Ceriodaphnia dubia Chronic Toxicity Testing Methods

1. SCOPE AND APPLICATION

1.1. This method is based on EPA 821-R-02-013 *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms,* 4th Edition, Test Method 1002.0. It describes the procedure used to estimate the chronic toxicity of whole effluent and receiving waters (freshwater aqueous samples) on <24 hour old (≤ 8-hour range in age) *Ceriodaphnia dubia* (water flea) neonates during a 7-day static renewal exposure. Tests covered by this procedure are BSY-CS-FCD and BSY-CD-FCD.

2. SUMMARY OF METHOD

2.1. Samples are received and prepared in the laboratory. Sample physical-chemical (phys-chem) parameters are measured and samples are adjusted as needed. Sample and control waters are placed into test vessels. Test organisms are collected, fed and held for observation prior to loading into test vessels. Organisms are then loaded into test vessels. Tests are incubated in laboratory incubators. While tests are in progress, survival/mortality, reproduction, and phys-chem parameters are measured and recorded daily before and after renewal. Tests are checked at ~24 hour intervals. The test endpoints are survival and reproduction. Results are entered into the Laboratory Information Management System (LIMS).

2.2. Test acceptability criteria: 80% or greater survival in the controls, 60% or more of control females must have produced their third brood, and the average number of young per surviving female must be 15 or greater (presumes all criteria for a valid test are met). Test duration is until a minimum of 60% of surviving females have three broods (maximum test duration of 8 days). (This presumes all criteria for a valid test are met).

2.3. Filter sample through a 60µm screen to remove debris and/or indigenous organisms if needed. Also, adjust pH (TA-08.06) and dissolved oxygen concentration; dechlorinate (TA-09.18), etc.

2.4. Interferences: Refer to EPA 821-R-02-013 Section 12.3. Includes toxic substances introduced by contaminants in dilution water or on hands, glassware, sample hardware, and testing equipment. Dissolved oxygen <4.0 mg/L; pH <6.0 SU or >9.0 SU; salinity >1 part per thousand; ammonia, chlorine, debris, indigenous organisms that may attack or be confused with the test organisms.

2.5. Definitions:

2.5.1. Neonates: Organisms less than 24-hours old

2.5.2. Diluent: Control water

2.5.3. Sample 1: Original (1st) sample taken from the facility; used for initial test set-up (0-hr and 24-hr)

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2.5.4. Sample 2: Sample collected 48 hours after the original sample; used for renewals (48-hr and 72-hr after test initiation)

2.5.5. Sample 3: Sample collected 96 hours after the original sample; used for renewals (96-hr, 120-hr, 144-hr and 168-hr (if needed) after test initiation)

3. EQUIPMENT AND SUPPLIES

3.1. 30 mL plastic test cups, soaked in deionized (DI) water for a minimum of 12 hours.

3.1.1. (Ten replicate cups) + (one parameter cup) =11 cups per test concentration.

3.2. 60-place Plexiglass tray to hold test cups and one smaller tray to hold parameter cups. One tray per sample or facility tested.

3.3. Autoclaved 250 mL graduated cylinder for making test solutions

3.4. Autoclaved 1000mL Erlenmeyer flask for mixing test dilutions

3.5. Autoclaved 6000mL Erlenmeyer flask to hold effluent sample

3.6. 60µm mesh to filter sample

3.7. Serological pipettes- 1mL and 10 mL, graduated

3.8. Pipetter for serological pipettes

3.9. Magnetic stir bars and stirrer

3.10. 0.2 mL Calibrated pipetter

3.10.1. Pipette tips

3.11. YCT and algal suspension combined 1:1 for daily feeding (YCT prepared per SOP TA-01.01; algal suspension of *P. subcapitata* prepared per SOP TA-01.04.)

3.12. Dissecting microscope or magnifying glass

3.13. Random loading sheet or a die.

3.14. Effluent samples to be tested (recommended to be warmed to 25 ± 1°C)

3.15. Meters to measure:

3.15.1. pH calibrated per SOP TA-06.23 or TA-06.25

3.15.2. Dissolved oxygen (DO) calibrated per SOP TA-06.04

3.15.3. Temperature using digital thermometer calibrated per SOP TA-06.13

3.15.4. Conductivity/ salinity calibrated per SOP TA-06.04

3.16. Hach ® kits to measure:

3.16.1. Total residual chlorine (TRC) per SOP TA-06.22

3.16.2. Alkalinity per SOP TA-06.08

3.16.3. Hardness per SOP TA-06.08

3.16.4. Ammonia per SOP TA-06.33

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3.17. *C. dubia* neonates meeting age requirements, obtained per SOP TA-05.02

3.18. Thermo Scientific Incubator (Incubator #1, #3 or #5)

3.18.1. Incubator should maintain test temperature at 25 ± 1ºC and a photoperiod of 16h light: 8hr dark. Light intensity should be ~50-100 ft. candles. Sample temperatures in cups must not deviate more than 3ºC for the duration of the test.

4. REAGENTS AND STANDARDS

4.1. Moderately Hard Freshwater (MHWCD), prepared per SOP TA-03.15.

5. SAMPLE COLLECTION, PRESERVATION, AND HANDLING

5.1. See SOP FS 1000 for sample collection preservation.

5.1.1. Sample criteria for use in a test: a) the sample should be held at 0-6 °C prior to test initiation; or be cooling if hand delivered on day of collection; and b) sample holding time should not exceed 36-hr from time of collection (last sample time when 4 grab samples are collected over a 24-hour period, or the end of the 24-hour collection period for a composite sample) to test initiation.

5.1.2. A series of three samples (designated as samples 1, 2, and 3) are collected and used upon receipt. Sample 1 is used for 2 days, sample 2 is used for 2 days, and sample 3 is used for 3 days if needed.

5.1.2.1. The remainder of each sample is stored in the walk-in cooler for solution renewal the following day or days.

5.1.2.2. Any variation in sample collection (from above) is not necessarily cause for test invalidation but the FDEP toxicity coordinator should always be notified/consulted for approval/acceptability.

5.2. See SOP TA-06.14 for bioassay sample log-in procedure.

6. TEST INITIATION

6.1. Using tape and a waterproof marker, label the Plexiglass tray containing soaked 30mL test cups with test concentration and facility sampled.

6.2. Calibrate meters and record readings in *Daily Physical-Chemistry Calibration Log* daily during test. Also, check meters at end of day and record values.

6.3. Filter sample through 60µm mesh into 6000mL Erlenmeyer flask

6.4. Fill disposable, 30 ml cup with effluent sample and take initial pH measurement

6.4.1. If pH is outside acceptable range of 6.0–9.0, then an additional test with a pH-adjusted aliquot will be run. The test with the original sample need now only be a screening test, regardless of whether the pH-adjusted aliquot is used for a screening or definitive test. Refer to SOP TA-08.06 for sample pH adjustment.

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6.5. In same plastic cup with sample, take initial DO measurement

6.5.1. If DO is <4.0 mg/L (low DO) or >9.0 mg/L (super saturated DO), aerate entire sample to ~ 90% saturation prior to test initiation. Samples with a potential DO problem generally show a downward trend in DO during the first few hours of the test. Monitor the DO closely. If DO begins to fall, set up gentle aeration at <100 bubbles/min to ALL test solutions for duration of test using micropipettes and the aeration manifold.

6.6. In same plastic cup with sample, measure the conductivity, temperature, and salinity (by direct measurement or conversion) of the sample.

6.6.1.1. The following web site: http://www.fivecreeks.org/monitor/sal.shtml can be used for salinity conversions from conductivity and temperature measurements.

6.7. Measure total residual chlorine (TRC) in sample per SOP TA-06.22. Check field data sheet supplied by sampler for field TRC and atypical field measurements.

6.7.1. If TRC ≥0.1 mg/L, and non-chlorine toxicity is to be measured, dechlorinate a portion of the sample using sodium thiosulfate per SOP TA-9.18. Test dechlorinated replicates of all chosen test concentrations concurrently with additional chlorinated (100% sample ONLY) replicates.

6.8. Measure alkalinity, hardness, and total ammonia of sample per SOP TA-06.08 and TA-06.17 or TA-06.33.

6.9. Preferred method for Definitive tests :

6.9.1. Warm sample to 25 ± 1ºC.

6.9.2. Empty DI water from soaking cups prior to filling with diluents (MHWCD) and sample test concentrations.

6.9.3. Using a graduated cylinder, pour 20 ± 1 mL of diluent into each control replicate cups, including parameter cup.

6.9.4. Pour 20 ± 1mL aliquots of the 100% sample into each eleven 100% sample concentration cups, including the parameter cup.

6.9.5. Place the eleventh cup of each concentration in a separate, small tray to measure physical parameters at a later point. A second tray is used to prevent possible probe contamination of the test cups.

6.9.6. Measure the desired volumes of diluent and sample into a 1000mL Erlenmeyer flask using two graduated cylinders. Swirl flask by hand to mix until homogenized. Measure 250mL of the mixture back into a 250mL cylinder. Transfer 20mL from the 250mL cylinder into test replicate cup A; repeat for replicates B through J for that particular concentration. Rinse cylinders with deionized water between concentrations. Repeat for each test concentration

6.9.6.1. For each sample tested, use ten replicates per concentration in a dilution series based on the Facility's NPDES permit, instruction

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from the appropriate FDEP ROC or, if applicable, previous knowledge of toxicity level. Usually a minimum of 5 test concentrations plus the control are used.

6.9.7. Example of sample volume (0.5 dilution) below for ten 20mL replicates and one parameter cup per replicate of each concentration in the *C.dubia* chronic toxicity test:

Concentration	Diluent	Sample	Total Volume
Control	250 mL	0 mL	250 mL
6.25%	250 mL	250 mL of 12.5%	500 mL
12.5%	250 mL	250 mL of 25%	500 mL
25%	250 mL	250 mL of 50%	500 mL
50%	250 mL	250 mL of 100%	500 mL
100%	0 mL	250 mL	250 mL

6.10. Perform QA standard reference toxicant (SRT) tests monthly, using 3g/L sodium chloride (NaCl). Other toxicants can be used.

6.10.1. Example of KCl (1 g/L toxicant) concentrations and diluent volumes for chronic SRT test.

Concentration	Diluent	Toxicant	Total Volume
Control	250 mL	0 mL	250 mL
0.3 g/L	210 mL	90 mL of 1 g/L KCl	300 mL
0.4 g/L	180 mL	120 mL of 1 g/L KCl	300 mL
0. 5 g/L	150 mL	150 mL of 1 g/LKCl	300 mL
0.6 g/L	120 mL	180 mL of 1 g/L KCl	300 mL
0.7 g/L	90 mL	210 mL of 1 g/L KCl	300 mL

6.11. Using calibrated 0.2 mL pipettor, or calibrated dropper, feed 0.2 mL of combined 1:1 YCT and algal suspension per test cup (recommended).

6.11.1. Feeding is done every day before transfer of organisms. Record feeding time in bench sheets comment section or in other designated space.

6.12. Measure pH, temperature, DO and conductivity of the solutions in the phys-chem (eleventh cup) cups in the separate tray.

6.12.1. Phys-chem parameters on “new sample” should be taken before transfer of organisms to ensure that concentrations were poured correctly. This allows the chance to interpret and correct mistakes without causing harm to the organisms.

6.12.2. Record all readings, and any comments pertaining to the condition or appearance of the sample, in their appropriate columns on the Chemistry

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page of the *Chronic Bioassay Bench Sheets for Ceriodaphnia dubia Definitive Tests* logbook.

6.12.2.1. Conductivity measurements are reported to 3 significant figures. pH, DO, and temperature measurements should be read to one decimal place. Take measurements from lowest to highest concentration.

6.12.3. Empty the phys-chem cups into the sink, rinse cups, and flush the drain with water.

6.12.4. All before renewal parameters should be taken using "A" replicates. If the "A" replicate is not available (removed due to mortality), measure the next available, alphabetically sequential aliquot of the same concentration.

6.13. Test initiation (0-hour and Day-0)

6.13.1. With Plastic repipetter, randomly load one *C. dubia* neonate from one chronic culture cup (collected specifically for chronic test from adults for neonates) into replicate A of the control and replicate A of each test concentrations. Repeat for the remaining 9 sets of replicate cups using a different cup of neonates per set of replicate cups (generally total of 6 cups/set). If necessary, same cup of neonates can be used to load more than 1 set of replicates/concentration.

6.13.1.1. Loading is done at a light table.

6.13.2. Use a random loading pattern similar to that shown in the EPA chronic manual.

6.13.2.1. Use one cup to load one row randomly (usually 6 cups). Loading is done across concentrations. (use randomization template)

6.13.2.2. In the logbook, record which cup was used to load a particular row.

6.14. A person other than the loader should verify that there is one neonate in each replicate (test initiation).

6.15. Place the tray of cups into the incubator and cover the tray with clear Plexiglass.

Note: Covers prevent accidental contamination and minimize evaporation.

6.16. Record counts and the date and time of test initiation on the Reproduction page of the *Chronic Bioassay Bench Sheets for Ceriodaphnia dubia Screening Tests* logbook.

7. INTERIM READINGS AND RENEWALS

7.1. Every day at 24 (± 2-3 hours) intervals, perform applicable Steps 6.2-6.12.4 to prepare new test cups/concentrations, transfer *C. dubia,* take parameters, feed, etc.into.

7.1.1. On days when a new set of effluent sample water arrives, follow Steps 6.2 through 6.8 for phys-chem readings. When two or more jugs of sample water were collected on the same day, only temperatures must be recorded from all jugs in *Sample Log-in* logbook. The remaining phys-chem parameters (pH,

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DO, and conductivity) should be checked on the day the sample is used, but need only be recorded from one jug in *Sample Log-in* logbook.

7.2. On days 1 until test termination (see 2.2), retrieve the previous day's cups from the incubator count and record of surviving *C. dubia* and record the number of neonates on the Reproduction page of the logbook.

7.2.1.1. If necessary use the lowest power of a dissecting scope (magnifying glass) or the light table.

7.2.1.1.1. Young will typically be present on days 3-8.

7.3. With Pasteur pipet, transfer ONE *C. dubia* adult from previous tray into the corresponding test cup on the new tray.

7.3.1.1. Transfers MUST be performed prior to measuring physical parameters in cups from the previous day.

7.3.1.2. Verify count under the lowest power dissecting scope (magnifying glass) or the light table.

7.3.2. Place new tray of cups into incubator and cover tray with clear Plexiglass.

7.4. Repeat applicable steps 6.2 -7.3.2 daily until test termination criteria (60% of surviving control females have 3rd brood) is met, proceed to Step 7.5. DO NOT CONTINUE TEST BEYOND 8 DAYS.

7.5. Test termination (when 60% of surviving control females have 3rd brood):

7.5.1. Count number of young produced and record in appropriate columns on bench sheet.

7.5.1.1. Adults and young MUST be counted prior to measuring physical parameters in cups from the previous day, but they don't need to be removed to take the final measurements.

7.5.1.2. At test termination, all observations on numbers of offspring should be completed within 2 hours. The rapid rate of development of *C. dubia* means that an extension of more than a few hours in the test period could be a significant part of the brood production cycle of the animals, and allow production of additional broods.

NOTE: Adults not producing young by end of the test are placed on a slide and examined to determine if they are male or female usually by a member of the invertebrate taxonomy group. Refer to EPA-821-R-02-013 Section 13.10.9-13.13.2.2.

7.5.2. Measure pH and DO of the solutions and record in appropriate columns on bench sheet.

7.5.3. Record any comments pertaining to the condition of the test animals.

7.5.4. Record test termination date and time.

7.5.5. Pour contents of all test cups into sink and flush drain with water.

7.5.6. Dispose of cups in trashcan and put tray in storage area.

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7.5.7. Enter test results into statistical program to obtain the NOEC, LOEC, or IC25. (Refer to SOP TA-07.16.) Data transcription (paper to electronic) should be verified by another analyst (if necessary Windows *speak* /*read aloud* function can be used)

8. DATA ARCHIVAL

8.1. Use a black, waterproof pen for all bench sheets and logbooks.

8.2. To correct raw data entries, place a single line through the incorrect or illegible entry. Write the corrected entry near the error, date and initial the correction. Do not overwrite entries. Write legibly. Do not attempt to put all information (correction/comments) into a small space.

8.2.1. The corrected entry is written as near its appropriate space as possible or else in the comment box on the appropriate bench sheet.

8.2.2. If the correction requires an explanation, write a comment and reference the error. Initial and date the explanation.

8.2.2.1. Use sequential alphabetical annotation to identify errors and corresponding comments (use A for first error and use A for corresponding explanatory comment; use B for second error, etc.).

8.3. Upon sample receipt, fill in the information requested in *Bioassay Sample Log-In* logbook, and the information requested at the top of the bench sheets as completely as possible, as per SOP TA-06.14.

8.4. Initial TRC, alkalinity, and hardness measurements should be recorded in their respective logbooks.

8.5. Record meter calibration readings in the *Daily Physical Chemistry Calibration Logbook*.

8.6. During sample prep and analysis, parameters and organism data should be entered into *Chronic Bioassay Bench Sheets for Ceriodaphnia dubia Screening Tests* log book.

8.7. Enter the EC50, NOEC, LOEC, and/or IC20 and IC25 results from the statistical programs (Refer to Step 7.5.7.) into the logbook and LIMS.

8.7.1. Have a second person verify data prior to uploading into LIMS.

9. QUALITY CONTROL

9.1. Refer to DEP Laboratory Quality Manual for corrective action and handling out-of-control data. Refer to EPA 821-R-02-013 Test Method 1002.0 for test conditions and acceptability criteria.

9.2. Sample should be held at 0-6 ºC prior to test initiation and between renewals. (Exception hand delivered sample day of collection - sample should be cooling relative to collection temperature)

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9.3. Sample holding time should not exceed 36 hours from time of collection until initial use of sample.

9.4. Incubator maintained at the selected test temperature of 25±1 ºC.

9.5. Test temperature must not deviate more than 3 ºC in the test cups for the duration of the test.

9.6. Incubator light intensity maintained at ~50-100 ft-candles for a photoperiod of 16 hr light**:**8 hr dark.

9.7. Perform QA standard reference toxicant (SRT) tests monthly since the *C. dubia* are cultured in-house (see Step 6.10).

9.8. If a problem occurs during the sample processing or testing, the following steps are performed: 1) Lab supervisor is notified. 2) Client is notified (client makes decision for additional testing). 3) Section administrator is notified. 4) A non-conformance report (NCR) is submitted describing the problem and the corrective action.

9.8.1.1. To create NCR: open LIMS and go to Laboratory/Administrative/Non-Conformance Report

9.9. Refer to http://www.dep.state.fl.us/water/sas/sop/index.htm for sample collection and preservation, and the Laboratory Quality Manual for corrective action and handling out-of-control data.

10. SAFETY/HAZARDOUS WASTE MANAGEMENT

10.1. Proper laboratory protection, including lab hood or ventilation system, lab coat, gloves and safety glasses, is required when working with chemicals and unprocessed samples.

10.2. Refer to DEP Laboratory Quality Manual for sample disposal

10.3. Refer to the Laboratory Health and Safety Plan

10.4. Read MSDS information for reagent handling and disposal.

Plan.

11. REFERENCES

11.1. EPA 821-R-02-013 *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, 4th Edition, Test Method 1002.0.*

11.2. DEP SOP TA-01.01, YCT (Yeast, Cereal Leaves, Flake-Fish Food, or Trout Chow) Preparation

11.3. DEP SOP TA-01.04, *Pseudokirchneriella subcapitata* Culture for *Ceriodaphnia dubia* Food

11.4. DEP SOP TA-03.01, Preparation of Synthetic Moderately Hard Freshwater

11.5. DEP SOP TA-05.02, Production of *Ceriodaphnia dubia* for Chronic Toxicity Testing

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11.6. DEP SOP TA-06.04, YSI Model 58 Dissolved Oxygen meter Calibration and Measurement

11.7. DEP SOP TA-06.13, Thermometer Accuracy Check and Use

11.8. DEP SOP TA-06.08, Measuring Total Alkalinity and Hardness

11.9. DEP SOP TA-06.01, Bioassay Sample Log-In Procedure DEP SOP TA-06.17, Measuring Total Ammonia

11.10. DEP SOP TA-06.22, Measuring Total Residual Chlorine using the Hach Pocket Colorimeter Test Kit

11.11. DEP SOP TA-06.23, Calibration and Use of Accumet Model AR10 pH Meter

11.12. DEP SOP TA-06.24, Calibration and Use of Mettler Toledo SevenMulti for Measuring pH

11.13. DEP SOP TA-06.25, Calibration and Use of Mettler Toledo SevenMulti for Measuring Conductivity, Salinity and TDS

11.14. DEP SOP TA-06.33, *Measuring Ammonia using Hach Ammonia Nitrogen Test Kits for Freshwater and Saltwater Samples.*

11.15. DEP SOP TA-07.16, Using ToxCalc for Statistical Analysis of Chronic Bioassays

11.16. DEP SOP TA 09.18, Dechlorination Procedure using Sodium Thiosulfate

11.17. DEP SOP TA-09.01, Sodium Chloride Reference Toxicant Stock Solutions

11.18. DEP Laboratory Quality Manual http://www.dep.state.fl.us/labs/library/index.htm

11.19. DEP Laboratory Health and Safety Plan https://www.fldepnet.org/content/dear/bureau-laboratories-safety-documents

APPENDIX OF CHANGES

07/25/2008	Moved *Scope and Application, Summary of the Method* and *Interferences* from comments to body of SOP. Added *References* and *Appendix of Changes*. Changed SOP TA-01.05 to TA-01.04 in Methods 6 comment. Removed SOP TA-06.06 form Materials # 11 comment.
07/13/2009	Inserted “surviving female” in the initial Materials comment. Changed “know” to ‘known” in *Daily* Step 5 comment. Changed “5” to “2” in Day 7 Step 5 comment (pg 3). Added “NOTE: If test termination criteria (60% of surviving control females have 3rd brood) proceed to Day 7” to Day 5 and Day 6 comment. Updated Quality Manual reference.
08/14/2009	Replaced reference to SOP TA -07.08 to SOP TA-07.16
07/21/2010	Updated reference to Biology Quality Manual. Removed reference to vinyl gloves in the Safety section comment and added a Caution statement.
05/18/2011	Updated reference to Quality manual and updated SOP link. Changed IC20 to IC25. Changed logbook names to match actual titles.

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04/11/2013	Reformatted the SOP.
02/11/2015	Formatted for 508 compliance.
03/15/2016	Updated links. Changed Table total volume. Re-arranged text.
03/08/2017	Updated links. Re-arranged text.
01/10/2018	Clarified test duration. Modified text to reflect clarification.

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