Shaded Text reflects February 2011 method amendment changes.

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

Test of Larval Growth and Survival Using Fathead Minnows

Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Sample/Preparation							
Filtering/Decanting	None; if indigenous organisms, filter through a sieve with 60 µm mesh openings **(Must)**; if high concentration of suspended solids, run a concurrent 2nd test with a portion of the sample treated by filtering or decanting						
D.O.	Measured in each sample/subsample prior to test initiation **(Must)**						
Pre-aeration	Only if D.O. of test solution is < 40% or > 100% upon preparation, in which case pre-aerate all solutions for the lesser of 20 min and attaining 40% of air saturation in the highest test conc.; test initiated at ≤ 20 min regardless of whether D.O. of 40 - 100% was achieved **(Must)**						
	Rate of pre-aeration minimal and controlled **(Must)**; not exceeding 100 bubbles/min per test vessel						
pH adjustment	pH measured in each sample/subsample prior to test initiation **(Must)**						
	No adjustment if pH of test solution is within 6.5 - 8.5; a second (pH adjusted) test might be required if pH is beyond this range						
Temp. Adjustment	T° to be measured in sample/subsample on arrival at lab **(Must)**						
	Sample/subsample adjusted to 25 ± 1°C before use **(Must)**						
	No use of immersion heaters **(Must)**; water bath recommended						
Test Conditions							
Facility	Isolated from general laboratory disturbances **(Must)**						
	Instruments available to measure basic water quality variables (T°, D.O., pH, conductivity) and lab prepared for other analysis (ie: hardness, alkalinity, ammonia and residual chlorine if municipal water) **(Must)**						
Test Type	Static Renewal						
Duration	7 days						
Temperature	Daily mean of 25±1°C (determined each day of test on all fresh and aged test solutions) with extreme fluctuations within range 23-27 °C **(Must)**						

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Test of Larval Growth and Survival Using Fathead Minnows

Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Lighting	Broad spectrum (fluorescent or equivalent); 100 - 500 lux at surface						
Photoperiod	16 ± 1h light; 8 ± 1h dark; gradual transition preferable						
	To coincide with that at which parent fish were held **(Must)**						
In-test pH	No adjustment if pH of test solution is between 6.5-8.5						
D.O. range	40-100% air saturation						
Aeration	Normally no aeration during test						
	If D.O. ≥ 40% is an objective of the study then aeration is minimal and controlled **(Must)**; and not exceeding 100 bubbles/min per test vessel						
Vessel Size & Type	Beakers, rectangular containers of borosilicate glass, perfluorocarbon plastic or disposable polystyrene; diameter of vessel approximate depth of test solution						
	Identical for each test solution in a given test						
	Covered during test						
Test Volume	≥250mL **(Must);** preferably 500 mL; water depth ≥ 3 cm						
Renewal of Solution	≥ 80% of solution renewed at 24 h intervals for test duration **(Must)**						
	Dead brine shrimp and detritus removed; new test solution added slowly and cautiously to avoid injury to the fish						
	Each test solution well mixed **(Must)**						
Dilution/Control Water	Uncontaminated ground, surface, or dechlorinated municipal water, or reconstituted water; D.O. 90 - 100% air saturation at time of use						
	Adjusted to 25 ± 1°C before use **(Must)**						
	Not supersaturated						
	For definitive test, control solution(s) are to be prepared at the same time as the experimental treatments, using an identical # of replicates **(Must)**						
	Dilution water used to prepare test conc. is also to be used for preparing one set of controls **(Must)**						
	If water other than lab water supply that is normally used to culture the breeding stock is used as control/dilution water, a second control is to be set up using the lab water supply **(Must)**						
Vessel Identification	Each vessel clearly coded or labelled to identify material or substance and concentration being tested, and date & time of test initiation **(Must)**						
# Test Conc.	≥7 plus control to calculate ICp for growth inhibition by regression analyses and LC50 **(Must)**; ≥8 plus control are recommended						
	1 plus control for single conc. test						

TEST SPECIFIC CHECKLIST

Test of Larval Growth and Survival Using Fathead Minnows

Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
# Replicates/Conc.	≥3 replicates per test conc. and per control(s)**(Must)**; ≥4 replicates per test conc. and per control(s) recommended						
	Test start with equal number of replicates for each test conc. including controls **(Must)**						
# Organisms/ Vessel	≥10 fish per test replicate with equal number in each replicate **(Must)**						
	Attempt made to achieve homogeneity of the experimental units in assigning fish to vessels **(Must)**						
	Randomization of each group of 10 or more larvae to each test vessel **(Must)**						
Vessel Randomization	Each group of replicate vessels for each treatment placed in random position in water bath **(Must)**						
Removal of Dead	Dead organisms discarded						
Feeding Regime	Feed 2-3 times/day during test with newly hatched brine shrimp nauplii (~1500-2500 per day); begin feeding schedule at test start and do not feed during final 12 h of the test **(Must)**						
Vessel Cleaning	All test vessels, measurement and stirring devices and fish transfer pails thoroughly cleaned and rinsed **(Must)**						
	Control/dilution water used in final rinse						
Chemical Testing	Solubilizing agent control solution is to be run, if used **(Must)**						
	Solubilizing agent concentration not exceed 0.1mL/L						
	Unstable stock solutions are prepared daily or as frequently as is necessary to maintain consistent concentrations for test solution renewal **(Must)**						
	Stock solution prepared by dissolving chemical in control/dilution water						
	Test conc. measured at beginning and end of renewal periods on the first and last days of the test, in high, medium and low strengths and in control						
Biological Endpoints	Survival based on increased mortality of the contaminant-exposed larval fish and growth based on the reduction in the biomass of contaminant-exposed larval fish; biomass is the total dry weight of fish surviving to test end in each vessel, divided by the # of larvae that were placed in the vessel at the start of the test, presumably 10 **(Must)**						
Statistical Endpoints	The (cumulative) Mean (± SD) % mortality for larvae for each treatment and control at test end **(Must)**						
	The (cumulative) Mean (± SD) biomass of live larvae for each treatment and control at test end **(Must)**						
	For multi conc. test, 7-day LC50 for mortality and 7-day ICp for decreased biomass for surviving fish (both with their 95% confidence limits) **(Must)**						

TEST SPECIFIC CHECKLIST
Test of Larval Growth and Survival Using Fathead Minnows

Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Statistical Endpoints cont.	A value of zero is assigned for biomass in a replicate if all fish in that replicate die during the test **(Must)**						
	If larva(e) are accidentally lost or damaged during exposure, it (they) are deducted from the initial number of larvae for the calculation of biomass for that replicate **(Must)**						
	All statistical tests used to derive endpoints enter concentrations as logarithms **(Must)**						
Calculation of ICp	Calculation of ICp by entering conc. as logarithms **(Must)**						
	Initial plot of raw data for biomass or % inhibition at each test concentration against log concentration						
	Regression analysis used for calculation of ICp & 95% confidence limits following guidance in Section 4.5.1 of EPS 1/RM/22 & EC 2005; assumptions for normality and homoscedasticity are met **(Must)**						
	Data from high test concentrations resulting in zero surviving larvae in all test replicates are removed before performing regression analyses **(Must)**						
	Data assessed for outliers and their removal justified **(Must)**						
	Lab attempts to fit more than one model to the data and the one with the best fit is chosen for calculation of ICp and confidence limits **(Must)**						
	Endpoints generated by regression analysis are bracketed by test concentrations; endpoint is not extrapolated beyond highest test concentration **(Must)**						
	Hormesis data entered directly for regression (no trimming of data points)						
	ICPIN used to derive ICp only if data do not allow regression statistics						
Observations and Measurements							
D.O., pH, Temp.	At least at start and end (just before or immediately after renewal) of each 24-hour exposure in representative concentrations (high, medium, low, and controls) in both the fresh and used solution **(Must)**						
Conductivity	Measured in each newly-prepared test solution prior to dispensing it to the test vessels						
Hardness	Control and highest test concentration, at least before starting test						
Mortality	Mortality in each test vessel determined from a count of swimming larvae at intervals of 24 h from the start until the end of test at 7 d of exposure **(Must)**						
	For controls- daily observations of combined incidence of fish dead, dying, showing loss of equilibrium or atypical swimming behaviour **(Must)**						
Growth	Mean dry weight of surviving fish at 7 d for each test vessel **(Must)**						
	Fish dried immediately at 100 °C for 6 h or at 60 °C for 24 h						
	Upon removal from oven, boats moved immediately to dessicator **(Must)**						

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		Y	N	NA	Y	N	NA
Growth cont.	Thereafter, the boats be individually and randomly removed from the dessicator and weighed on a balance the measures consistently to 10 µg						
	Rapid weighing and standard timing among weigh boats is necessary						
	Growth, mortality and swimming behaviour of fish in the lab control water be compared to that in the sample of receiving water, if receiving water used as control/dilution **(Must)**						
Test Organism							
Species	*Pimephales promelas*						
Source	Disease-free stock from another laboratory, captured in the wild if special care taken in identifying species and eliminating disease						
	If test organisms received from a supplier, confirmation of species made by qualified taxonomist **(Must)**						
	Embryos or larvae (hatched for ≤ 24h) imported for immediate testing, follows EC's 1999 procedures for test organism importation **(Must)**						
	If test organisms imported, temp. & D.O. within shipping containers measured & recorded upon departure and on arrival **(Must),** Temp. maintained at/near test conditions and no change >3°C during any 24-h period in transit, D.O. ≥80% saturation **(Must)**						
	If imported, recommended they are transported as newly-eyed embryos rather than young						
	Each shipment of imported test organisms contains written statement with age, date and time of shipment **(Must)**						
Age	Larval fathead minnows hatched for ≤ 24h, and inflated swim bladder evident at start of test **(Must)**						
	Test organisms should represent ≥3 spawnings						
	All larval fish used in a test must be from the same batch **(Must)**						
	Larvae not fed until after transfer to test vessels (i.e., test start)						
	Fish appearing abnormal are not selected for the test **(Must)**						
Health Criteria	If test organisms are imported, mortality rate for larval fish ≤10% **(Must)**. To determine either count # larvae hatched from batch (if eyed eggs shipped) or total # live & dead (if young larvae shipped) and compare to # live prior to transfer to test vessel						
	Mortalities < 5% of general population and of fish in individual tanks during 7 days preceding eggs collection **(Must)**						

TEST SPECIFIC CHECKLIST
Test of Larval Growth and Survival Using Fathead Minnows

Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Health Criteria cont.	If the 7-d mortality rate is between 5 and 10%, holding of fish extended for at least another 7d before collection of eggs, until <5% mortality in 7d is realized **(Must)**						
	If combined incidence of mortalities and disease of adult breeding stock >10% per week at any time, that stock not to be used to produce test fish **(Must)**						
	Two dozen pairs of spawning adults should provide ≥ 200 embryos per day on average and 500 or more per day under good conditions						
Culture/Holding Conditions							
Temperature	Holding 4-26°C; culture 25°C (22-26°C); rate of change ≤ 3°C/day						
pH	6.8 - 8.5 (7.0 - 8.5 preferred)						
D.O.	Culture water aerated to maintain 80-100% saturation; not supersaturated						
Lighting	Broad spectrum (fluorescent or equivalent); 100 - 500 lux at water surface						
Photoperiod	16 ± 1h light; 8 ± 1h dark; gradual transition between light and dark						
Feeding	Adults: 1 time daily; frozen brine shrimp supplemented by commercial pelleted or flaked food; rate judged by amount consumed in 10 min (~1-5% wet body weight)						
	Newly hatched fish to be raised as future breeding pairs:≥2 times daily with nauplii of brine shrimp; at 30 days, weaned to frozen brine shrimp						
Cleaning	Siphoning of debris daily or as required						
	Tanks disinfected before introducing new batch of fish						
	Spawning tiles disinfected, scaled and rinsed before reuse						
Water	Uncontaminated ground, surface, dechlorinated municipal water, or reconstituted water; not supersaturated						
	Remedial measures taken if dissolved gases >100% saturation **(Must)**						
	Surface water filtered (≤ 60 µm), if used						
	If reconstituted water is to be used as control/dilution water, adult fish must be acclimatised to that reconstituted water for ≥5 days immediately before embryos are obtained for the test **(Must)**						
	Flow to culture aquaria ≥ 1.4 L/g fish per day						
	Total Residual Chlorine ≤ 0.002 mg/L; Un-ionized ammonia ≤ 0.02 mg/L; nitrite ≤ 0.06 mg/L; all measured weekly						
	Temperature, D.O., pH and flow monitored in each tank daily						
Morbidity/Mortality	Adult and pre-adult fish being cultured inspected daily for signs of disease						
	Mortality and disease monitored and recorded at least 5 d/w **(Must)**						
	Dead and moribund individuals removed immediately						

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Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Disease Control	Strongly recommended that groups of fish showing signs of disease be discarded rather than treated						
	If the use of chemically-treated fish can not be avoided, allow ≥ 2 weeks before collecting eggs for use in test						
	Records kept of any treatment of breeding stock for disease prevention or control **(Must)**						
Acclimation	Fish kept at 22-26℃ for ≥2 weeks before using their embryos to obtain larvae for test						
Obtaining Eggs	One spawning substrate per male fish in breeding tanks						
	Daily inspection of tiles mid-morning recommended						
	If embryos, tile should be removed and placed in hatching tray						
	Fish replaced if 3-week period without eggs						
	Automatic replacement of fish on fixed scheduled (eg: 3 to 6 months)						
Hatching Eggs	Aerate tiles in hatching tray						
	Inspect incubating embryos daily **(Must)**						
	Remove and discard dead embyros or those with fungus **(Must)**						
	Minimal disturbance on days 3-5						
Gene pool	Larvae for future spawning stock selected from different parents; gene pool supplemented every 2 years with larvae from another lab						
Facility & Apparatus	Vessels and accessories contacting organisms and culture media made of non-toxic material **(Must)**						
	Culture facility located away from physical disturbances and preferably separate from test containers						
QA/QC							
Validity Criteria	Test is terminated and declared invalid if, for controls, the combined (for all replicates) and cumulative (over time) incidence of any mortalities, moribund fish, or fish showing loss of equilibrium, or any other signs of atypical swimming behaviour is >20% at any period of observation, including that at test end **(Must)**						
	Test results are also invalid if average dry weight of surviving control fish is < 250 µg at test end **(Must)**						
	If solvent control used, the test is declared invalid if above validity criteria for survival and dry weight are not met for either the solvent control solutions or those comprised solely of untreated control water **(Must)**						

TEST SPECIFIC CHECKLIST

Test of Larval Growth and Survival Using Fathead Minnows

Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Reference Toxicant	Started within 14 days before or after the start of the definitive test, or run concurrently with definitive test; following the same method and procedures used for the definitive test **(Must)**						
	Ideally with larvae from the same stock of brood animals used to generate larvae for the definitive test						
	For reference toxicant test run concurrently with definitive test, the same batch of larvae and experimental conditions are used for both tests **(Must)**						
	Larvae from each batch of imported test organisms are tested in a reference toxicant test run concurrently with the definitive test, and both tests are run under the same experimental conditions **(Must)**						
	Standard test with LC50 and ICp endpoints **(Must)**						
	Reagent-grade sodium chloride, phenol, and/or zinc sulphate						
	LC50 for survival and ICp for biomass are evaluated for at least one reference toxicant chemical **(Must)**						
	Using the control/dilution water that is customary at the lab, or reconstituted water if a greater degree of standardization is desired						
Warning Chart	Prepared for each reference toxicant and continually updated **(Must)**						
	LC50 and/or ICp is/are acceptable if within warning limits (± 2 SD on log scale)						
	Log concentration used for mean and SD calculations and plotting **(Must)**						
	If LC50 or ICp are outside warning limits, a thorough check of culture health and conditions is carried out, and depending on findings the reference toxicity test is repeated, new breeding stock is obtained and/or new culture is established before undertaking any further toxicity tests						
Sample Handling							
Sample Collection	For off-site effluent and leachate tests either ≥ 3 subsamples from a single sample or, if toxicity of wastewater is known to change if stored up to 7-10d, ≥ 3 fresh samples collected every 2-3 d or less **(Must)**						
	For on-site effluent and leachate tests, samples collected daily and used within 24 h						
Containers	Non-toxic materials for sample and transport containers **(Must)**						
	New containers or thoroughly rinsed if used containers **(Must)**						
	Collapsible polyethylene or polypropylene containers recommended						
Volumes	Volumes of 8-10L						
Labelling	Upon collection, sample containers filled, sealed and labelled/coded **(Must)**						

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Test of Larval Growth and Survival Using Fathead Minnows

Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Labelling cont.	Include at least sample type, source, date and time of collection and name of sample collectors						
Holding Time	Test to be initiated within 3 days after sample collection or elutriate extraction **(Must)**						
	Recommend test initiation within 1 day of sample collection or elutriate extraction						
Holding Conditions	Make effort to keep samples cool throughout their period of transport **(Must)**, in the dark, at 1 - 7°C (preferably 4 ± 2°C) using regular ice or frozen gel packs						
	Upon collection, if sample > 7 °C, cool to 1 - 7°C with regular ice or frozen gel packs (not dry ice) **(Must)**						
	Sample are kept from freezing during transport or storage **(Must)**						
	The portion of sample/subsamples required for test and solution renewals are stored in darkness in sealed containers without air headspace at 4± 2°C **(Must)**						
Sample Aliquots	Each sample or subsample in a collection container are agitated thoroughly just before pouring **(Must)**						
EPS 1/RM/22	Have February 2011 amendments been incorporated into Standard Operation Procedures (SOPs)?						
Test Report							
Sample/subsample	Brief description of sample type and volume or weight (if a dry chemical) if and as provided to the lab **(Must)**						
	Information on labelling or coding, for each sample/subsample **(Must)**						
	Date of sample/subsample collection; date and time sample(s)/subsample(s) received at test facility **(Must)**						
	Dates or days during test when individual samples or subsamples used **(Must)**						
	For effluent or leachate, temperature of sample upon receipt at lab **(Must)**						
	D.O. and pH of sample just before its preparation and use **(Must)**						
	Date of elutriate generation and procedure for preparation **(Must)**						
Test Organism	Species and source of breeding stock and test larvae **(Must)**						
	Age of larvae (i.e., hours since hatched) at start of test **(Must)**						
	Brief statement confirming larvae have inflated swim bladders **(Must)**						
	Any unusual appearance, behaviour, or treatment of larvae, before their use in the test **(Must)**						
	Data for breeding stock (including that if test organisms are imported) showing combined incidence of mortalities and disease on a weekly basis, up to and including the 7-d period preceding the test **(Must)**						

TEST SPECIFIC CHECKLIST

Test of Larval Growth and Survival Using Fathead Minnows

Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Test Organism cont.	Larval mortality rate (Must be <10% over period between receipt of shipment and just prior to transfer to test vessel) for any batch of embryos/larvae imported **(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/Dilution Water	Type and source of water used as control and dilution water **(Must)**						
	Type and quantity of any chemical(s) added to control or dilution water **(Must)**						
Test Method	Citation of biological test method used **(Must)**						
	Description of procedures in those instances in which a sample, subsample, or test solution has been filtered, settled and decanted, or adjusted for hardness or pH **(Must)**						
	Design and description if specialized procedure **(Must)**						
	Frequency and type of all observations/measures made during test **(Must)**						
	Programs 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, conc., volume, and depth of solutions in test vessels including controls **(Must)**						
	Number of individuals per test vessel, and number of replicates **(Must)**						
	Procedure, rate, and duration if any pre-aeration of test solutions **(Must)**						
	Rate and duration of aeration of test solutions **(Must)**						
	Dates when test was started and ended **(Must)**						
	All measures of temp., pH and D.O. in solutions, during the test **(Must)**						
	Statement indicating whether reference toxicity test performed under same experimental conditions as test sample & description of any deviation from test method document **(Must)**						
Test Results	# and % of mortalities in each replicate test vessel as recorded during each 24-h observation period over the 7 days **(Must)**						
	Mean (± SD) % mortality for each treatment at the end of the test **(Must)**						
	Combined and cumulative (over time) mean (± SD) % of control fish that either died, appeared moribund, displayed loss of equilibrium, or showed clearly-atypical swimming behaviour, at each period of observation including at the end of test; avg. dry weight per surviving control larva at test end, as used for the dry-weight test validity criterion **(Must)**						
	Mean (± SD) biomass for each treatment including the controls, at test end, as used for the ICp calculation **(Must)**						

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Test of Larval Growth and Survival Using Fathead Minnows

Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Test Results cont.	LC50 and 95% confidence limits and indication of quantal method used; ICp and 95% confidence limits for the biomass data; details regarding any transformation of data that was required, and indication of quantitative method used **(Must)**						
	Any outliers and the justification for their removal **(Must)**						
	Results/duration of any toxicity tests with reference toxicants performed at the same time or within 14 days of the start 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 **(Must)**						
	Anything unusual about the test, any problems encountered, any remedial measures taken **(Must)**						
Original Data Sheets	Original data sheets are signed or initialled, and dated by the laboratory personnel conducting the test						
Information Kept On-File	Do Lab SOPs indicate that the information on Section 8.2 of the EPS 1/RM/22 method must be kept on file for 5 years? **(Must)** *For details on this information, see EPS 1/RM/22, section 8.2.						

Notes: