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 is used to estimate the chronic toxicity of effluents and receiving waters (freshwater aqueous samples) to the water flea, *Ceriodaphnia dubia*, using <24 hour old neonates in a 7 (seven) 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 manipulated as needed. Samples and controls are placed into test vessels. 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. Tests are checked at ~24 hour intervals for survival and reproduction. Survival and reproduction are recorded daily. Phys-chem parameters are measured daily in aliquots of new test solutions from each concentration and on old test solutions from each concentration (after test day 0). The test endpoints are survival and reproduction. Results are entered into the Laboratory Information Management System (LIMS).

2.2. Interferences include 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.

2.3. Definitions:

2.3.1. Neonates: Organisms less than 24 hours old

2.3.2. Diluent: Control water

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

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

2.3.5. Sample 3: Sample collected 98 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. Daily

3.1.1. 30 mL plastic test cups, soaked in deionized (DI) water for a minimum of 12 hours.

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

3.1.2. 60-place Plexiglass tray to hold test cups and one smaller tray to hold parameter cups. One tray per sample or facility tested.

3.1.3. 0.2 mL Calibrated pipettor

3.1.4. YCT and algal suspension combined 1:1 for daily feeding (YCT prepared per SOP TA-01.01; algal suspension prepared per SOP TA-01.04.)

3.1.5. 250 mL graduated cylinders, one per sample and one for the diluent

3.1.6. 1000 mL beaker

3.1.7. Plastic repipettor

3.1.8. Dissecting microscope or magnifying glass

3.1.9. pH meter, calibrated per SOP TA-06.23 or TA-06.24

3.1.10. Dissolved oxygen (DO) meter, calibrated per SOP TA-06.04

3.1.11. Digital Thermometer, calibrated per SOP TA-06.13

3.1.12. Conductivity meter, calibrated per SOP TA-06.25

3.1.13. Thermo Scientific Incubator (Incubator #1, #3 or #5), maintained at 25±1 ºC and 16-hr light 8-hr dark cycle

3.1.14. Random loading sheet or a die (one of two dice) (refer to EPA-800-R-02-013 for use and preparation).

3.2. Test Day 1

3.2.1. Daily materials

3.2.2. *C. dubia* neonates (<24 hours old ≤ 8-hr range in age), obtained per SOP TA-05.02

3.2.3. Sample 1, warmed to ~ 25ºC prior to use

3.3. Test Day 2

3.3.1. Daily materials

3.3.2. Sample 1, warmed to ~ 25ºC prior to use

3.3.3. Denver Instruments® Model 225 pH/ion meter with ammonia probe, calibrated per SOP TA-06.17

3.4. Test Day 3

3.4.1. Daily materials

3.4.2. Sample 2 collected, warmed to ~ 25ºC prior to use

3.5. Test Day 4

3.5.1. Daily materials

3.5.2. Sample 2, warmed to ~ 25ºC prior to use

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3.5.3. Denver Instruments® Model 225 pH/ion meter with ammonia probe, calibrated per SOP TA-06.17

3.6. Test Day 5

3.6.1. Daily materials

3.6.2. Sample 3, warmed to ~ 25ºC prior to use

3.7. Test Day 6

3.7.1. Daily materials

3.7.2. Sample 3, warmed to ~ 25ºC prior to use

3.7.3. Denver Instruments® Model 225 pH/ion meter with ammonia probe, calibrated per SOP TA-06.17

3.8. Test Day 7 (if test finished)

3.8.1. Meters to measure pH, DO, temperature, and conductivity, calibrated per their respective SOPs

3.9. Test Day 7 (if test continued)

3.9.1. Daily materials

3.9.2. Sample 3, warmed to ~ 25ºC prior to use

3.10. Test Day 8 (if necessary)

3.10.1. Meters to measure pH, DO, temperature, and conductivity, calibrated per their respective SOPs

4. REAGENTS AND STANDARDS

4.1. Daily

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

4.1.2. Hach® kits to measure total residual chlorine (TRC), alkalinity, and hardness (Refer to SOPs TA-06.22 andTA-06.08.)

