This checklist is a summary of the requirements and recommendations in the Environment Canada test method. As a summary, it will not contain all supplementary information. If there is a discrepancy between the checklist and the Environment Canada test method, the test method is taken as the definitive source.

Y= Yes, meets requirements; N= No, does not meet requirements; NA= not applicable.

TEST SPECIFIC CHECKLIST

RM 58 – July 2014 Edition - Toxicity of Sediment to Echinoid Embryos and Larvae (Sea Urchins and Sand Dollars)

Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Sample/Preparation							
Filtering	Never wet-sieved **(Must)**						
	≥ 2 mm particles and indigenous macro-organisms removed by forceps or press-sieve if necessary						
Homogenization	Porewater separated during transport/storage mixed back into the sediment **(Must)**						
Characterization	whole sediment - % coarse-grained, % sand, % silt, % clay **(Must)**						
	% water content **(Must)**						
	TOC **(Must)**						
	Total ammonia **(Must)**						
	Sulphide **(Must)**						
	pH **(Must)**						
Test Conditions							
Test Facility	Isolated from general laboratory disturbances						
	Equipment and supplies that contact sediments, water, or stock solutions must not contain substances which can be leached or dissolved in amounts that adversely affect organism health. **(Must)**						
	Instruments available to measure basic water quality variables ((T°, D.O., pH, salinity) and be prepared for other analyses (e.g.: ammonia and particle size) **(Must)**						
Test Type	Static **(Must)**						
Test Duration	Embryos exposed to each treatment for the same period of time (48 to 120 hours depending on species and development rate) then preserved in glutaraldehyde. **(Must)**						
	48 h if *L. pictus*, 72 h if *D. excentricus*, or 96 h if *S. purpuratus* to determine % normal larvae: 6 "water only" vials preserved 1 h before putative test end - test terminated if ≥70% or continued for an additional 24 h if < 70%. **(Must)**						

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Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Test T°	Daily mean temperature of 15 ± 1°C for *S. purpuratus* and *D. excentricus*; and 20 ± 1°C *L. pictus* **(Must)**						
	Instantaneous temperature within 3°C of daily mean temperature **(Must)**						
Lighting	Normal laboratory lighting – 500 to 1000 lux at water surface and photoperiod of 16-hour light and 8-hour dark recommended						
Salinity	Overlying seawater from a natural source **(Must)**						
	Salinity of 30 ± 2 g/kg **(Must)**						
	Salinity not adjusted on Day -1, Day 0 (at setup), nor at any time during the test **(Must)**						
pH	Not adjusted before or during the test **(Must)**						
Aeration	None **(Must)**						
Vessel Size & Type	20 mL disposable glass scintillation or shell vials						
	Vials loosely covered with plastic (new) film **(Must)**; individual vials should be tightly sealed if volatiles are present						
Test Volume	0.5 ± 0.05 g of wet sediment; 10 mL of overlaying water						
Renewal of Sediment or Overlaying Water	None						
Dilution/ Control Water	Filtered (60 µm) uncontaminated natural seawater **(Must)**						
	Salinity: 28 - 32 g/kg **(Must)**						
	Water used in any given test from same source **(Must)**						
	Water should be used within 3 days of filtration						
	pH: 7.5 - 8.5 **(Must)**; recommend 8.0 - 8.2						
	Adjusted to ± 1°C of the test T° before starting the test **(Must)**						
	D.O: 90 - 100 % air saturation **(Must)**; not supersaturated						
Vessel Identification	Each vessel clearly labelled or positions coded so that samples/treatments and replicates can be identified **(Must)**						
Field Replicates	≥5 replicate samples collected from each sampling station and depth of interest **(Must)**						
Number of Test Chambers	≥8 for each field replicate and control sediment **(Must)**						
	≥23 "water only" vials **(Must)**						
	Test start with equal # of test chambers for each replicate and controls **(Must)**						

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Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Organisms	~200 embryos per vessel of which ≥90% are newly fertilized **(Must)**						
	Transfer to the test vials completed within 4 hours of fertilization **(Must)**; preferably within 2 hours.						
	Fertilized egg pool must be kept homogenous during the transfer to test vessels (e.g. by gently swirling with a glass rod immediately before removing each aliquot) **(Must)**						
Feeding Regime	None						
Vessel Cleaning	Disposable recommended						
	All test vessels, measurement devices and stirring equipment and pails for transferring organisms thoroughly cleaned/rinsed in accordance with good laboratory practice **(Must)**; Control/dilution water used in final rinse						
Endpoints	Number of normal larvae produced in each replicate and the associated % normal larvae calculated for each treatment **(Must)**						
Reference Toxicant Endpoints	Initial plot of raw data (% normal) against log highly recommended; any major disparity between graphic and computer derived ICp resolved **(Must)**						
	Calculation of ICp and it's 95% CL via non-linear regression analysis provided assumptions of normality and homoscedasticity are met **(Must);** data assessed for outliers						
	More than one model fit to data and model with best fit chosen for generation of ICp and 95% CL (**Must)**						
	Endpoints generated by regression analysis are bracketed by test concentrations; endpoint is not extrapolated beyond highest test concentration **(Must)**						
	Lowest test concentration inducing zero (or near-zero) normal development kept in data set, subsequent high test concentration(s) removed before regression analyses **(Must)**						
	Hormesis data entered directly for regression (no trimming of data points)						
	Linear interpolation (ex. ICPIN) used to derive ICp if data do not allow regression statistics						
	If data exhibited hormesis and ICPIN is used, control responses entered for those concentrations which demonstrated hormesis **(Must)**						

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Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Observations and Measurements							
Fertilization Success Rate	6 "water only" vials preserved at hour zero to confirm fertilization success rate and provide an estimate of the number of fertilized eggs added to each treatment **(Must)**						
D.O. + pH + T° + salinity + ammonia	Measured in one vial (controls and each treatment) on day 0 and one vial at test termination **(Must)**						
Recovery of Embryos and Larvae	Overlying water transferred to new vials at test termination - this includes "water only" controls that are paired with sediment samples (transfer is not necessary for vials that are part of a reference test); 1 mL of 0.5% glutaraldehyde added **(Must)**; vials capped and stored at room temperature until scored **(Must)**						
	Each technician responsible for counting and scoring echinoid embryos and larvae trained and experienced in how to do so **(Must)**						
	All embryos and larvae recovered from each replicate at test end are scored **(Must)**						
Scoring	Counting and scoring of preserved organisms accomplished via inverted microscope or Sedgwick-Rafter cell (or similar chamber)						
	Embryos and larvae sink after preservation so careful discarding of excess water through decanting or pipette is permitted						
	Number of normal larvae (prism or pluteus) and abnormal larvae are counted and documented; unfertilized eggs are not counted or scored **(Must)**						
Test Organisms							
Species	Test carried out with one of the following species: *Strongylocentrotus purpuratus* (Pacific purple sea urchin); *Dendraster excentricus* (eccentric sand dollar) or; *Lytechinus pictus* (white sea urchin) **(Must)**						
	For a given test, animals representing a single species, all test organisms derived from gametes retrieved from the same population of sexually mature adults **(Must)**						
Source	Wild population; biological supply houses; commercially; or from other laboratories						
Shipping Test Organisms	All information needed to properly identify adult echinoids to species provided with each shipment **(Must)**						

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Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Shipping Test Organisms cont.	Records accompanying each batch of test organisms must include quantity and source, supplier's name, date of shipment and arrival, arrival condition, and species identification **(Must)**						
Age	Gametes obtained from mature and gravid adults						
Health Criteria	Holding conditions satisfactory if organisms can deliver viable gametes that meet needs and validity criteria of the test						
Culture/ Holding Conditions for Echinoids Maintained in Lab for Extended Time (>3 days)							
T°	10 ±2°C for Pacific purple urchins, 13 ±2°C for the eccentric sand dollar, and 13 ±2°C for white sea urchins						
pH	7.5 - 8.5 **(Must)**; 8.0 ± 0.2 preferred						
D.O.	80 – 100% air saturation						
	Remedial measures taken if dissolved gases > 100% saturation **(Must)**						
Salinity	Not less than 25 g/kg or greater than 36 g/kg **(Must)**; 28 - 34 g/kg (preferably 30 - 32 g/kg)						
	Rate of salinity change ≤5 g/kg/day **(Must),** should be ≤3 g/kg/day						
Lighting	Low intensity normal laboratory light; 16-h light: 8-h dark; normal daylight, seasonal photoperiod						
Feeding	Sea urchins: kelp (or other macroalga) or romaine lettuce, spinach and carrots; always available; old or decomposing food removed						
	Sand dollars: provide sediment with detritus and alga, use lighting to encourage growth of alga, if necessary, add shredded eel grass, spinach, flaked fish food, cultured alga or algal paste						
Cleaning	Tanks cleaned and disinfected (if necessary) before introducing new adults						
	Removal of old alga, fecal material and debris daily or as required						
Disease/ Mortality	Monitor daily, mortality should be ≤2%/d averaged over 7 d preceding collection of gametes, and cumulative mortality over the same 7-d period ≤20% **(Must)**; remove dead, diseased or moribund animals; discard groups of diseased animals						
Holding Water	Reconstituted or clean natural seawater						
	Renewed continuously (flow-through: flow of 5-10 L/d for each organism, and equal to the tank volume in 6-12 h) or periodically to prevent buildup of metabolic wastes (target values ≤0.02 mg/L unionized ammonia, ≤0.06 mg/L nitrite)						

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Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Holding Water cont.	If reconstituted sea water is used, made up by adding HSB, commercially-available dry ocean salts or reagent-grade salts to fresh water **(Must)** ; if made using dry salts is aerated for ≥24 h **(Must);** aeration for ≥3 days recommended						
	Any artificial HSB prepared using commercial or reagent grade salts is filtered (≤1 μm) and aerated for ≥24h **(Must)** aeration for ≥3 d recommended; HSB from natural seawater should be filtered (≤1 μm)						
	Unused portions of prepared natural or artificial HSB should be capped and stored in the dark at 4±2°C until used						
Water Depth	≥ 20 cm for sea urchins; ≥ 10 cm water with 2 - 3 cm of sediment or sand rich in detritus for sand dollars						
Acclimation	Recommend gradual acclimation and holding adults a minimum of 3 - 4 days at test T° and salinity and in same water as control/dilution water to be used in test prior to gamete collection						
	Temperature rate of change ≤ 5°C /d						
Facility & Apparatus	Aquaria, troughs or tanks made of nontoxic materials (~ 50 - 150 L water)						
	Holding tanks located away from physical disturbances, preferably separate from area used for testing						
	Held in groups of ≤20 to avoid mass spawning						
Specific Conditions for Holding Adults for Immediate Use (≤3d)							
Gamete Collection	Gametes collected ≤3 d after adults arrive at lab						
	Confirmation should be obtained from supplier that adults are mature and eggs are viable						
T°	T° at shipping at/near test T°						
Acclimation	Gradual exposure to testing lab's holding conditions and/or control/dilution water						
	Adults spawned on same day as arrival at lab held for minimum of 3 h to observe health and move to testing conditions **(Must)**						
Lighting	Recommend lighting representing test conditions						
Salinity	Adjustment should be <5 g/kg/day, can be higher if test validity criteria is met and sensitivity of gametes in reference toxicant tests not affected						
Feeding	Not required						
Mortality	Adults cumulative mortality ≤20% for 7-d period prior to shipment **(Must)**						

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Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Gamete Handling							
Gamete Collection	Adults are stimulated to spawn using 0.5 to 1 mL of 0.5 *M* KCl through the peristomal membrane into the coelom for sea urchins; and 0.5 mL of the solution through the mouth for sand dollars						
	A second injection used if no spawning in 5 or 10 min						
	Collection of spawn terminated within 15 min of start of steady spawning						
	Perform gamete check to ensure only good quality gametes are selected for test						
	Sperm should represent ≥3 male adults and eggs ≥3 female adults						
Gamete Check	"Good quality" eggs from each female fertilized with 5-7 drops of diluted sperm (i.e. 20-50 µL dry sperm in 10 mL filtered seawater) from each of the "good-quality" batches of sperm						
	Mixture of sperm and eggs observed microscopically after 10 min; Sperm quality assessed for motility, activity, clumping and fertilization success, and egg quality for shape, colour, size and fertilization success						
Spawning Males	"Dry spawning" recommended; collect sperm from bottom of petri dish or beaker or from surface of animal (sand dollars should be spawned in a minimal amount of seawater)						
	Care taken collecting dry sperm to avoid contamination with water or KCl solution **(Must)**						
	Semen collected "dry" held on ice for 4 h before "activation" in seawater then used in test in the subsequent 60 - 120 min						
	Semen collected "wet" used to start test in a period of ≥ 0.5h to ≤ 2h.; in interim, sperm stored on ice in minimal control/dilution water						
Spawning Females	"Wet spawning" recommended; animal placed aboral side down on small beaker (50 - 250 mL) filled with control/dilution water at test T°; water decanted from gametes after spawning complete. Alternatively, animal placed aboral side up in a vessel with control/dilution covering test (shell) by 1 cm; Eggs collected off surface and placed in beaker/appropriate vessel						
	Eggs washed 3 X by diluting with 100 mL control/dilution water, mixing, settling for 10 min and decanting						
	Eggs can be held for 4 h at test T° before use (recommend gentle aeration)						

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Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Sperm Density	Sperm density estimated with hemocytometer (diluted 100x to 10,000x, using 10% glacial acetic acid made up with control/dilution water); count sperm in middle 400 small squares						
	# sperm/mL in initial suspension = (dilution factor) x (# sperm counted) x (hemocytometer conversion factor) x (conversion of mm^3 to mL) ÷ (# squares); for standard Neubauer hemocytometer: # sperm/mL = 100 x (# sperm) x 4000 x 1000 ÷ 400						
	Sperm density adjusted to desired conc. (determined by sperm:egg ratio selected) using control/dilution water						
	Alternative technique is to use turbidity or optical density as indication of # sperm/mL						
Egg Density	Eggs counted using a Sedgwick-Rafter cell (add ≤ 1 mL of mixed suspension, as required, diluted 10-, 100-, or 1000-fold for counting)						
	Egg suspension adjusted to 1000 eggs/mL by adding control/dilution water or decanting water, as necessary						
Sperm:Egg Ratio	Optimum sperm:egg ratio determined in each lab to give ≥90% fertilization under control conditions						
Preparation of Fertilized Eggs	Within 15 – 30 min of fertilization of the batch of eggs intended for use, a minimum of 5 replicate subsamples examined for fertilization success rate **(Must)**; Minimum of 100 eggs from each replicate scored for presence of a fertilization membrane						
	If mean fertilization is ≤90% a second addition of sperm can be made and the fertilization rate reassessed. If fertilization rate remains ≤90% after second sperm addition eggs are not used and new animals are spawned **(Must)**						
QA/QC							
Test Validity Criteria	Average of ≥60% normal larvae by test end in: 1) "water only" controls, and 2) laboratory control sediments **(Must)**						
	Average of ≥60% normal larvae by test end in "water only" controls of reference toxicant test run in conjunction with sediment samples **(Must)**						
Reference Toxicant	Multi-concentration "water only" reference toxicity test performed in conjunction with each batch of organisms used in one or more sediment toxicity tests conducted per day **(Must)**; concurrent tests use same batch of newly fertilized embryos as definitive test **(Must)**						

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Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Reference Toxicant cont.	IC50 endpoint based on % normal larvae – results calculated and reported as µg Cu/L (if copper is the reference toxicant chosen) **(Must)**						
	Copper is recommended						
	Using same water as control/dilution water **(Must)**						
	If aliquots of test solutions are stored for future chemical analysis they are held in the dark at 4 ± 2°C **(Must)**						
Warning Chart	Prepared for each reference toxicant and continually updated **(Must)**						
	Within acceptable warning limits (± 2 SD on log scale)						
	Separate warning chart for each echinoid species used **(Must)**						
	All data on a warning chart generated using the same reference toxicant **(Must)**						
	If IC50 outside control limits (mean ±3 SD), or if endpoints fell between control and warning limits >5% of the time, test should be repeated and aspects of test carefully scrutinized						
Sample Handling							
Containers	Non-toxic materials for sample and transport containers, new containers or thoroughly rinsed used containers **(Must)**						
	Upon collection, sample containers filled, sealed and labeled/coded **(Must)**						
Labeling	At least a code that can be linked to supporting documentation which includes: sample type, source, precise location, replicate number, and date of collection **(Must)**						
Holding Time	Test to be initiated within 6 weeks after sampling **(Must)**						
	Recommend test initiation within 2 weeks after sampling						
Holding Conditions	Upon collection, if sample > 7 °C, cool to 1 - 7°C with regular ice or frozen gel packs						
	Make effort to keep samples in darkness and at 1 - 7°C during transport						
	Sample be kept from: freezing (including partial freezing) or drying during transport and storage **(Must)**						
	Sample temperature and date of receipt recorded on bench sheet or chain-of-custody form upon arrival at the laboratory **(Must)**						
	Any remaining portion(s) of sample held for possible additional testing be stored in darkness in sealed containers without air headspace at 4 ± 2°C **(Must)**						

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Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Subsample Mixing	Subsample (i.e.: a sample divided between 2 or more containers) mixed together to ensure their homogeneity **(Must)**						
Sample Aliquots	Each sample in a collection container thoroughly mixed just before separating into test vials **(Must)**						
Test Report							
Sample Data	Brief description of sample type (dredged, reference sediment, etc.) or coding as provided to the lab **(Must)**						
	Information on labelling or coding, for each sample **(Must)**						
	Date of sample collection; name of sample collector; date and time sample received at lab **(Must)**						
Test Organism	Species, source, and date of collection **(Must)**						
	Brief description of holding time and conditions for adults **(Must)**						
	% mortalities among adults shipped and held ≤3 d before spawning **(Must)**						
	Average daily and cumulative 7-day % mortalities among adults being acclimated and held >3 d **(Must)**						
	Any unusual appearance, behaviour, or treatment of adults or gametes, before the test is started **(Must)**						
Test Facilities	Name and address of test laboratory **(Must)**						
	Name of person(s) performing the test **(Must)**						
	Brief description of test vessels (size, shape, type of material) **(Must)**						
Control Sediment and Control/ Dilution Water	Type and source of water used as control and dilution water **(Must)**						
	Source(s) of sediment used as control sediment **(Must)**						
	Type & quantity of any chemical(s) added to control or dilution water **(Must)**						
Test Method	Citation of biological test method used **(Must)**						
	Brief description of frequency and type of all observations and all measurements made during test **(Must)**						
	Program(s) and methods used for calculating statistical endpoints **(Must)**						
Test Conditions	Design and description if any deviation from or exclusion of any of the procedures and conditions specified in test method document **(Must)**						
	Number of discrete samples per treatment; number of replicate test chambers for each treatment; number and description of treatments in each test including the control(s) **(Must)**						
	Volume of sediment and overlying water in each test chamber **(Must)**						

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Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Test Conditions cont.	Brief statement indicating that gamete viability check was performed, and whether a pre-test was performed **(Must)**						
	Number of males and females used to pool sperm and eggs **(Must)**						
	Sperm:egg ratio used in testing, including estimated initial sperm stock density **(Must)**						
	Number of eggs per test chamber and egg density **(Must)**						
	Pre-test fertilization period (minutes) **(Must)**						
	Mean fertilization (%) at test start **(Must)**						
	Time between fertilization and test initiation **(Must)**						
	Period of time test vessels are stored prior to enumerating results **(Must)**						
	Date when test was started and ended; statement of test duration **(Must)**						
	Date when each test chamber was scored **(Must)**						
	Each sediment sample: % coarse-grained, % sand, % silt, % clay, % water content, total organic carbon content, total ammonia, sulphide, and pH **(Must)**						
	Indicate is any samples of test sediment (including reference sediment) were press-sieved – including the procedure and mesh size used **(Must)**						
	From test start and end: temperature, salinity, DO, and pH in the overlying water from each treatment **(Must)**						
	From test start and end: total and un-ionized ammonia in all sediment exposures including the corresponding "water only" control tested in conjunction with the sediment samples **(Must)**						
	For reference toxicant test - from test start and end: total and un-ionized ammonia in all sediment exposures including the corresponding "water only" control tested in conjunction with the sediment samples **(Must)**						
	Date when reference toxicity test was performed and brief statement indicating whether it was performed under the same experimental conditions as test sample; description of any deviation/ exclusion of procedures/ conditions specified in test method document **(Must)**						

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Parameter	**Specification**	**Document Review**			**Implementation**		
		Y	**N**	**NA**	**Y**	**N**	**NA**
Test Results	At test start – number (and percentage) of fertilized and unfertilized eggs in each of six replicate "water only" vials, including the mean (± SD) **(Must)**						
	At test end - % normal larvae (± SD) in "water only" controls; for all treatments and "water only" control, number of 1) normal larvae including mean (± SD) and 2) abnormal larvae in each replicate including mean (± SD); % normal larvae in all treatments and controls **(Must)**						
	Mean recovery success rate for each treatment **(Must)**						
	Type and results from all statistical analysis and comparisons of the data **(Must)**; interpretation criteria "pass/fail" are used appropriately to classify the sediment						
	Duration and results of any toxicity tests with the reference toxicant(s) performed at the same time of the test, together with the geometric mean value (± 2 SD) for the same reference toxicant(s) as derived at the test facility in previous tests with the same species **(Must)**						
	Any outliers and the justification for their removal **(Must)**						
	Anything unusual about the test, any problems encountered, any remedial measures taken **(Must)**						
Information Kept On-File	Do lab SOPs indicate that the information on Section 7.2 of 1/RM/58 method must be kept on file for 5 years? **(Must)** *For details on this information, see 1/RM/58, section 7.2						

Notes: