This checklist is a summary of the requirements and recommendations in the Environment and Climate Change Canada test method. As a summary, it will not contain all supplementary information. If there is a discrepancy between the checklist and the Environment and Climate Change 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.
DO = dissolved oxygen; temp = temperature; conc = concentration(s); sal = salinity; min = minute(s); h = hour; # = number (of);
SD = standard deviation; ‰ = parts per thousand, equivalent to g/kg

TEST SPECIFIC CHECKLIST

Reference Method for Determining Acute Lethality Using *Acartia tonsa*

Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Sample Handling: Effluent							
Salinity	Salinity of effluent is > 4‰ and is discharging directly to estuarine or marine receiving waters **(must)**	☐	☐	☐	☐	☐	☐
Containers	Made of nontoxic material; new or thoroughly cleaned and rinsed with clean water before use **(must)**; then rinsed with sample to be collected	☐	☐	☐	☐	☐	☐
	Each sample container is filled completely to exclude air	☐	☐	☐	☐	☐	☐
Volumes	≥ 500 mL for single- and multi-conc test	☐	☐	☐	☐	☐	☐
Labelling	Immediately after filling, each sample container is sealed and labeled or coded **(must)**	☐	☐	☐	☐	☐	☐
	Label and/or records include a code or sample identifier, sample type, source, sampling method, date and time of collection, and name of sampler(s) **(must)**	☐	☐	☐	☐	☐	☐
Holding Time	Test is initiated within 5 days after termination of sampling **(must)**; recommend within 3 days after termination of sampling	☐	☐	☐	☐	☐	☐
	Date and time of receipt of the sample(s) at lab is recorded **(must)**	☐	☐	☐	☐	☐	☐
Holding Conditions	Temp of sample in each container is measured and recorded upon receipt at lab **(must)**	☐	☐	☐	☐	☐	☐
	Samples are kept between 1 and 8 °C if more than 2 days in transit or when ambient temp is extreme (i.e., > 30 °C or < 1 °C), and in darkness throughout transport	☐	☐	☐	☐	☐	☐
	Samples are kept from freezing during transport or storage **(must)**	☐	☐	☐	☐	☐	☐
	Options for sample storage prior to testing include: held in the dark at 4 ± 2 °C for a brief period in full, sealed container(s) within a refrigerated facility; or held in full, sealed container(s) at 20 ± 2 °C overnight if test to be started the next day **(must)**	☐	☐	☐	☐	☐	☐
Sample Handling: Chemicals							
Containers	Sealed and coded or labelled upon receipt **(must)**	☐	☐	☐	☐	☐	☐
Labelling	Label and/or record(s) includes a code or sample identifier with required information (i.e., chemical name, supplier, date received) **(must)**	☐	☐	☐	☐	☐	☐

TEST SPECIFIC CHECKLIST

Reference Method for Determining Acute Lethality Using *Acartia tonsa*

Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Properties	Information on the properties of the test chemical is obtained, including: concentration of major ingredients, solubility in seawater (natural or artificial), vapour pressure, chemical	☐	☐	☐	☐	☐	☐
	stability, dissociation constants, toxicity to humans and aquatic organisms, biodegradability and data-sheets on safety aspects (e.g., Safety Data Sheets)	☐	☐	☐	☐	☐	☐
	Acceptable procedures for preparing aqueous solutions of the chemical are obtained and reported and/or solubility in control/dilution water is determined experimentally where aqueous solubility is in doubt or problematic	☐	☐	☐	☐	☐	☐
	Other available information such as structural formulae, degree of purity, nature and percentage of significant impurities, presence and amounts of additives, and n-octanol:water partition coefficient is obtained and recorded.	☐	☐	☐	☐	☐	☐
	An acceptable analytical method for measuring the chemical in seawater at concentrations intended for the test is known along with the precision and accuracy of the analysis	☐	☐	☐	☐	☐	☐
Holding Conditions	Storage conditions (e.g., temp, protection from light), as dictated by the nature of the chemical, and standard operating procedures for chemical handling are followed	☐	☐	☐	☐	☐	☐
Sample Preparation: Effluent							
Mixing and Subsampling	Contents of each sample container are thoroughly agitated before pouring and subsamples are combined prior to use for preparing aliquots **(must)**	☐	☐	☐	☐	☐	☐
DO, pH, Salinity	Measured in unadjusted, undiluted effluent before preparation of test solutions **(must)**	☐	☐	☐	☐	☐	☐
Temp	Measured in unadjusted, undiluted effluent before preparation of test solutions and adjusted to 20 ± 2 °C if outside that range **(must)**	☐	☐	☐	☐	☐	☐
	No use of immersion heaters or microwaves **(must)**	☐	☐	☐	☐	☐	☐
Pre-aeration	None if DO measured in test sample just before test start is between 70% and 100%; if DO <70% or >100%, test sample is pre-aerated for ≤30 min at a rate of 25 to 50 mL/min·L through an air stone[1] at the end of which test solutions are prepared, organisms are introduced, and test initiated immediately, regardless of DO level **(must)**	☐	☐	☐	☐	☐	☐
Filtering	Samples are not normally filtered prior to testing; sample must be filtered if it contains organisms that might be mistaken for or predate on test organisms or if solids interfere with observation of test organisms **(must)**; filter is 1 µm; parallel tests using filtered and unfiltered samples are carried out	☐	☐	☐	☐	☐	☐
pH Adjustment	No pH adjustment of sample or test solution **(must)**	☐	☐	☐	☐	☐	☐

[1] Air stones acceptable for use are: (i) Marina®, 2.5 cm length × 1.5 cm diameter, cylindrical (one use only); (ii) AS1 silica glass, 3.8 cm length × 1.3 cm width, rectangular (re-usable after proper cleaning); or (iii) alternate air stone that has been shown to perform equivalently to the Marina® or AS1 air stone.