5. SAMPLE COLLECTION, PRESERVATION, AND HANDLING

5.1. See SOP FS 1000 for sample collection preservation. 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.

5.1.1. For a valid test: a) the sample should be held at 0-6 °C prior to test initiation, 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. The remainder of each sample is stored for solution renewal the following day or days.

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5.2. See SOP TA-06.14 Bioassay Sample Log-in Procedure for bioassay sample log-in.

6. SAMPLE PREPARATION

6.1. Place new, rinsed, 30 mL test cups in large, Plexiglass tray.

6.1.1. Eleven cups for each set of control replicates for each sample tested, plus eleven cups per concentration for each sample tested (60 cups go on large tray for test solutions, 6 cups go on small tray for parameters.

6.2. Using tape and a waterproof marker, label the tray beside each column of cups to identify the test concentration and facility sampled.

6.3. Measure total residual chlorine in sample

6.3.1. 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.

6.3.2. Test dechlorinated replicates of all chosen test concentrations concurrently with additional chlorinated (100% sample ONLY) replicates.

6.3.3. Check field data sheet supplied by sampler for field TRC and atypical field measurements.

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

6.4. Calibrate meters per their respective SOPs.

6.4.1. Record calibration readings in the Daily Physical Chemistry Calibration Log Book.

6.5. Empty cups prior to filling with diluent (MHWCD) and sample test concentrations.

6.6. Preferred method for Definitive tests (0.5 dilution factor): (Refer to Table 1)

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

6.6.2. Using a graduated cylinder, pour 20 ± 1 mL of diluent each of the 11 control replicate cups.

6.6.3. Pour 20 ± 1mL aliquots of the 100% sample into each of the eleven 100% sample concentration cups.

6.6.4. <u>50% sample concentration</u>: Measure and pour 250 mL of diluent and 250 mL of 100% sample in the 500 mL flask and swirl to mix. Remove and set aside 250 mL. Dispense 20 ± 1 mL of the solution from the flask into each of the eleven cups labeled “50%.” Discard excess from flask.

6.6.5. <u>25% sample concentration</u>: Add 250 mL of the diluent and the 250 mL of 50% concentration to the flask. Swirl to mix. Remove and set aside 250 mL. Dispense 20 ± 1 mL of the solution from the flask to each of the eleven cups labeled “25%.” Discard excess from flask.

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6.6.6. 12.5% sample concentration: Add 250 mL of the diluent and the 250 mL of the 25% concentration to the flask. Swirl to mix. Remove and set aside 250 mL. Dispense 20 ± 1 mL of the solution from the flask to each of the eleven cups labeled "12.5%." Discard excess from flask.

6.6.7. 6.25% sample concentration: Add 250 mL of the diluent and the 250 mL of the 12.5% concentration to the flask. Swirl to mix. Remove and set aside 250 mL. Dispense 20 ± 1 mL of the solution from the flask to each of the eleven cups labeled "6.25%." Discard the excess.

6.6.8. 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).

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

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

6.8.1. Record all readings, and any comments pertaining to the condition or appearance of the sample, in their appropriate columns on the Chemistry page of the *Chronic Bioassay Bench Sheets for Ceriodaphnia dubia Definitive Tests* logbook.

6.8.2. Empty the phys-chem cups into the sink and flush the drain with water.

6.8.3. Fill the cups with DI water to soak for use during the next renewal.

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

6.9.1. With Plastic repipetter, RANDOMLY load ONE *C. dubia* neonate from 1 chronic culture cup (collected specifically for chronic test) into replicate 1 of the control and replicate 1 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.

6.9.1.1. Loading is done at a light table.

6.9.1.2. (1 *C. dubia* per replicate) x (10 replicates per test concentration) = 10 *C. dubia* per concentration tested.

