THREE BROOD STATIC RENEWAL TOXICITY TEST USING *Ceriodaphnia dubia*

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THREE BROOD STATIC RENEWAL TOXICITY TEST USING *Ceriodaphnia dubia*

1.0 SCOPE AND APPLICATION

The procedure for conducting a 7-day toxicity test using *Ceriodaphnia dubia (C. dubia)* is described below. This test is applicable to effluents, leachates, and liquid phases of sediments which require a chronic toxicity estimate. This method uses reproductive success and mortality as endpoints.

These are standard (i.e., typically applicable) operating procedures which may be varied or changed as required, dependent on site conditions, equipment limitations, or limitations imposed by the site specific procedure. In all instances, the ultimate procedures employed must be documented and associated with the final report.

Mention of trade names or commercial products does not constitute United States Environmental Protection Agency (U.S. EPA) endorsement or recommendation for use.

2.0 METHOD SUMMARY

Neonatal *Ceriodaphnia dubia* are placed in individual exposure chambers containing approximately 15 mL of test solution. Survival and reproduction are recorded over the six to eight day duration of the test. These data are used to determine the Lowest Observable Adverse Effect Concentration (LOAEC), the No Observable Adverse Effect Concentration (NOAEC), the Inhibitory Concentration (IC_{25}), the Effective Concentration (EC_{50}) and the Chronic Value of the test media.

3.0 SAMPLE PRESERVATION, CONTAINERS, HANDLING, AND STORAGE

The selected environmental matrix will be sampled utilizing the methods detailed in Environmental Response Team/Scientific, Engineering, Response, and Analytical Services (ERT/SERAS) Standard Operating Procedures (SOP) #2012, *Soil Sampling*, #2013, *Surface Water Sampling*, #2016, *Sediment Sampling*, SERAS SOP #2003, *Sample Storage, Preservation and Handling,* and any other procedure applicable for the media sampled.

Once collected, the samples will be placed in containers constructed from materials suitable for the suspected contaminants. Because test organisms will be directly exposed to the sample material, or exposed to an elutriate of the test material, no chemical preservatives are to be used. The preservation and storage protocol is therefore limited to holding the samples on ice, or at 0.1 to 4 degrees Celsius ($^{\circ}$C) for the holding time specified by the analytical method. Prior to shipping, the laboratory performing the toxicity tests will be notified of any potential hazards that may be associated with the samples.

4.0 INTERFERENCES AND POTENTIAL PROBLEMS

1. Non-target chemicals (e.g., residual chlorine) may cause adverse effects to the test organisms, giving false results.

2. Dissolved oxygen depletion due to biological oxygen demand, chemical oxygen demand, and/or metabolic wastes is a potential problem.

3. Loss of toxicants through volatilization or adsorption to exposure chambers may occur (Weber 1993).

5.0 EQUIPMENT/APPARATUS

5.1 Apparatus

1. Laboratory culture of *C. dubia*
2. Approximately 60 exposure chambers/day, 30 milliliter (mL) or larger, glass, polypropylene (PP), or polycarbonate (PC), labeled
3. Temperature controlled chamber (incubator, water bath, etc.)
4. Trays and covers for exposure chambers
5. Fire polished, borosilicate glass 1 mL pipettes, for organism transfer
6. Dilution water, approximately 1.5 Liter (L)/day
7. Test media, approximately 500 mL/day
8. Graduated cylinders, 500 mL, 100 mL and 10 mL
9. Mixing chambers, 500 mL or larger
10. Borosilicate glass pipettes
11. Beakers, 250 mL (or similar)
12. Dissolved Oxygen (DO) meter
13. pH meter
14. Conductivity meter
15. Thermometer
16. Suitable food
17. Waste containers

5.2 Test Organisms

Test organisms must be reared "in house" by the laboratory. Positive identification of the laboratory's culture of *C. dubia* is required (Berner 1986). *C. dubia* designated for use in testing must be less than or equal to 24 hours old, and all neonates must have been released within eight hours of each other. Only neonates from third brood to eighth brood, and only those from healthy adults producing eight or more progeny in third and successive broods are acceptable for testing. All organisms utilized in a single test must be from the same source. *C. dubia* taken from culture vessels having more than ten percent males, or producing ephippeal cysts are considered unfit for testing. *C. dubia* should be cultured individually to ensure progeny of known age and brood number.

C. dubia "starter" cultures may be obtained from reputable commercial suppliers. However, the organisms must be held in quarantine through at least two generations before progeny are suitable for use as test organisms.

C. dubia may be fed a number of suitable diets (Yeast/Algae/Trout Chow, Yeast/Cerophyll/Trout Chow (YCT) and algae, Yeast/Algae, Algae alone). The diet chosen must be used consistently for the culture as well as testing. The preferred diet is the Yeast/Cerophyll/Trout Chow (YCT) and algae diet described by U.S. EPA (Lewis et al. 1994). Other diets are acceptable, provided performance criteria are met, and side-by-side comparison tests confirm acceptability.

Feeding of test organisms must be performed using a minimal volume of food. U.S. EPA recommends 0.1mL of YCT and 0.1 mL of algae suspension per 15 mL of test solution (Lewis et

al. 1994). Insufficient food will stress test organisms, and yield poor reproduction rates. Excessive feeding may decrease the dissolved oxygen in the test chambers, and may interfere with the toxicity of the test media.

5.3 Equipment for Physical/Chemical Analysis

1. Properly calibrated laboratory meters are required for the measurement of DO, temperature, pH, and conductivity. Meters must have calibration checked according to the manufacturers' instructions prior to use.

2. DO meter calibration must be checked daily (or prior to use), and must be verified at least weekly (or prior to use) via the iodometric titration (Winkler) method (American Public Health Association, 1992).

3. pH meters must have calibration verified daily (or prior to use), using certified pH buffers. During instrument use, calibration must be verified every three hours, to ensure that it has not drifted.

4. Conductivity meters must have calibration verified daily (or prior to use), using certified conductivity standards.

5. Thermometers and temperature recording devices must have calibration verified at least annually against a National Institute of Standards and Technology (NIST) traceable thermometer.

6. Alkalinity and hardness should be measured, at a minimum, in the laboratory dilution water and in the highest tested concentration of the test media, at test initiation, and upon test renewal with fresh test solution.

7. All physical/chemical measurements must be recorded on properly labeled laboratory data sheets, or in a laboratory notebook.

6.0 REAGENTS

1. Dilution Water

Unless specified otherwise, standard laboratory dilution water shall be U.S. EPA moderately hard, reconstituted water. See Weber (1993) for the preparation of synthetic fresh water. The dilution water utilized for testing should be the same type used to culture and acclimate the test organisms (e.g., surface water).

2. Test Medium

If the test medium is aqueous, test dilutions may be made directly for the desired concentrations. If the test medium is a sediment, preliminary filtration and dilutions will be required to produce a liquid phase.

7.0 PROCEDURES

11. Select a range of concentrations to bracket the expected toxic range (if possible). Optimally, the test concentrations should span those causing no adverse effects, to those causing adverse effects in all exposed organisms. The example concentrations listed below may be adjusted to meet the needs of a specific situation. A geometric or logarithmic range of concentrations also may be used (Sprague 1973). The example below provides enough test solution for ten (10) exposure chambers per concentration, each containing 15 mL, along with 150 mL extra volume for physical/chemical analyses. Other concentration ranges may be used at the investigators discretion.

Example 1. Test Dilutions

Test Concentrations (% test media)	Diluent	Volume (mL) Test Media
0.00 (control)	300.00	0.00
6.25	281.25	18.75
12.50	262.50	37.50
25.00	225.00	75.00
50.00	150.00	150.00
100.00	0.00	300.00

2. Rinse all exposure chambers in dilution water.

3. To prepare test solutions, measure the appropriate volume of test media into a beaker and dilute to 300 mL using dilution water.

4. Add an appropriate volume of a suitable food to each exposure chamber just prior to addition of test solution. Food volume should be small, in order to prevent further dilution of the test solutions. Addition of food can alternately be made to each of the test solutions, prior to distribution to test chambers. This ensures consistent food concentrations in each test chamber.

5. Using a pipette, graduated cylinder, or other suitable volumetric device, pour 15 mL of test solution into each exposure chamber. Reserve the remainder for physical/chemical analyses.

6. Continue these steps for all concentrations. Always work from lowest concentration to the highest in order to minimize the risk of cross contamination.

7. Using the blunt end of a fire polished, borosilicate glass pipette, randomly transfer one neonatal *Ceriodaphnia dubia* into each test chamber, releasing the organism below the surface of the test media and gently expelling the animal into the test chamber. Use a minimum of ten replicate test chambers per exposure treatment. Randomization of test chambers and of test organism distribution shall be performed in accordance with U.S. EPA guidelines (Lewis et al 1994).

8. Measure and record temperature, DO, pH, and conductivity daily on all new test solutions. Measure alkalinity and hardness on the control water and on the highest tested concentration of test solution daily, or upon use of fresh samples.

9. Count and record survival after one hour of exposure.

10. Place a cover loosely over the exposure chambers to minimize evaporation.

11. Place the exposure chambers into a temperature control device (incubator, water bath, temperature controlled room, etc.) and maintain test temperature at 25° ± 2°C.

12. On the second and successive days, prepare fresh test solutions and a new set of exposure chambers.

13. DO and pH should be measured and recorded daily from both old and new test solutions. Measurements of the fresh test solution shall be made in the mixing chamber. Measurement of the spent test solution shall be performed after the test organisms have been transferred to fresh solution, and the spent solution has been pooled by concentration.

14. Transfer adult *C. dubia* by carefully removing with a wide bore pipette and transferring into the new exposure chamber.

15. Count the number of broods, the brood size, and the number of live or dead organisms daily. Count the number of males at test termination. *C. dubia* usually start to produce offspring after the third day of the test, and should have three broods by the completion of the test. The test is terminated when 60% of the control organisms have at least three broods.

8.0 CALCULATIONS

The common statistical endpoints of the short-term chronic toxicity test using *C. dubia* differ depending on the results of the test. For lethality endpoints (Lethal Concentration [LC_{50}], Effective Concentration [EC_{50}]), if there are no partial responses in any replicate (i.e., all alive or all dead), then the Graphical Interpolation Method may be used. If there are two or more test concentrations exhibiting partial response, and the data exhibits a significant chi square test, the Probit Method should be used. If there are one or more test concentrations exhibiting partial response, and the chi square test is not significant, the Spearman-Karber Method or the Trimmed Spearman-Karber Method may be used. All four of these methods are detailed in Weber (1993). Computer programs for Probit Analysis and Spearman-Karber are also available from U.S. EPA's internet site (http://www.epa.gov/nerleerd/stat2.htm).

The Lowest Observable Adverse Effect Concentration (LOAEC), and the No Observable Adverse Effect Concentration (NOAEC) are calculated for both survival and reproduction. The LOAEC/NOAEC for survival is calculated first, using Fisher's Exact Test (Lewis et al. 1994). Test concentrations exhibiting a significant difference from the control (The LOAEC and higher concentrations)are not used for calculation of reproductive effects.

Prior to determining the LOAEC/NOAEC for reproduction, the data set (excluding data with significantly affected survival) is checked for normality using the Shapiro-Wilk's Test. If the data set is normally distributed, it is then checked for homogeneity of variance, using Bartlett's Test. If the data set meets the assumptions of normality and homogeneity, it is tested for LOAEC/NOEAC using Dunnett's Test for data sets with equal number of replicates, or the Bonferroni T-test for data sets with unequal number of replicates. If the data set does not meet the assumptions of normality or homogeneity of variance, it is tested for LOAEC/NOAEC using the Steel's Many-One Rank Test for data sets with equal number of

replicates, or the Wilcoxon Rank Sum Test with Bonferroni Adjustment for data sets with unequal number of replicates. All of these methods are detailed in Lewis et al. (1994). Several commercially available computer programs will also perform these calculations.

The Inhibition Concentration (IC) at which there is a reduction in reproduction, usually is calculated for a 25% reduction (IC_{25}) using the U.S. EPA's IC_p program. The program is available from U.S. EPA Duluth, or it can be downloaded from U.S. EPA's site on the internet (http://www.epa.gov/nerleerd/stat2.htm).

9.0 QUALITY ASSURANCE/QUALITY CONTROL

To ensure a valid test, quality control should encompass the guidelines in this text and in Appendix A, as well as the following: test media sampling, test organism history, facilities and equipment, test media and dilution water preparation, test conditions, and SRT testing.

10.0 DATA VALIDATION

The following criteria are a basis for rejecting the results of this test:

1. Greater than 20 percent control mortality
2. Greater than 20 percent non-concentration related mortality in any concentrations
3. Temperature variation greater than 25 ± 2°C
4. Test media stored more than 72 hours prior to test initiation
5. Criteria in Appendix A not met
6. Less than 60 percent of the controls release third brood within 8 days
7. Less than an average of 15 progeny produced in the control group after third brood release.

11.0 HEALTH AND SAFETY

When working with potentially hazardous materials, follow U.S. EPA, Occupational Safety and Health Administration (OSHA), and corporate health and safety procedures.

12.0 REFERENCES

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APPENDIX A
Summary of Test Conditions for *Ceriodaphnia dubia* Survival and Reproduction Test
SOP #2025
June 2002

THREE BROOD STATIC RENEWAL TOXICITY TEST USING *Ceriodaphnia dubia*

Summary of Test Conditions for *Ceriodaphnia dubia* Survival and Reproduction Test.

1.	Test type:	static, daily renewal
2.	Temperature:	25.0 ± 2°C
3.	Illumination:	Cool white fluorescent lights, approximately 50 to 100 foot-candles at the test surface
4.	Photoperiod:	16 hours light, 8 hours dark
5.	Test chamber size:	30 mL containers (or similar)
6.	Test solution volume:	15 mL per exposure chamber
7.	Renewal:	Daily
8.	Age of test organisms:	≤ 24 hours old, all released within 8 hours
9.	Replication:	One organism per replicate Ten replicates per concentration
10.	Feeding:	Feed minimal volume of suitable food daily
11.	Aeration:	None
12.	Dilution water:	U.S. EPA moderately hard reconstituted water, unless otherwise specified
13.	Test media concentrations:	Minimum of five and one control
14.	Test duration:	Until at least 60% of control organisms release third brood, maximum eight days
15.	Effects measured:	Survival and reproduction
16.	Test acceptability:	≥80% control survival ≥60% of controls release third brood ≥15 progeny per control organism (average)