green sea urchin fertilization, when the test was done at high temperature (17 °C) or low temperature (7 °C), while there was little salinity effect at a near-optimal temperature of 12 °C (Dinnel *et al*., 1982). Similar effects of decreased salinity were shown with a South African sea urchin (genus *Parechinus*) for which fertilization success dropped off steadily as salinity decreased from that of normal seawater. The eggs had an optimal salinity in the range 28 to 37 g/kg, and were more sensitive than sperm (Greenwood and Bennett, 1981).

16 to 24 h at 4 ± 2°C in the dark and within a sealed container with minimal air space (and without any aeration). Sample pH should be measured and recorded before salt addition and after salt addition but before aging. Following this aging period, the effluent sample should be stirred, warmed to the test temperature, its pH checked and recorded, test concentrations prepared, and the toxicity test started (EC, 2001).

If the second approach is chosen, sample salinity must be adjusted to the test salinity (30 ± 2 g/kg) by the addition of the required amount of hypersaline brine (and, as necessary, deionized water). HSB must be used for this purpose and it should have salinity of 90 ± 1 g/kg . Guidance provided in EC 2001 and Section 2.3.4 for preparing HSB must be followed. If HSB with a salinity of 90 g/kg is used to adjust the salinity of a freshwater sample to 30 g/kg (see Sections 3.4 and 4.1.1), the maximum concentration of the sample that could be tested would be 67%.

Samples of effluent, leachate, receiving water, elutriate, *produced water,* or other aqueous extract from sediment could also be tested without adjusting salinity of the sample, if it were desired to assess the total effect, including divergent salinity. It should be realized that if the sample is essentially fresh water (salinity <5 g/kg) or is a brine (e.g., produced water), the results of the toxicity test will probably reflect unfavourable salinity rather than any toxic substance(s) in the sample. If an unadjusted sample were tested, it would be desirable to run a set of salinity controls using parallel concentrations of distilled water (Section 4.1.4), or to conduct a second test with salinity of the sample adjusted, or both, in order to understand the contribution of salinity to toxicity.

4.3.3 Dissolved Oxygen and Aeration

If (and only if) calculations from the dissolved oxygen measured in the sample to be tested indicated that one or more of the test concentrations would be outside the 40 to 100% range of air saturation, the sample or an aliquot of sample should be aerated before starting the test ("pre-aeration"). To achieve this, oil-free compressed air should be dispensed through airline tubing and a disposable plastic or glass tube of small aperture (e.g., capillary tubing or a pipette with an Eppendorf tip, with an opening of about 0.5 mm). The rate of pre-aeration must be at a minimal and controlled rate, which should not exceed 100 bubbles/min. Duration of pre-aeration must be the lesser of 20 minutes and attaining 40% saturation (or 100% saturation, if supersaturation is evident).[43] Any pre-aeration must be discontinued at ≤20 minutes and the test initiated, whether or not 40 to 100% saturation was achieved in the aliquot of sample, or would be expected in all test solutions. Dissolved oxygen must then be recorded for the start of the test in representative aliquots of the test solutions including the highest concentration. Any pre-aeration must be reported, including the duration and rate (Section 9).

If oxygen in one or more test vessels is below 40% of saturation, the test becomes invalid as an assessment of the toxic quality, *per se*, of the material or substance being tested. The test would still be a valid assessment of the total effect of the material (e.g., effluent) or substance (e.g., chemical) including its deoxygenating influence.[44] The required use of oxygen-

[43] Aeration can strip volatile chemicals from solution, or increase their rate of oxidation and degradation to other substances or materials. However, aeration of a sample before exposure of gametes might be necessary due to the oxygen demand of the test substance or material (e.g., oxygen depleted in the sample during storage). Because of the small volumes of test solutions for the fertilization assay, aeration of individual concentrations is not practical, and aeration of an aliquot of sample is carried out if necessary.

[44] The lower limit of 40% saturation for dissolved oxygen in test solutions is an arbitrary value, because oxygen levels well above that are stressful to most aquatic organisms and probably affect gametes also. Stress from low oxygen might interact with any stress from toxicants, and be measured as part of the effect of the sample, be it effluent or other test material or substance. Any such interaction at DO >40% saturation has been accepted in this test procedure, as part of the impact being measured.

saturated control/dilution water will, in most instances, result in dissolved oxygen levels that should not have a large influence on test results.

4.3.4 pH

The pH must be measured in aliquots of the control(s), high, medium, and low concentrations before beginning the test.

Toxicity tests for regulatory or *monitoring* purposes would normally be carried out without adjustment of pH. However, if the sample of test material or substance causes the pH of any test solution to be outside the 7.5 to 8.5 range, results might reflect effects due to pH alone.[45] If it is desired to assess toxic chemical(s) *per se* rather than the deleterious or modifying effects of pH, then the pH of the solutions or sample should be adjusted, or a second, pH-adjusted test should be conducted concurrently.[46] For an adjusted test, the initial pH of the sample, or of each test solution[47] could, depending on objectives, be adjusted to within ± 0.5 pH units of that of the control/dilution water, before exposure of the gametes. Another acceptable approach for this second, pH-adjusted test is to adjust each test solution, including the control, upwards to pH 7.5 to 8.0 (if the solution has pH <7.5), or downwards to pH 8.0 to 8.5 (if the solution has pH >8.5). Solutions of hydrochloric acid (HCl) or sodium hydroxide (NaOH) at strengths ≤1 *N* should normally be used for all pH adjustments. Some situations (e.g., effluent samples with highly buffered pH) might require higher strengths of acid or base.[48]

In some circumstances it might be desired to carry out the most sensitive test possible for detecting toxic chemicals, rather than including pH as part of the total effect of a chemical, effluent, leachate, elutriate, or liquid extracted from sediments or other solid materials (such as pore water). In such a case, any effect of low or high pH, in changing viability of gametes and success of fertilization, should be eliminated by adjusting pH of test solutions as necessary, to the preferred range of 8.0 ± 0.2.[49]

Abernethy and Westlake (1989) provide useful guidelines for adjusting pH. Aliquots of samples or test solutions receiving pH adjustment should be allowed to equilibrate after each incremental addition of acid or base. The amount of time

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[45] Reproduction in the green sea urchin is known to be sensitive to pH (Starr, 1990). Although a European sea urchin showed that sperm viability was prolonged when pH ranged from 6 to 7.5, compared to about pH 8, there was nevertheless clearly evident damage to the sperm below pH 8, since subsequent developmental abnormalities increased at pH 7.5 and mitotic abnormalities increased at pH 7 (Pagano *et al*., 1985).

[46] The usual justification for not adjusting the pH of the sample or solution is that pH might have a strong influence on the toxicity of a substance or material being tested. Thus, for the (generally) low concentrations of waste found in receiving water after dilution, any change from the natural pH, with concomitant modification of toxicity, should be accepted as part of the pollution "package". That leads to the rationale that the pH should not adjusted in tests, and that is the requirement to be followed in most instances, if test solutions are in the pH range 7.5 to 8.5.

[47] Tests with a chemical, effluent, leachate, elutriate, or aqueous extract of a sediment, which are to receive pH adjustment, might require the separate adjustment of each test solution including the control. Tests with receiving water would normally adjust an aliquot of the undiluted sample, before preparing the test concentrations.

[48] The rationale for making these adjustments is not really contradictory to the previous rationale of not adjusting pH of wastewaters, but depends on the purpose of the test. Some chemicals and wastewaters will create levels of pH that have direct sublethal or lethal effects, especially in monitoring or compliance tests with full-strength effluent. An investigator might not be primarily interested in whether extreme pH is toxic, because such a pH would be unlikely after even moderate dilution in receiving seawater, which is naturally well-buffered. If pH *per se* were of primary interest, it could be economically assessed by physicochemical measurements. An investigator would often wish to know if toxic substances were present in a wastewater, and detecting them would require elimination of any masking by toxic action of pH. That rationale leads to the use of pH-adjusted samples or test solutions, in a parallel manner to the standardization of temperature, salinity, and dissolved oxygen at favourable levels when testing for toxic substances.

[49] Of the six existing procedural documents which indicate pH requirements for the fertilization assay, five of them specify values in the range 7.8 to 8.2 (Appendix D).

required for equilibration will depend on the buffering capacity of the solution/sample. For effluent samples, a period of 30 to 60 min is recommended for pH adjustment (Abernethy and Westlake, 1989). For an echinoid test, the adjustment would be made on aliquots used to prepare test concentrations, the pH in each would be recorded (Section 4.4), and the test started with no further attempt at adjustment.

If the purpose of the toxicity test is to gain an understanding of the nature of the toxicants in the test substance or material, pH adjustment is frequently used as one of a number of techniques (e.g., oxidation, filtration, air stripping, addition of chelating agent, etc.) for characterizing and identifying sample toxicity. These "Toxicity Identification Evaluation" (TIE) techniques provide the investigator with useful methods for assessing the physical/chemical nature of the toxicant(s) and its (their) susceptibility to detoxification (USEPA, 1991a, 1991b).

4.4 Test Observations and Measurements

At the end of the exposure, preserved eggs are taken from each test vessel after mixing[50], and an equal number from each vessel, in the range of 100 to 200 eggs, is counted and classified as either fertilized or not fertilized (Figure 3).[51] The count is made under a microscope at 100 × magnification, preferably by phase-contrast microscopy. A counting cell such as a Sedgwick-Rafter chamber might be useful, although the count can be made using an etched petri plate. Microscopic technique is important, and can affect the accuracy of the counts. Consistency of counting should therefore be checked by trials, especially among different people who might be involved in counting.

The criterion of fertilization is a raised fertilization membrane, and this includes full, partial or collapsed membranes (see Figure 3), none of which are seen in unfertilized eggs (NCASI, 1991).[52]

Eggs of Arbacia, when fertilized, have much smaller fertilization membranes than those of the other 4 species. This could lead to greater uncertainty in the counting of fertilized vs. unfertilized eggs, even by experienced analysts. Adding several drops of a 150 - 200 g/kg NaCl brine to the microscope slide containing the test eggs causes the eggs to shrink temporarily, leaving a greater space between the fertilization membrane and the egg and allowing the fertilization membrane, if present, to be more easily discerned.

Artifacts such as partial collapse of membrane or movement of the egg to one side of the hyaline sphere, can occur during preservation after the test. Clearly abnormal eggs, or dead ones, are simply omitted from the count, whether they are fertilized or not. The counts are recorded for each test vessel.

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[50] Most of the eggs are normally in the lower part of the test vessel and could be sampled from there. However, unfertilized eggs are sometimes adhesive and might clump together on the glass. This could bias the results, whether the clumps happen to be over-sampled or under-sampled. A remedy is to pipette off much of the overlying test solution, in order to concentrate the suspension of eggs, then mix and sample for counting.

[51] The eggs of the Pacific purple sea urchin have very large, easily discernable membranes which makes test counting very easy, however, care must be taken when removing samples from sample vials as the membranes might easily break, releasing eggs from the membranes, and resulting in false negative results in the counts.

[52] High or low pH, or high temperature, can cause false fertilization membranes, and if that were of concern for a particular test sample, a separate vessel or vessels at high concentration could be carried through without preservation, and checked the following day for normal development into larval stages. A set of unfertilized "blanks" (i.e., test vessels with unfertilized eggs added to the test solutions but containing no sperm) might also be included in the test in order to identify false fertilization membranes. Control blanks (i.e., eggs in control/dilution water that are included in the test without any sperm added; see Section 4.1.4) should have little to no eggs fertilized. Those demonstrating >10% fertilization might be indicative of accidental contamination of stocks of eggs with sperm, in which case investigators are encouraged to use their professional judgement to determine whether the test should be invalidated or not.

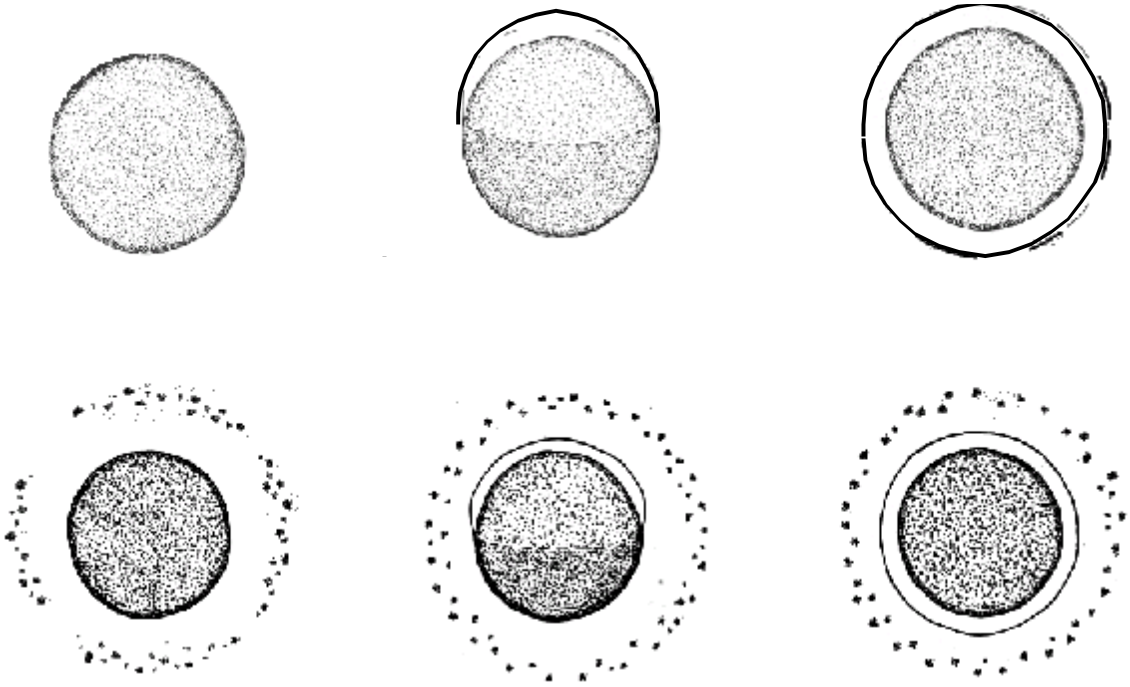

Figure 3 Discriminating Between Fertilized and Unfertilized Eggs.

Outlines of eggs as seen under a dissecting microscope. The three drawings in the upper row represent a sea urchin such as the green sea urchin. The egg on the left is not fertilized. The middle egg has a fertilization membrane that is partially raised and is considered fertilized. The right-hand egg has a completely raised fertilization membrane. The Pacific purple sea urchin is similar but within the outer fertilization membrane, an inner hyaline membrane might be evident. The three drawings in the lower row represent the eccentric sand dollar, from left to right, unfertilized, fertilized with a partially raised membrane, and with a completely raised membrane. The jelly-like coating of the sand dollar contains pigment granules and usually disappears during later development of the egg. Drawn by M.A. White, from prepared slides from McGibbon and Moldan (1986), and from drawings of Kelley Battan of NCASI, Anacortes, Washington.

4.5 Test Endpoints and Calculations

The biological endpoint of the test is adverse effect on success of fertilization, assessed by comparison with the controls. Percent fertilization is calculated for each test vessel.

The *inhibiting concentration for a specified percent effect (ICp)* is the required endpoint for a multi-concentration test. Regression analysis must be used to for the calculation of the ICp, if possible, following the guidance provided in Section 4.5.2 and in EC, 2005. The 95% confidence limits must be given for any ICp reported.

4.5.1 Validity of Test

The test is invalid if the mean fertilization rate for all replicates of the control water is <60%, or ≥98%. [53] Also, a positive and logical dose-effect curve should have been attained, for the results to be considered valid, i.e., the effect on fertilization must become generally greater at higher concentrations.

If dissolved oxygen in one or more test vessels was less than 40% saturation, the test should be considered an invalid assessment of the toxic quality, *per se*, of the substance or material being tested. The test would still be a valid assessment of the total effect of the test substance or material (Section 4.3.3).

4.5.2 Multi-Concentration Tests

Echinoid fertilization data presents a unique case in toxicity data analysis, for the following reasons:

1. While the data is, by nature, binomial (an egg is either fertilized or not fertilized), because the number of replicates is 100, the data often meet the assumption of normality.[54]

2. One of Environment Canada's test validity criterion limits the control response to ≥ 60%, or < 98%. As a result, control response will not be 100% (by design), and this needs to be accounted for in the data analysis. In addition, because the control response will not be maximized, there is the possibility that fertilization may be enhanced (stimulated) at low doses of test substances (i.e., hormesis may occur).

Probit analysis would be the usual choice for multi-concentration binomial data; however, non-linear regression techniques (specifically, the parameter estimate procedure used) provide several advantages over probit analysis.[55] These include:

1. The ability to directly estimate the control response (Abbott's correction[56] is not needed)

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[53] Although 80% control fertilization is a target for the test, and fertilization at that rate may be optimal for the control, the lower limit for validity has been set at ≥60% control fertilization, since useful information might still be obtained from such a test. The upper limit of validity has been set at *less than 98%*, since high fertilization rates are indicative of over-sperming and are associated with loss of test sensitivity. In the first edition of this test method document, the lower limit for test validity was ≥50%. This has been raised to ≥60% herein due to the fact that the option of spawning adults without full acclimation has been added to this test method, on the premise that such a practice is acceptable as long as the test validity criteria are met. With the addition of this option, a slightly more stringent criterion for test validity, based on the fertilization rate in the control water, was justified. Also, all Canadian and US laboratories surveyed recently (Section 1.1) indicated that they were able to achieve well over 60% fertilization in the control most of the time. For this criterion, the upper limit of test validity has been lowered from <100% to <98% in order to provide greater assurance that oversperming is not occurring.

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

[55] Probit analysis here refers to the basic model familiar to toxicologists. This model is defined by a straight-line slope and intercept where control mortality is assumed to be 0%. More advanced models incorporate additional parameters that can estimate control response directly, and more advanced interpretations of probit models may include any model which uses a probit transformation (Zajdlik, personal communication, 2010; Ives, personal communication, 2010).

[56] Abbott's correction has been widely used to correct control mortality in probit analysis; however, some researchers have found that use of Abbott's correction can induce bias in the estimated control mortality (Zajdlik & Associates Inc., 2010).

2. A wider variety of model choice, including the potential to model hormesis,[57] if it exists

3. Avoiding the unjustified rejection of analysis based on the chi-square heterogeneity test[58]

Non-linear regression is usually applied to continuous data, however, weighting techniques can be used to accommodate the binomial nature of the data and correct variance heterogeneity.[59] Arcsin square root transformation, which has historically been used to transform binomial data for analysis, is not recommended.[60]

Given the rationale above, in a multi-concentration test, the required statistical endpoint for percent fertilization is an ICp and its 95% confidence limits via non-linear regression analysis.

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

Regression analysis is the principal statistical technique and must be used for the calculation of the ICp, provided that the assumptions below are met (Figure 4). A number of models are available to assess fertilization data via regression analysis. Use of regression techniques requires that the data meet assumptions of *normality* and *homoscedasticity*. For this test, binomial weighting techniques must be applied to all data. The data are also assessed for outliers using one of the recommended techniques (see Section 10.2 in EC, 2005). An attempt must be made to fit more than one model to the data. Finally, the model with the best fit[61] must be chosen as the one that is most appropriate for generation of the ICp and associated 95% confidence limits. The

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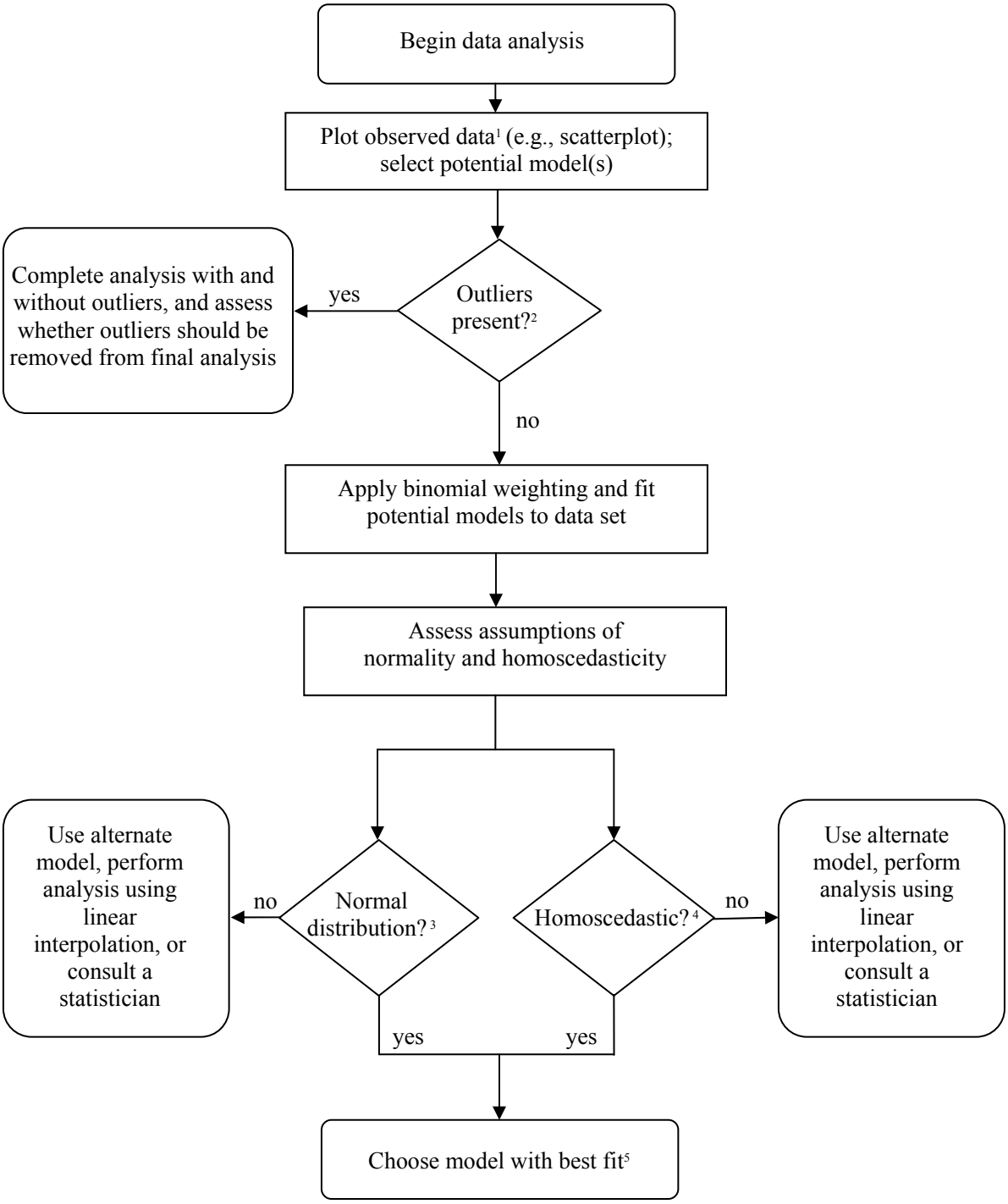
[57] In the most common software packages used by toxicologists, there is at least one model form present in non-linear regression that can accommodate hormesis.

[58] In probit analysis, the chi-square test for heterogeneity is used to determine the suitability of the model (EC, 2005). However, for the special case where the number of subjects is "high" (e.g., 100 as it is for echinoid fertilization), significant heterogeneity is a likely outcome. The estimation procedures used in non-linear regression (as recommended here, with binomial weighting) limits this problem (Ives, personal communication, 2008).

[59] More specifically, parameter estimates that use iteratively reweighted least squares with inverse binomial variances can accommodate for the binomial nature of the data. This is referred to in some software programs as "binomial weighting". Weighting is particularly important with respect to the estimation of the confidence limits (Carroll and Ruppert, 1988).

[60] Before desktop computers became widely available, the arcsin square root transformation was used to transform binomial data to meet the assumptions (equal variance, normality) of a test. Drawbacks of the arcsin approach include that the procedure is not always successful at inducing homogeneous variance and/or normality, and that a transformation bias is introduced (Zajdlik & Associates Inc., 2010). From a pragmatic perspective, analysis of echinoid fertilization sample data sets have shown that significant deviation from normality is not common, and that application of binomial weighting will often induce homogeneity (AquaTox Testing and Consulting Inc., 2009). As a result, the need for an additional transformation is expected to be rare.

[61] As described in Section 6.5.8 of EC (2005), Environment Canada's current guidance on statistical methods for environmental toxicity tests specifies the use of the following five models for regression analysis, when estimating the ICp: linear, logistic, Gompertz, exponential and *hormesis* (logistic adapted for hormetic effect at low doses). Specific mathematical expressions of the model, including worked examples for a common statistics package, are also provided in that guidance document (Section 6.5.8 and Appendix O in EC, 2005). Given the binomial nature of the echinoid fertilization data, the analyst may choose to place emphasis on models which acknowledge this feature (e.g., cumulative normal model, logistic model).



[1] If zero or near-zero values are recorded at high concentrations, see main text for guidance
[2] Outliers may be assessed at other points in the analysis; for example, outliers may be identified on residual plots
[3] Use Shapiro-Wilks test and normal probability plots
[4] Use Levene's test and examine residual plots
[5] Use lowest residual mean square error or alternate (e.g., AIC or BIC)

Figure 4 Flowchart summarizing steps in statistical analysis of a multi-concentration test to derive an ICp.

lowest residual mean square error (or alternate measure of fit, such as AIC or BIC[62]) is recommended to determine best fit. Endpoints generated by regression analysis must be bracketed by test concentrations; extrapolation of endpoints beyond the highest test concentration is not an acceptable practice.

With some highly toxic test materials or substances, it is possible to record zero or near-zero[63] percent fertilization at one or more exposure concentration(s). In these cases, the results from the high test concentration(s) provide no further information on the response of the organism, and the repetitive zeroes may interfere with regression assumptions of *normality* and *homoscedasticity*.[64] The lowest test concentration inducing zero or near-zero percent fertilization is kept in the data set, but data from any subsequent high test concentration(s) must be removed before the regression analyses.

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

In the event that the data do not lend themselves to regression analysis (i.e., assumptions of *normality* and *homoscedasticity* cannot be met), linear interpolation (e.g., ICPIN; see Section 6.4.3 in EC, 2005) can be used to derive an ICp. In this case, the log values of the concentration data would be used, but binomial weighting is not applied. If the data exhibited *hormesis* and ICPIN is used, control responses must be entered for those concentrations which demonstrated *hormesis* (Option 4, Section 10.3.3 in EC, 2005).

For each test concentration, including the control treatment(s), the mean (± SD) percent fertilization as determined at the end of the test, must be reported.

4.5.3 Single Concentration Tests

The most commonly-used test design with echinoid fertilization is a multi-concentration test design. If investigators wish to consider a single-concentration test (e.g., to evaluate sediments from different sample locations), the echinoid contact test method (in preparation, "Biological Test Method : Reference Method for Determining Sublethal Toxicity of Embryo/Larval Echinoids (Sea Urchins or Sand Dollars) in Contact with Sediment") may be more appropriate, as sediment is incorporated into this test design.

In a single-concentration test, the response in one or more full-strength test solutions (e.g., from multiple sites) are compared with the control response. Echinoid fertilization data presents a unique case in toxicity data analysis, since although the data is, by nature, binomial (an egg is either fertilized or not fertilized), because the number of replicates is 100, the data often meet the assumption of normality.[65] Accordingly, the recommendations made here emphasize techniques for quantitative (continuous) data.

If percent fertilization is assessed for a single test solution and the control, a t-test[66] is normally the

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[62] Akaike's information criterion (AIC) or Bayesian information criterion (BIC). Typically, the model with the lowest AIC or BIC is chosen.

[63] For this test, near zero is defined as ≤2% fertilization, calculated as the treatment mean. This value for near zero was empirically derived by examining sample data sets.

[64] In practice, application of binomial weighting can minimize the test assumption problems associated with the repetitive zeros at the high concentrations. However, as a precaution, the removal of these non-informative data remains a requirement.

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

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

appropriate statistical test. In situations where more than one test site is under study, and the investigator wishes to compare multiple treatments with the control, or compare treatments with each other, a variety of multiple comparison tests exist (Section 3.3. in EC, 2005). Choice of the test to use depends on:

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

Correction of fertilization using Abbott's formula will, in most cases, not be necessary.[67]

For each test solution or treatment, including the control treatment(s), the mean (± SD) percent fertilization as determined at the end of the test, must be reported.

4.6 Reference Toxicant

The routine use of a *reference toxicant* or toxicants 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) (EC, 1990d).

For adults that are gradually acclimated to test conditions and held in the laboratory for an extended period of time (i.e., >3 days), sensitivity of gametes to the reference toxicant(s) must be determined by performing a *reference toxicity test* within 14 days before or after the date that the definitive toxicity test is performed, or by performing this test concurrently with the definitive one. When a reference toxicity test is performed at the same time as the definitive toxicity test, the same *batch* of gametes must be used for each of these two tests.

If gametes are collected from adults on the day of arrival or within 3 days of arrival at the laboratory, a portion of the gametes collected for use in a definitive test must be tested for its tolerance to the reference toxicant(s). The reference toxicant test must be performed under the same experimental conditions as those used with the test sample(s). Testing of the reference toxicant must be performed concurrently with the actual toxicity test.

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.

[67] Abbott's formula would only be recommended if: (i) the difference in percent fertilization 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 percent fertilization for each treatment are required (instead of the difference in fertilization) (Zajdlik & Associates Inc., 2010). If labs 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 investigator is focussed on the difference in percent fertilization between sites, and accordingly, (ii) would not be expected to occur.

Copper is recommended for use as the reference toxicant for this test.[68] Gamete sensitivity must be evaluated by tests following the standard methods and conditions given in this document, to determine the ICp for copper. As for all multi-concentration tests described herein, regression analysis must be used for the calculation of the reference toxicant ICp and its 95% confidence limits, if possible, following the guidance provided in Section 4.5.2 and in EC, 2005. 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 mg Cu^{++}/L.

Natural or reconstituted seawater is to be used for controls and dilution. To provide a high degree of standardization for this reference toxicity test, the salinity of the control/dilution water should be adjusted to a consistent value that is favourable to the gametes, in the range 28 to 32 g/kg, preferably 30 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 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 are available (EC, 1990d), 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)[69] are recalculated with each successive ICp until the statistics stabilize (USEPA, 1989, 2002; EC, 1990d).

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[68] No clear choice of a standard reference toxicant seems to have been made by the various groups carrying out echinoid fertilization assays, and most do not specify a reference toxicant or deal with the subject (Appendix D). Copper and sodium azide have both been used by two groups in the United States and have been selected for use in B.C. provincial laboratories, along with silver (van Aggelen, pers. comm., 1992). For copper, IC50s in the vicinity of 20 to 26 µg Cu/L and coefficients of variation from 23% to 46% have been reported (Chapman, pers. comm., 1992b). Less information is available for sodium azide and it is not mentioned in the guide to reference toxicants (Environment Canada, 1990d). *Stock solutions* of sodium azide can be stored for a maximum of three months.

Other chemicals might be tried as potential reference toxicants. Cadmium has been found by IGATG (1991) to have a threshold-effect concentration of 18 µg Cd/L for green and white sea urchins. Cadmium is not, perhaps, the most desirable chemical for standard use since it is a dangerous bioaccumulative toxicant. Cadmium has also been reported to be associated with a high proportion of deformed eggs which are difficult to classify when counting (van Aggelen, pers. comm., 1992). Reagent-grade phenol might be useful since it would be an organic chemical to complement the inorganic copper, and since phenol is already recommended for other standard toxicity tests of Environment Canada (1990a, 1990b, 1992a, 1992b). Phenol stock solutions should be made up on the day of use. Sodium dodecyl sulphate has also been used with fertilization assays, but has some undesirable features as a reference toxicant (EC, 1990d).

[69] 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.

If a particular ICp falls outside the warning limits, the sensitivity of the gametes 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.

One check that might be made in such circumstances is the fertilization success for various sperm:egg ratios, compared with the range of values previously obtained. That assessment should provide a useful indication of decreasing viability of gametes, as might occur, perhaps, at the end of a spawning season. Depending on the findings, it might be necessary to repeat the reference toxicity test with new gametes, and/or a new *batch* of adults, before undertaking further toxicity tests.

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 ICp 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.

4.7 Legal Considerations

Care must be taken to ensure that samples collected and tested with a view to prosecution will be admissible in court. For this purpose, legal samples must be: representative of the material or substance being sampled; uncontaminated by foreign substances or materials; identifiable as to date, time, and location of origin; clearly documented as to the chain of custody; and analyzed as soon as possible after collection. Persons responsible for conducting the test and reporting the findings must maintain continuity of evidence for court proceedings (McCaffrey, 1979), and ensure the integrity of the test results.

Specific Procedures for Testing Chemicals

This section gives particular instructions for testing chemicals, in addition to the procedures in Section 4.

5.1 Properties, Labelling, and Storage of Sample

Information should be obtained on the properties of the chemical, formulated product, or chemical mixture to be tested, including the concentration of the major ingredients, water solubility, vapour pressure, chemical stability, dissociation constants, n-octanol:water partition coefficient, and biodegradability. Data-sheets on safety aspects of the test substance(s) (e.g., Material Safety Data Sheets) should be consulted, if available. Where aqueous solubility is in doubt or problematic, acceptable procedures used previously for preparing aqueous solutions of the chemical(s) should be obtained and reported, and/or chemical solubility in test water should be determined experimentally.[70] Other available information such as structural formula, degree of purity, nature and *percentage* of significant impurities and additives, handling precautions, and estimates of toxicity to humans and/or aquatic organisms, should be obtained and recorded.[71] An acceptable analytical method for the chemical in water at concentrations intended for the test should also be known, together with data indicating the precision and accuracy of the analysis.

Chemical containers must be sealed and coded or labelled upon receipt to indicate at least the chemical name, supplier, and date received. Storage conditions are to be dictated by the nature of the chemical, and often include temperature restrictions and the need for protection from light. Standard operating procedures for chemical handling and storage should be followed.

5.2 Preparing Test Solutions

Test solutions of the chemical should be prepared if possible, by adding aliquots of a stock solution made up in control/dilution water.[72] If deionized water, distilled water, or fresh water was used to make the stock solution, commercially-available dry ocean salts, reagent-grade salts, or hypersaline brine (HSB) at 90 ± 1 g/kg salinity should be added as necessary to adjust the salinity of each test solution to within the acceptable range (i.e., 30 ± 2 g/kg). For aqueous samples (e.g., chemical formulations in water), test solutions may also be prepared by adding appropriate quantities of commercially available dry ocean salts, reagent-grade salts, or HSB (or deionized water, if necessary) to the sample or each of the test solutions (see Sections 2.3.4 and 4.3.2). Alternatively, for strong solutions or large volumes, weighed (analytical balance) quantities of chemical may be added to control/dilution water to give the nominal strengths for testing. Nominal test concentrations must be prepared and reported in consideration of any salinity adjustments. A set of controls comprised solely of HSB and deionized water (i.e., *HSB control*) must be included in any test in which HSB is added to the sample or test solutions (see Section 4.1.1). Likewise, a set of controls comprised solely of commercially-available dry ocean salts or reagent-grade salts, and deionized water (i.e., *salt controls*) must be included in any test in

[70] Information regarding chemical solubility and stability in seawater and fresh water will also be useful in interpreting test results.

[71] Knowledge of the properties of the chemical will assist in determining any special precautions and requirements necessary while handling and testing it (e.g., testing in a well-ventilated facility, need for a solvent, etc.).

[72] The concentration and stability of the test chemical in the stock solution should be determined before the test. Stock solutions subject to photolysis should be shielded from light, and unstable solutions must be newly prepared as necessary.

which these salts are added to the sample or test solutions (see Section 4.1.1).

For chemicals that do not dissolve readily in water, stock solutions may be prepared using the generator column technique (Billington *et al*., 1988; Shiu *et al*., 1988) or, less desirably, by ultrasonic dispersion.[73] Organic solvents, *emulsifiers*, or *dispersants* should not be used to increase chemical solubility except in instances where they might be formulated with the test chemical for its normal commercial purposes. If used, an additional control solution must be prepared containing the same concentration of solubilizing agent as in the most concentrated solution of the test chemical. Such agents should be used sparingly, and should not exceed 0.1 mL/L in any test solution. If solvents are used, the preferred ones (USEPA, 1985; ASTM, 1990) are triethylene glycol and dimethyl formamide. Methanol, ethanol, and acetone could be used but are more volatile.

5.3 Control/Dilution Water

Control/dilution water may be reconstituted (artificial) seawater, the laboratory's supply of natural "uncontaminated" seawater, or a sample of particular receiving water if there is special interest in a local situation. The choice of control/dilution water depends on the intent of the test (see Section 3.4).

Reconstituted seawater should be used if a high degree of standardization is required, such as for measuring toxicity of a chemical relative to values derived elsewhere for this chemical and others. The salinity of the control/dilution water used for such comparative tests should be common to all tests and used for all dilutions. This salinity should be within the range 28 to 32 g/kg. Additionally, the salinity of all test concentrations should be within 1 g/kg of the controls.

If the toxic effect of a chemical on a particular receiving water is to be assessed, sample(s) of the receiving water could be used as the control/dilution water by taking them from an area that was not contaminated by the chemical. Examples of such situations include appraisals of the toxic effect of chemical spills (real or potential) or intentional applications of a chemical (e.g., spraying of a pesticide) on a particular waterbody. If a sample of receiving water is to be used as control/dilution water, a separate control solution must be prepared using the control/dilution water that is normally used for the echinoid fertilization test and is able to achieve valid test results on a routine basis (see Section 4.5.1).

The laboratory supply of uncontaminated natural seawater, or reconstituted seawater, may also be used to appraise the toxic effect of a chemical on a particular receiving water, especially if there is already an interfering toxicity in the receiving water, or its collection and use is impractical (see Section 4.1.1). The laboratory seawater in which adults are held is also appropriate for use in other instances (e.g., preliminary or intra-laboratory assessment of chemical toxicity).

If information is desired on the influence of salinity on toxicity of the chemical under investigation, separate tests should be conducted concurrently at two or more salinities. However, it should be kept in mind that salinities outside the 28 to 32 g/kg range might in themselves affect success of fertilization.[74] Control/dilution water for such tests should be from a single source, either reconstituted seawater (Section 2.3.4) or natural seawater adjusted for salinity as necessary using hypersaline brine, dry salts, deionized water, distilled water, or an uncontaminated fresh water.

[73] Ultrasonic dispersion may also be used but is less desirable since it can produce droplets that differ in size and uniformity, some of which might migrate towards the surface of the liquid, or vary in biological availability, creating variations in toxicity.

[74] Outside the limited range favourable for gametes, the effect of salinity on toxicity of a substance would be evaluated more successfully by using some other test with a *euryhaline* organism, rather than the fertilization assay.

5.4 Test Observations and Measurements

In addition to the observations on toxicity described in Section 4.4, there are other observations and measurements to be made during testing with chemicals.

During preparation, each solution should be examined for evidence of chemical presence and change (e.g., odour, colour, opacity, *precipitation*, or *flocculation* of chemical). Any observations should be recorded.

It is desirable and recommended that aliquots of test solutions be analyzed to determine the concentrations of chemicals to which gametes are exposed, in at least the high, medium, and low test concentrations, and the control(s).[75]

All samples should be preserved, stored, and analyzed according to proven methods with acceptable detection limits for determining the concentration of the particular chemical in aqueous solution. Toxicity results for any tests in which concentrations are measured should be calculated and expressed in terms of those measured concentrations, unless there is good reason to believe that the chemical measurements are not accurate. In making calculations, each test solution should be characterized by the geometric average of the measured concentration to which the gametes are exposed.

5.5 Test Endpoints and Calculations

ICp is the recommended statistical endpoint for a multi-concentration test performed using a chemical (see Section 4.5.2).

If a solvent control is used, the test is rendered invalid if the fertilization success in this control is decreased significantly from that for the control using only water.

[75] Such analyses need not to be undertaken in all instances, due to analytical limitations, cost, or previous results indicating chemical stability under conditions similar to those in the test. Chemical analyses are particularly advisable if the test solutions are aerated, the test substance is volatile, insoluble, or precipitates out of solution, or if the test chemical is known to sorb to the material(s) of the test vessels (USEPA, 1985). Some situations (e.g., testing of pesticides for purposes of registration) can require the measurement of chemical concentrations in test solutions.

Specific Procedures for Testing Samples of Effluent, Leachate, and Elutriate

This section gives particular instructions for the collection, preparation, and testing of effluents, leachates, and elutriates, in addition to the procedures listed in Section 4.

6.1 Sample Collection, Labelling, Transport, and Storage

Containers for transportation and storage of samples of effluent, elutriate, or leachate must be made of nontoxic material. Collapsible polyethylene or polypropylene containers manufactured for transporting drinking water (e.g., Reliance™ plastic containers) are recommended. The volume of these containers can be reduced to fit into a cooler for transport, and the air space within kept to a minimum when portions are removed in the laboratory for the toxicity test or for chemical analyses. The containers must either be new or thoroughly cleaned, and rinsed with uncontaminated water. They should also be rinsed with the sample to be collected. Containers should be filled to minimize any remaining air space.

Most tests with effluent, leachate, or elutriate will be performed "off-site" in a controlled laboratory facility. Testing of effluents and leachates should commence within 1 day of sampling whenever possible, and must commence no later than 3 days after sampling. Samples of sediment or other solid material collected for extraction and subsequent testing of the elutriate should also be tested as soon as possible. Extraction procedures followed by testing should begin within two weeks of sampling (preferably within one week), and testing must start no later than six weeks after collection (EC, 1994). Procedures given in Environment Canada (1994) for the preparation of elutriates should be followed. Testing of elutriates must commence within 3 days of their preparation, or as specified in a regulation or *protocol*.

Generally, a two-litre sample is adequate for an off-site multi-concentration test, associated routine chemical analysis, and any necessary adjustments or repeat tests. Smaller amounts are required for single-concentration tests (see Section 4.5.4). Upon collection, each sample container must be filled, sealed, and labelled or coded. Labelling should include at least sample type, source, date and time of collection, and name of sampler(s). Unlabelled or uncoded containers arriving at the laboratory should not be tested, nor should samples arriving in partially filled containers be routinely tested, because volatile toxicants escape into the air space. However, if it is known that volatility is not a factor, such samples might be tested at the discretion of the investigator.

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

Upon arrival at the laboratory, the temperature of the sample must be measured and recorded. An aliquot of effluent or leachate required at that time may be adjusted immediately or overnight to the test temperature and used in the test. Any remaining portion(s) of sample held for possible additional testing must be stored in darkness in sealed containers, without air headspace, at 4 ± 2 °C.

Temperature conditions should also be as previously indicated for transportation and storage of elutriates, as well as for samples

intended for aqueous extraction and subsequent testing of elutriate, unless otherwise specified.

6.2 Preparing Test Solutions

Each sample in a collection container must be agitated thoroughly just before pouring, to ensure the re-suspension of settleable solids. Subsamples (i.e., a sample divided between two or more containers) must be mixed together to ensure their homogeneity. The dissolved oxygen content and pH of each sample must be measured just before its use. As necessary, the sample should be pre-aerated (see Section 4.3.3) before the test solutions are prepared and distributed to replicate test chambers.

The salinity of each test sample should be measured before the test is started. If it is outside the range considered acceptable for the test (i.e., 28 to 32 g/kg), the salinity of the sample or each test solution should be adjusted to within this range using natural or artificial hypersaline brine, commercially-available dry ocean salts, reagent-grade salts (see Sections 2.3.4 and 4.3.2), or deionized water. Nominal test concentrations must be prepared and reported in consideration of any such salinity adjustments.

Filtration of samples is normally not required nor recommended. However, if a sample of effluent or leachate contains debris or indigenous organisms which might be confused with or attack gametes or fertilized eggs, the sample must be filtered through a sieve with 60-µm mesh openings, before use (USEPA, 1994). Such filtration could remove some suspended or settleable solids that are characteristic of the sample and might otherwise contribute to part of the toxicity or modify the toxicity. For instance, high concentrations of biological solids in certain types of treated wastewater might contribute to sample toxicity due to ammonia and/or nitrite production (Servizi and Gordon, 1986). Alternatively, the presence of high concentrations of suspended solids in a sample might inhibit fertilization or damage the gametes directly. In instances where concern exists regarding the effect of this filtration on sample toxicity, a second test should be conducted concurrently using an unfiltered portion of the sample.

6.3 Control/Dilution Water

Tests conducted with samples of effluent or leachate for *monitoring* and regulatory *compliance* purposes should use, as the control/dilution water, either a supply (source) of the laboratory seawater shown previously by the testing laboratory to routinely enable valid test results, or a sample of the receiving water. Because results could be different for the two sources of water, the objectives of the test must be decided before a choice is made. Shipping difficulties and costs should also be considered; the use of receiving water for dilutions and controls increases the volume of liquid to be shipped, although that might not be a major factor for this small-scale assay.

The use of uncontaminated receiving water as the control/dilution water can be desirable if site-specific information is required on the potential toxic impact of an effluent, leachate, or elutriate on a particular receiving water (see rationale in Section 4.3.2). An important example of such a situation would be testing for sublethal effect at the edge of a mixing zone, under site-specific regulatory requirements. Conditions for the collection, transport, and storage of such receiving-water samples should be as described in Section 6.1. Any sample of receiving water used as the control/dilution water for testing effluents or leachates should be filtered according to the standard recommendation for natural control/dilution water, through a 60-µm mesh sieve (USEPA, 1994; Section 3.4). If a sample of receiving water is to be used as control/dilution water, a separate control solution must be prepared using the laboratory seawater that is normally used for performing fertilization tests (i.e., holding water or other suitable laboratory water; see Section 4.1.1).

Tests requiring a high degree of standardization should use reconstituted (artificial) seawater as control/dilution water (see Section 3.4). An example of such a situation might be a test

intended to compare the toxicity of a particular effluent, leachate, or elutriate with that of samples collected and/or tested elsewhere.

If any artificial or natural hypersaline brine (*HSB*; Section 2.3.4) is added to the sample or test solutions (see Section 6.2), the toxicity test must include a set of controls (i.e., *HSB controls*) which is prepared and adjusted to the test salinity (i.e., 30 ± 2g/kg) using only this HSB and deionized water. Likewise, if any commercially-available dry ocean salts or reagent-grade salts are added to the sample or test solutions, the toxicity test must include a set of controls (i.e., *salt controls*) which is prepared using the same source, batch, and concentration of dry salts as that added to the test sample. A second set of controls (i.e., *dilution-water controls*), comprised of 100% dilution water, is required if any water used to dilute the sample differs in any respect from the *HSB controls* or *salt controls* (Section 4.1.1). The salinity of all test concentrations should be within 1 g/kg of the controls.

If it is desired to assess the total effect of the wastewater including its low (or high) salinity, for regulatory compliance or other (e.g., research) purposes, the test could be run without adjusting the salinity of the control/dilution water or the sample/test solutions to 30 ± 2 g/kg. For instance, salinity could be adjusted to that of the receiving water, or the control/dilution water adjusted to 30 ± 2 g/kg without adjusting the salinity of sample or test solutions. Such an evaluation should include a second, salinity-adjusted test and/or a set of salinity controls (Section 4.1.4).

6.4 Test Observations and Measurements

Success of fertilization should be observed as in Section 4.4.

Colour, *turbidity*, odour, and homogeneity (i.e., the presence of floatable or settleable solids), should be observed in the sample of effluent, leachate, or elutriate at the time of preparing test solutions. A record should be made of any reactions or overt changes upon dilution with water or during the test, such as *precipitation*, *flocculation*, foaming, odour, and change in colour or turbidity.

For effluent samples having appreciable solids content, it is desirable to measure total suspended and settleable solids (APHA *et al*., 1989, 2005) upon receipt, as part of the overall description of the effluent, and as sample characteristics that might influence the results of the toxicity test.

6.5 Test Endpoints and Calculations

Tests for *monitoring* and for *compliance* with regulatory requirements should normally include, as a minimum, three or more replicates per concentration (including the control) if a multi-concentration design is used and an ICp is calculated. A test which compares full-strength solutions to a control (single-concentration) via hypothesis testing should normally include, as a minimum, four or more replicates. Test procedures for regulatory compliance might specify that a single concentration (100% sample unless otherwise specified) be used, or might require determination of the ICp.

Toxicity tests can have other objectives such as determination of in-plant sources of toxicity, or toxicity changes resulting from waste treatment or process changes. Such tests might be multi-concentration tests or single-concentration tests (100% or an appropriate dilution, plus a control). Single-concentration tests are often cost-effective for determining the presence or absence of measurable toxicity or as a method for screening a large number of samples for relative toxicity. Endpoints for these tests would again depend on the objectives of the undertaking, but could include arbitrary “pass” or “fail” ratings, or percent reduction in fertilization at a specified concentration. Items in Section 4.5.3 provide instructions that are relevant here, on statistical analysis and reporting of results from a set of tests on different samples, each tested at only one concentration.

Specific Procedures for Testing Receiving-Water Samples

Instructions for testing samples of receiving waters, additional to those provided in Section 4, are given here.

7.1 Sample Collection, Labelling, Transport, and Storage

Procedures for the collection, labelling, transportation, and storage of samples of receiving water should be as described in Section 6.1. The test should commence within 1 day of sampling, whenever possible, and must start within 3 days after sampling.

7.2 Preparing Test Solutions

Samples in the collection chambers should be agitated before pouring to ensure their homogeneity. Compositing of sub-samples, preparation and use of controls, and adjustment of the salinity of sample, test solutions, and/or control/dilution water should be as described in Sections 4.1.1, 4.3.2, 6.2, and 6.3.

Each receiving-water sample should be filtered through a sieve with a 60-µm mesh opening before use, to enable the removal of potential predators or suspended material which might otherwise interfere with the test results (USEPA, 1994). If there is concern about the contribution of suspended solids to sample toxicity or of toxicity reduction due to sample filtration, a second test, without sample filtration, should be carried out concurrently, as described in Section 6.2.

7.3 Control/Dilution Water

For receiving-water samples collected in the vicinity of a wastewater discharge, chemical spill, or other point-source of possible contamination, "upstream" water may be sampled concurrently and used as control water and diluent for the "downstream" samples. Discussion in Section 4.1.1 is relevant here, on the implications and possible effects of using such water for the control and for dilution. This control/dilution water should be collected as close as possible to the contaminant source(s) of concern, but outside its zone of influence. Water current or dispersal tracer studies might be necessary to establish an acceptable sampling location. All control/dilution water from a natural source should be filtered (Section 3.4).

If uncontaminated receiving water is used as control/dilution water, a separate control solution must be prepared using the laboratory seawater shown previously by the testing laboratory to routinely enable valid test results in an echinoid fertilization test. Test conditions and procedures for preparing and evaluating each control solution should be identical, and as described in Sections 4.1 and 5.3.

Logistic constraints, lack of on-site information, expected toxic effects, or other site-specific practicalities might prevent or rule against the use of "upstream" water as the control/dilution water. In such cases, a suitable laboratory seawater supply (i.e., holding water or a laboratory seawater known to routinely achieve valid test results using this biological test method) should be used as control/dilution water. This water may be adjusted in salinity to partially simulate "upstream" water (Section 4.1.1), but the salinity limitations of this echinoid assay preclude major manipulations.

7.4 Test Observations and Measurements

Observations and measurements of test samples and solutions for colour, *turbidity*, foaming, *precipitation*, etc. should be made as described in Section 6.4, both during the preparation of test solutions and subsequently during the tests. These are in addition to the primary toxicity observations described in Section 4.4.

7.5 Test Endpoints and Calculations

Statistical endpoints for tests using samples of receiving water should be consistent with the options and approaches identified in Sections 4.5 and 6.5, and would again be based on success in fertilization compared to the control(s).

Testing of each receiving-water sample should include a minimum of three replicates per concentration (including the control) if a multi-concentration design is used and an ICp is calculated. Endpoints for tests with receiving-water samples might often be restricted to data on fertilization of gametes exposed to sample of full-strength receiving water in single-concentration tests (see Section 4.5.3). In this case, a minimum of four replicates should be used.

If toxicity of receiving-water samples is likely, and information is desired concerning the degree of dilution necessary to permit normal fertilization in echinoids, a multi-concentration test to determine the ICp should be conducted as outlined in Section 4. The undiluted (100%) sample should be included in the test as the highest concentration of the series.

Certain sets of tests might use a series of samples such as seawater from a number of locations, each tested at full strength only. Statistical testing and reporting of results for such tests should follow the procedures outlined in Section 4.5.3.

Specific Procedures for Testing Samples of Liquid Extracted from Sediment or Similar Material

General instructions are given here for testing liquids derived from samples of sediment or similar solids such as sludge or soil. These are in addition to the general instructions provided in Section 4. In this section, the word "sediment" is used for convenience but should be taken to include other similar solid materials such as soils and industrial or municipal sludges, which might contribute pollutants to natural waters or require testing for other reasons.

8.1 General Aspects of Procedure

When the first edition of this test method document was published, assessing the toxicity of sediments was becoming widely recognized as an important part of environmental protection, notably when considering ocean dumping, such as under Part VI of the *Canadian Environmental Protection Act*. Since its publication in 1992, this sublethal test has been used on a regular basis on samples of sediment pore water, as part of the Disposal at Sea Program, to help evaluate the suitability of dredged material for disposal at sea (CEPA, 1999; Government of Canada, 2001). However, the potential influence of confounding factors (e.g., ammonia and sulphides) in sediment pore water, on the interpretation of results, has contributed to uncertainty with the application of this test from a regulatory perspective (Scroggins *et al*., 2003). As a result, Environment Canada initiated some method-improvement and method-development research which has led to further guidance for the improvement of the echinoid fertilization assay for porewater testing, described herein. Additionally, Environment Canada is currently developing a new sediment-contact *embryo*/larval test for measuring the toxicity of samples of solid-phase sediment to the early life stages of echinoids (Buday, 2006; Jackman and Doe, 2004, 2006; McLeay, 2007).

The echinoid fertilization assay described herein is suitable for testing the toxicity of liquids derived from sediments. It provides a rapid method for comparing extracts of contaminated sediments (Long *et al.*, 1990). Tests on sediment-derived liquids showed that an echinoderm *embryo* test and the bacterial Microtox test were the most sensitive of seven sediment tests evaluated (Pastorok and Becker, 1989). Sublethal toxicity tests including a fertilization assay using Arbacia and interstitial water from sediments, were considerably more sensitive than a standard test on the whole sediment using amphipods (Carr and Chapman, 1992).

General guidance is given here on application of the echinoid fertilization assay for testing liquids derived from sediments. It is not the purpose of this report to provide instructions for carrying out a field survey of sediments, sampling them, or extracting aqueous or other material from them. Detailed guidance for the collection, handling, transport, and storage of sediment samples (Section 8.1.1) is provided in Environment Canada (1994). The same Environment Canada document provides procedural details for the extraction of liquids [i.e., pore water, elutriate, or solvent extract (Section 8.2)] from sediments for subsequent toxicity tests and chemical analyses. This guidance document should be consulted and followed, in addition to the guidance provided here. Detailed information regarding the basis of such guidance can be found in books (e.g., Mudroch and MacKnight, 1991) and in primary literature cited in Environment Canada (1994) or related reviews of this subject matter (e.g., Geisy and Hoke, 1989; McLeay and Sprague, 1991).

This fertilization assay is not suitable for testing the whole sediments themselves (i.e., the solids), but is useful for liquids derived from

those solids, whether leachate, elutriate, solvent extract, or pore water.

Based on the results of an inter-laboratory study carried-out in 2008 to investigate the improvement of the porewater testing component of EPS 1/RM/27, the following sections of this second edition method document offer some additional guidance for the testing of samples of sediment pore water (Miller, 2008). These procedures may be included to help reduce the potential influence of confounding factors on test results in porewater fertilization tests. This additional guidance is based on methodologies developed at the Columbia Environmental Research Centre, Marine Ecotoxicology Research Station (Carr, Nipper, and Biendenbach, pers. comm., 2008; Carr and Chapman, 1992, 1995; Carr *et al*., 2006). It includes guidance on the following: the use of a control pore water in the pre-test to establish the sperm:egg ratio to be used in the definitive test (see Section 4.2.3); and the use of both a control pore water and one or more samples of reference pore water, for determining the toxicity (or lack thereof) in porewater samples from contaminated sites or sites of interest (see Sections 8.1.4, 8.2.1, 8.2.2 and 8.2.3).

8.1.1 Sample Labelling, Transport and Storage

General procedures for labelling, transporting, and storing sediment samples should be as described in Section 6.1. Temperature limits are those described in Section 6.1, and samples must not freeze or partially freeze, or be allowed to dry (ASTM, 1991b; EC, 1994).

For the liquids derived from sediments, containers and handling procedures should be the same as those given in Section 6.1 for elutriates. If a non-aqueous solvent has been used to extract substances or materials, a glass container should be used to store the liquid, so that it will not be affected by the solvent or leach substances into the sample.

Testing of samples should start as soon as possible after collection. Extraction procedures should begin within two weeks of sampling, and preferably within one week. Testing must start no later than six weeks after collection of samples (EC, 1994).[76] Testing of the liquid obtained from sediments should begin within 1 day of making such preparations (EC, 1994) and must begin within 3 days, unless specified otherwise in a regulation or other designated procedure.

8.1.2 Preparing Samples

Depending on the nature of the sample and the objectives of the test, homogenization of a sample might or might not be required before testing. If mixing is carried out, it must be thorough. Sub-samples (i.e., a sample divided between two or more containers) must be mixed together (i.e., composited). If further sample storage is required, the composited sample, or a portion of it, should be returned to the sub-sample containers and stored.

8.1.3 Observations and Measurements on Sample

Observations of the colour, *turbidity*, foaming, *precipitation*, etc. should be made on both the sediment and any liquid derived from it, during preparation of test solutions, as described in Section 6.4.

8.1.4 Liquid from Control and Reference Sediments

Liquid (pore water) extracted from one or more samples of control and/or *reference sediment*

[76] The toxicity and geochemistry of contaminated sediments from Hamilton Harbour were reported to change with storage for longer than one week, although the data supporting that statement were not provided (Brouwer *et al*., 1990). Testing within two weeks conforms with current standardization in U.S. procedures (ASTM, 1991b). A maximum permissible storage time of six weeks was included in draft reports of Environment Canada (1990e, 1990f) in view of practical difficulties for shorter times, including time required if initial chemical analyses are to be performed.

(unpolluted)[77] must be assessed in the same manner as that extracted from the sediment under investigation. The liquids derived from the reference sediment and the *control sediment* serve different purposes. Pore water extracted from a reference sediment serves as a field reference or a site reference, for a comparison of the biological effects observed in the test sediment pore water. The reference pore water should be used for comparative purposes whenever possible or appropriate, because this provides a site-specific evaluation of toxicity. Using other kinds of tests, however, Environment Canada and other laboratories have frequently recorded apparent toxic effects with pore water from unpolluted reference sediments, or the reference pore water is unidentified in the case of coded or blind samples, making it unsuitable for comparison. In such cases, it would be necessary to compare the biological effects for the test pore water with those for the control sediment pore water. Accordingly, pore water derived from one or more control ("clean") sediments should be included as a sample, with each test of pore water(s) derived from sediment (or series of sediments), to help establish a baseline or "normal" level. It would be desirable to establish a standard, clean, "*control sediment*" for this purpose, or ideally a series of reference sediments of differing characteristics that could be matched with those for the sediments being tested.

Ideally, the physicochemical characteristics of a reference sediment(s) should be similar to those of the sediment(s) being investigated, and should account for the confounding factors (i.e., not the contaminants of concern) that might be present in the sample pore waters (Scroggins *et al*., 2003)[78]. However, it is also important that some type of clean (i.e., control) pore water be used routinely in porewater tests to provide a basis for interpreting data derived from the test. A control pore water might be collected from a sediment that is low in contaminants, and contains low levels of ammonia, and sulfides. Once a good (i.e., clean, stable, previously shown to support good fertilization etc.) control sediment is located, large volumes of pore water can be collected and stored (i.e., frozen) for future porewater tests. The control sediment pore water serves a different purpose than the laboratory seawater control (i.e., control/dilution water) which provides a direct measure of the test validity. Typically, both types of controls (i.e., the laboratory seawater control and the porewater control using pore water extracted from a suitable control sediment) should be used in toxicity tests for pore water from contaminated sediments (Scroggins *et al*., 2003), along with a suitable porewater sample from one or more reference sites, since experience indicates that control water (i.e., laboratory seawater) alone might not be sufficient for an acceptable evaluation of porewater toxicity.

There is no single procedure for making use of the results from the control sediment and reference sediment (i.e., pore water), however, if a control pore water and a reference pore water are included in the test, are handled identically to the test pore water (see Sections 8.2.1 and

[77] A *reference sediment* is a field-collected sample of presumably clean (uncontaminated) sediment, selected for properties (e.g., particle size, etc.) representing sediment conditions that closely match those of the test sediment, except for the degree of chemical contamination. It is often selected from a site that is in the general vicinity of the site(s) where the samples of test sediment are collected. It might or might not prove to be toxic due to the presence of naturally occurring chemicals or unanticipated presence of contaminants from human influence. A *control sediment*, on the other hand, is an uncontaminated (clean) sediment which definitely does not contain any contaminants that could affect the fertilization of echinoid eggs. The physicochemical properties of a control sediment might not match those of the test sediment, nor is it necessarily collected in the same vicinity as the test sediment. It simply provides a basis for interpreting data derived from toxicity tests using test sediment(s). A reference sediment that is in fact clean (uncontaminated) may be used as a control sediment.

[78] Ideally, an attempt should be made to match the distribution of particle sizes and organic/inorganic matter balance (ASTM, 1991a, 1991b; McLeay and Sprague, 1991).

8.2.2), and run concurrently with the test pore water, tests for significant differences between the results for the reference/control and test sediments can be carried out (i.e., the control pore water can be used as a baseline for determining the presence or absence of toxicity at each treatment level [see Section 8.2.3]; and the reference pore water, if not blind or coded, can provide a site-specific evaluation of toxicity). Caution should be exercised in interpreting findings for the test substance or material, and such tests should be carried out with guidance from a statistician. Sometimes, neither a reference pore water nor a control porewater sample are available. In these situations, it might be necessary to use the laboratory water control (i.e., control/dilution water) for comparison with porewater test results for environmental samples, however this approach is less preferred.[79] If only control/dilution water is used as a basis of comparison, there is no control for confounding factors, and interpretation of test results might require an understanding of the tolerance limits of the test species for potential confounding factors in the test samples. Also, any site-specific evaluation of toxicity would be limited.

8.2 Testing Liquids Extracted from Sediments and Similar Solids

Toxicants from sediments or soils can enter an aqueous phase and affect organisms in natural waters. The aqueous phase might be a liquid derived from a soil or sediment (e.g., interstitial or pore water), or a liquid used to treat the sample and extract potential toxicants (e.g., an elutriate).

A liquid obtained from sediment for toxicity testing would be expected to fall into one of four broad categories.

(1) Interstitial water, which fills the spaces between particles (i.e., pore water), and could exchange with the overlying water making up the estuary, bay, etc. It is normally obtained from a sediment by centrifuging or squeezing it (ASTM, 1991b).

(2) Water that is essentially fresh water, used to obtain an aqueous extract of materials from the sediment (i.e., elutriate), for example by shaking a sample with added clean water. This category is not very appropriate for the echinoid fertilization assay.

(3) Control/dilution water or other clean water which has a salinity equivalent to seawater, used to obtain an aqueous extract as in (2).

(4) Solvents other than water (e.g., organic solvents), used to remove substances or materials from the sample of sediment (Schiewe *et al*., 1985; True and Heyward, 1990).

The water of the first three categories could be tested as a normal liquid sample, following the universal procedures given in Section 4 and the procedures for effluents, leachates, and elutriates as given in Section 6. For the fourth category (i.e., solvents), the preferred option is to have the same concentration of solvent in each of the test vessels. The control/dilution water to be used in the test is brought to the same concentration of solvent as that in the highest concentration of sample that will be tested. Lower concentrations are prepared using the modified control/dilution water, so that the solvent effect, if any, should be the same in all vessels. There should be a control without solvent, as well as the one with solvent. It would be desirable to run a separate test to determine the ICp of the solvent.

[79] Clean pore water and filtered seawater are very different matrices that often behave quite differently in fertilization assays (e.g., clean porewater samples often yield a lower [but still acceptable] % fertilization than that achievable using filtered seawater). For porewater testing, therefore, clean pore water (i.e., control pore water) is a more appropriate matrix for establishing the sperm:egg ratio to be used in the test (see Section 4.2.3) and for a basis of comparison (i.e., negative control) to determine whether a test pore water is toxic or not.

8.2.1 Preparing Test Samples

Specific guidance is given in Environment Canada (1994) for the extraction of liquids (i.e., pore water, elutriate, or solvent extract) from sediment. This guidance should be consulted and followed when preparing sediment extracts for echinoid fertilization assays, and also applies to the preparation of control and reference pore water.[80]

Compositing of "sub-samples" of liquid obtained from the sediment (e.g., successive extractions) should be as described in Section 6.2. Sub-samples should not be composited if the relative toxicity of successive extractions was to be ascertained. Samples or sub-samples of elutriate or pore water should be centrifuged to remove suspended solids (EC, 1994). Only control porewater samples may be frozen for the purpose of long-term storage. Control porewater samples may be frozen at -20°C and stored at that temperature for up to one year as long as they are particle-free before freezing. To obtain particle-free pore water, control porewater samples should be centrifuged twice (e.g., centrifuge sediment sample at ~3000 × g for 15 min at 4°C, remove pore water being careful not to remove particles near sediment layer, and then centrifuge pore water at ~3000 × g for 15 min at 4°C). Samples should be thawed the day before they are to be used in a test, and should be centrifuged after thawing to remove any precipitated suspended particulate matter which can inhibit the ability of the echinoid sperm to locate and fertilize eggs (Carr and Chapman, 1995). The pH and dissolved oxygen content of the sample should be checked with regard to the limits in Sections 4.1.2 and 4.1.3.

Once the liquid has been obtained, test concentrations are prepared in the standard manner (Section 4.1.2). As in testing effluents, leachates, and elutriates, there could be a single-concentration test (plus control) for regulatory purposes, or a multi-concentration test to determine the ICp (Section 6.5). The procedures for obtaining and testing liquid from samples of control or reference sediment should be identical to those used for the test sediment.

8.2.2 Control/Dilution Water

If the sediment sample is marine or estuarial, and the water derived from it is essentially seawater in the standard range of salinity for this test (i.e., 28 to 32 g/kg), the Universal procedures of Section 4 would be followed.

For freshwater samples derived from sediment, or if the water derived from the sediment has salinity lower than that of full-strength seawater (i.e., similar to estuarial water), its salinity would normally be adjusted upwards. The standard procedure would be to adjust the salinity of all test and control solutions to the same value (within 1 g/kg of each other in the range 28 to 32 g/kg), usually accomplished by adjusting an aliquot of the sample as described in Section 4.3.2. Procedures using commercially-available dry ocean salts, reagent-grade salts, or natural or artificial hypersaline brine are recommended, as outlined in Section 2.3.4.

The same control/dilution water (e.g. laboratory seawater) must be used for the reference and/or control pore water, and the test pore water dilutions and controls in multi-concentration porewater tests. In addition, the reference and/or control pore water, and the test pore water must include the same treatments (i.e., porewater dilutions) and should include the same number of replicates for each treatment (i.e., to facilitate tests for significant differences between the results for the pore waters).

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[80] A study carried out by Environment Canada's Atlantic Laboratory for Environmental Testing confirmed EC's 1994 recommendation to collect porewater samples at 4°C and 10,000 × g. The study showed that samples of pore water collected by centrifugation at 4°C resulted in greater toxicity than those collected at room temperature. The toxicity of the pore water was not affected by the speed of centrifugation (2700 vs. 10000 × g), however approximately double the volume of pore water was produced at the higher centrifugation speed (i.e., 10000 × g) (Jackman and Doe, 2004).

For the calculation of an ICp, at least three replicates per treatment (recommend 5) and seven test concentrations (more recommended) must be included (see Sections 4.1.2, 4.1.3, and 4.5.2).

8.2.3 Endpoints and Calculations

Endpoints for tests with liquids derived from sediment should be consistent with the options and approaches identified in Sections 4.5 and 6.5.

To test for significant differences between the reference and/or control pore water and the test pore water, percent fertilization in each test porewater treatment is compared to the equivalent reference and/or control porewater treatment using Dunnett's t-test.[81]

[81] The fertilization result for a 100% test porewater sample is compared to the fertilization result for a 100% reference or control pore water. Likewise, the fertilization result for a 50% dilution of the sample pore water is compared to the fertilization result for a 50% dilution of the reference or control pore water. The diluent for both the sample and the reference or control pore waters is the same (i.e., filtered seawater).

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 8 of this biological test method, and, if so, provide details as to the deviation. The reader must be able to establish from the test-specific report whether the conditions and procedures preceding and during the test rendered the results valid and acceptable for the use intended.

Section 9.1 provides a list of the items which must be included in each test-specific report. Section 9.2 gives a list of those items which must either be included in the test-specific report, provided separately in a general report, or held on file for a minimum of five years. Specific *monitoring* programs or related test *protocols* might require selected test-specific items listed in Section 9.2 to be included in the test-specific report, or might relegate certain test-specific information (e.g., details regarding the test material and/or explicit procedures and conditions during sample collection, handling, transport, and storage) as "data to be held on file".

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

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

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

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

9.1 Minimum Requirements for Test-Specific Report

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

9.1.1 Test Substance or Material

- Brief description of sample type (e.g., chemical or chemical substance, effluent, elutriate, leachate, receiving water, or liquid extracted from sediments or similar solids) and volume or weight (if dry chemical), if and as provided to the laboratory personnel;
- information on labelling or coding for each sample;
- date of sample collection; date and time sample received at test facility;
- for effluent or leachate, measurement of temperature of sample upon receipt at test facility;
- measurements of pH and dissolved oxygen of sample of wastewater or receiving water, just before its preparation and use in toxicity test; and

- for a test with elutriate or any liquid extracted from sediments or similar solids, dates for sample generation and use; description of procedure for preparation.

9.1.2 Test Organisms

- species and source;
- brief description of holding time and conditions, for adults;
- percentage of mortalities among adults shipped and held briefly (i.e., ≤3 d); and/or weekly percentage of mortalities among the adults being acclimated and held for longer periods (i.e., >3 d); and
- any unusual appearance, behaviour, or treatment of adults or gametes, before the test is started.

9.1.3 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).

9.1.4 Control/Dilution Water

- type(s) and source(s) of water used as control and dilution water;
- type(s),source(s), and collection procedure of control and/or reference pore water used, if any; and
- type and quantity of any chemical(s) added to control or dilution water.

9.1.5 Test Method

- citation of biological test method used (i.e., as per this document), and options selected;
- design and description if specialized procedure (e.g., test performed with and without filtration of sample; preparation and use of elutriate; preparation and use of solvent and, if so, solvent control);
- brief description of procedure for preparation of hypersaline brine and duration of aging;
- brief description of procedure(s), products used, and duration of aging for any salinity adjustments of control/dilution water, sample, or test solutions;
- brief description of procedure(s) if any sample or test solutions received filtration and/or pH adjustment;
- 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.

9.1.6 Test Conditions and Procedures

- design and description if any deviation from or exclusion of any of the procedures and conditions specified in this document;
- number, concentration, volume, and depth of solutions in test vessels, including controls;
- number of replicates per treatment;
- brief statement indicating that the gamete viability check was performed, and whether a pre-test was performed;
- estimated number of sperm per vessel and sperm:egg ratio;
- number of males and females used to pool sperm and eggs;
- brief statement concerning aeration (if any, give rate, duration) of sample or test solutions before starting the test;
- measurements of temperature, salinity, pH, and dissolved oxygen (mg/L and percent saturation) in aliquots of test solutions (including controls), at the start of the test;
- period of time test vessels (i.e., preserved eggs) are stored prior to enumerating results;

- date when test was started; statement of test duration; 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.

9.1.7 Test Results

- for each replicate test solution (including each of the control replicates): the number of fertilized and unfertilized eggs counted in each vessel at the end of the test;

- mean (± SD) percent fertilized eggs or proportion fertilized for each test vessel and each treatment (e.g., each concentration), including the controls;

- ICp (together with its 95% confidence limits) for the percent fertilization or proportion fertilized data; details regarding any weighting techniques and indication of the quantitative statistics used;

- any outliers, and the justification for their removal;

- the duration and results of any toxicity tests with the reference toxicant(s) performed at the same time or within 14 days 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.

9.2 Additional Reporting Requirements

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

9.2.1 Test Substance or Material

- identification of person(s) who collected and/or provided the sample;

- 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.

9.2.2 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 and dissolved oxygen concentration 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 maturation of gonads, spawning success and time, fecundity, and fertilization success rates before test; and

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

9.2.3 Test Facilities and Apparatus

- description of systems for regulating light and temperature within the test facility; and
- description of procedures used to clean or rinse test apparatus.

9.2.4 Control/Dilution Water

- sampling and storage details if the control/dilution water was "upstream" receiving water;
- details regarding any water *pre-treatment* (i.e., procedures and conditions for salinity adjustment, filtration, sterilization, temperature adjustment, de-gassing, aeration); and
- measured water quality variables (Section 2.3.4) before and/or at time of starting the test.

9.2.5 Test Method

- description of laboratory's previous experience with this biological test method for measuring toxicity using the same procedures, conditions, and test species;
- procedures used in preparing and storing stock and/or test solutions of chemicals; description and concentration(s) of any solvent used;
- methods used (with citations) for chemical analyses of sample or test solutions; details concerning sampling, sample/solution preparation and storage, before chemical analyses; and
- use and description of preliminary or range-finding test.

9.2.6 Test Conditions and Procedures

- photoperiod, light source, and intensity adjacent to surface of test solutions;
- conditions, procedures, and frequency for toxicity tests with reference toxicant(s);
- water quality measurements for water supply used for holding and control/dilution water (see Section 2.3.4);
- any other chemical measurements on sample, stock solutions, or test solutions (e.g., concentrations of one or more specific chemicals; suspended solids content), before and/or at time of the test; and
- appearance of sample or test solutions; changes in appearance noted during test.

9.2.7 Test Results

- results for any range-finding test(s) conducted in conjunction with the definitive test;
- results of the gamete viability check and pre-test;
- graphical presentation of dose-response data;
- results for any statistical analyses conducted both with outliers and with outliers removed; for regression analyses, file information indicating sample size (e.g., number of replicates per treatment), parameter estimates with variance or standard error, any ANOVA table(s) generated, plots of fitted and observed values of any models used, results of outlier tests, results of tests for normality and homoscedasticity, and how the model of best fit was chosen;
- 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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Appendix A

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

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

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

Title of Biological Test Method or Guidance Document	Report Number	Publication Date	Applicable Amendments
A. Generic (Universal) Biological Test Methods (cont'd.)			
Test for Survival and Growth in Sediment Using the Freshwater Amphipod *Hyalella azteca*	EPS 1/RM/33	December 1997	—
Test for Measuring the Inhibition of Growth Using the Freshwater Macrophyte, *Lemna minor*	EPS 1/RM/37 2nd Edition	January 2007	—
Test for Survival and Growth in Sediment Using Spionid Polychaete Worms (*Polydora cornuta*)	EPS 1/RM/41	December 2001	—
Tests for Toxicity of Contaminated Soil to Earthworms (*Eisenia andrei, Eisenia fetida*, or *Lumbricus terrestris*)	EPS 1/RM/43	June 2004	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	September 2007	—
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	—

Appendix B

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Appendix D

Review of Procedural Variations Used by Previous Authors and Groups for Fertilization Assays Using Sea Urchins and Sand Dollars

Based on documents available to the authors in March, 1992. The following elements of procedure are omitted because they were common to all tests, or could be easily adapted to all methods covered here.

1. **Static tests** - All exposure and fertilization was in small vessels without renewal of solutions.

2. **Test substance** - All methods could be used for pure chemicals, formulations, wastewaters, or samples of seawater, by adjusting salinity as is common practice in the methods reviewed.

3. **Endpoints** - The usual endpoint was reduced fertilization compared to control. All methods appear suitable for estimating ICp and NOEC/LOEC by usual statistical techniques.

Explanation of authors or originating agency.

Beak 1988 is the Canadian consulting company, listed in the references.

EVS 1989 is the Canadian consulting company, listed in the references.

B.C. MOE 1990 is British Columbia Ministry of Environment, and includes van Aggelen (1988).

IGATG 1991 includes that reference and Jonczyk *et al.* (1991).

Dinnel *et al.* 1987 with co-authors, represents a major school or approach in echinoid testing.

USEPA 1988 is in the book of methods published by the Cincinnati office of EPA.

ASTM 1990 is a subcommittee developing a standard method, chairman G.A. Chapman.

NCASI 1991 and 1992 are in reference list; a scientific group sponsored by pulp and paper industry.

USEPA (Pac. 91) is in reference list as Chapman (1991), a Pacific-coast method for interlaboratory comparisons, which prompted documents from U.S. consultants (see following).

USEPA (Pac. 92) is Chapman (1992) in reference list, a draft Pacific-coast method of EPA.

Kobayashi 1971 represents the early methods used by this productive researcher.

Kobayashi 1984 represents a later synopsis of methods by this researcher.

S. Calif. Project was a regional pollution research agency, in references as Oshida *et al*., 1981.

Nacci *et al*. 1986 is a publication cited by others as a source of methods.

Cherr *et al.* 1987 are authors from the Bodega Marine Lab.

BML 1991 is in references, and is Bodega Marine Lab, part of Univ. of California.

ERCEES 1990 is a U.S. consulting company in California and is in reference list.

MECAS 1990 is a U.S. consulting company in California and is in reference list.

NWAS 1990 is a U.S. consulting company on the west coast and is in reference list.

The order of listing is (1) Canadian laboratories, (2) major committees, government agencies, laboratories and schools (which happen to be in the United States), and (3) consulting laboratories and major authors. Detailed methods of Pagano and colleagues were not clear from papers and have been omitted.

Abbreviations:
lab. = laboratory
N.I. = not indicated
c/d water = control/dilution water
Pac. = Pacific
reconst. = reconstituted
s.u. = sea urchin(s)
s.d. = sand dollars(s)

1. Species and Availability of Adults

Document	Species, Information Given on Location, Collection, Spawning Season
Beak 1988	*Lytechinus pictus* Californian urchin, purchased. Spawning condition year-round. *Strongylocentrotus droebachiensis* green sea urchin, Canadian Atlantic, Pacific, Arctic. Said to spawn March to April.
EVS 1989	*S. purpuratus* Pacific purple sea urchin. Collect from clean locations or purchase. Spawns Dec. to March. *S. droebachiensis* as above. *S. franciscanus* red sea urchin, Pacific. Spawns April-May. *Dendraster excentricus* "eccentric sand dollar" of the Pacific, said to spawn late spring and summer
B.C. MOE 1990	*D. excentricus* eccentric sand dollar as above, but said to spawn June to Nov.
IGATG 1991	*S. droebachiensis* as above, but spawns Feb. to March or April *L. pictus*, as above.
Dinnel *et al.* 1987	*S. purpuratus* Ripe Dec. to March, longer in lab. *S. droebachiensis* as above. Ripe Jan. to April, longer in lab. *S. franciscanus* as above. *D. excentricus* as above. Ripe May to October.
USEPA 1988	*Arbacia punctulata* "Arbacia", or Atlantic purple sea urchin. May be purchased.
ASTM 1990	*A. punctulata* as above. *D. excentricus* as above. *S. purpuratus* as above. *S. droebachiensis* as above. Other species may be used if necessary
NCASI 1991, 1992	*D. excentricus* as above. Spawn all year except late Dec. to late Jan. by arranging laboratory holding conditions. *S. purpuratus* Spawns Jan. to June by lab. holding. *S. droebachiensis* as above. Also Jan. to June by lab. holding.
USEPA (Pac. 91)	*S. droebachiensis* as above.
USEPA (Pac. 92)	*S. purpuratus* as above.
Kobayashi 1971	*Hemicentrotus pulcherrimus* a sea urchin, Japan. Spawns Jan. to March. *Anthocidaris crassispina* a sea urchin, Japan. Spawns May to Aug. *Temnopleurus toreumaticus* a sea urchin, Japan. Spawns July to Oct. *Pseudocentrotus depressus* a sea urchin, Japan. Spawns Oct. to Nov.
Kobayashi 1984	Same as Kobayashi 1971 except *T. toreumaticus* not mentioned.
S. Calif. Project	*S. purpuratus* as above. Collect by hand.
Nacci *et al*. 1986	*A. punctulata* as above.
Cherr *et al*. 1987	*S. purpuratus* as above.
BML 1991	*S. purpuratus* as above.

ERCEES 1990	*S. purpuratus* as above. Collect or purchase. *A. punctulata* as above. Collect or purchase. *Lytechinus sp.* as above. Collect or purchase. *D. excentricus* as above. Collect or purchase.
MECAS 1990	N.I.
NWAS 1990	*S. purpuratus* as above. *D. excentricus* as above. Purchased as necessary.

2. Holding Adults in the Laboratory

Document	Duration	Water	Feeding
Beak 1988	5 d	reconstituted seawater	romaine lettuce? s.u. given macroalga
EVS 1989	≤9 wk	flowing seawater at 0.1 L/min per shallow tray, or static with monthly replacement	s.u.with brown macroalga s.d. with eel grass
B.C. MOE 1990	N.I.	unfiltered flowing seawater	s.d. not fed
IGATG 1991	≥7 d	green s.u., flowing seawater white s.u. in reconst. seawater	green s.u., brown macroalga s.u. kelp or romaine lettuce
Dinnel *et al.* 1987	N.I.	flowing seawater, filtered recirculation with filter	s.u. macroalga s.d. plankton and detritus
USEPA 1988	N.I.	filtered seawater, 5 L/min, for 20-L tank with 20 adults, or recirculated reconst. seawater	s.u. kelp or romaine lettuce
ASTM 1990	N.I.	reconstituted seawater, or unfiltered seawater	s.u., macroalga, R. lettuce s. d., microalgae
NCASI 1991, 1992	N.I.	unfiltered seawater, 1 to 2 L/min to 160-L tank	s.d. algal growth flake food s.u. macroalga, romaine lettuce
USEPA (Pac. 91)	N.I.	N.I	N.I.
USEPA (Pac. 92)	N.I.	filtered seawater, 5 L/min, or recirculated reconst. seawater	kelp or romaine lettuce
Kobayashi 1971	≤2 d	N.I.	N.I.
Kobayashi 1984	≤2 d	N.I.	N.I.
S. Calif. Project	N.I.	recirculated seawater	brown alga
Nacci *et al.* 1986	N.I.	N.I.	N.I.
Cherr *et al*. 1987	N.I.	flowing seawater	macroalga
BML 1991	N.I.	N.I.	N.I.
ERCEES 1990	N.I.	seawater brought in weekly	giant kelp
MECAS 1990	0 to 2 d	flowing seawater	N.I.
NWAS 1990	days/mos.	seawater, flowing or partly recirculated	s.u. kelp or lettuce s.d. plankton and detritus

3. Holding Conditions for Adults

Document	Species	Temperature (°C)	Salinity (g/kg)	Oxygen (% sat'n)	Lighting
Beak 1988	*Lytechinus anamesus*	15	30	N.I.	N.I.
EVS 1989	various spp. s.u. *D. excentricus*	~10 15	28 "	airstones "	constant dark photoperiod
B.C. MOE 1990		N.I.	27 to 30	N.I.	N.I.
IGATG 1991	*S. droebachiensis* *L. pictus*	9 15	30 "	N.I. "	N.I. "
Dinnel *et al.*, 1987	*Strongylocentrotus* *D. excentricus*	natural seasonal	≥27	N.I.	N.I.
USEPA 1988	*A. punctulata*	15 ± 3	30	N.I.	N.I.
ASTM 1990	*Strongylocentrotus* *D. Excentricus* *A. punctulata*	8 to 10 12 to 14 15	25 to 35 " "	50 to 100% " "	N.I. N.I. high lighting
NCASI 1991, 1992	*Strongylocentrotus, D. excentricus*	7 to 14	N.I.	N.I.	ambient lab.
USEPA (Pac. 91)	*S. purpuratus*	N.I.	N.I.	N.I.	N.I.
USEPA (Pac. 92)	*S. purpuratus*	12 (10 to 14)	>30 (32 preferred)	N.I.	N.I.
Kobayashi 1971		N.I.	N.I.	N.I.	N.I.
Kobayashi 1984		N.I.	N.I.	N.I.	N.I.
S. Calif. Project	*S. purpuratus*	12	N.I.	N.I.	N.I.
Nacci *et al.* 1986		N.I.	N.I.	N.I.	N.I.
Cherr *et al.* 1987		N.I.	N.I.	N.I.	N.I.
BML 1991		N.I.	N.I.	N.I.	N.I.
ERCEES 1990		N.I.	N.I.	N.I.	N.I.
MECAS 1990		12	N.I.	N.I.	N.I.
NWAS 1990	*S. purpuratus, D. excentricus*	10 ± 2	≥25	N.I.	12 Light 12 Dark

4. Type of Control/Dilution Water

Document	Recommended Type of Water and Treatment
Beak 1988	deionized water with sea salts
EVS 1989	clean seawater filtered at 1 µm, UV sterilization optional
B.C. MOE 1990	seawater
IGATG 1991	deionized water with sea salts, or seawater filtered at 0.45 µm
Dinnel *et al.* 1987	seawater, filtered at 5 µm, activated carbon optional, or recirc'n with filter
USEPA 1988	deionized water plus sea salts or brine; seawater may be additional control
ASTM 1990	reconstituted from sea salts or formula, filtered 0.45 µm, TOC and TSS ≤5 mg/L, UV sterilization if pathogens likely, must achieve 70% fertilization with sperm held in water for 1 h
NCASI 1991, 1992	seawater, filtered 1 µm and UV sterilization, aerated, held 0 h
USEPA (Pac. 91)	seawater, filtered 1 µm
USEPA (Pac. 92)	seawater, or reconstituted, preferably from brine
Kobayashi 1971	N.I., presumed seawater
Kobayashi 1984	N.I., presumed seawater
S. Calif. Project	N.I.
Nacci *et al*. 1986	brine prepared from seawater, diluted to salinity 30 g/kg with distilled water
Cherr *et al*. 1987	seawater, filtered 0.45 µm
BML 1991	seawater, filtered and UV sterilized
ERCEES 1990	seawater, supply renewed weekly, filtered 20 µm and 5 µm
MECAS 1990	seawater, filtered 0.45 µm
NWAS 1990	seawater, unfiltered, adjusted to 32 g/kg salinity with deionized water

5. Temperature and Salinity During Test

Document	Temperature (°C)	Salinity (g/kg) and Method of Adjustment
Beak 1988	20 ± 1	30 ± 2
EVS 1989	15	adjust to unspecified salinity with salts if testing seawater samples, no adjustment for freshwater samples
B.C. MOE 1990	10	N.I.
IGATG 1991	20 ± 1	30 ± 2
Dinnel *et al*. 1987	s.u. 8 to 10 s.d. 12 to 16	30 ± 3, adjust with sea salt or deionized water
USEPA 1988	20 ± 1	30 ± 2, adjust effluent as necessary
ASTM 1990	12, but 20 for *A. punctulata*, and ≤2 °C variation between, within vessels	>25 and <32, within 1 g/kg of control, 30 recommended; adjust with the brine or salts
NCASI 1991, 1992	12	30, adjust test solutions with brine or salts
USEPA (Pac. 91)	12	32 ± 1
USEPA (Pac. 92)	12 ± 1	32 ± 2, adjust sample to 32
Kobayashi 1971	N.I.	N.I., adjust low-salinity samples with brine or by boiling
Kobayashi 1984	N.I.	N.I.
S. Calif. Project	N.I.	N.I., apparently not adjusted. Some tests 31 to 32.6
Nacci *et al*. 1986	N.I.	N.I.
Cherr *et al.* 1987	N.I.	N.I.
BML 1991	15	32, adjust both sample and water if necessary
ERCEES 1990	"appropriate"	N.I., adjusted if necessary with brine or deionized water
MECAS 1990	12 ± 1	30 ± 2, adjust test solutions with brine or spring water
NWAS	12 ± 1	32 ± 2, adjust sample as needed with brine

6. Dissolved Oxygen and Lighting During Test

Document	Initial DO (% saturation) and Adjustment	Lighting
Beak 1988	N.I.	normal lab., nominal 1100 lux
EVS 1989	aerate samples enough to attain acceptable DO	N.I.
B.C. MOE 1990	N.I.	N.I.
IGATG 1991	N.I.	normal lab., nominal 1100 lux
Dinnel *et al.* 1987	N.I.	N.I.
USEPA 1988	N.I.	normal lab., 540 to 1080 lux
ASTM 1990	90 to 100% in c/d water	N.I.
NCASI 1991, 1992	N.I.	normal lab. fluorescent
USEPA (Pac. 91)	N.I.	N.I.
USEPA (Pac. 92)	N.I.	normal lab., 540 to 1100 lux
Kobayashi 1971, 1984	N.I.	N.I.
S. Calif. Project	Not controlled	N.I.
Nacci *et al.* 1986	N.I.	N.I.
Cherr *et al*. 1987	N.I.	N.I.
BML 1991	N.I.	N.I
ERCEES 1990	N.I.	N.I.
MECAS 1990	N.I.	N.I.
NWAS 1990	N.I.	normal lab., no photoperiod required

7. Hydrogen-ion Concentration at Start of Test

Document	pH, for Test Water Unless Otherwise Specified, and Adjustments
Beak 1988	N.I.
EVS 1989	adjust sample to pH 7.5 if necessary; pH of test water N.I.
B.C. MOE 1990	N.I.
IGATG 1991	N.I.
Dinnel *et al.* 1987	adjusted if required; levels not indicated
USEPA 1988	N.I.
ASTM 1990	7.8 to 8.1 for Pacific purple s.u., "similar" for other species; adjust c/d water
NCASI 1991, 1992	N.I.
USEPA (Pac. 91)	8.1 ± 0.1 for c/d water
USEPA (Pac. 92)	N.I.
Kobayashi 1971, 1984	N.I.
S. Calif. Project	Not controlled. Some tests averaged 7.8 to 7.9
Nacci *et al.* 1986	N.I.
Cherr *et al.* 1987	N.I.
BML 1991	8.0, adjust both sample and c/d water if necessary, ensure pH is stable
ERCEES 1990	N.I.
MECAS 1990	8.0 ± 0.2, adjust test solutions as necessary
NWAS 1990	8.0

8. Volume of Test Water, Vessels Used, and Number of Replicates

Document	Volume (mL)	Vessel	Replicates
Beak 1988	5	20-mL scintillation vials, disposable	four
EVS 1989	10	test tubes, 16 × 150 mm with caps	three
B.C. MOE 1990	2	borosilicate glass tubes, disposable	three
IGATG 1991	5	20-mL scintillation vials, disposable	three
Dinnel *et al.* 1987	10	borosilicate glass test tubes, 16 × 100 mm, disposable, unwashed	≥3
USEPA 1988	5	20-mL scintillation vials, disposable	≥3, normally four
ASTM 1990	N.I.	glass vials, 15 to 22 mL, or other	recommend 4, usually ≥3
NCASI 1991, 1992	2	borosilicate glass culture tubes, 13 × 100 mm disposable	four
USEPA (Pac. 91)	5	borosilicate glass tubes, 16 × 100 mm	three
USEPA (Pac. 92)	5	disposable glass test tubes, 16 × 100/125 mm	≥3
Kobayashi 1971	N.I.	glass finger bowl, 5 cm diam., 3 cm deep	N.I.
Kobayashi 1984	N.I.	finger bowl filled with test medium	N.I.
S. Calif. Project	50 (sperm) 900 eggs	polypropylene cup 1-L beaker	N.I. N.I.
Nacci *et al.* 1986	10	glass vials	N.I.
Cherr *et al.* 1987	2	borosilicate culture tubes, 13 × 100 mm	N.I.
BML 1991	2	N.I.	three
ERCEES 1990	10	20-mL scintillation vials	four
MECAS 1990	5	25-mL scintillation vials	≥3
NWAS 1990	10	borosilicate culture tubes, 18 × 150 mm	four

9. Exposure Times for Sperm, for Eggs Plus Sperm, and for Experimental Controls

Document	Sperm exposure	Eggs + Sperm	Control Vessels
Beak 1988	60 min	60 min	4 c/d water
EVS 1989	30 min (s.u.) 60 min (s.d.)	20 min 20 min	3 with c/d; freshwater samples with duplicate salinity controls made with distilled water, concentrations same as for the sample
B.C. MOE 1990	10 min	10 min	3 seawater
IGATG 1991	60 min	20 min	3 c/d water
Dinnel *et al.* 1987	60 min	20 min	≥3 c/d water
USEPA 1988	60 min	20 min	≥3, normally 4, c/d water
ASTM 1990	60 min	20 min	c/d water; solvent control if used
NCASI 1991, 1992	10 min	10 min	4 c/d water
USEPA (Pac. 91)	20, 60 min	20, 60 min	diverse, to assess alternate methods in this exploratory round-robin
USEPA (Pac. 92)	60 min	20 min	≥3c/d water; unfertilized eggs in c/d water and high concentration; optional seawater and receiving water controls; salinity controls if samples <30 or >34 g/kg
Kobayashi 1971	none, sperm and eggs together in 3-min fertilization exposure		yes, assumed in c/d water
Kobayashi 1984	N.I., assume sperm and eggs together in 3-min fertilization exposure, or option with "aged" gametes pre-exposes sperm to test water for 5 min, pre-exposes eggs for several hours		N.I.
S. Calif. Project	15 min	eggs pre-exposed 30 min, then with sperm	4 seawater, plus salinity controls to match effluent concentrations
Nacci *et al.* 1986	60 min	20 min	N.I.
Cherr *et al.* 1987	10 min	10 min	yes, details unspecified
BML 1991	10 min	10 min	N.I., assumed c/d water
ERCEES 1990	60 min	20 min	N.I., 4 assumed in c/d water
MECAS 1990	N.I.	N.I.	≥3 seawater
NWAS 1990	60 min	20	4 c/d water

10. Stimulation of Spawning and Collecting Gametes

Document	Stimulus Used	Collecting
Beak 1988	0.5 mL of 0.5*M* KCl	5 mm seawater in petri dishes
EVS 1989	0.5 mL of 0.5*M* KCl (2nd injection if needed)	c/d water in 150-mL beaker
B.C. MOE 1990	s.u., 1.0 mL of 0.5*M* KCl s.d., 0.5 mL of 0.5*M* KCl	as above seawater at 10 °C in 250 mL beaker
IGATG 1991	0.5 mL of 0.5*M* KCl	5 mm seawater in petri dishes
Dinnel *et al.* 1987	s.u. 1.0 mL of 0.5*M* KCl s.d. 0.5 mL	seawater in 100-mL beaker
USEPA 1988	12 volts D.C. for 30 sec	bowl, shallow c/d water, use syringe
ASTM 1990	most species, 0.5 to 1.0 mL of 0.5*M* KCl, 2nd injection if no spawn in 10 min; use 12 volts D.C. for *Arbacia*	seawater in small beaker
NCASI 1991, 1992	s.u. 1.0 mL s.d. 0.5 mL of 0.5*M* KCl	collect with pipet to tubes at12°C c/d water in 50-mL beaker (s.u. 100-mL beaker)
USEPA (Pac. 91)	0.5 to 1.0 mL of 0.5*M* KCl, 2nd injection if needed	c/d water in 100-mL beaker
USEPA (Pac. 92)	0.5 mL of 0.5*M* KCl, 2nd injection if needed	eggs in c/d water in 100-mL beaker, semen "dry"
Kobayashi 1971	KCl injection for ♀	testes removed, "dry" sperm to seawater
Kobayashi 1984	"KCl method"	N.I.
S. Calif. Project	0.5 mL of 0.5*M* KC1	eggs into seawater in 100-mL beaker, semen "dry" with pipet to tubes at, <5 °C
Nacci *et al.* 1986	electrical	N.I. moistened (♂), collect with pipet to vials on ice
Cherr *et al.* 1987	0.5 mL of 0.5*M* KCl	seawater in 50-mL beaker
BML 1991	0.5 to 1.0 mL of 0.5*M* KCl	shake and place on fingerbowl with seawater (♀)
ERCEES 1990	0.5 mL of 0.5*M* KCl	in small beaker, "dry" for sperm, water for eggs
MECAS 1990	0.5 mL of 0.5*M* KCl, 2nd inject'n in 5 min if needed	eggs into 100-mL beaker with 20 mL water, sperm "dry" with syringe to vial on ice
NWAS 1990	s.u. 1.0 mL of 0.5*M* KCl, s.d. 0.5 mL	on empty 100-mL beaker, collect eggs to cold c/d water, semen "dry" with pipet to cooled test tube

11. Holding Gametes

Document	Conditions and Limitations for Holding
Beak 1988	sperm composited from several males
EVS 1989	s.u. sperm on ice, wash eggs 3 times, pool gametes from ♂, ♀
B.C. MOE 1990	sperm composited from ≥2 males, used ≤4 h, eggs stored ≤24 h
IGATG 1991	composite sperm, hold on ice, use ≤20 min, eggs from 4 animals
Dinnel *et al.* 1987	sperm activation for ≤1.5 h did not affect test, wash eggs 3 times, compositing optional
USEPA 1988	sperm used in <1 h, kept on ice, eggs keep several hours at lab. temperature
ASTM 1990	sperm in cool seawater keep several hours, keep "dry" and refrigerated for many hours, rinse eggs 2 or 3 times, keep sperm separate and use block design for test or composite
NCASI 1991, 1992	sperm usually ≤1 h, eggs normally ≤2h, hold at 12 °C
USEPA (Pac. 91)	collect for ≤30 min, wash eggs twice, composite sperm
USEPA (Pac. 92)	collect for ≤30 min, wash eggs twice, store in water at standard strength, sperm in separate vials on ice and use in ≤4 h
Kobayashi 1971	use ≤1 h
Kobayashi 1984	use gametes ≤1 h, wash eggs several times
S. Calif. Project	"dry" semen stored at <5 °C, pool eggs from 6 ♀, wash twice
Nacci *et al.* 1986	N.I.
Cherr *et al.* 1987	gametes on ice for ≤2 h
BML 1991	eggs and "dry" sperm to vials on ice, wash eggs twice
ERCEES 1990	pool sperm, eggs
MECAS 1990	"dry" sperm to vial on ice, wash eggs twice, hold in dark at 12 °C
NWAS 1990	"dry" semen to refrigerated tube, wash eggs twice and use fresh

12. Numbers of Gametes Used Per Test Vessel and Sperm-to-egg Ratios

Document	Sperm/Vessel	Eggs/Vessel	Sperm:Egg Ratio
Beak 1988	7 or 5 million?	2000	2500 or 3500:1?
EVS 1989	s.u. 4 million s.d. 2.4 million	2000 2000	2000:1 1200:1
B.C. MOE 1990	N.I.	500	N.I.
IGATG 1991	~5 million	2000	~2500:1
Dinnel *et al.* 1987	various	2000	determine appropriate ratio, commonly purple s.u. 200:1, red s.u. 1000:1, green s.u. 2000, s.d. (*D. excentricus*) 1200:1
USEPA 1988	5 million	2000	2500:1
ASTM 1990	empirical to give 70% to 90% fertilization	200/mL of test solution	commonly 200:1 for purple s.u. s.d. 1200:1, others 2000 to 2500:1
NCASI 1991, 1992	s.d. 20 000 to 60 000 s.u. empirical	500 500	40:1 to 120:1 determine appropriate ratio
USEPA (Pac. 91)	various	1120?	various, to assess methods
USEPA (Pac. 92)	560 000	1120	500:1 (fixed ratio)
Kobayashi 1971	N.I.	N.I.	N.I.
Kobayashi 1984	N.I.	N.I.	N.I.
S. Calif. Project	N.I. (1.2 mL of standard preparation)	31 500	N.I.
Nacci *et al.* 1986	0.1 million	1000	100:1 (authors say 1000:1)
Cherr *et al.* 1987	0.5 million	500	1000:1
BML 1991	N.I. (0.1 mL “dry”)	N.I. (0.1 mL)	1000:1
ERCEES 1990	empirical	2000	determine appropriate ratio
MECAS 1990	1 million?	empirical	determine ratio needed for 70 % to 90% fertilization
NWAS 1990	empirical	2000	determine ratio needed for 70% to 90% fertilization, commonly ratios from 200:1 to 2000:1

13. Adjustment of Results for Degree of Fertilization in Controls*

Document	Method of Adjustment
Beak 1988	Abbott's formula
EVS 1989	Abbott's formula
B.C. MOE 1990	Abbott's formula
IGATG 1991	Abbott's formula:** $A = (O - C) \cdot (100) / (100 - C)$
Dinnel *et al.* 1987	Abbott's formula
USEPA 1988	Abbott's formula
ASTM 1990	"Adjusted percent Fertilization" = $AF = 100 \cdot OF/CF$ ** [symbols changed, this gives same result as Abbott's formula, but is calculated for fertilization]
NCASI 1991, 1992	N.I.
USEPA (Pac. 91)	N.I.
USEPA (Pac. 92)	as in USEPA (1988)
Kobayashi 1971	N.I.
Kobayashi 1984	N.I.
S. Calif. Project	IC50 not mentioned as a statistic to be estimated
Nacci *et al.* 1986	N.I.
Cherr *et al.* 1987	"normalized" for control fertilizations, method not stated
BML 1991	N.I.
ERCEES 1990	N.I.
MECAS 1990	N.I.
NWAS 1990	Abbott's formula

* The kinds of adjustment shown here are not used to estimate NOEC/LOEC; the unmodified (i.e., raw) values for percent fertilization are used in those calculations. The adjustments shown above produce the numerical equivalents of the Percent Reduction in Fertilization calculated in the first edition of this document as a preliminary to estimating an ICp. In this, the 2nd edition, correcting for the degree of fertilization in the controls is no longer necessary as non-linear regression models explicitly contain an intercept/asymptote term to directly account for control performance. Note: that in order to account for control effects the control data must be part of the dataset analyzed.

** A = Adjusted percentage of unfertilized eggs for the exposure in a given test vessel
O = Observed percentage of unfertilized eggs for the test exposure in a given test vessel
C = Control percentage of unfertilized eggs in dilution/control water
AF = Adjusted percentage of **fertilized** eggs in a given test vessel
OF = Observed percentage of **fertilized** eggs in a given test vessel
CF = Control percentage of **fertilized** eggs in dilution/control water

14. Requirements for Valid Test

Document	Percent Fertilization in Control	Other Requirements
Beak 1988	N.I.	
EVS 1989	N.I.	
B.C. MOE 1990	N.I.	
IGATG 1991	N.I.	
Dinnel *et al.*1987	≥50	
USEPA 1988	≥70 (>90 might mask toxicity)	
ASTM 1990	≥50, desirable 70 to 90, best 80 to 95	
NCASI 1991, 1992	50 to 100 acceptable, 50 to 90 preferred	
USEPA (Pac. 91)	desirable to attain 80 to 95	
USEPA (Pac. 92)	fertilization ≥50 in control; sperm concentration within a factor of two if desired; essentially zero fertilization in egg controls in c/d water and effluent	
Kobayashi 1971	N.I.	
Kobayashi 1984	pre-test check ≥85) ("aged gametes" ≥91)	membrane to elevate within 3 min of fertilization
S. Calif. Project	N.I.	
Nacci *et al.* 1986	≥60m; ≤90	
Cherr *et al.* 1987	N.I.	
BML 1991	N.I.	
ERCEES 1990	≥70, ≤90	positive and logical dose-effect curve; physical and chemical requirements met
MECAS 1990	N.I.	
NWAS 1990	≥70, ≤90	

15. Reference Toxicant

Document	Chemical	Required?	Test Type or Endpoint*
Beak 1988	N.I.		
EVS 1989	sodium dodecyl sulphate	yes	in duplicate, 5 concentrations 1.0 to 10 mg/L
B.C. MOE 1990	N.I.		
IGATG 1991	cadmium chloride	no	
Dinnel *et al.* 1987	silver	no	
USEPA 1988	copper sulphate	yes	with each batch of gametes
ASTM 1990	N.I.	no	"might assess sensitivity of a spawning"
NCASI 1991, 1992	N.I.		
USEPA (Pac. 91)	copper	no?	
USEPA (Pac. 92)	copper, sodium dodecyl sulphate, or other	yes	with each set of tests
Kobayashi 1971	N.I.		
Kobayashi 1984	N.I.		
S. Calif. Project	N.I.		
Nacci *et al.* 1986	N.I.		
Cherr *et al.* 1987	sodium azide	no	
BML 1991	N.I.		
ERCEES 1990	N.I.		
MECAS 1990	N.I.		
NWAS 1990	sodium azide	yes	concurrent with main test

* Unless otherwise indicated, the test is the standard type with the endpoints used in the main test, and estimation of ICp and NOEC/LOEC.

Appendix E

Bibliography. Additional Papers Directly Relevant to Canadian Echinoid Fertilization Assay

This list could assist laboratories wishing to enter the wider literature on echinoid testing. Many of these publications contain data on toxic concentrations of various pollutants to echinoid gametes, or compare findings for other stages of development or other organisms. Some annotations have been added in square brackets.

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Appendix F

Logarithmic Series of Concentrations Suitable for Toxicity Tests*

Column (Number of concentrations between 100 and 10, or between 10 and 1) **						
1	**2**	**3**	**4**	**5**	**6**	**7**
100	100	100	100	100	100	100
32	46	56	63	68	72	75
10	22	32	40	46	52	56
3.2	10	18	25	32	37	42
1.0	4.6	10	16	22	27	32
	2.2	5.6	10	15	19	24
	1.0	3.2	6.3	10	14	18
		1.8	4.0	6.8	10	13
		1.0	2.5	4.6	7.2	10
			1.6	3.2	5.2	7.5
			1.0	2.2	3.7	5.6
				1.5	2.7	4.2
				1.0	1.9	3.2
					1.4	2.4
					1.0	1.8
						1.3
						1.0

* Modified from Rocchini *et al.* (1982)

** A series of successive concentrations (minimum of seven; recommend 10 or more) may be chosen from a column. Mid-points between concentrations in column (×) are found in column (2× + 1). The values listed can represent concentrations expressed as percentage by volume or weight, mg/L, or µg/L. As necessary, values could be multiplied or divided by any power of 10. Column 2, which spans two orders of magnitude in concentration might be used if there was considerable uncertainty about the degree of toxicity. More widely spaced concentrations should not be used. For effluent testing, there is seldom much gain in precision by selecting concentrations from a column to the right of columns 3 or 4; the finer gradations of columns 4 to 7 might occasionally be useful for testing chemicals that have an abrupt threshold of effect.

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