TEST SPECIFIC CHECKLIST

Reference Method for Determining Acute Lethality Using *Acartia tonsa*

Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Salinity Adjustment	No salinity adjustment of sample **(must)**	☐	☐	☐	☐	☐	☐
Solution Preparation	Same water is used for preparing control and all test concentrations less than 100% **(must)**	☐	☐	☐	☐	☐	☐
Sample Preparation: Chemicals							
Solution Preparation	Test solutions are typically prepared by adding aliquots of a stock solution in control/dilution water; alternatives include adding quantities of chemical directly to control/dilution water to give nominal strengths for testing; or by salinity adjustment of aqueous samples (i.e., chemical formulations in water) by adding dry ocean salts directly to the sample or test solutions to adjust the salinity to within the desired range	☐	☐	☐	☐	☐	☐
	If stock solutions are used, conc and stability of test chemical in solution is determined before the test	☐	☐	☐	☐	☐	☐
	Unstable stock solutions are newly prepared **(must)**; and stock solutions subject to photolysis are shielded from light	☐	☐	☐	☐	☐	☐
	If deionized, distilled, or fresh water is used to prepared the stock solution, dry ocean salts are used to adjust the salinity of each test solution to within the desired range	☐	☐	☐	☐	☐	☐
	Nominal concentrations are prepared and reported in consideration of any salinity adjustment **(must)**	☐	☐	☐	☐	☐	☐
	Pre-aeration normally not performed	☐	☐	☐	☐	☐	☐
Solvent	Water is the preferred solvent for preparing stock solutions; emulsifiers or dispersants are not used unless formulated with the test chemical; organic solvent is used only if no other method of test solution preparation is available	☐	☐	☐	☐	☐	☐
	Solubilizing agent is used sparingly and does not exceed the conc that affects the survival of *A. tonsa* or a maximum of 0.1 mL/L in any test solution; preliminary solvent only test is conducted if toxicity of solubilizing agent is unknown	☐	☐	☐	☐	☐	☐
	If solvent (or equivalent) is used, an additional control solution (i.e., solvent control) is prepared with the conc of solubilizing agent that is present in the most concentrated solution of the test chemical **(must)**	☐	☐	☐	☐	☐	☐
Test Conditions							
Facility and Apparatus	Tests are isolated from general disturbances **(must)**	☐	☐	☐	☐	☐	☐
	Test area is ventilated and free from physical disturbances or airborne contaminants; dust and fumes are minimized; test area is isolated from areas where test solutions are prepared or equipment is cleaned	☐	☐	☐	☐	☐	☐

TEST SPECIFIC CHECKLIST

Reference Method for Determining Acute Lethality Using *Acartia tonsa*

Parameter	Specification		Document Review			Implementation		
			Y	N	NA	Y	N	NA
Facility and Apparatus cont.	All apparatus and supplies that contact test/stock solutions or control/dilution water do not contain substances that can be leached or dissolved in amounts that adversely affect the test organism **(must)**; and minimize sorption of materials from water		☐	☐	☐	☐	☐	☐
	Lab has the instruments to monitor basic water quality (e.g., temp, sal, DO, and pH) and is prepared to accurately and promptly analyze other variables (e.g., ammonia) **(must)**		☐	☐	☐	☐	☐	☐
	Lab has a microscope and lens that allow for clear observation of nauplii and copepod eggs **(must)**		☐	☐	☐	☐	☐	☐
	All non-disposable test vessels, measurement devices, stirring equipment, and copepod-transfer equipment are clean and rinsed in accordance with standard laboratory practice **(must)**		☐	☐	☐	☐	☐	☐
	Facilities are appropriate for degree of hazard associated with samples and risk of sample and apparatus contamination		☐	☐	☐	☐	☐	☐
Test Type	Static (no renewal of test solutions) **(must)**		☐	☐	☐	☐	☐	☐
Duration	48 h **(must)**		☐	☐	☐	☐	☐	☐
Temperature	20 ± 2 °C; measured in test solutions **(must)**		☐	☐	☐	☐	☐	☐
Lighting	Same as that defined for culturing (i.e., cool white; 400 to 800 lux) **(must)**		☐	☐	☐	☐	☐	☐
Photoperiod	16 ± 1 h light: 8 ± 1 h dark **(must)**		☐	☐	☐	☐	☐	☐
DO range	70 to 100% air saturation		☐	☐	☐	☐	☐	☐
	Test is initiated after pre-aeration regardless of whether DO range is achieved		☐	☐	☐	☐	☐	☐
Aeration	Test solutions are not aerated during the test **(must)**		☐	☐	☐	☐	☐	☐
Test Vessels	24-well flat-bottom polystyrene microplate that accommodates a 1.5 to 2.2 mL per well working volume (e.g., Falcon™ Fisher Scientific, Catalogue No. 08-772-51, with a non-treated surface; 3.5-mL well volume); covered **(must)**		☐	☐	☐	☐	☐	☐
	Two test concentrations per microplate with 4 empty wells in the middle two columns of the microplate		☐	☐	☐	☐	☐	☐
	Test vessels (e.g., type, size, shape) are identical for all test solutions **(must)**		☐	☐	☐	☐	☐	☐
	Each test vessel is clearly coded or labeled as to conc and start-date and -time **(must)**		☐	☐	☐	☐	☐	☐
# Test Conc	Single-conc Test	1 (100% effluent or test solution) plus control(s) **(must)**	☐	☐	☐	☐	☐	☐
	Multi-conc Test	≥ 5 plus control(s) **(must)**	☐	☐	☐	☐	☐	☐
		Highest conc is full-strength effluent; each successive conc must have at least 50% of the strength of the next higher one **(must)**	☐	☐	☐	☐	☐	☐
# Replicates/ Conc	Single-conc Test	Minimum 30 wells (replicates) per conc **(must)**	☐	☐	☐	☐	☐	☐
	Multi-conc Test	Minimum 10 wells (replicates) per conc **(must)**; additional replicates may be used (e.g., chemical testing)	☐	☐	☐	☐	☐	☐

TEST SPECIFIC CHECKLIST

Reference Method for Determining Acute Lethality Using *Acartia tonsa*

Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
# Organisms/ Well	1 egg per well **(must)**	☐	☐	☐	☐	☐	☐
Egg Distribution	A portion of eggs are placed into Petri dishes containing test solution (i.e., concentration –specific test solution), prior to distribution to test wells containing matching test solution (i.e., test concentration) to prevent excessive dilution	☐	☐	☐	☐	☐	☐
	Eggs are added to control(s) first, and working toward highest test concentration to avoid cross-contamination	☐	☐	☐	☐	☐	☐
	Test initiation, or the time at which eggs have been added to all wells for a given concentration (i.e., rolling start time) is recorded for each concentration **(must)**	☐	☐	☐	☐	☐	☐
	All wells are checked using a microscope to confirm that only a single egg has been added to each well and appropriate action is taken if more than one egg is in each well **(must)**;	☐	☐	☐	☐	☐	☐
Randomization	Order of concentrations on the microplate are randomized for multi-conc test **(must)**	☐	☐	☐	☐	☐	☐
	Microplates are randomly positioned within the test facility **(must)**	☐	☐	☐	☐	☐	☐
	Eggs are randomly selected for transfer to each test well **(must)**	☐	☐	☐	☐	☐	☐
Test Volume/ Loading Density	Test volume is 1.5 mL per well and identical for each well and all test solutions **(must)**	☐	☐	☐	☐	☐	☐
	Test solutions are prepared and well mixed just before use **(must)**	☐	☐	☐	☐	☐	☐
Control/Dilution Water	Same type(s) as described for culturing; preferably identical to culture water	☐	☐	☐	☐	☐	☐
	Artificial water, if used, is prepared as described for artificial culture water **(must)**	☐	☐	☐	☐	☐	☐
	Same water is used for preparing control(s) and all test solutions less than 100% **(must)**	☐	☐	☐	☐	☐	☐
	Adjusted to 20 ± 2°C prior to use **(must)**	☐	☐	☐	☐	☐	☐
	DO is 90 to 100% air saturation and not supersaturated **(must)**; aerated if necessary using vigorous aeration with oil-free compressed air and acceptable air stones	☐	☐	☐	☐	☐	☐
Control/Dilution Water (Chemical Testing)	As per effluent test; additional option includes receiving water; artificial seawater is recommended if a high degree of standardization is needed and the salinity of all test concentrations should be within 1‰ of the controls.	☐	☐	☐	☐	☐	☐
	If receiving water used as control/dilution water, a separate control using the lab's normal culture/control/dilution water is included **(must)**	☐	☐	☐	☐	☐	☐
	For multiple concurrent tests at various salinities control/dilution water is from a single source with salinities adjusted using dry salts or fresh water	☐	☐	☐	☐	☐	☐
# Controls/Test	One or more dilution-water control solutions are prepared per test **(must)**	☐	☐	☐	☐	☐	☐
	Control solution(s) and its control organisms are used for only one toxicity test and/or one effluent sample **(must)**	☐	☐	☐	☐	☐	☐

TEST SPECIFIC CHECKLIST

Reference Method for Determining Acute Lethality Using *Acartia tonsa*

Parameter	Specification		Document Review			Implementation		
			Y	N	NA	Y	N	NA
# Controls/Test cont.	Salinity Control	A salinity control (with salinity adjusted to within 1‰ of the effluent sample or highest test concentration) is included in the test if the salinity of the sample (or highest test concentration) is > 5‰ higher or lower than the salinity to which the adult copepods supplying eggs have been acclimated **(must)**	☐	☐	☐	☐	☐	☐
		Prepared as described for control/dilution water **(must)**	☐	☐	☐	☐	☐	☐
		Salinity is >4‰ and ≤ 35‰ **(must)**	☐	☐	☐	☐	☐	☐
Feeding Regime	Test organisms are not fed during the test **(must)**		☐	☐	☐	☐	☐	☐
Endpoint	Single-conc Test	Percent mortality at 48 hours reported for 30 replicates of test sample and 30 replicates of control(s) **(must)**	☐	☐	☐	☐	☐	☐
	Multi-conc Test	Mortality; 48-h LC50 and its 95% confidence limits **(must)**	☐	☐	☐	☐	☐	☐
		Dilution-water control is used for calculations in effluent tests **(must)**	☐	☐	☐	☐	☐	☐
		Method of LC50 calculation is reported **(must)**	☐	☐	☐	☐	☐	☐
Calculations (Chemical Testing)	Percent mortality for test organisms at the end of the test is calculated and reported for each test concentration, if more than 10 replicate wells are used **(must)**		☐	☐	☐	☐	☐	☐
	If solvent used: only the data from the solvent control is used to calculate the LC50, or for calculating other statistical endpoints		☐	☐	☐	☐	☐	☐
Observations and Measurements								
Monitoring Vessel	A beaker containing test solution is prepared for east test solution for measurement of required water quality parameters (temp, DO, pH and sal) at start and end of test **(must)**		☐	☐	☐	☐	☐	☐
Sample/Solutions	Appearance of sample or test solution and any obvious changes during the test are recorded		☐	☐	☐	☐	☐	☐
	Initial measurements are done after the pre-aeration period, if applied		☐	☐	☐	☐	☐	☐
Temp	At start and end of test in each test solution including control(s) as a minimum **(must)**; daily measurement is recommended		☐	☐	☐	☐	☐	☐
DO	At start and end of test in each test solution including control(s) as a minimum **(must)**		☐	☐	☐	☐	☐	☐
pH	At start and end of test in each test solution including control(s) as a minimum **(must)**		☐	☐	☐	☐	☐	☐
Salinity	At the start of the test in each test solution including control(s) as a minimum **(must)**		☐	☐	☐	☐	☐	☐
	Measured using conductivity or refractometry **(must)**		☐	☐	☐	☐	☐	☐
	Instruments for measuring salinity are properly operated and maintained as required by accreditation programs and are calibrated and verified routinely **(must)**		☐	☐	☐	☐	☐	☐
	Further investigation of effluent ion composition is done where high total dissolved solids are suspected		☐	☐	☐	☐	☐	☐

TEST SPECIFIC CHECKLIST

Reference Method for Determining Acute Lethality Using *Acartia tonsa*

Parameter	Specification		Document Review			Implementation		
			Y	N	NA	Y	N	NA
Salinity Method QA	Performance-based approach used to confirm suitability/acceptability of method **(must):**		☐	☐	☐	☐	☐	☐
	Conductivity	Calibrated daily when in use with certified conductivity standard **(must)**;	☐	☐	☐	☐	☐	☐
		A conductivity standard close to the conductivity of the effluent sample and a conductivity cell with a cell constant appropriate for use in high ionic strength solutions are used	☐	☐	☐	☐	☐	☐
		Verified to accurately measure seawater salinity using a certified seawater standard; tolerance limit for accuracy is within 1‰ **(must)**	☐	☐	☐	☐	☐	☐
		Reported conductivity accounts for temperature **(must)**	☐	☐	☐	☐	☐	☐
	Refractometry	Calibrated daily when in use with purified water at 0‰ **(must)**	☐	☐	☐	☐	☐	☐
		Verified to accurately measure seawater salinity using a certified seawater standard; tolerance limit for accuracy is within 1‰ **(must)**	☐	☐	☐	☐	☐	☐
	Verification for accuracy is carried out after calibration		☐	☐	☐	☐	☐	☐
Chemical Concentration (Chemical Testing)	Chemical conc is measured in aliquots from high, medium, and low test conc and control at beginning and end of test, as minimum; samples are preserved, stored and analyzed using appropriate methods for analysis in seawater		☐	☐	☐	☐	☐	☐
	If concentrations are measured, results are calculated and expressed in terms of measured concentrations; test solutions are characterized by the geometric mean measured concentrations to which test organisms were exposed		☐	☐	☐	☐	☐	☐
	Appearance of test solutions during preparation, and at each prescribed observation period and any obvious changes during the test are noted and recorded		☐	☐	☐	☐	☐	☐
Appearance	Any differences in appearance or behaviour when comparing exposed organisms with control organisms are noted (e.g., impaired mobility)		☐	☐	☐	☐	☐	☐
Mortality	At 24 and 48 hours using a microscope and appropriate lens **(must)**		☐	☐	☐	☐	☐	☐
	Egg hatching, copepod mobility, and missing eggs and/or nauplii are recorded **(must)**		☐	☐	☐	☐	☐	☐
	Procedures and characteristics for determining hatched eggs (clear perforation observed), copepod mobility (immobile if lacks any movement within 30 seconds of observation once located), and missing test organisms as defined in the method are followed **(must)**		☐	☐	☐	☐	☐	☐

TEST SPECIFIC CHECKLIST

Reference Method for Determining Acute Lethality Using *Acartia tonsa*

Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Mortality cont.	A test organism is considered dead if **(must)**: i) the egg is seen to be unhatched; or ii) the nauplius is immobile (as determined from a 30-second observation after locating the nauplius); or iii) the test organism is missing.	☐	☐	☐	☐	☐	☐
	Results for individual wells are pooled (each concentration with 10 replicate wells is given a score out of 10, e.g., 8/10; and with 30 replicate wells a score out of 30, e.g., 24/30) **(must)**	☐	☐	☐	☐	☐	☐
Missing Organisms	The number of missing test organisms is ≤10% of the total number of test organisms introduced at the beginning of the test	☐	☐	☐	☐	☐	☐
Multiple Test Organisms in a Well	If more than one test organism is found in a given well, each organism is evaluated independently and both (all) are included in the data analysis **(must)**; additional test organisms are reported **(must)**	☐	☐	☐	☐	☐	☐
Disposal	All surviving copepods used in the test (including controls) are disposed of at the end of the test **(must)**	☐	☐	☐	☐	☐	☐
Test Organism							
Species	*Acartia tonsa* **(must)**	☐	☐	☐	☐	☐	☐
	Taxonomic identification of species is provided and documented by qualified taxonomist or barcoding for each batch of *A. tonsa* introduced into the lab **(must)**	☐	☐	☐	☐	☐	☐
Source	Test organisms are cultured and maintained in the testing lab facility **(must)**	☐	☐	☐	☐	☐	☐
	All eggs used in a test are derived from the same population **(must)**	☐	☐	☐	☐	☐	☐
	All eggs used in a test originate from cultures that have met culture health criteria **(must)**	☐	☐	☐	☐	☐	☐
	Records accompanying each batch include: approximate quantity and source of test organisms, supplier's name(s), date of shipment, date of arrival at lab, and arrival condition (i.e., temp, DO, pH, sal and general observations on water quality and behaviour) **(must)**	☐	☐	☐	☐	☐	☐
Acclimation	New batches of *A. tonsa* are acclimated to specified physicochemical conditions (Section 2.4) and fed	☐	☐	☐	☐	☐	☐
	Copepods are acclimated to test conditions (Section 2.4) prior to testing and acclimation period immediately precedes use in a test **(must)**	☐	☐	☐	☐	☐	☐

TEST SPECIFIC CHECKLIST

Reference Method for Determining Acute Lethality Using *Acartia tonsa*

Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Acclimation cont.	Temperature: 20 ± 2°C for ≥ 2 weeks prior to testing **(must)**; rate of change ≤ 3°C/day	☐	☐	☐	☐	☐	☐
	Salinity: within 5‰ of salinity for control/dilution water to be used in the test, for ≥ 2 weeks prior to testing **(must)**	☐	☐	☐	☐	☐	☐
	Low Salinity: for testing at salinities of >4 to ≤ 10 g/kg, *A. tonsa* are acclimated to a lower salinity (e.g., 10 g/kg), and health checks at lower salinities are met prior to use for egg production **(must)**	☐	☐	☐	☐	☐	☐
	DO: 80 to 100% saturation	☐	☐	☐	☐	☐	☐
	pH: 7.5 to 8.5, assuming seawater with approximate salinity of 26 – 31 g/kg	☐	☐	☐	☐	☐	☐
	Photoperiod: constant 16 ± 1 hours light: 8 ± 1 hours dark for ≥ 2 weeks prior to testing **(must)**; Light: cool white; 400 to 800 lux	☐	☐	☐	☐	☐	☐
Age/Size	Test is initiated with eggs that are ≤ 24 hours old **(must)**	☐	☐	☐	☐	☐	☐
	Eggs are obtained from laboratory cultures that are 14-28 days old, or older cohorts if culture health criteria are met	☐	☐	☐	☐	☐	☐
	<24 hours before testing adult copepods are isolated in vessels containing clean control/dilution/culture water (Temp: 20 ± 2°C and DO: 90 – 100% **(must)**) with food (double concentration) and at stocking densities of 20-200 copepods/100 mL.	☐	☐	☐	☐	☐	☐
Health Criteria	Survival of test organisms in culture health check is ≥80% **(must)**	☐	☐	☐	☐	☐	☐
	Culture health check is based on individual eggs (≤ 24 hours old) in each of 20 wells containing 1.5 mL of fresh culture water for 48 hours **(must)**	☐	☐	☐	☐	☐	☐
	After 48 hours of incubation, egg hatching, naupliar mobility, and missing egg and/or nauplius are assessed and recorded for each well **(must)**	☐	☐	☐	☐	☐	☐
	The test organism is considered dead if the egg is unhatched, the nauplius is immobile (based on a 30-second observation after locating the nauplius), or the test organism is missing **(must)**	☐	☐	☐	☐	☐	☐
	During culture health check, microplates are kept under testing conditions **(must)**	☐	☐	☐	☐	☐	☐
	Adults used to produce eggs are cultured under similar loading conditions and feeding rates as those used to produce eggs for definitive test **(must)**	☐	☐	☐	☐	☐	☐
	A microscope is used to confirm that each well contains a single egg (≤24 hours old) **(must)**; appropriate action is taken if more than one egg is in each well	☐	☐	☐	☐	☐	☐
	The health of an age-class culture (e.g., the "14-21 days" culture) is assessed at least once and meets the health criteria before eggs from that culture are used in the test **(must)**; where there are multiple vessels of the same age-class, the health check may be carried out using only one of the culture vessels	☐	☐	☐	☐	☐	☐
	Eggs used in a definitive test are traceable back to a valid culture health check **(must)**	☐	☐	☐	☐	☐	☐

TEST SPECIFIC CHECKLIST

Reference Method for Determining Acute Lethality Using *Acartia tonsa*

Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Culture Conditions							
Facility and Apparatus	Culture vessels and accessories contacting organisms, water, or culture media are made of nontoxic materials **(must)**	☐	☐	☐	☐	☐	☐
	Glass aquaria, beakers, or wide-mouth jars (e.g., 500 mL to 2 L) are used as culture vessels and are loosely covered	☐	☐	☐	☐	☐	☐
	Culturing is isolated from physical disturbances and separated from test area	☐	☐	☐	☐	☐	☐
Water Temperature	20 ± 2 °C **(must)**	☐	☐	☐	☐	☐	☐
DO and Aeration	Continuous gentle aeration of cultures **(must)**; DO maintained at 80 to 100%; aeration using filtered, oil-free compressed air; vigorous aeration is avoided; supersaturation (if any) is remedied	☐	☐	☐	☐	☐	☐
Salinity	Cultured at a salinity that is appropriate for culture health and acclimation to the salinity of test samples	☐	☐	☐	☐	☐	☐
pH	7.5 to 8.5, assuming seawater with approximate salinity of 26 – 31 g/kg	☐	☐	☐	☐	☐	☐
Lighting	Cool white; 400 to 800 lux	☐	☐	☐	☐	☐	☐
Photoperiod	16 ± 1 h light: 8 ± 1 h dark **(must)**	☐	☐	☐	☐	☐	☐
Water Quality	Consistently supports good survival, reproduction, and health of *A. tonsa* **(must)**; Uncontaminated natural or reconstituted/artificial seawater	☐	☐	☐	☐	☐	☐
	Natural seawater is filtered (e.g., ≤ 1µm) to remove particulates and indigenous organisms **(must)**; aerated, if necessary	☐	☐	☐	☐	☐	☐
	Artificial seawater is made up to desired salinity by adding commercially available dry ocean salts to suitable fresh water and by mixing thoroughly during addition **(must)**	☐	☐	☐	☐	☐	☐
	Sources of water for preparing artificial seawater are deionized or distilled water; uncontaminated ground or surface water; or dechlorinated municipal drinking water	☐	☐	☐	☐	☐	☐
	Dechlorinated water, if used, is free of any harmful concentration of chlorine or chlorinated compounds upon organism exposure **(must)**[2]	☐	☐	☐	☐	☐	☐
	Water is not supersaturated with gases **(must)**	☐	☐	☐	☐	☐	☐
	Artificial seawater is aerated continuously and vigorously for ≥12 h before use **(must)**; longer periods (≥3 days) are recommended; and may be filtered prior to use to remove undissolved salts	☐	☐	☐	☐	☐	☐

[2] The CCME guideline value for total residual chlorine (TRC) for the protection of marine life is ≤0.5 µg/L. Analytical techniques used to measure TRC in the treated supply of dechlorinated water should ideally have detections limits low enough to assure that TRC is below the guideline, however this might be unrealistic for methods used in the laboratory for routine measurements. STB 1/RM/60 indicates that the use of equipment that can measure TRC down to 20 µg/L is acceptable as this level has been shown not to affect *A. tonsa* health.

TEST SPECIFIC CHECKLIST
Reference Method for Determining Acute Lethality Using *Acartia tonsa*

Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Water Quality cont.	Salinity is measured using conductivity or refractometry **(must)**	☐	☐	☐	☐	☐	☐
	Commercially available sea salts used to prepare artificial seawater have previously been shown to support good survival, reproduction and health of *A. tonsa*	☐	☐	☐	☐	☐	☐
	Seawater supply is monitored as frequently as required to document quality and variation for: sal, pH, DO, and TRC (if using dechlorinated water), as a minimum; and as appropriate suspended solids, total organic carbon, ammonia, metals, and pesticides	☐	☐	☐	☐	☐	☐
	Method and duration for storage of batches of seawater (natural and artificial) are appropriate	☐	☐	☐	☐	☐	☐
Handling/Transfer	Minimal and appropriate handling is practiced to minimize damage or drying out	☐	☐	☐	☐	☐	☐
	Adults can be handled by gently pouring or by careful pipetting or siphoning (3 – 5 mm opening); Eggs can be transferred using a 1-2 mL pipette with narrow opening (~ 1mm)	☐	☐	☐	☐	☐	☐
	Tip of pipette is under surface when copepods or eggs are released and transfers are done quickly with minimal carryover of “old” water	☐	☐	☐	☐	☐	☐
Feeding	Copepods in all culture vessels are fed with *Rhodomonas salina* **(must)**	☐	☐	☐	☐	☐	☐
	Culture vessels are fed ≥ 3 times weekly with an amount of *R. salina* that supports continual growth and reproduction **(must)**; daily feeding is recommended	☐	☐	☐	☐	☐	☐
	Ration for daily feeding is 6 to 60 million *R. salina* cells per L of *A. tonsa* culture water; ration for 3 times weekly feeding is 14 to 140 million cells per L of *A. tonsa* culture water; culture water has slight pink or red colour after feeding	☐	☐	☐	☐	☐	☐
	A double feed ration prior to testing is provided to promote a larger production of eggs	☐	☐	☐	☐	☐	☐
	Guidance for culturing *R. salina* for *A. tonsa* provided in Appendix E is followed	☐	☐	☐	☐	☐	☐
Water/Culture Renewal	Cultures are not renewed or sorted during first week following arrival at the lab	☐	☐	☐	☐	☐	☐
	Culture vessels are renewed weekly by starting new cultures with 100% renewal of culture water **(must)**	☐	☐	☐	☐	☐	☐
	During renewal copepods are separated into age- and size classes (i.e., age-class cultures) in new and labelled culture vessels **(must)**; eggs from all age classes can be combined to start a new 0-7-day culture	☐	☐	☐	☐	☐	☐
	Sieves are used (stacked or sequentially) to separate age-class cultures; *A. tonsa* are kept moist during renewal; water velocity passed through sieves is minimal	☐	☐	☐	☐	☐	☐
	Older cohorts (i.e., ≥ 28 day-old age class) are discarded, held as a back-up culture, or may be used as a continued source of eggs if repeated culture health check continues to meet health criteria	☐	☐	☐	☐	☐	☐
	Organisms density is typically 100 to 500 organisms per L, but can be higher (e.g., 2000/L)	☐	☐	☐	☐	☐	☐
	Mixed-age mass culture vessels may be maintained as backup; during renewal of mass cultures all age classes can be combined	☐	☐	☐	☐	☐	☐

TEST SPECIFIC CHECKLIST

Reference Method for Determining Acute Lethality Using *Acartia tonsa*

Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Monitoring	Water temp, DO, sal, pH, aeration, culture density, and light intensity are monitored in each culture vessel at regular intervals **(must)**	☐	☐	☐	☐	☐	☐
	Copepods in cultures are observed periodically for normal swimming behaviour and reasonable body size	☐	☐	☐	☐	☐	☐
QA/QC							
Validity Criteria	Test is invalid if >20% control organisms die **(must)**	☐	☐	☐	☐	☐	☐
	Results for each set of controls used in a test are examined to determine if they independently meet the test validity criteria **(must)**	☐	☐	☐	☐	☐	☐
	Tests using salinity control: test is invalid if results in either salinity control or dilution-water control fail to meet validity criteria **(must)**	☐	☐	☐	☐	☐	☐
	Tests using solvent control: test is invalid if results in either solvent control or dilution-water control fail to meet validity criteria **(must)**	☐	☐	☐	☐	☐	☐
Reference Toxicant	Reagent grade nickel; 48-h LC50 is determined and expressed as mg/L	☐	☐	☐	☐	☐	☐
	Nickel stock solutions are prepared on day of use or shown to remain stable if stored	☐	☐	☐	☐	☐	☐
	Frequency is within 14 d of test start of toxicity test using the lab's established cultures, and upon acclimation of a new batch of *A. tonsa* **(must)**	☐	☐	☐	☐	☐	☐
	Test is performed using the same conditions, procedures and culture/control/dilution water as that used in the effluent test **(must)**	☐	☐	☐	☐	☐	☐
	Concentrations of stock solutions and the control, low, medium, and high test concentrations are measured chemically using appropriate methods, or stored for future analysis	☐	☐	☐	☐	☐	☐
	If stored, ref. tox. aliquots are held in the dark at 4 ± 2°C **(must)**; nickel solutions are acidified before storage, and stored aliquots analyzed promptly if required	☐	☐	☐	☐	☐	☐
	LC50 calculations are based on measured concentrations if they differ (i.e., ≥ 20%) from nominal ones and if the accuracy of the analyses is satisfactory	☐	☐	☐	☐	☐	☐
Warning Chart	Prepared using 48-h LC50 results and continually updated with each new reference toxicity test **(must)**	☐	☐	☐	☐	☐	☐
	Log conc used in all calculations of mean and standard deviation **(must)**; and in all plotting procedures	☐	☐	☐	☐	☐	☐
	Each new LC50 for the reference toxicant is compared with previously established limits of the chart	☐	☐	☐	☐	☐	☐
	LC50 is acceptable if within warning limits (± 2 SD on log scale)	☐	☐	☐	☐	☐	☐

TEST SPECIFIC CHECKLIST

Reference Method for Determining Acute Lethality Using *Acartia tonsa*

Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Warning Chart cont.	Laboratory observes trends (e.g., increasing or decreasing) and variation (i.e., reasonable spread of warning limits) for warning chart; and appropriate actions are taken if an LC50 falls outside the warning limits and/or outside control limits (± 3 SD on log scale)	☐	☐	☐	☐	☐	☐
Test Report (all items here are required, i.e. must be reported)							
Effluent or Chemical	Name and location of operation generating the effluent	☐	☐	☐	☐	☐	☐
	Date and time of sampling	☐	☐	☐	☐	☐	☐
	Type of sample (e.g., whole effluent, final mill effluent, etc.) or coding as provided to the laboratory personnel	☐	☐	☐	☐	☐	☐
	Information on labelling or coding for each sample	☐	☐	☐	☐	☐	☐
	Brief description of sampling point	☐	☐	☐	☐	☐	☐
	Sampling method (e.g., grab, batch, 24-h composite etc.)	☐	☐	☐	☐	☐	☐
	Name of person(s) collecting sample	☐	☐	☐	☐	☐	☐
	Date and time sample received at test facility and temp of sample upon receipt	☐	☐	☐	☐	☐	☐
Test Facilities and Conditions	Test type and method (e.g., single-concentration as specified in STB 1/RM/60)	☐	☐	☐	☐	☐	☐
	Name and city of testing laboratory	☐	☐	☐	☐	☐	☐
	Species of test organism	☐	☐	☐	☐	☐	☐
	Date and time for start of toxicity test	☐	☐	☐	☐	☐	☐
	Person(s) performing the test and verifying the results	☐	☐	☐	☐	☐	☐
	The pH, temp, DO, and salinity of unadjusted, undiluted effluent, just before preparing test solutions	☐	☐	☐	☐	☐	☐
	Method used (with citation) for measuring salinity of effluent (or chemical sample), control/dilution water, and test solutions	☐	☐	☐	☐	☐	☐
	Indication if sample or solution was filtered; indication if any parallel tests with unfiltered sample or solution were performed (see Section 4.3)	☐	☐	☐	☐	☐	☐
	Confirmation that no adjustment of sample or solution pH occurred; indication of procedure used for any pH adjustment if both pH-adjusted and non-adjusted tests were run (see Section 4.2)	☐	☐	☐	☐	☐	☐
	Confirmation that no adjustment of sample or solution salinity occurred; indication if any parallel test run using salinity-control water as dilution water (see Section 4.2)	☐	☐	☐	☐	☐	☐
	Indication of aeration of test sample (rate and time) before introduction of test organisms	☐	☐	☐	☐	☐	☐
	Concentrations and volumes tested, including control(s)	☐	☐	☐	☐	☐	☐
	Number of eggs added to each microplate well; number of microplate wells per concentration	☐	☐	☐	☐	☐	☐

TEST SPECIFIC CHECKLIST
Reference Method for Determining Acute Lethality Using *Acartia tonsa*

Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Test Facilities and Conditions cont.	Indication if any additional test organisms were observed in a microplate well at the end of the test and, if so, how the data were analyzed	☐	☐	☐	☐	☐	☐
	Measurements of DO, pH, and temp determined for each test solution including control(s) at the beginning and end of the test, as a minimum; as well as salinity of each test solution at the beginning of the test	☐	☐	☐	☐	☐	☐
	Results of culture health check(s) (i.e., % mortality) conducted for the age-class culture to be used as the source of eggs in the definitive test	☐	☐	☐	☐	☐	☐
	Age of adults (i.e., age-class culture) used as source of eggs for the test and age of eggs at the start of the test	☐	☐	☐	☐	☐	☐
Results	Numbers of unhatched eggs, immobile nauplii, and missing test organisms in each concentration, including the control(s), at 24 hours	☐	☐	☐	☐	☐	☐
	Number of dead test organisms (report numbers of unhatched eggs, immobile nauplii, and missing test organisms) in each concentration, including the control(s), at 48 hours	☐	☐	☐	☐	☐	☐
	Percent mortality of *A. tonsa* in test concentration(s) and control(s), at 48 hours, for a single-concentration	☐	☐	☐	☐	☐	☐
	Estimate of 48-h LC50 and 95% confidence limits in multi-conc tests, if statistically achievable; methods used for calculating statistical endpoints	☐	☐	☐	☐	☐	☐
	Most recent 48-h LC50 (with 95% confidence limits) for reference toxicity test(s); reference chemical(s); date test initiated; historic geometric mean LC50 and warning limits (± 2 SD)	☐	☐	☐	☐	☐	☐
	Anything unusual about the test, any problems encountered, and any remedial measures taken	☐	☐	☐	☐	☐	☐
Deviations	Deviations from any “must” requirements are reported and details provided	☐	☐	☐	☐	☐	☐
Information Kept On-File	Do lab SOPs indicate that the additional reporting requirements in Section 9.2 of the STB 1/RM/60 method must be kept on file for 5 years? For details of this information, see STB 1/RM/60, section 9.2.	☐	☐	☐	☐	☐	☐

Notes: