

TEST SPECIFIC CHECKLIST

Revised: July 2014

Test of Reproduction and Survival Using the Cladoceran *Ceriodaphnia dubia*

N.B. *Shaded text* reflects February 2007 2nd edition changes

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Parameter	Specification	Met Specifics?		
		Y	N	NA
2 nd edition EPS 1/RM/21	Has the laboratory incorporated all of the changes in the 2 nd edition of EPS 1/RM/21 into their SOPs?
Sample Preparation				
Filtering	None; if indigenous organisms, filter through a sieve with 60 µm mesh openings (Must)
D.O. Measurement ...	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 not exceeding 100 bubbles/min per test vessel; minimal and controlled (Must)
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
T° 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
Hardness Adjustment	No adjustment; a second adjusted test could be run
Test Conditions				
Test Facility	Isolated from general disturbances and from culture area
	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; at least once daily (Must)
Test Duration	Test ends when 60% or more of first generation adults in control solutions have had 3 broods, or at the end of 8 days, whichever occurs first (Must)
	2 or more neonates in any test vessel during any day of the test constitutes a brood (Must)
	When a single neonate is produced the day before or the day after one or more neonates are counted (as a brood) in the same control vessel, it is scored as part of the neonate count for the following or previous day (Must)
Test T°	Daily mean temperature of 25 ± 1°C (Must)
Light Quality	"Cool White" fluorescent
Light Intensity	100 - 600 lux at surface
Photoperiod	16 ± 1h light; 8 ± 1h dark; coincides with culture photoperiod (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 ..	30 mL or ≥ 20 mL plastic cups, glass beakers or glass test tubes
	Covered with sheets of glass
Test Volume	≥ 15 mL; identical volume in each test vessel
Renewal of Solution ..	≤ 24 h for test duration (Must) ; more frequently for tests with volatile or unstable materials
	Any brood organism in the process of releasing young is given sufficient time to do so before changing that test solution (Must)
	First generation daphnids transferred to the respective new solution and live progeny counted, recorded and discarded (Must)
	Care taken to prevent any carryover of neonates from the aged test solution to a fresh one (Must)
	Used solution held for phys/chem measurement
	Each test solution well mixed (Must)

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Dilution/Control Water	Ground, surface, or dechlorinated municipal water, or reconstituted water; D.O. 90 - 100% air saturation at time of use; hardness within $\pm 20\%$ of value for water used for culturing organisms Adjusted to $25 \pm 1^\circ\text{C}$ before use (Must) Not supersaturated For each definitive test, control solution(s) are 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 that in which organisms have been cultured is used as control/dilution water, a second control is to be set up using the culture water source (Must)
Vessel Randomization	Test treatments of 10 replicates are to be randomly assigned to a position on a test board using a template or a table of random numbers (Must)
# Test Conc.	≥ 7 plus control to calculate ICp and LC50 (Must) ; ≥ 10 are recommended .. 1 plus control for single conc. test
# Replicates/Conc. ...	≥ 10 replicate vessels per test conc. and control(s) (Must) Equal number of replicates among treatments
# Organisms/Vessel ..	Tests are initiated using a single neonate organism per 15 mL volume of test solution in each of 10 replicate test vessels (Must) For multi-concentration tests, up to 20 brood cups, each with 8 or more young are identified on one or more brood boards One neonate from first one or two brood cups is transferred to each of 8-10 test vessels (i.e.,: 7-9 test solutions & 1 control for 1st replicate); one neonate from second one or two brood cups is (are) transferred to 2nd replicate of 8-10 test vessels etc.
Feeding Regime	Daily (Must)
Vessel Cleaning	Food type and ration identical to that provided for individual cultures All test vessels, measurement and stirring devices and daphnid transfer apparatus thoroughly cleaned/rinsed in accordance with good laboratory practice (Must) ; Control /dilution water used in final rinse
Substance Testing ...	Solubilizing agent control solution be run, if used (Must) Agent concentration not exceed 0.1mL/L
Biological Endpoints ..	Survival based on increased mortality of the first-generation daphnids; and reproduction, based on reduction in the number of live neonates produced by each first-generation daphnid during its first three broods (Must)
Statistical Endpoints ..	Mean (cumulative) % mortality for the ten first-generation adults at test end (Must) Mean (cumulative) number (\pm SD) of live neonates produced per first-generation daphnid during its first three broods only (Must) A value of zero is assigned for number of neonates in a replicate if the adult female died before producing young (Must) If female died during test after producing neonates, the number of neonates produced is still used in the analyses (Must) Data from any high test concentrations resulting in zero neonates in all test replicates is removed before regression analyses (Must) For multi conc. test, LC50 for mortality and ICp for decreased reproduction (both with their 95% confidence limits) are calculated (Must)

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Calculation of ICp	Calculation of ICp by entering conc. as logarithms (Must) Initial plot of raw data (number of live neonates) against the log conc. Regression analysis used for calculation of ICp & 95% confidence limits (Must) All requirement for regression analysis outlined in Section 4.6.1 of 2 nd ed. EPS 1/RM/21 are met (Must) Endpoints generated by regression analysis are bracketed by test concentrations (i.e., extrapolation of endpoints beyond the highest test concentration is not acceptable) (Must) ICPIN analyses used only if regression fails to provide meaningful ICps
<u>Observations & Measurements</u>				
D.O. + pH + T°	At least at start and end (before renewal) of each 24-hour exposure in representative concentrations (Must) T° monitored throughout test (Must) If T° records are based on measurements other than in test vessels, relationship between readings and temperatures within test vessels to be established (Must)
Conductivity	Recommend daily measurement of each newly-prepared test solution (prior to dispensing new solutions)
Hardness	Control and highest test concentration, at least before starting test
Mortality	Daily (magnifying device recommended) (Must) Death of any first generation daphnid recorded (Must)
Reproduction	Daily observation of # live neonates produced by each 1st generation daphnid during first three broods (Must) ; counting of dead neonates not required Any neonates produced as a 4th or subsequent brood are not included in the total # of neonates produced for any treatment (including controls) during the test (i.e., discarded without recording their numbers) (Must) Mortality and reproduction rates for <i>C. dubia</i> held in 10 replicate solutions of culture water compared to those for test organisms held in the 10 replicate solutions of receiving water, if receiving water used as control/dilution water (Must)
<u>Test Organism</u>				
Source	Commercial biological supply house or government laboratory; taxonomy ideally verified by microscopic examination The parentage of all organisms used to start a test originate from the same mass culture (Must)
Age	Neonates <24h old and within 12h of the same age (Must) ; <12h old and within 6 h of the same age preferable Neonates taken from individual cultures
Health Criteria	Average mortality rate for brood organisms in individual cultures must not exceed 20% during the 7d period prior to test initiation (Must) Neonates used to start a test must be taken only from individual brood cultures containing at least 8 young that were produced during the third or subsequent brood (Must) Within the 7d period prior to testing, brood organisms in individual cultures must produce ≥15 young per adult during their first three broods(Must) No ephippia present in culture (Must)

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<u>Culture/Holding Conditions</u>				
T°	25 ± 1°C; rate of change ≤ 3 °C/day
pH	6.0 - 8.5 (7.0 - 8.5 preferred)
D.O.	Culture water aerated before use as required to provide 90-100% saturation; not supersaturated
Hardness	Within range of ± 20% of that of control/dilution water for ≥ 2 generations of daphnids preceding test organisms
Light Quality	"Cool White" fluorescent
Light Intensity	100 - 600 lux at water surface
Photoperiod	16 ± 1h light; 8 ± 1h dark
Handling	Minimal, by pipetting
Feeding	Daily (Must) Yeast, Cerophyll™ and trout chow (YCT) plus algae Food added to fresh culture median immediately before or after transfer of organisms Algal concentrate & YCT thoroughly mixed by shaking before dispensing (Must) Thawed YCT stored in refrigerator and unused portions discarded after 2 weeks (Must) Unused algal concentrate stored in fridge & discarded after 1 month (Must)
Cleaning	Water replaced ≥ 2 (for mass culture) or ≥ 3 (individual culture) times/ week
Culture Water	Uncontaminated ground, surface or dechlorinated municipal water, reconstituted water; TRC ≤ 0.002 mg/L
Acclimation	Culture started ≥3 weeks before brood animals needed
Mass Cultures	Established and maintained to ensure supply of neonates for individual cultures
Individual Cultures	Neonates from mass cultures not to be used in tests (Must) Cultures from a single brood organism to provide test organisms (Must) Young produced from first 2 broods are discarded Young produced from 3rd and subsequent broods used for toxicity tests provided that adults are ≤ 14 days old
Facility & Apparatus	Daphnids cultured under controlled-temperature conditions (Must) Culture facility isolated from test facility, solution preparation/storage and equipment cleaning areas Materials of vessels and accessories contacting organisms and culture media be nontoxic (Must) Materials such as copper, brass, galvanized metal, lead and natural rubber not come in contact with culture vessels or media, test samples, test vessels, dilution water or test solutions (Must) New glass beakers used as culture or test vessels be cleaned and acid-soaked before use (Must) Culture vessels covered

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QA/QC				
Test Validity Criteria . . .	<p>Test is valid if:</p> <ul style="list-style-type: none"> • Mean mortality of first generation control adults is $\leq 20\%$; (Must) . . . • Average of ≥ 15 live young are produced per surviving adult in the control solutions upon $\geq 60\%$ of the adults achieving their third brood; and (Must) • 3 broods are produced by $\geq 60\%$ of control organisms by the end of the eighth day of the test (Must) <p>Test is terminated early and declared invalid if, at any time during the test the mean mortality rate for first-generation control daphnids is $\geq 20\%$ or if ephippia are evident in one or more control solutions</p>
Reference Toxicant . . .	<p>Within 14 d before or after the toxicity test is started or during it and following the same procedure as the definitive test (Must)</p> <p>Standard test with LC50 and ICp endpoints (Must)</p> <p>One or more of sodium chloride, phenol or zinc sulphate</p> <p>LC50 for survival and ICp for reproduction evaluated for at least one of these chemicals (Must)</p> <p>Using same water as culture dilution/water</p>
Warning Chart	<p>Prepared for each reference toxicant and continually updated (Must)</p> <p>Within acceptable warning limits (± 2 SD on log scale)</p> <p>Log concentration used for mean and SD calculations and plotting (Must)</p>
Sample Handling				
Sample Collection . . .	For off-site effluent and leachate tests, either 3 subsamples from a single sampling or ≥ 3 separate samples are collected (Must) ; for on-site tests, samples are collected daily and used within 24 h
Volumes	Volumes of ≥ 1 L for single conc. test or ≥ 2 L for multi conc. test
Containers	Non-toxic materials for sample and transport containers, new containers or thoroughly rinsed used containers (Must)
Labeling	Collapsible polyethylene or polypropylene containers recommended
	Upon collection, sample containers filled, sealed and labeled/coded (Must)
	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 sampling (or following preparation of elutriate) (Must)
	Recommend test initiation within 1 day after sampling
Holding Conditions . . .	Make effort to keep samples cool throughout their period of transport (Must) at 1 - 7°C (preferably $4 \pm 2^\circ\text{C}$) using regular ice or frozen gel packs
	Upon collection, if sample $> 7^\circ\text{C}$, cool to 1 - 7°C with regular ice or frozen gel packs (not dry ice) (Must)
	Sample be kept from freezing during transport or storage (Must)
	The portion(s) of sample/subsamples required for solution renewals are stored in darkness in sealed containers without air headspace at $4 \pm 2^\circ\text{C}$ (Must)
Sample Aliquots	Each sample or subsample in a collection container be agitated thoroughly just before pouring (Must)

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Test Report					
Sample Data	Brief description of sample type 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/subsamples used (Must)	
	For effluent or leachate, T° 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 brood organisms (Must)
		Confirmation that the parentage of all organisms that were taken from a series of individual cultures to initiate the test, originated from the same mass culture (Must)
		Range of age at start of test (Must)
Test Facilities	Any unusual appearance, behaviour, or treatment of test organisms, before their use in the test (Must)	
	Data showing health of individual brood cultures, including: mean % mortality of brood organisms during 7-day period preceding test; mean # of surviving young produced within the first three broods of each adult during the 7-day period preceding the test; # of young produced by each brood organism in its third or subsequent brood; and any observations of ehippia (Must)	
	Name and address of test laboratory (Must)	
	Name of person(s) performing the test (Must)	
Control/Dilution Water	Brief description of test vessels (size, shape, type of material) (Must)	
	Type and source of water used as control and dilution water (Must)	
Test Method	Type and quantity of any chemical(s) added to control/dilution water (Must)	
	Citation of biological test method used (Must)	
	Description of procedure(s) in those instances in which a sample, subsample, or test solution has been filtered/adjusted for hardness or pH (Must)	
	Design and description if specialized procedure (Must)	
	Brief description of frequency and type of all observations and all measurements made during test (Must)	
Test Conditions	Name and citation of program(s) and methods used for calculating statistical endpoints (Must)	
	Name and citation of program(s) and methods used for calculating statistical endpoints (Must)	
	Design and description of any deviation from or exclusion of any of the procedures and conditions specified in test method document (Must)	
	Number, concentration, volume, and depth of solutions in test vessels, including controls (Must)	
	Number of individuals per test vessel, and number of replicates per treatment (Must)	
	Brief statement (including procedure, rate, and duration) of any pre-aeration of test solutions (Must)	

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Test Conditions (continued)	Brief statement concerning aeration (if any, give rate, duration) of test solutions during exposure of test organisms (Must) Confirmation that all neonates produced in each test solution (including control(s)) by a brood organism during its 4 th or subsequent broods were discarded without including them in counts of number of young produced per replicate or treatment (Must) Dates when test was started and ended (Must) All required measurements of T°, pH and D.O. in test solutions (including controls), made during the test (Must) Brief statement indicating whether the reference toxicity test was performed under the same experimental conditions as those used with the test sample(s); and description of any deviation(s) from or exclusion(s) of any of the procedures and conditions specified for the reference toxicity test in this document (Must)
Test Results	For each replicate test solution (including each control replicate): the presence or absence of mortality of the first-generation adult in that test solution at each period of observation including at the end of the test (Must) For each treatment including the control treatment(s): the (cumulative) mean % mortality for the first-generation daphnids at test end (Must) For each replicate test solution (including each control replicate): the number of live neonates produced by each first-generation daphnid during its first three broods only as noted during each observation period and test end (Must) For each treatment including the control treatments: the (cumulative) mean number (including its SD) of live neonates produced by each first-generation daphnid during its first three broods only (Must) LC50 and 95% confidence limits and indication of quantal method used; ICp and 95% confidence limits for the reproduction data; details regarding any weighting techniques applied to the data; and indication of quantitative method used (Must) Any outliers and the justification for their removal (Must) Results and duration of any toxicity tests with the reference toxicant(s) performed within 14 days of the test start, 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 must be signed or initialled, and dated by the laboratory personnel conducting the tests (Must)
<u>Info Kept On-File</u>	Do Lab SOPs indicate that the information on Section 8.2 of the 2 nd edition of EPS 1/RM/21 method must be kept on file for 5 years? (Must) For details of this information, see the 2 nd edition of EPS 1/RM/21, Section 8.2.