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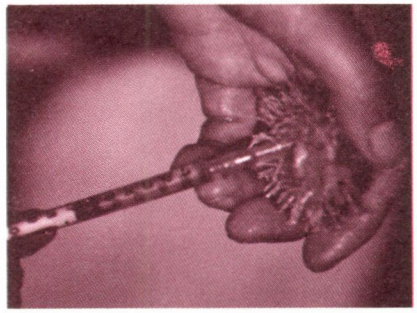

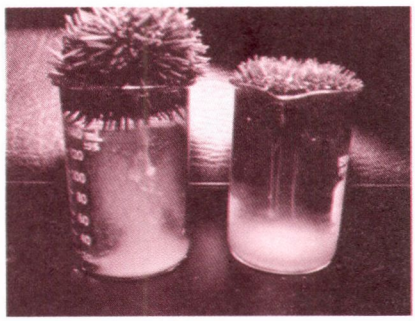

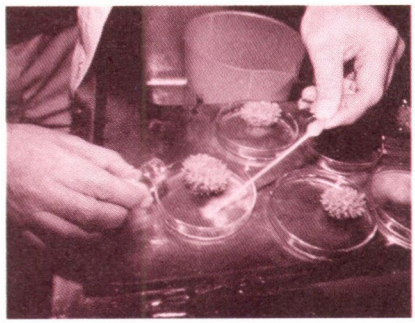

Biological Test Method: Fertilization Assay Using Echinoids (Sea Urchins and Sand Dollars)

Report EPS 1/RM/27
December 1992

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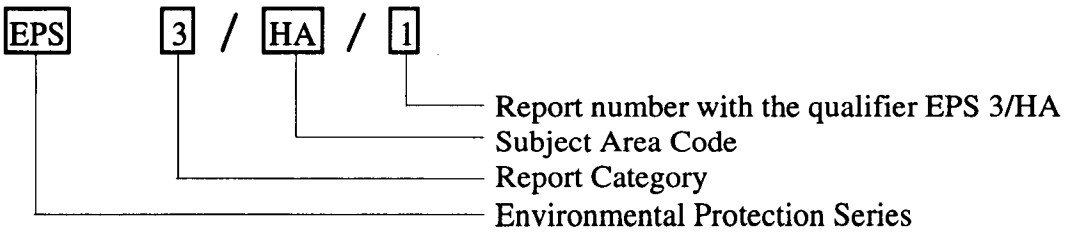

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Abstract

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. In the test, sperm are exposed to the substance being tested. Eggs are then added, and the success of fertilization under continued exposure to the same concentration of test substance is measured. The endpoint is decreased success of fertilization, described in terms of the concentration estimated to cause a specified percent inhibition (ICp), or the lowest-observed-effect concentration (LOEC) and no-observed-effect concentration (NOEC). 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 five concentrations of test substance 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 (*S. purpuratus*), or the eccentric sand dollar (*Dendraster excentricus*) from the Pacific. Provided that requirements for test validity are met, other local species may be used, or species from other geographic locations if a permit can be obtained to bring in specimens. Possible species to import include the Atlantic purple sea urchin commonly called Arbacia (*Arbacia punctulata*), and the white sea urchin from California (*Lytechinus pictus*).*

Procedures are given for holding adult echinoids in the laboratory, and obtaining sperm and eggs for a test. General or universal conditions and procedures are outlined for testing a variety of substances. 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 substance. 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, and validation of the test.

Résumé

Le présent document expose les méthodes recommandées par Environnement Canada en vue de l'exécution d'un essai de toxicité sublétale en milieu marin sur les gamètes d'oursins verts ou d'oursins plats. Dans le cadre de cet essai, on expose d'abord le sperme à la substance étudiée. On ajoute ensuite des oeufs et on mesure le taux de fécondation, tout en maintenant l'exposition à une concentration constante de la substance étudiée. On obtient comme résultat une diminution du taux de fécondation que l'on exprime par la concentration estimée de la substance étudiée causant un pourcentage précis d'inhibition (CI_p), ou par la concentration minimale avec effet observé (CMEO) et par la concentration sans effet observé (CSEO). 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 de vie, cet essai constitue un instrument de mesure puissant et significatif de la sublétalité. On peut utiliser, dans le cadre de cet essai, cinq concentrations de la substance étudiée afin de déterminer la concentration seuil, ou encore une seule concentration, comme dans les essais réglementaires ou à résultat unique.

Pour cet essai, on recommande d'utiliser les espèces suivantes : l'oursin vert (Strongylocentrotus droebachiensis) que l'on trouve sur les côtes atlantique, pacifique et arctique du Canada, l'oursin violet du Pacifique (S. purpuratus) ou le clypéaster excentrique (Dendraster excentricus) qui vit dans le Pacifique. Dans la mesure où elles satisferaient aux exigences relatives à la validité des essais, on peut également utiliser d'autres espèces locales ou encore des espèces d'autres pays si l'on obtient un permis d'importation. Parmi les espèces que l'on peut importer, mentionnons l'oursin violet de l'Atlantique, communément appelé Arbacia (Arbacia punctulata), et l'oursin blanc de Californie (Lytechinus pictus).

Il présente également les méthodes permettant de conserver des échinides adultes en laboratoire et d'obtenir le sperme et les oeufs nécessaires à un essai. On y indique également les conditions et méthodes générales ou universelles qui doivent être mises en oeuvre pour réaliser des essais sur un large éventail de substances. D'autres conditions et méthodes sont propres à l'évaluation d'un ou de plusieurs échantillons de produits chimiques, d'effluents, de milieux récepteurs, de lixiviats, d'élutriats ou de liquides provenant de sédiments et de substances solides similaires. Le lecteur y trouvera des directives concernant les installations d'essai, la manipulation et le stockage d'échantillons, la préparation de solutions d'essai et la mise en route d'essais, les conditions d'essai particulières, les observations et mesures appropriées, les résultats d'essais, les méthodes de calcul et la validation de l'essai.

Foreword

This is one of a series of ***recommended methods*** *for measuring and assessing the aquatic biological effects of toxic substances. Recommended methods are those which have been evaluated by Conservation and Protection (C&P), and are favoured:*

- *for use in C&P aquatic toxicity laboratories;*
- *for testing which 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 on the basis of their acceptability for the needs of programs for environmental protection and management, 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 toxic effects of samples of chemical, effluent, leachate, elutriate, receiving water, and sediment or similar solid substance.

Mention of trade names in this document does not constitute endorsement by Environment Canada; other products with similar value are available.

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List of Abbreviations and Chemical Formulae

°C . . . . . degree(s) Celsius

cm . . . . . centimetre(s)

d . . . . . day(s)

DO . . . . . dissolved oxygen (concentration)

g . . . . . gram(s)

g/kg . . . . . grams per kilogram

h . . . . . hour(s)

HCl . . . . . hydrochloric acid

H_2O . . . . . water

ICp . . . . . inhibiting concentration for a (specified) percent effect

L . . . . . litre(s)

LOEC . . . . lowest-observed-effect concentration

m . . . . . metre(s)

mg . . . . . milligram(s)

min . . . . . minute(s)

mL . . . . . millilitre(s)

mm . . . . . millimetre(s)

mS . . . . . millisiemen(s)

MSD . . . . . minimum significant difference

N . . . . . Normal

NaOH . . . . sodium hydroxide

NOEC . . . . no-observed-effect concentration

SD . . . . . standard deviation

SI . . . . . Système internationale d'unités

sp. . . . . species

TEC . . . . . threshold-effect concentration

TIE . . . . . toxicity identification evaluation

TM (™) . . Trade Mark

µg . . . . . microgram(s)

µm . . . . . micrometre(s)

> . . . . . greater than

< . . . . . less than

≥ . . . . . greater than or equal to

≤ . . . . . less than or equal to

± . . . . . plus or minus

% . . . . . percentage or percent

Terminology

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

Grammatical Terms

Must is used to express an absolute requirement.

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

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

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

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

General Technical Terms

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

Compliance means in accordance with governmental permitting or regulatory requirements.

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 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 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 animals.

Larva is a recently hatched individual which has physical characteristics other than those seen in the adult of the species.

Minimum Significant Difference (MSD) means the difference between groups (in this fertilization assay, the difference in average percent fertilization) that would have to exist before it could be concluded that there was a significant difference between the groups. MSD is provided by Dunnett's multiple-comparison test, a standard statistical procedure.

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 or marine/estuarine receiving water for toxicity.

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

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

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 also be measured directly using a salinity/conductivity meter or other means (see APHA *et al.*, 1989). Salinity is reported here as g/kg. The term "parts per thousand" ($^{o}/_{oo}$) 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 Substances

Brine is a solution of sea salts in water, in stronger concentration than in oceanic water. It can be obtained from 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 to fresh or distilled water. The strength of brine used for this fertilization assay should be 60 to 90 g/kg.

Chemical is, in this report, any element, compound, formulation or mixture of a 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 an aquatic toxicity test, the control must duplicate all the conditions of the exposure treatment(s), but must contain no test substance. The control is used to determine 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 substance, or for the control test, or both.

Control sediment is a sediment that is essentially free of contaminants and is used routinely to assess the acceptability of a test.

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 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 substance (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, residing in a coastal body of ocean water that is measurably diluted with fresh water derived from land drainage.

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

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

Pore 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.

Receiving water is 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 to which commercially-available dry ocean salt has been added in a quantity that provides the salinity (and pH) desired for the water in the test.

Reference sediment is sediment collected from the field and selected to match the sediment being tested in properties such as particle size, compactness, total organic content, but "clean", i.e., without chemical contaminants. It is often selected from a site in the general vicinity of sediments being tested for toxicity, but uninfluenced by the source(s) of contamination.

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 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 substance is evaluated, and the precision of results obtained by the laboratory for that chemical.

Sediment is particulate material normally lying below water or formulated for experimental purposes.

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

Substance is a particular kind of material having more or less uniform properties.

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

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

Toxicity Terms

Acute means within a short period 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.

Chronic value is the geometric mean of the NOEC and LOEC in tests which have a chronic exposure. See also *TEC* as a recommended term.

Endpoint means the variables (i.e., time, reaction of the organisms, etc.) that indicate the termination of a test, and also means the measurement(s) or value(s) derived, that characterize the results of the test (NOEC, ICp, etc.).

ICp is the inhibiting concentration for a (specified) percent effect. It represents a point estimate of the concentration of test substance that would cause a designated percent impairment in a quantitative biological function such as 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 *median effective concentration* (EC50) is not appropriate in tests of this kind because it is limited to quantal measurements, i.e., an estimate that 50% of the individual organisms exposed to that concentration would show a particular effect, while the other 50% would not show the effect. The echinoid fertilization assay does not completely conform with the requirements for an EC50, because a major part of the exposure involves only the sperm and the chief effect might be on the sperm, but no direct quantal measurements are made of the numbers or proportions of sperm affected.

Similarly no specific observations are made on the eggs themselves at the end of their exposure. The measurements of reduced fertilization represent the end result of various effects on either sperm or eggs, or both. Accordingly, the measurements are best treated as quantitative observations, and described as the ICp for impaired fertilization.

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 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. LOEC is generally reserved for sublethal effects.

NOEC is the no-observed-effect concentration. This is the highest concentration of a test material 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.

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

Sublethal means detrimental to a living organism, but below the level that directly causes death within the test period.

TEC is the threshold-effect concentration. It is calculated as the geometric mean of NOEC and LOEC. *Chronic value* or *subchronic value* are alternative terms that might be appropriate depending on the duration of exposure in the test.

Toxicity is the inherent potential or capacity of a substance to cause adverse effects on living organisms. The effect 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 on a group of 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 *(graded or quantitative)*, after exposure to specific concentrations of chemical, effluent, receiving water, leachate, elutriate, or interstitial water derived from sediment or similar solid substance. The echinoid fertilization assay must be considered a graded toxicity test since there is no measurement of the proportions of either sperm or eggs that are directly affected, only the degree of effect resulting from damage to one or the other.

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Section 1

Introduction

1.1 Background

Aquatic toxicity tests are used within Canada and elsewhere to measure, predict, and control the discharge of substances that could be harmful to aquatic life. Recognizing that no single test method or test organism can be expected to satisfy a comprehensive approach to environmental conservation and protection, the Inter-Governmental Aquatic Toxicity Group (see Appendix A) 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 were selected to help meet Environment Canada's testing requirements.

Universal procedures for a fertilization assay with echinoid gametes are described in this report. Also presented are specific sets of test conditions and procedures, required or recommended when using the test to evaluate different types of substances (namely, samples of chemical, effluent, receiving water, leachate, elutriate, or interstitial water derived from sediment or similar solid substance) (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 procedures, an attempt was made to balance scientific, practical, and financial considerations, and to ensure that the results will be accurate and precise enough for the majority of situations in which they will be applied. The authors assume that the user has a certain degree of familiarity with aquatic toxicity tests. Guidance regarding test options and applications is provided here. For regulatory use of the test, the choice of test options and applications is to be decided by the regulatory agency.

1.2 General Aspects of Echinoids and Their Use in Tests

Sea urchins and sand dollars belong to the Phylum Echinodermata and Sub-phylum (formerly 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 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

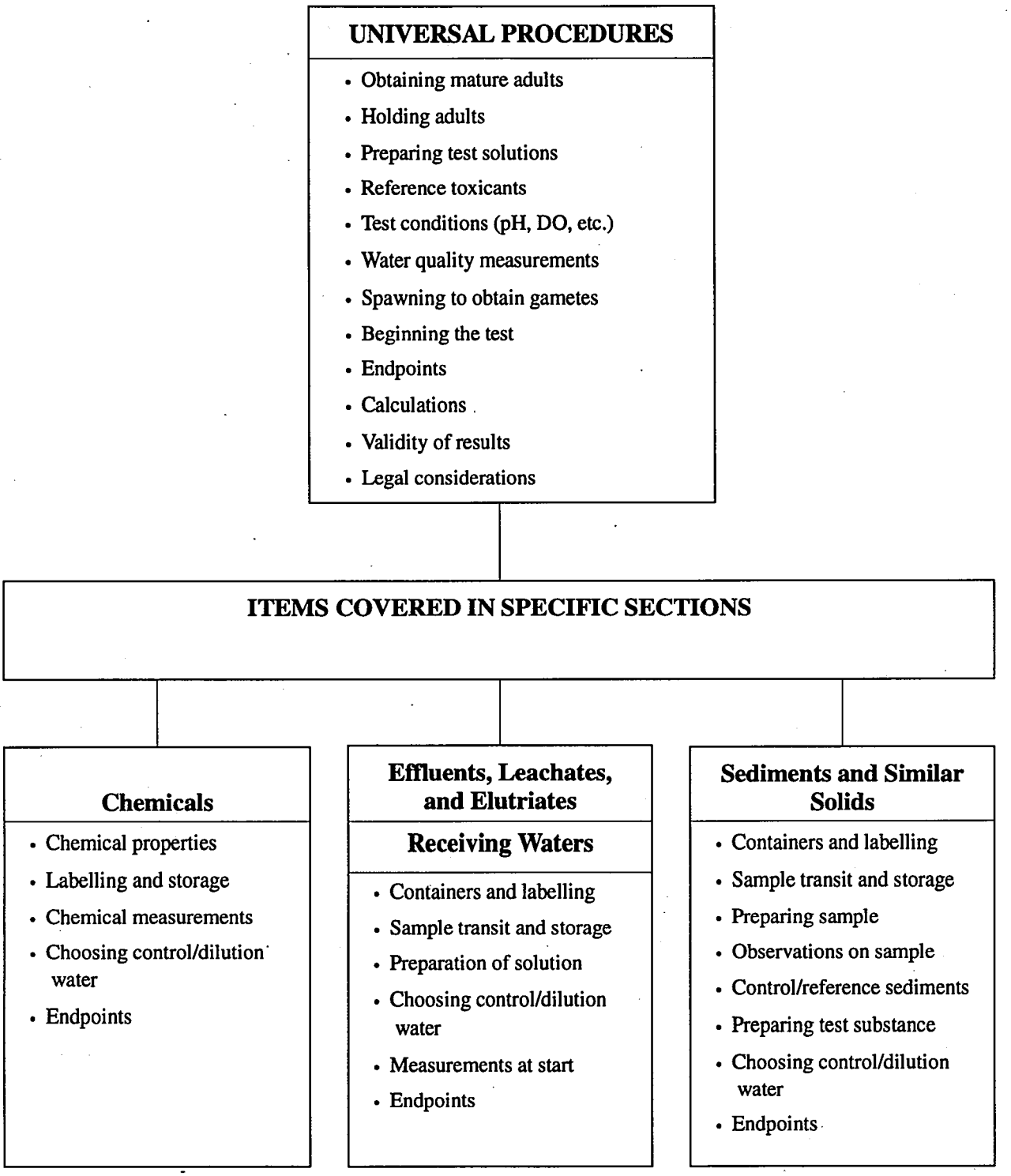

Figure 1 **Diagram of Approach Taken in Delineating Test Conditions and Procedures Appropriate for Various Types of Substances**

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 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 this 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 was more sensitive to the effluent from a municipal waste 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

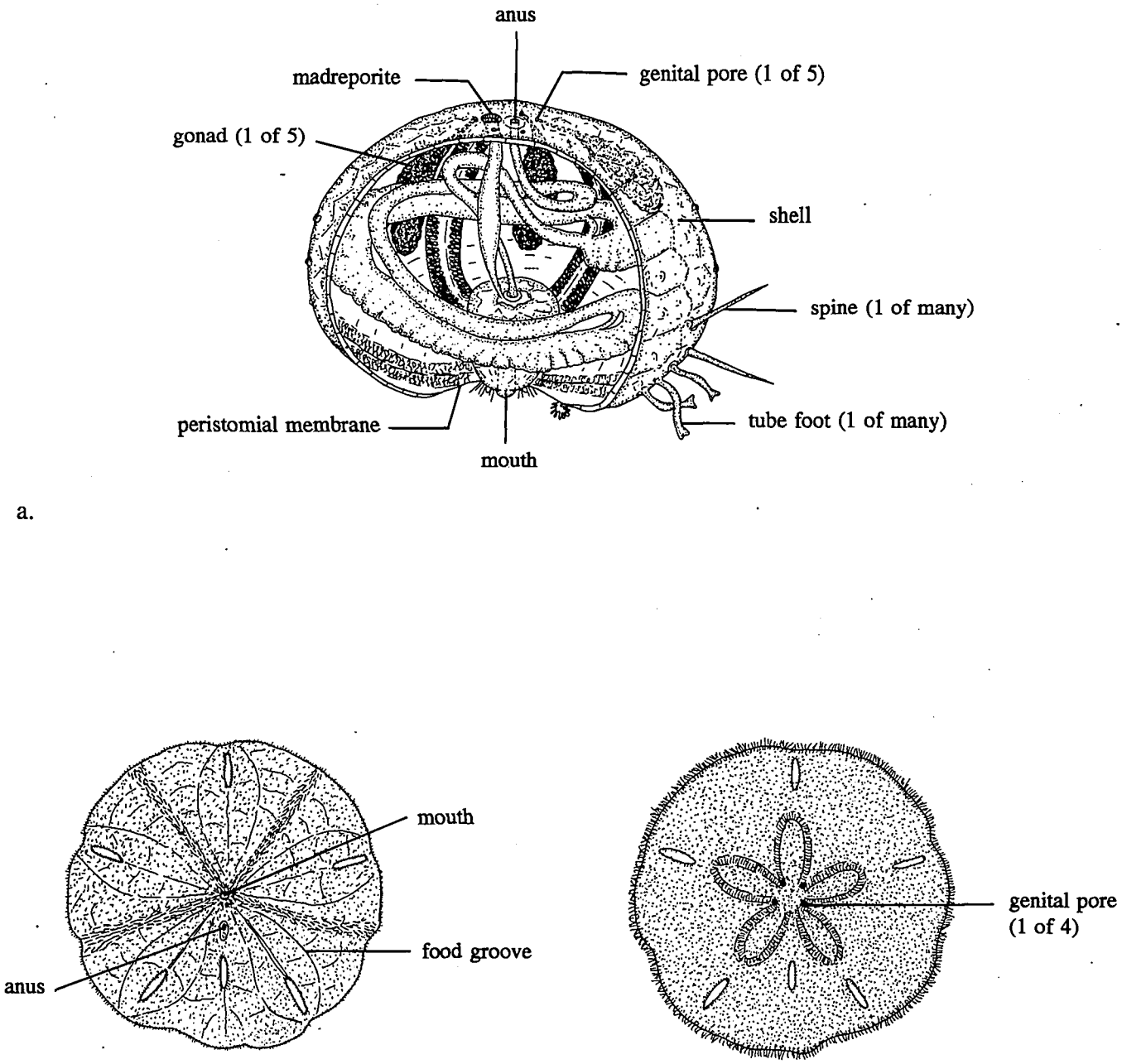

Figure 2 **General Appearance of Echinoids.**
a. Cut-away view of a typical sea urchin, *Arbacia*, showing location of genital pores on the aboral (upper) side. Only two or three of the numerous spines and tube feet are indicated. **b.** Oral side (normally down) of a typical sand dollar. **c.** Aboral side of a sand dollar showing location of the genital pores. (Drawings by M.A. White, after Storer *et al.* (1979) and Barnes (1974)).

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 and sperm 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., Environment Canada, 1990a; 1990b).

Precision of the test appears to be satisfactory. The U.S. EPA (1988) determinated that within-laboratory coefficients of variation for IC50s using reference toxicants and one species of sea urchin (*Arbacia punctulata*) were 30% to 48%. A coefficient of variation of 74% was found for IC50s of copper tested by six laboratories using four species of echinoids in an effluent testing program, compared to coefficients 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 coefficients of variation 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 coefficients of variation, with values 23%, 48%, 57%, 80% and 94% (Miller *et al.*, 1992). These interlaboratory coefficients, averaging 79% for IC25s and 63% for IC50s, are similar to the precision for chemical analyses, e.g., an average coefficient of 60% found in an interlaboratory comparison of chemical analyses of priority pollutants (Rue *et al.*, 1988). Recent unpublished results for an interlaboratory round-robin sponsored by the U.S. EPA are apparently similar, with coefficients of variation of 57% for 40-min fertilization assays and 86% for 80-min assays (NCASI, 1992).

The echinoid fertilization assay has been used in several Canadian aquatic toxicity laboratories, both governmental and industrial. Standard test methods have 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 has been carried out by certain Environment Canada laboratories (see Appendix B), 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 have been reviewed and recommended by an Environment Canada scientist (Wells, 1982; 1984), but no standard method has been published by a Canadian federal government agency.

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 (U.S. EPA, 1988) and Pacific (Chapman, 1991; 1992) coasts. Methods are also being developed by the American Society for Testing and Materials (ASTM, 1990). A critique of recent methodology has been provided by NCASI (1992), with special relevance to pulp and paper effluents. In addition, a number of consulting companies and other marine labs have written procedures for their own organizations (see Appendix C).

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 D).

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 becoming 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 U.S. EPA (1991a, 1991b).

The purpose this "generic" report is to provide standardized Canadian methods for testing sublethal toxicity of various substances 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. If a Canadian regulatory test using echinoid fertilization success were to be promulgated, it would have a rigidly-defined method, selected from the alternatives presented in this report. The echinoid test procedures in existing documents vary in their coverage of endpoints, and of issues such as pH adjustment, alternative methods 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 substances. 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 (Environment Canada, 1990a; 1990c; 1992a; 1992b).

Section 2

Test Organisms

2.1 Species

The test may be done with any of the species listed below. Other native species may be used if they are found to be suitable.

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 (Eshscholtz), 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. *Arbacia* and white sea urchins can be purchased from biological supply houses and shipped to the test laboratory. Additional species might be available commercially or through interlaboratory arrangements, and might meet the needs of testing when local species are not in spawning condition.

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 C). In general, toxicity results from fertilization assays using echinoids appear to be similar among species (Kobayashi, 1984; Nacci *et al.*, 1986). There might be small differences in sensitivity; 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).

The common sand dollar, *Echinarachnius purma* (Lamarck), has not been used frequently in toxicity tests, and performed poorly in a recent 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 occurence 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).

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 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 B). 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.

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 population 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.

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. 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 can 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.

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 (Subsection 4.2.1) and examining the gametes. 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

Table 1 General Features of Echinoids Recommended for Use in Tests

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	**9**, ≤12
Pacific Purple Sea Urchin	- generally January to May, optimally January to March for feral animals (December, June)		10	**12**, ≤17
Eccentric Sand Dollar	- May through summer to October (February to December)		9	**12**, ≤17
Arbacia	- June to August		5.1	**15**, ≤22
White Sea Urchin	- March through summer to November	♂ ♀	2.8 3.2	**15**, ≥8 ≤24

* 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 C; from Meinkoth, 1981; NRC, (1981); Strathmann, (1987); Starr, (1990); and from information provided by reviewers listed in the Acknowledgments.

** 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 recommended standard temperatures to obtain gametes in normal fashion, as derived from Appendix C and NRC (1981). Other temperatures could be used to speed or slow the maturation process, or the seasonal temperature of incoming natural seawater could be accepted. Maxima and minima are indicated in some cases.

spawning. In sea urchins, mature ovaries are coloured yellow to red depending on species, and testes are white.

2.3 *Holding 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. Adult green and Pacific purple sea urchins and eccentric sand dollars have been fed and kept satisfactorily for months. A minimum holding time of 3 or 4 days is desirable, at the test temperature and in the water to be used for controls and dilution. The criterion of satisfactory holding conditions is the delivery of viable gametes that meet the needs of the test.

Echinoids should 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 can 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 can induce others to spawn, so such animals should be isolated immediately upon detection, to prevent mass spawning.

The recommended conditions for holding echinoids, 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. Recommended conditions have been drawn, in general, from Appendix C. Further details and rationale are given in some of the publications included in Appendix C, and in the References, particularly ASTM (1990), U.S. EPA (1988), NCASI (1991), and papers of Dinnel and colleagues listed in the References and in the Bibliography of Appendix D.

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

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 customary. For sand dollars, overhead fluorescent lighting at the equivalent of bright office lighting encourages algal growth on the sediment, which can result in desirable nutritional self-sufficiency for the tray of sand dollars.

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 that has been previously demonstrated 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.

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

Source of adults	- collected from clean-water areas or purchased from supply houses
Water	- uncontaminated natural seawater or reconstituted 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 35 g/kg; 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 9 to 15°C depending on species, somewhat lower or higher to delay or speed spawning, see Table 1; rate of change from one temperature to another ≤3°C/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 to 8.2
Water quality	- monitor temperature, salinity, dissolved oxygen, pH, and flow to each holding tank, preferably daily
Lighting	- normal laboratory lighting at low intensity; not considered critical
Feeding	- for sea urchins: kelp, other macroalga, or romaine lettuce; for sand dollars: provide sediment with detritus and alga, use lighting to encourage growth of algae, and if necessary add cultured alga
Cleaning	- removal of old alga, fecal material, and debris, daily or as required, unless intended as food
Disease/mortality	- monitor mortality daily; it should be ≤2%/d averaged over 7 d preceding collection of gametes; remove diseased or moribund animals; groups of diseased animals should be discarded

As a general guideline for the flow rate of seawater in "once-through" systems, the investigator should aim at providing 5 to 10 L/d or more for each organism held, and also at having 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 amounts of water and exchange times in the existing methods (Appendix C). 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.

The average salinity of the water should be 28 to 34 g/kg, preferably 30 to 32 g/kg. Extreme salinity values should not be less than 25 or more than 35 g/kg during holding of echinoids.[1]

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 seawater is to be used as dilution and control water (see Subsection 4.1.1 and Section 5.3), 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 a limited seawater supply can 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. 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).[2]

Reconstituted seawater can be prepared by adding brine, commercially-available dry ocean salts or salt formulations (e.g. Instant Ocean™) to a suitable fresh water, in quantities sufficient to provide the desired salinity. If ocean salts are used, the suitability and consistency among batches of a particular formulation of salts should be verified by testing, since some investigators feel that specific batches of sea salt can produce unwanted toxic effects or sequester test substances. If necessary, reconstituted

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1 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 C). 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 also Subsection 4.3.2).

2 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.

seawater could also be formulated from its individual component salts, with various degrees of sophistication for special situations that might be encountered. In such cases, a specialized reference should be consulted (Bidwell and Spotte, 1985).

Ocean salts may be added to seawater, samples, or other liquids to increase the salinity to the level desired for testing. A preferred alternative is to prepare a brine (very saline water) and add that as required. A brine can be prepared by freezing seawater at -10° to -20°C, and collecting the high-salt water under the ice when it reaches the desired salinity of ≤100 g/kg. That is conveniently done in a polyethylene carboy with a bottom-draining spigot (Chapman, 1992). Alternatively, the seawater could be completely frozen, then partially melted, with collection of the brine which melts first. Brine can also be prepared by slow heat-concentration of seawater at about 40°C until salinity reaches a value in the range 60 to 90 g/kg. Higher temperatures and stronger final salinity have been found undesirable in this evaporation method; when the brine is diluted back to normal salinity for seawater, it does not produce satisfactory conditions for aquatic organisms (ASTM, 1990). The seawater should be filtered through a pore size ≤10 µm before the freezing or evaporation process. Brine has been stored successfully for a year (NCASI, 1992). Using a brine of 90 g/kg to adjust the test concentrations to 30 g/kg means that the maximum concentration of effluent (or other freshwater sample) that could be tested would be 67%.[3] Adjusting higher concentrations of a freshwater sample to 30 g/kg would require the use of ocean salts.

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 Subsection (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 can 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.[4]

2.3.5 Temperature

Echinoids may be held at standard temperatures (Table 1) or, if desired, at normal seasonal temperatures, e.g., using the temperature of the incoming natural seawater supplied to the laboratory. Temperatures should be adjusted to standard levels and held there for ≥3 d before the animals are

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

4 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.

used to supply gametes for tests. A pre-spawning standard temperature of 9°C should be maintained for green sea urchins, 12°C for Pacific purple urchins and the two sand dollars, and 15°C for *Arbacia* and the white sea urchin.[5]

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. Similarly, temperature may be raised to encourage early development of gametes. Excessively high temperatures must be avoided, and recommended upper limits are 12°C for green sea urchins, 17°C for Pacific purple urchins and eccentric sand dollars, 22°C for *Arbacia*, and 24°C for white sea urchins, a species which should not be held at less than 8°C. Water temperatures may be changed to the desired value at a rate not exceeding 3°C per day.

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 to 8.2, and must be within limits of 7.5 to 8.5. 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 C).

2.3.8 Feeding

Sea urchins are to be fed with kelp or macroalga (*Laminaria, Nereocystis, Macrocystis, Egregia, Hedophyllum*) or, alternatively, with romaine lettuce. Food should be added frequently enough (weekly, daily) that it is always available to the urchins, and old or decomposing food should be removed. 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

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5 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. For example, white sea urchins obtained in March might be held at 12°C rather than 15°C until spawning is desired. Advice should be sought from the people collecting at a particular site.

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 might be added to the sediment from a culture, 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

Adults should be inspected daily for signs of disease. Dead individuals should be removed immediately. In groups of animals which are intended to provide gametes, mortality should not exceed 2% per day, averaged over the seven days preceding collection of gametes.

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.

Section 3

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 (see Subsection 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 C). 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 C, 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 C). A standard size should be selected and used within a laboratory.[6]

The 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

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6 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.

rinsing, but that technique has been known to result in measurable toxicity[7], 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. Vessels of plastic are not recommended since there is evidence of deleterious effects on fertilization success (Dinnel *et al.*, 1987). In descriptions of existing procedures (Appendix C) 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 substance and intent (Sections 5 to 8), the control/dilution water may be "uncontaminated" natural seawater, reconstituted seawater, or a sample of receiving water collected "upstream" of the source of contamination. If receiving water is used, conditions for collection, transport, and storage should be as described in Section 6.1. 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 1 µm.

Salinity of control/dilution water should be 30 g/kg and must be in the range 28 to 34 g/kg. Lower salinities should be adjusted upwards with brine or sea salts (Subsection 2.3.4) and higher ones should be adjusted downwards with deionized water or uncontaminated fresh water.[8]

The pH of control/dilution water must be in the range 7.5 to 8.5, and should normally be 8.0 to 8.2. Those values would usually be obtained because of the natural buffering capacity of seawater. If not, adjustment should be made with acid or base (Subsection 4.3.4).

Control/dilution water must be adjusted to the test temperature before use. It must not be supersaturated with excess gases (see Subsection 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.

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7 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.

8 Gametes of some or all of the test species would presumably tolerate salinities outside the recommended limits (see Subsection 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 C).

Section 4

Universal Test Procedures

Procedures described in this section apply to all the tests of particular chemicals and wastewaters described in Sections 5, 6, 7, and 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 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 C). 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 Subsection 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 should be well mixed with a glass rod, Teflon™ stir bar or other nonreactive device. toxic material Temperatures should be adjusted as required to within ±1°C of the test temperature. It might be necessary to adjust the salinity or

pH of the sample of test substance or the test solutions (see Subsections 4.3.2 and 4.3.4), or to provide preliminary aeration (Subsection 4.3.3).

If reconstituted seawater is to be used as the dilution and control water, the prepared solution should be homogeneous and aged for 1 to 2 weeks before use (Venables, 1986). If dry salts have been added, the water should be stirred gently for a minimum of 60 minutes (preferably with a magnetic stirrer) to ensure that the salts are in solution. Aging of reconstituted seawater should be done in a clean, covered vessel at the test temperature and with aeration. The salinity should be monitored during stirring and aging to make certain that the desired value is obtained and stable.

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

The control/dilution water should be filtered to remove solids that might interfere with sperm counts, and the filtration is particularly important for natural seawater. A filter of pore size approximately 1.0 µm is recommended, to remove most suspended solids (see also Section 6.2). 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 Subsection 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.[9] In such a case, reconstituted seawater (Subsection 2.3.4) or the laboratory's natural seawater should be used as control/dilution water. The laboratory water could also be used if the collection and use of receiving water is impractical.[10]

4.1.2 Concentrations

For tests that are intended to estimate the NOEC/LOEC, the ICp, or both, at least five concentrations plus a control solution (100% control/dilution water) are to be prepared. An appropriate geometric series may be used, in which each successive concentration is about 0.5 of the previous one (e.g., 100, 50, 25, 12.5, 6.3). Concentrations may be selected from other appropriate logarithmic series (see Appendix E). There is not usually a great improvement in precision of the test

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9 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 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 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 on a specific receiving water, it does not matter if that receiving water modifies sample toxicity by the presence of additional toxicants, or conversely by the presence of substances that reduce toxic effects, such as humic acids. 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.

10 An alternative that could partially simulate receiving water would be to adjust the salinity (and perhaps the pH) of the laboratory seawater or reconstituted water 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 Subsection 2.3.4.

Table 3 Checklist of Recommended 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	- "uncontaminated" laboratory seawater; reconstituted seawater; "upstream" receiving water to assess toxic impact at a specific location, with additional control of laboratory seawater; filtered (1.0 µm) before use; dissolved oxygen (DO) content 90 to 100% saturation at time of use; salinity 28 to 34 g/kg, preferably 30 g/kg, and pH 7.5 to 8.5, preferably 8.0 to 8.2
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 by trial as that which gives 90% fertilization under control conditions; this is normally in the range 200:1 to 2500:1, but could be much higher in some seasons
Replicates	- each concentration must have three replicates, and four are recommended
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	- as standard salinity 30 g/kg, limits 28 to 34 g/kg; each test solution in that range and also within 1 g/kg of the control; adjust as necessary with brine, sea salts, deionized or uncontaminated fresh water; samples of effluent, leachate, receiving water, and elutriate or other sediment-derived liquid should be adjusted to the salinity of control/dilution water; if it were desired to evaluate the total effect of a wastewater, including its salinity, an assay could be run without adjusting sample salinity, but a second adjusted test should be run, or salinity controls, or both
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 at a minimal effective rate, before making up concentrations and starting the test

Table 3 Checklist of Recommended Test Conditions and Procedures (Cont'd)

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 to 8.2, apply for minimizing direct effects of pH on the gametes, and maximizing the potential for detecting toxic chemicals
Lighting	- normal laboratory lighting
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, NOEC/LOEC for fertilization success; IC25 is a recommended option; in single-concentration tests, percent fertilization and whether significantly lower than control
Reference toxicant	- copper is recommended; determine NOEC/LOEC and/or ICp, at least monthly when tests are being run
Test validity	- average success of fertilization in control must be ≥50% and <100%
Chemicals	
Solvents	- 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, the laboratory seawater
Effluents, Leachates, and Elutriates	
Sample requirement	- two litres should be adequate for the assay and for routine chemical analyses

Table 3 Checklist of Recommended Test Conditions and Procedures (Cont'd)

Transport and storage	- if warm (>7°C), cool to 1 to 7°C with ice or frozen gel packs; transport in the dark at 1 to 7°C (preferably 4 ±2°C) using frozen gel packs as necessary; store in dark at 4 ±2°C; sample must not freeze; use in test should start within 24 h and must start within 72 h of sampling/extraction; extraction of sediment should occur within 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	- standard treatment is filtration of sample at 5 µm; if there is concern for loss of toxicity with the solids, a second test with unfiltered sample is an option
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
Control/dilution water	- as for effluents, leachates, and elutriates

from the use of concentrations closer together than those obtained with the 0.5 factor. In routine tests, concentrations should not be more widely spaced than those obtained using a factor of 0.3 (e.g., concentrations 100, 30, 9, etc.), because that leads to poor precision of the toxicity estimate. More widely spread concentrations might be used, however, if there is considerable uncertainty about the toxic levels.

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.[11] 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.[12]

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 greatly 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 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

There must be at least three replicates prepared for each concentration including the controls. They are required for statistical analysis of results, specifically by Dunnett's test (Gulley *et al.*, 1989). It is recommended but not absolutely required that four replicates be used, because four are required for nonparametric statistical analysis, if results of the test do not satisfy requirements for normality and

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11 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 × 100% = 99.0%. No allowance is made for that slight difference.

12 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".

homogeneity.[13] The test must start with an equal number of replicates for each concentration including controls. If there is accidental loss of a replicate during the test, unbalanced sets of results can be analyzed with less power (Gulley *et al.*, 1989).

4.1.4 Controls

A control exposure is required for all tests, employing the same control/dilution water that is used to make up the test concentrations. The control must have the same number of replicates as in the rest of the test, i.e., at least three.

Salinity controls should be run if test salinity is, for any reason, outside the required range of 28 to 34 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 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 in the sample (see Subsection 4.3.2).

If a solvent is used in testing a chemical that is sparingly soluble, then a "solvent control" must be run in replicate, 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 that was used for holding the adults (see Subsection 2.3.4).

Additional kinds of controls are not required, but would 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).

4.2 Beginning and Performing the Exposure

Semen containing sperm is collected from several echinoids by forced spawning. Semen from each individual is pooled before use. Eggs are collected, and pooled in the same fashion. Sperm are exposed to the test substance 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 or 20 min to allow fertilization. Preservative is added to each vessel to end the exposure.

4.2.1 Collecting Gametes for the Test

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

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

must 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. If gametes from three adults of each sex cannot be obtained, it is permissable to use fewer.[14]

The adults are stimulated to spawn by injecting potassium chloride.[15] Sea urchins are injected with 1.0 mL of 0.5 *M* KCl through the peristomial membrane into the coelom (Figure 2). 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, often used with *Arbacia*, is stimulation of the shell for 30 seconds by electrodes supplied with 12 volts D.C.

The preferred technique for collecting semen from male sea urchins is called "dry spawning". One technique for dry-spawning male urchins is to place an individual in a beaker or petri dish rinsed with control/dilution water, 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 barely cover it with control/dilution water. 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. 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 produce insufficient volumes of sperm when spawned "dry". Sand dollars can be spawned in a minimal amount of seawater (5 mL); 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 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.

If there is no spawning in 5 or 10 minutes, a second injection may be used. 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

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14 An optional procedure that could help ensure quality of the test would be to examine the gametes after they have been obtained from five or more individual adults, and to select and pool the batches that appeared most viable. The semen from each male would be stored separately on ice, a small portion would be diluted with control/dilution water on a microscope slide, and motility of the sperm would be judged. Sperm from the three most active samples would be pooled and used. Eggs from five or more individual females would be similarly examined under a microscope for poor quality as shown by small size, irregularity, or vacuolization. Eggs from three or more good batches would be pooled and used in the test. If good-quality gametes are not available from three adults, fewer batches should be used. "It is more important to use high quality [gametes] than it is to use a pooled population of [gametes]" (Chapman, 1992).

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

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. 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 for 4 h before "activation" in seawater, then used in a test in the subsequent 30 to 120-min period.[16] 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 is important to rinse them soon after collection, since the substance can be toxic with the Pacific purple sea urchin and perhaps with other species. 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.

4.2.2 Preparing Standard Suspensions of Gametes

Semen is pooled to produce a concentrated suspension of sperm. If sperm were collected in beakers of water, pipet 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.[17] 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

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16 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 coefficient of variation 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" condition as released from the adult. The time limits for holding gametes, given in Subsection 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, 1992).

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

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.[18] The concentration of this standard sperm suspension is determined by the sperm:egg ratio that is selected (Subsection 4.2.3).

An alternative counting technique that may be used, is to use turbidity as an indication of the number of sperm/mL, without a hemocytometer count. The advantage is a saving of time, since the turbidimetric measurement takes only one minute compared to 20 or 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 coefficient of variation (C.V.) of about 9% for repeated hemocytometer counts of single dilutions of sperm, and a C.V. 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 optimum of 90% (Subsection 4.2.3), minimum of ≥50%, and maximum of <100% fertilization (Subsection 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.)

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

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[18] 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.

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 (Subsection 4.2.3), although it may 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 one million to 50 million per mL.[19]

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

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

[19] 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 by about 5%, however, and such a change is large enough that it should be allowed 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.

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.

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 trial in each laboratory, as that which gives 90% fertilization under control conditions. 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 too high indicate an excess of sperm that can mask an effect by compensating for part of the toxicity, thus reducing the sensitivity of the test and raising the IC25 and/or NOEC.[20] 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, whether it be the optimum of 90% fertilization, or between the limits of ≥50% and <100% for a valid test (Subsection 4.5.1).[21]

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 C). The following sperm-to-egg ratios have been reported to achieve a fertilization range of 70 to 90%:

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20 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. Similary, 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.

21 The target of 90% fertilization success was selected since it helps avoid the problems of too many and too few sperm. 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 90% fertilization often requires a fairly delicate balance of the gamete ratio and other factors, and suitable procedures must be established within each laboratory.

green sea urchin, 2000:1; Pacific purple sea urchin, commonly 200:1 but up to 2000:1; eccentric sand dollar, often about 1200:1 but also reliably reported in the range 50:1 to 200:1; 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).

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 trial might 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.

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 less than 50% fertilization in the control, or 100% fertilization (Subsection 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 (personal communication, G.A. Chapman, U.S. EPA, Newport, Oregon).

Because of the normal variation in percent fertilization for controls, a pre-test trial of this is highly recommended ("control pre-trial"). Investigators familiar with the echinoid fertilization assay find that the time spent in a control pre-trial for each test has, in the long run, saved considerable time, money, and sometimes irreplaceable samples. For example, one laboratory routinely runs control pre-trials "on a matrix of gametes from at least two females and two males with at least three different sperm dilutions per male" (Carr and Chapman, 1992).

An alternative approach to circumvent control pre-trials 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 closest to 90% in the control would be used in calculating the NOEC/LOEC, or ICp, or both (Section 4.5). NCASI (1992) points out that this actually requires less of the investigator's time than running a control pre-trial 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 control pre-trial, or arbitrarily selected, the strength of the suspension of sperm is also fixed (Subsection 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.[22] Each vessel must be clearly labelled or positions coded so that concentrations and replicates can be identified.

Temperature, salinity, dissolved oxygen, and pH levels in aliquots of the test solutions should be checked and adjusted, if required/permitted, 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.

Either of two longer exposures might 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 of 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.[23]

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 initial test volume of 10 mL is the usual standard and is described here.[24] 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 (Subsection 4.1.2). At the end of the sperm exposure, the egg preparation is mixed and 1.0 mL is added to each test

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22 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.

23 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 (personal communication, G.C. van Aggelen, B.C. Ministry of Environment).

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 coefficient of variation by a factor of 2.0 (NCASI, 1992).

24 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.

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 sould 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, 1992).

At the end of the sperm-plus-eggs exposure, the test is terminated by adding either 2 mL of 1% glutaraldehyde, or 2 mL of 10% buffered formalin to each test vessel.[25] (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 is a static test without renewal of test solutions. The test is carried out at 15°C for the four native species, and 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 34 g/kg. An attempt is made, if necessary, to raise the dissolved oxygen of all test solutions above 40% saturation.

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 should be measured at the beginning of the test, and at representative times during the test.

The test temperatures recommended here are 3° to 6°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

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[25] 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. Formalin is a hazardous substance, must be handled in a fume hood, and counting of eggs must take place with similar positive ventilation. 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.

sensitive in detecting some toxicants.[26] 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 C).

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 34 g/kg range, and they should also be within 1 g/kg of the salinity of the control.[27]

If a chemical is being tested, it should be made up to the test concentrations using a control/dilution water (Subsection 4.1.1) which has a salinity in the required range. Salinity of aqueous samples (e.g., effluents) should be measured before the test, and if necessary, should be adjusted using brine, sea salts, or deionized or uncontaminated fresh water, as appropriate (see Subsection 2.3.4).

Samples of effluent, leachate, receiving water, and elutriate 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, 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 (Subsection 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 indicate 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"). Oil-free compressed air should be dispensed through a disposable glass pipette, with bubble size 1 to 3 mm, at a minimal rate for

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26 In fertilization assays at a favourable salinity of 28 g/kg, the IC50s for silver decreased appreciably over the temperature series 7°, 12°, and 17°C. For green sea urchins the IC50s were respectively 215, 110, and 38 µg/L, 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.

27 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). 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).

effective aeration of the particular vessel and volume of fluid being used. Duration of pre-aeration should be the lesser of 20 minutes and attaining 40% saturation (or 100% saturation, if supersaturation is evident).[28] Any pre-aeration should 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 (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 substance being tested. The test would still be a valid assessment of the total effect of the substance (e.g., effluent) including its deoxygenating influence.[29] The required use of oxygen-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.[30] However, if the sample of test 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.[31] If it is desired to assess toxic chemicals *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. For an adjusted test, the initial pH of the sample, or of each test solution may, 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 an adjusted test is to change each test solution, including the control,

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28 Aeration can strip volatile chemicals from solution, or increase their rate of oxidation and degradation to other substances. However, aeration of a sample before exposure of gametes might be necessary due to the oxygen demand of the test substance (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.

29 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 substance. Any such interaction at DO >40% saturation has been accepted in this test procedure, as part of the impact being measured.

30 A justification for not changing the pH of the sample or solution is that pH can have a strong influence on the toxicity of a substance being tested. Thus, any change caused in the pH of the receiving water, with concomitant modification of toxicity, could be accepted as part of the pollution "package". That leads to the rationale that the pH should not be adjusted in tests.

31 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).

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.[32]

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, or elutriate. 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 to 8.2.[33]

Abernethy and Westlake (1989) provide useful guidelines for adjusting pH. Aliquots of samples or test solutions receiving pH-adjustment[34] should be allowed to equilibrate after each incremental addition of acid or base. The amount of time required for equilibration will depend on the buffering capacity of the solution/sample.

For effluent samples, a period of 30 to 60 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, pH adjustment is frequently used as one of a number of techniques (e.g., oxidation, filtration, air stripping, addition of chelating agent) 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 their susceptibility to detoxification (U.S. EPA, 1991a; 1991b).

4.4 Test Observations and Measurements

At the end of the exposure, preserved eggs are taken from each test vessel after mixing[35], and an equal number from each

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32 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.

33 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 C).

34 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.

vessel, in the range of 100 to 200 eggs, is counted and classified as either fertilized or not fertilized (Figure 3). 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).[36]

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.

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.

Various statistical endpoints can be calculated from percent fertilization, and the rationale and methods of calculation follow and are discussed in detail in U.S. EPA (1988). The *NOEC* and *LOEC* can be derived statistically by the hypothesis-testing approach, and this is recommended as a primary technique. Calculation of the *inhibiting concentration for a specified percent effect (ICp)* is also recommended. Advice should be sought from a statistician in carrying out the analyses of results.

4.5.1 Validity of Test

The test is invalid if fertilization rate in the control water is less than 50%, or if complete fertilization is achieved, i.e., 100% fertilization in all replicates of the control.[37] A positive and logical dose-effect curve must have been attained, for the results to be considered valid, i.e., the effect on fertilization must become generally greater at higher concentrations. Additionally, the required physical and chemical conditions must have been met.

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

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35 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.

36 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.

37 Although 90% fertilization is aimed for in the test, and that would be a satisfactory result for the control, the lower limit for validity has been set at ≥50% control fertilization, since useful information might still be obtained from such a test. The upper limit of validity has been set at *less than 100%*, since high fertilization rates are indicative of over-sperming and are associated with loss of test sensitivity.

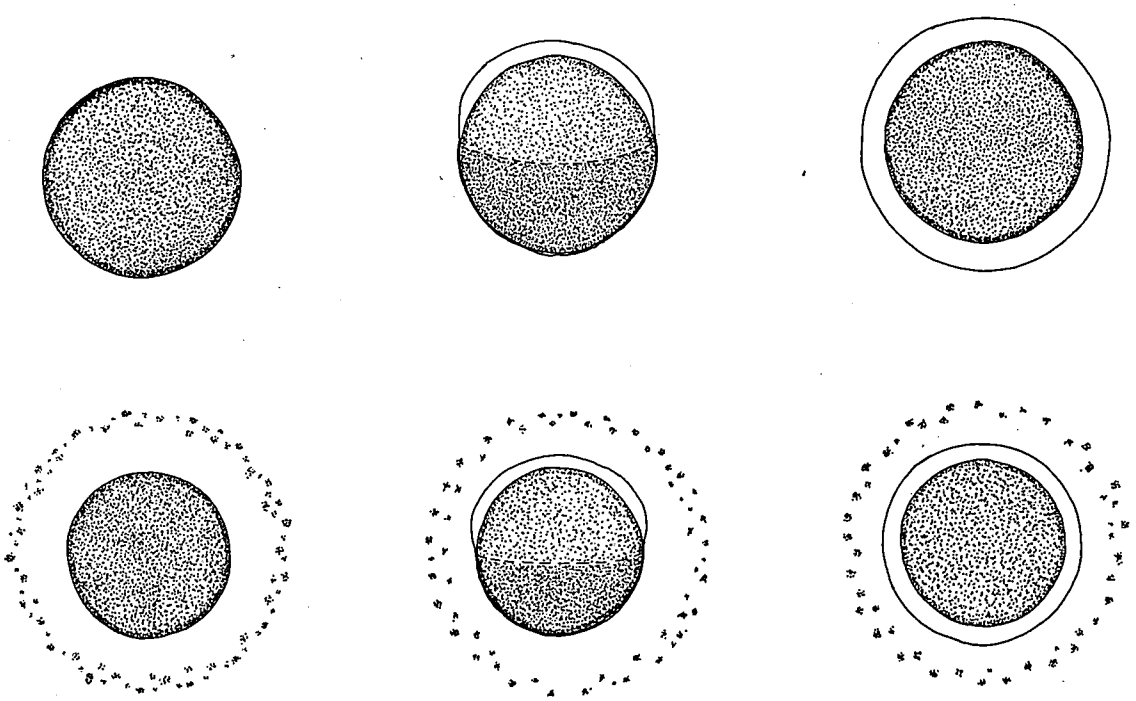

Figure 3 **Discriminating Between Fertilized and Unfertilized Eggs.**
Outlines of eggs as seen under a dissecting microscope. The three drawings in the upper row represent a sea urchin such as the green sea urchin. The egg on the left is not fertilized. The middle egg has a fertilization membrane that 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.

being tested. The test would still be a valid assessment of the total effect of the test substance (Subsection 4.3.3).

4.5.2 No-Observed-Effect Concentration

The NOEC and LOEC are determined from the values for percent fertilization in each replicate of the control and the various concentrations. If there is zero fertilization in all the replicates of a given concentration, that concentration is excluded from calculations to determine the NOEC/LOEC.

The statistical procedures to be followed are given in TOXSTAT[38] (Gulley *et al.*, 1989). An up-to-date version of TOXSTAT is available on computer disk (see Appendix B). The methods in TOXSTAT start with a check of normality and homogeneity of variance, and provide suitable tests of significance for particular types of distribution.

If the data are regular or can be made so by suitable transformation, an analysis of variance is carried out. Differences of each concentration from the control can be assessed by Dunnett's test, a standard multiple-comparison test. Dunnett's test provides estimates of the Minimum Significant Difference (MSD), which is the magnitude of the difference in average success of fertilization, that would have to exist between the control and a test concentration, before a significant effect could be concluded for that concentration. Dunnett's test is not a particularly powerful way of discriminating effects in toxicity tests since it ignores the information on the ordering of the test concentrations by magnitude (Masters *et al.*, 1991). Williams' test is also available in TOXSTAT and is designed to be sensitive to the association between the degree of effect and the ordering of concentrations by magnitude (Gulley *et al.*, 1989). Williams' test (Williams, 1971; 1972) is recommended as an alternative to Dunnett's test. If there are unequal numbers of replicates because of accidental loss or other cause, the *Bonferroni t-test* is substituted for Dunnett's or Williams' test.

If a set of data cannot meet the requirements for normality or homogeneity, and cannot be transformed to do so, there are nonparametric tests provided in TOXSTAT which may be substituted (*Steel's many-one rank test*, or the *Wilcoxon rank sum test* in the case of unequal replicates). Those nonparametric options may be used, and are powerful tools for data that are not normally distributed. The nonparametric tests are less powerful than parametric tests, however, when used on normally-distributed data, and in that situation they might fail to detect real differences in effect, i.e., an underestimate of toxicity might result. It should also be remembered that four replicates are required to make use of the nonparametric methods.

A geometric average of the NOEC and LOEC is often calculated for the convenience of having one number rather than two. Such a value may be used and reported, recognizing that it represents an arbitrary estimate of an effect-threshold that might lie anywhere in the range between the LOEC and NOEC. The calculated value of the geometric mean is governed by whatever concentrations the investigator happened to select for the test. No confidence limits can be estimated statistically for the NOEC and

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[38] The methods of TOXSTAT are not detailed here because the instructions are best followed in the written description that accompanies the programs on computer disk. Briefly, data are tested for normality by the *Shapiro-Wilks* test, and for homogeneity by *Bartlett's test*. If the data do not meet the requirements, it might be possible to transform them with logarithms or arc-sine to meet the requirements. The transformation can reduce the sensitivity of the analysis and the ability of the toxicity test to detect differences.

LOEC, and that is also the case for their geometric mean, although the NOEC and LOEC indicate the outer limits of the estimate. The geometric mean of NOEC and LOEC is often called the *chronic value* in the United States, or sometimes the *subchronic value*, but those terms are misleading for this test with gametes which represents an extremely small fraction of an echinoid's lifetime and therefore does not approach a chronic exposure. A recommended term for the geometric average of NOEC and LOEC is ***TEC*** signifying *threshold effect concentration*. The use of "threshold" is in the dictionary sense of "point at which an effect begins to be produced".

4.5.3 Inhibiting Concentration

As an alternative or additional endpoint which is becoming widely used, the ICp (inhibiting concentration for a specified percent effect) may be calculated as a point-estimate of the concentration causing a certain degree of effect. The percentage is selected by the investigator, and is customarily 25% or 20% reduction in performance rate compared to the control.[39] In this test, the IC25 is recommended and would be the concentration estimated to cause a 25% reduction in success of fertilization compared to the control.

The ICp is often a more useful and desirable measure of effect than estimates of NOEC/LOEC because confidence limits can be calculated, allowing statistical comparisons with ICps in other tests (Suter *et al.*, 1987). Such comparisons cannot be approached in as logical a manner, or with tests for significant differences, by means of the hypothesis-testing approach used for NOEC/LOEC.

The observation of percent fertilized eggs at each concentration is used to calculate the inhibition of fertilization, as a percent reduction compared to the control. The *Percent Reduction in Fertilization* for a given concentration is calculated as:

$$PRF = \frac{CF-OF}{CF}$$

Where:

PRF = Percent Reduction in Fertilization;

OF = Observed percent Fertilized eggs in a given test vessel; and

CF = Control percent Fertilized eggs in dilution/control water.[40]

An analysis of percent reduction in fertilization, to determine an ICp, should begin with a hand plot of PRF values against the logarithm of test concentration. The IC25 (or other ICp) would be read from an eye-fitted line. This graphic estimate would serve to check whether results from mathematical computations were reasonable. The graph would also show whether a positive and logical relationship was obtained between concentration and effect,

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39 Some work with other sublethal tests has indicated that the IC25 or IC20 is a useful measure of sublethal effect that is similar in magnitude to the LOEC.

40 This formula gives the same result as Abbott's formula applied to the proportion of *unfertilized* eggs. Abbott's formula is a correction for control performance that is commonly used in aquatic toxicology (Appendix C, part 13). The formula shown here gives the same numerical result as a calculation of reduced fertilization based on the corrected values derived from the formula of ASTM (1990), shown in Appendix C, part 13. Calculation by the formula shown here provides percent reductions that can be directly plotted, or used in calculations to estimate an ICp.

one of the requirements for validity of the test (Subsection 4.5.1).

A mathematical estimate of the ICp can be obtained by a straightforward *linear interpolation* method (U.S. EPA, 1989, Appendix J). Confidence limits about the ICp can be estimated by a "bootstrap" method on computer (BOOTSTRP), which incorporates linear interpolation to estimate the ICp itself (Norberg-King, 1988; U.S. EPA, 1989). The BOOTSTRP program can be obtained on a user-supplied disk from an Environment Canada regional office (Appendix B).[41] Use of this program requires a computer with a math co-processor. Probit analysis to estimate an effective concentration (EC50 or EC25) is not valid for reasons given under ICp in the Terminology section.

Some common-sense limitations must be applied to estimates of ICp. It should not be estimated on the basis of an extrapolation, i.e., the data should extend above and below the percent effect of interest. For example, if it were desired to estimate the IC25, there should be at least one concentration causing greater than 25% effect (but less than 100% effect), and at least one causing less than 25% effect (but greater than 0% effect). If, for some reason, it is desired to estimate an extreme value such as IC05 or IC95, the foregoing restriction may be relaxed, but there must be at least one concentration causing a partial effect near the ICp of interest, say an effect that did not differ by >5% from the one of interest.[42] Variability is great near the extremes of the relationship, and in particular, observed effects of 0% and 100% often add little information to the estimate of ICp.

4.5.4 Other Test Designs and Purposes

In a single-concentration test, a t-test is normally the appropriate method of comparing the data from the test concentration with those of the control, and the procedure for a t-test can be taken from any statistics textbook. If percent fertilization in the test concentration is significantly lower than the same statistic for the control, it is accepted that the test substance is having an effect.

Requirements for homogeneity of variance and normality must be satisfied (Appendix H of U.S. EPA, 1989; Gulley *et al.*, 1989) before using the standard t-test. If the data do not satisfy the requirements, a nonparametric test could be selected with advice from a statistician; no particular test appears to have become standard practice as yet.

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41 At present, linear interpolation using ~~BOOTSTRP~~ ~~appears to be~~ the only method that is in common use and easily available, for obtaining confidence limits on an ICp. Investigators should be alert for improved methods which might become available, however, since there are some undesirable features of linear interpolation, with or without ~~BOOTSTRP~~. First, there is a requirement that "the responses are monotonically non-increasing" (U.S. EPA, 1989), i.e. in the echinoid assay, a better fertilization rate should not prevail at a high concentration than at a lower concentration. That is not always the case in toxicity assays, and the correction procedure in linear interpolation can bias the estimate of ICp. Second, the ICp is interpolated between two bracketing concentrations, and the rest of the relationship between concentration and effect is not used, as it would be, say, in ~~using probit analysis to estimate a lethal concentration by fitting a~~ line to the entire distribution of data. Third, the ~~interpolation to~~ estimate the ICp ~~is done~~ on an arithmetic basis of concentration instead of a logarithmic one, which would also affect the value derived.

42 The quality and distribution of other data in the test also influence the value of the estimate of an extreme ICp and no firm guideline can be given for the required closeness of an observed data-point to the effect of interest. The spread of the confidence limits will always indicate the reliability of the ICp.

In some cases, the test will not involve various concentrations of a single sample of test substance, but rather a set of different samples, such as full-strength effluents from different industries, or samples of seawater from different places. It might be desired to test whether each sample is different from the control, and that could be done using one option in the statistical program TOXSTAT, with Dunnett's test as the appropriate one for comparison with the control. It might also be an objective, to determine whether the samples were different from each other, and the appropriate statistical test in that case would be Tukey's test. Such sets of tests cannot report ICp or NOEC/LOEC for each sample tested, but should report the observed percent fertilized eggs, and whether that number was significantly different from the control(s).

4.6 Reference Toxicant

The routine use of a reference toxicant or toxicants is necessary 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) (Environment Canada, 1990d).

Sensitivity of gametes to the reference toxicant(s) must be evaluated at least once each calendar month in which the fertilization assay is performed; assessments every two weeks would be more satisfactory and are recommended. The test using the reference toxicant is most useful when carried out simultaneously with an actual toxicity test, and ideally, one would be carried out for each batch of gametes used in toxicity tests.

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

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

Copper is recommended for use as the reference toxicant for this test.[43] Gamete sensitivity should be evaluated by tests following the standard methods and conditions given in this document, to determine the ICp, or NOEC/LOEC for

copper. 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 the reference toxicant tests, 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 34 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). Upon preparation of the test solutions, aliquots should be taken from at least the control, low, middle, and high concentrations, and analyzed directly or stored for future analysis should the ICp or NOEC be atypical (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). Stored aliquots requiring chemical measurement should be analyzed promptly upon completion of the toxicity test. Calculations of ICp or NOEC 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.

A warning chart (Environment Canada, 1990d) should be prepared and updated for each reference toxicant used. Successive ICps, ~~or NOECs~~ 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 ~~or NOEC~~ together with its upper and lower warning limits (± 2 SD calculated on a geometric [logarithmic] basis)[44] are recalculated with each successive ICp ~~or NOEC~~ until the statistics stabilize (U.S. EPA, 1989; Environment Canada, 1990d).

If a particular ICp ~~or NOEC~~ falls outside the warning limits, the sensitivity of the gametes

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43 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 C). 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 (personal communication, G.C. van Aggelen, B.C. Ministry of Environment). For copper, IC50s in the vicinity of 20 to 26 µg Cu/L and coefficients of variation from 23% to 46% have been reported (personal communication, G.A. Chapman, U.S. EPA; U.S. EPA, 1988). 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 (TEC) 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 (personal communication, G.C. van Aggelen). 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; 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 (Environment Canada, 1990d).

44 If the ICps or NOECs fail to show a lognormal distribution, an arithmetic mean and SD might prove more suitable. Use of the ICp is recommended, in preference to the NOEC or even the TEC, since it is a point estimate and less influenced by the particular concentrations and dilution factor selected for the test.

and the test system are suspect. Inasmuch as this can 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 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.

Test endpoints 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 data-point could be within the warning limits but still represent undesirable variation in results. A coefficient of variation of 20% or 30% is tentatively suggested as a limit by Environment Canada (1990d). That seems a reasonable range but the matter has not been studied for the echinoid fertilization assay.

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 substance being sampled; uncontaminated by foreign substances; 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.

Section 5

Specific Procedures for Testing Chemicals

This section gives particular instructions for testing chemicals, in addition to the procedures in Section 4. For chemicals, a multi-concentration test is usually performed to determine the ICp and/or NOEC/LOEC. Three replicates, the minimum required for statistical analysis of results, might also be required under regulations for registering a pesticide or similar category of chemical.

5.1 Properties, Labelling, and Storage of Sample

Information should be obtained on the properties of the chemical to be tested, including water solubility, vapour pressure, chemical stability, dissociation constants, and biodegradability. Data sheets on safety aspects of the substance should be consulted, if available. Such information on the chemical will assist in determining any special requirements for handling and testing it, for example the possible need for a ventilated facility. Information on solubility and stability in seawater and fresh water will also be useful for interpreting test results. If aqueous solubility is in doubt or problematic, acceptable procedures used previously for preparing aqueous solutions of the chemical should be obtained and reported. Other available information such as structural formula, degree of purity, nature and percentage of significant impurities, presence and amounts of additives, and n-octanol:water partition coefficient should be obtained and recorded. An acceptable analytical method should also be known for the chemical in water at concentrations intended for the test, together with data on precision and accuracy.

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. If fresh or distilled water was used to make the stock solution, brine or ocean salts would normally be added to the test concentrations to bring them to the standard salinity (Subsections 2.3.4 and Section 3.4). 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.

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). Ultrasonic dispersion may 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. 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 should 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 (U.S. EPA, 1985a; 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 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.

Reconstituted seawater may 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. There should be one or more salinities common to all tests and used for all dilutions and as the control water as described in Subsection 4.3.2 (i.e., salinity in all concentrations within 1 g/kg of the control, in the range 28 to 34 g/kg).

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 real or potential spills of chemical(s) or intentional applications of a chemical such as spraying of a pesticide. The laboratory supply of natural seawater, or reconstituted water, may also be used as a partial simulation of the receiving water, especially if there is already an interfering toxicity in the receiving water, or its collection and use is impractical (see Subsection 4.1.1). The laboratory seawater in which adults are held is also appropriate for use in other instances such as 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 34 g/kg range might in themselves affect success of fertilization.[45] Control/dilution water for such tests should be from a single source, either reconstituted seawater (Subsection 2.3.4) or natural seawater diluted appropriately with "uncontaminated" fresh water.

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, such as odour, colour, opacity, precipitation, or flocculation. Any observations should be recorded.

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[45] 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.

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 concentrations, and the control(s).[46]

The aliquots should be preserved, stored, and analyzed according to best proven methodologies for measuring the 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.

5.5 Test Endpoints and Calculations

The statistical endpoints for tests performed using chemicals will be the standard ones described in Subsections 4.5.2 and 4.5.3, i.e., NOEC/LOEC, or ICp, or both.

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

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[46] Such analyses need not be undertaken in all instances, due to analytical limitations, cost, or previous data 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 (U.S. EPA, 1985a). Some situations (e.g., testing of pesticides for purposes of registration) can require the measurement of chemical concentrations in test solutions.

Section 6

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

This section gives particular instructions for testing samples of effluent, leachate, and elutriate, 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, leachate, or elutriate must be made of nontoxic material. Glass or Teflon™-coated containers are preferred as they are inert and reduce sorption of chemicals. Polyethylene or polypropylene containers manufactured for transporting drinking water are less desirable but may also be used. 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 air space.

Most tests with effluent, leachate, or elutriate will be performed "off-site" in a laboratory. Testing of effluent and leachate samples should commence within 24 h of sampling whenever possible, and must commence no later than 72 h after sampling. Samples of sediment collected for aqueous extraction and subsequent testing of the elutriate should also be tested as soon as possible; extraction procedures should begin within two weeks of sampling (preferably within one week), and testing must start no later than six weeks after collection (Environment Canada, 1994). Procedures given in Environment Canada (1995) for the preparation of elutriates should be followed. Testing of such elutriates must commence within 72 h of their preparation, or as specified in a regulation or protocol.

A two-litre sample is adequate for an off-site multi-concentration test (e.g., concentrations of 100, 50, 25, 12.5, 6.3, 3.2, 1.6%), associated routine chemical analysis, and any necessary adjustments or repeat tests. Smaller amounts are required for single-concentration tests (Subsection 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.

All samples of effluent or leachate should be kept cool (1 to 7°C, preferably 4 ± 2°C) throughout their period of transport and storage. Upon collection, warm (>7°C) samples should be cooled to 1 to 7°C with ice or frozen gel packs. As necessary, gel packs or other means of refrigeration should be used to assure that sample temperature remains within 1 to 7°C during transit. Samples must not freeze during transport.

Upon arrival at the laboratory, 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 should be stored in darkness in sealed containers 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 extraction and subsequent testing of elutriate, unless otherwise specified.

6.2 *Preparing Test Solutions*

Samples in the collection containers must be agitated thoroughly just before pouring to ensure the re-suspension of settleable solids. Sub-samples (i.e., a sample divided between two or more containers) must be mixed together to ensure their homogeneity. If further sample storage is required, the composited sample (or a portion thereof) should be returned to the sub-sample containers and stored (Section 6.1) until used.

Samples may be passed through a 5-µm filter before use, and although optional, that treatment should be regarded as the standard practice. The presence of high concentrations of suspended inorganic or organic solids in a sample might inhibit fertilization or damage the gametes directly. High concentrations of biological solids in certain types of treated wastewaters can also contribute to sample toxicity due to ammonia and/or nitrite production (Servizi and Gordon, 1986). Filtration would also remove filter-feeding organisms that might be present in treated wastewater, or other organisms that might eat gametes.

Filtration will remove some suspended solids that are characteristic of the sample and might otherwise contribute part of the toxicity or modify the toxicity. If there is concern about the removal of toxicity by the filtration process, two tests should be carried out concurrently, one without filtration and a second with filtered sample. The two tested aliquots should be treated in an identical manner, except for the filtration.

6.3 *Control/Dilution Water*

Tests with samples of effluent or leachate, intended to assess compliance with regulations, should use as the control/dilution water, either the laboratory seawater normally supplied to the adults, 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 "~~upstream~~" receiving water as the control/dilution water can be desirable if information is required on the potential toxic effect of an effluent, leachate, or elutriate on a particular receiving water (see rationale in Subsection 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 recommendations for all control/dilution water, ~~at 1.0 µm~~ (Subsection 4.1.1). If a sample of receiving water is to be used as control/dilution water, a separate control solution should be

prepared using the laboratory seawater supplied to the adults.

Tests requiring a high degree of standardization should use reconstituted seawater as control/dilution water. 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.

As a standard procedure for assessing toxic substances in the wastewater, the salinity of all test and control solutions should be adjusted to the same value, usually by adjusting an aliquot of the sample as described in Subsection 4.3.2 (making test solutions within 1 g/kg of each other in the range 28 to 34 g/kg). Procedures using brine or dry commercial sea salt are recommended, as described in Subsection 2.3.4. If it is desired to assess the total effect of the wastewater including its low (or high) salinity, for regulatory or compliance purposes, the test could be run without adjustment of salinity. An evaluation could be made for both purposes (presence of toxic substances, and compliance) by running the adjusted test and a second test with unadjusted salinities, and/or a set of salinity controls (Subsection 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 material, should be observed in the sample of effluent, leachate, or elutriate at the time of preparing test solutions. A record should be made of 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) 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 undiluted portions of the sample, and three or more replicate control solutions. Test procedures for regulatory compliance could specify that a single concentration (100% sample unless otherwise specified) be used, or might require determination of the NOEC/LOEC and/or 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 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.

Section 7

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 labelling, transportation, and storage of samples should be as described in Section 6.1. Testing of samples should commence as soon as possible, preferably within 24 h, and no later than 72 h after sampling.

7.2 Preparing Test Solutions

Samples in the collection containers should be agitated before pouring to ensure their homogeneity. Compositing of sub-samples should be as described in Section 6.2.

Samples may be filtered at 5 µm before use. If there is concern about changes in toxicity from the filtering process or removal of solids, both filtered and unfiltered tests could be run 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 Subsection 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 should be filtered (~~Subsection 4.1.1, and Sections 6.2 and~~ 6.3).

If "upstream" water is used as control/dilution water, a separate control solution should be prepared using the laboratory seawater that is normally supplied to the adult echinoids. 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, the laboratory seawater supply normally used for holding adults should be used as control/dilution water. This water may be adjusted in salinity to partially simulate "upstream" water (Subsection 4.1.1), but the salinity limitations of this echinoid assay preclude major manipulations.

7.4 Test Observations and Measurements

Observations of sample and solution colour, turbidity, foaming, precipitation, etc. should be made as described in Section 6.4, both during preparation of 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).

Tests for monitoring and compliance should normally include, as a minimum, three or more undiluted portions of the sample, and three or more replicate control solutions. 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 full test to determine ~~NOEC/LOEC and/~~or ICp should be conducted as outlined in Section 4, with undiluted sample as the highest concentration in the series tested.

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 Subsections 4.5.2 and 4.5.3.

Section 8

Specific Procedures for Testing Samples of Sediment and Similar Substances

General instructions are given here for testing liquids derived from samples of sediment or similar solids such as sludges and soils. 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 substances 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

Assessing the toxicity of sediments is 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.*

The echinoid fertilization assay 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 substances from them. Detailed guidance for the collection, handling, transport, and storage of sediment samples (Subsection 8.1.1) is provided in Environment Canada (1993). Also in Environment Canada (1993) are procedural details for the extraction of liquids (i.e., pore water, elutriate, or solvent extract; (Subsection 8.2) from sediments for subsequent toxicity tests and chemical analyses. This recent 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 (1993) 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 sediments themselves (i.e., the solids), but is useful for liquids derived from those solids, whether leachate, elutriate, solvent extract, or interstitial water. For economy of wording, the following sections sometimes refer to "testing sediments", but that should be taken as testing the liquids derived from the sediments.

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; Environment Canada, 1993).

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, 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 (Environment Canada, 1993).[47] Testing of the liquid obtained from sediments should begin within 72 h of making such preparations (Environment Canada, 1993), or as specified in a regulation or other designated procedure.

8.1.2 Preparing Sample

Depending on the nature of the sample and the objectives of the test, homogenization of samples 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. 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 Control or Reference Sediments

One or more samples of control or reference (unpolluted) sediment must be assessed in the same manner as the sediment under investigation. Although the test procedures include a blank or control which does not contain substances from the sediment being studied, experience indicates that such a control might not be sufficient for an acceptable evaluation of toxicity. Using other kinds of tests, Environment Canada and other laboratories have frequently recorded apparent toxic effects with unpolluted sediments. Accordingly, one or more control or reference ("clean") sediments should be included as a sample, with each test of a sediment or series of sediments, to help establish a baseline or "normal" level. It would be desirable to establish a standard, clean "reference sediment" for this purpose, or a series of reference sediments of differing characteristics that could be matched with those of the sediments being tested. The control or reference sediment(s) should be similar in general physical and chemical characteristics to the sediment(s) being investigated. In particular, an attempt should be made to match the distribution of particle sizes and organic/inorganic balance (ASTM, 1991a; 1991b; McLeay and Sprague, 1991).

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47 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 permissable 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.

There is no single procedure for making use of the results from the control or reference sediment. If the control sediment shows no toxicity, then results for the test sediment (i.e., the one under investigation) are accepted as valid. If the reference sediment shows toxicity, no standard method appears to have been developed, as yet, to adjust the results for the test sediment.[48]

Caution should be exercised in interpreting findings for the test substance. It would be desirable to test for significant differences between results for the reference and test sediments. Such tests could be carried out with guidance from a statistician, if paired observations or replicate tests were available.

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 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, i.e., that which fills the spaces between particles, 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 substances 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 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, 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 NOEC/LOEC and/or ICp of the solvent.

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[48] Brouwer *et al.* (1990) incorporated such a control by expressing the performance measured for the test sediment as a percentage of that from the control (reference) sediment, using a bacterial test procedure. However, only one concentration of each was tested, and the technique does not appear to have been extended to a multi-concentration test.

8.2.1 Preparing Test Substances

Specific guidance is given in Environment Canada (1993) 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.

Compositing of "sub-samples" of liquid obtained from the sediment (e.g., successive extractions) should be as described in Section 6.2. Sub-samples would not be composited if the relative toxicity of successive extractions was to be ascertained. Filtration at 5 µm is recommended, as for standard samples of natural seawater. The pH and dissolved oxygen content of the sample should be checked with regard to the limits in Subsections 4.1.2 and 4.1.3.

Once the liquid has been obtained, test concentrations are prepared in the standard manner (Subsection 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 NOEC/LOEC or ICp, or both (Section 6.2). 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 (28 to 34 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 34 g/kg), usually accomplished by adjusting an aliquot of the sample as described in Subsection 4.3.2. Procedures using brine or dry commercial sea salt are recommended, as outlined in Subsection 2.3.4.

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.

Section 9

Reporting Requirements

The test report should describe the materials and methods used, as well as the results. A reader should be able to establish from the report whether the conditions and procedures rendered the results acceptable for the use intended.

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 report may be referred to by citation or by attachment of a general report that outlines standard laboratory practice. Where choices exist, the approach selected should be specified. The general report should convey the procedural information included in Sections 9.2 to 9.5. A report giving the findings of an individual test should contain the information indicated in Sections 9.1, 9.6 and 9.7. Specific monitoring programs might require other selected items in the report (e.g., procedures and results for tests requiring pH adjustment or modified aeration/oxygenation). Other details pertinent to the conduct and findings of the test, that are not conveyed by the reports, should be kept on file by the laboratory, so that the appropriate information can be provided if an audit is required.

9.1 Test Substance

- sample type, source, and description (chemical, effluent, leachate, elutriate, or receiving water; sampling location and method; specifics regarding nature, appearance and properties, volume and/or weight);
- information on labelling or coding of the test substance;
- details on manner of sample collection, transport and storage (e.g., batch, grab or composite sample, description of container, temperature of sample upon receipt and during storage);
- identification of person(s) collecting and/or providing the sample; and
- dates and times for sample collection, receipt at test facility, start and end of definitive test.

9.2 Test Organisms

- species and source;
- description of holding conditions for adults (facilities, lighting, water source and quality, water pre-treatment, water exchange rate and method, density of animals in tanks, temperatures in those tanks, food type and frequency of feeding, disease incidence); and
- weekly percentage of mortalities among the adults being held.

9.3 Test Facilities and Apparatus

- name and address of test laboratory;
- name of person(s) performing the test;
- description of systems for regulating temperature within test facility; and

- description of test vessels (usual name, size, shape, type of material).

9.4 Control/Dilution Water

- type(s) and source(s) of water used as control and dilution water;
- type and quantity of any chemical(s) added to control or dilution water;
- sampling and storage details if the control/dilution water was "upstream" receiving water;
- water pre-treatment (temperature adjustment, de-gassing, aeration rates, duration, etc.); and
- measured water quality variables (Subsection 2.3.4) before and/or at time of commencement of toxicity test.

9.5 Test Method

- brief mention of method used if standard (e.g., as per this document), and options selected;
- design and description if specialized procedure or modification of standard method;
- procedure used in preparing stock and/or test solutions of chemicals;
- any chemical analyses of test solutions and reference to analytical procedure(s) used; and
- use of preliminary or range-finding test.

9.6 Test Conditions

- statement on aeration of sample before the test (if any, give rate, duration, and manner);
- number, concentration, volume, and depth of test solutions including controls, and number of replicates;
- estimated number of sperm per vessel and sperm:egg ratio;
- general nature of lighting of test facility;
- description of any test solutions receiving pH adjustment or filtration, including procedure;
- any chemical measurements on test solutions (e.g., chemical concentration, suspended solids content);
- temperature, salinity, pH, and dissolved oxygen (mg/L and % saturation) as measured in aliquots of control, and high, medium and low test solutions at start; and
- conditions and procedures for measuring the ICp or NOEC/LOEC of the reference toxicant(s).

9.7 Test Results

- appearance of test solutions and changes noted during test;
- numbers of fertilized and unfertilized eggs counted for each vessel at the end of the test, and percent fertilized;
- results for range-finding test, if conducted;
- the NOEC/LOEC or ICp, or both, as measures of fertilization success;

Minimum Significant Difference in % fertilization, if NOEC/LOEC are determined; the statistical test(s) used, and any transformation of data that was required; and

- the results of the relevant toxicity test(s) with the reference toxicant(s), together with the geometric mean value (± 2 SD) for the same reference toxicant(s) as derived at the test facility in previous tests.

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Appendix A

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* As of November, 1992.

Appendix B

Conservation & Protection, Regional and Headquarters Offices

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* The statistical programs of TOXSTAT and BOOTSTRP are available from the Laboratory Division at this address by providing a formatted computer diskette.

Appendix C

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.

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

U.S. EPA 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.

U.S. EPA (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).

U.S. EPA (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 dollar(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.
U.S. EPA 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.
U.S. EPA (Pac. 91)	S. droebachiensis as above.
U.S. EPA (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, white s.u., romaine lettuce
Dinnel *et al.* 1987	N.I.	flowing seawater, filtered recirculation with filter	s.u. macroalga s.d. plankton and detritus
U.S. EPA 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
U.S. EPA (Pac. 91)	N.I.	N.I.	N.I.
U.S. EPA (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.	NI
EVS 1989	various spp s.u.	~10	28	airstones	constant dark
	D. excentricus	15	"	"	photoperiod
B.C. MOE 1990		N.I.	27 to 30	N.I.	N.I.
IGATG 1991	*S. droebachiensis*	9	30	N.I.	N.I.
	L. pictus	15	"	"	"
Dinnel *et al.* 1987	*Strongylocentrotus*	natural			
	D. excentricus	seasonal	≥ 27	N.I.	N.I.
U.S. EPA 1988	*A. punctulata*	15 ± 3	30	N.I.	N.I.
ASTM 1990	*Strongylocentrotus*	8 to 10	25 to 35	50 to 100%	N.I.
	D. excentricus	12 to 14	"	"	N.I.
	A. punctulata	15	"	"	high lighting
NCASI 1991, 1992	*Strongylocentrotus, D. excentricus*	7 to 14	N.I.	N.I.	ambient lab.
U.S. EPA (Pac. 91)	*S. purpuratus*	N.I.	N.I.	N.I.	N.I.
U.S. EPA (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
U.S. EPA 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
U.S. EPA (Pac. 91)	seawater, filtered 1 µm
U.S. EPA (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
U.S. EPA 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 brine or salts
NCASI 1991, 1992	12	30, adjust test solutions with brine or salts
U.S. EPA (Pac. 91)	12	32 ±1
U.S. EPA (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 1990	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.
U.S. EPA 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
U.S. EPA (Pac. 91)	N.I.	N.I.
U.S. EPA (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
U.S. EPA 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.
U.S. EPA (Pac. 91)	8.1 ± 0.1 for c/d water
U.S. EPA (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 mL 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, disposable, unwashed	≥3
U.S. EPA 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 disposable	four
U.S. EPA (Pac. 91)	5	borosilicate glass tubes, 16 × 100 mm	three
U.S. EPA (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 water; 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
U.S. EPA 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
U.S. EPA (Pac. 91)	20, 60 min	20, 60 min	diverse, to assess alternate methods in this exploratory round-robin
U.S. EPA (Pac. 92)	60 min	20 min	≥3 c/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 min	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
U.S. EPA 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 at 12°C c/d water in 50-mL beaker (s.u. 100-mL beaker)
U.S. EPA (Pac. 91)	0.5 to 1.0 mL of 0.5*M* KCl, 2nd injection if needed	c/d water in 100-mL beaker
U.S. EPA (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* KCl	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 (♀) or
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
U.S. EPA 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 ≤2 h, hold at 12 °C
U.S. EPA (Pac. 91)	collect for ≤30 min, wash eggs twice, composite sperm
U.S. EPA (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
U.S. EPA 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
U.S. EPA (Pac. 91)	various	1120?	various, to assess methods
U.S. EPA (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
U.S. EPA 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.
U.S. EPA (Pac. 91)	N.I.
U.S. EPA (Pac. 92)	as in U.S. EPA (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 present document (Subsection 4.5.3) as a preliminary to estimating an ICp.

** 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	
U.S. EPA 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	
U.S. EPA (Pac. 91)	desirable to attain 80 to 95	
U.S. EPA (Pac. 92)	fertilization ≥50 in control; sperm concentration within a factor of two of 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	≥60, ≤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	
U.S. EPA 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.		
U.S. EPA (Pac. 91)	copper	no?	
U.S. EPA (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 D

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 E

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 Rochinni *et al.* (1982).

** A series of five or more successive concentrations should 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 1 might be used if there was considerable uncertainty about the degree of toxicity. More widely spaced concentrations (differing by a factor <0.3) should not be used. For effluent testing, there is seldom much gain in precision by selecting concentrations from a column to the right of column 3; the finer gradations of columns 4 to 7 might occasionally be useful for testing chemicals that have an abrupt threshold of effect.