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

6.9.2.1. Use one cup to load one row randomly (row (usually 6 cups) is across concentrations)

6.9.2.2. Record which cup was used to load a particular row.

6.10. A second person should verify that 1 organism is in each cup.

6.10.1. Verification can be done at a light table or at a dissecting scope.

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

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6.12. 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. SAMPLE ANALYSIS

7.1. Day 1 (t=24 hours)

7.1.1. Perform Steps 6.1.-6.10. (Skip Step 6.3.)

7.1.2. Retrieve remainder of Sample 1 from refrigerator and warm to ~ 25ºC.

7.1.3. Retrieve the previous day's cups from the incubator and verify the count of surviving animals.

7.1.3.1. Use the lowest power of dissecting scope (magnifying glass) or the light table.

7.1.3.2. Record the survival counts on the Reproduction page of the *Chronic Bioassay Bench Sheets for Ceriodaphnia dubia Screening Tests* logbook.

7.1.3.2.1. Young will typically be present on days 3-8. Record counts on the Reproduction page of the logbook.

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

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

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

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

7.2. Day 2 (t=48 hours)

7.2.1. Follow Steps 6.1.-6.10.

7.2.1.1. Use Sample 2 and warm to ~25ºC.

7.2.2. Follow Steps 7.1.3.-7.1.5.

7.3. Day 3 (t=72 hours)

7.3.1. Follow Steps 6.1.-6.10.

7.3.1.1. Use Sample 2 and warm to ~25ºC.

7.3.2. Follow Steps 7.1.3.-7.1.5.

7.4. Day 4 (t=96 hours)

7.4.1. Follow Steps 6.1.-6.10.

7.4.1.1. Use Sample 3 and warm to ~25ºC.

7.4.2. Follow Steps 7.1.3.-7.1.5.

7.5. Day 5 (t=120 hours)

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7.5.1. Follow Steps 6.1.-6.10.

7.5.1.1. Use Sample 3 and warm to ~25ºC.

7.5.2. Follow Steps 7.1.3.-7.1.5.

7.6. Day 6 (t=144 hours): If test termination criteria (60% of surviving control females have 3rd brood) is met, proceed to Day 7. If not, continue with Day 6.

7.6.1. Follow Steps 6.1.-6.10.

7.6.1.1. Use Sample 3 and warm to ~25ºC.

7.6.2. Follow Steps 7.1.3.-7.1.5.

7.7. Day 7 (t=168 hours)

7.7.1. If 60% or more of the surviving females in the controls have produced their third brood, proceed with test termination (Step 8).

7.7.2. If less than 60% of the surviving females in the controls have produced their third brood, follow Steps 6.1.-6.10. (Skip Step 6.3.)

7.7.2.1. Use Sample 3 and warm to ~ 25ºC.

7.7.3. Follow Steps 7.1.3.-7.1.5.

7.8. Day 8 (t=192 hours), or test termination

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

7.8.1.1. 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.

7.8.1.2. Counting should be done under a dissecting scope or over a light table.

7.8.1.3. 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 would be a significant part of the brood production cycle of the animals, and could result in 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. Refer to EPA-821-R-02-013 Section 13.10.9-13.13.2.2.

7.8.2. Measure pH and DO of the solutions in all cups and record in appropriate columns on bench sheet.

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

7.8.4. Record test termination date and time.

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

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

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7.8.7. Enter test results into statistical program to obtain the NOEC, LOEC, or IC25. (Refer to SOP TA-07.16.)

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 and initial the correction. Do not overwrite entries.

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 7.8.7.) into LIMS.

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

9. QUALITY CONTROL

9.1. Sample should be held at 0-6 ºC prior to test initiation and between renewals.

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

9.3. Test acceptability criteria:

9.3.1. 80% or greater survival in the controls.

9.3.2. A minimum of three broods in 60% of surviving female control organisms.

9.3.3. An average of fifteen neonates/surviving female in the controls.

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

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9.5. Test temperature must not deviate (maximum minus minimum) 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. Chronic standard reference toxicant (SRT) tests are performed monthly since the *C. dubia* are cultured in-house. (Refer to **Table 3** for example of SRT concentrations.)

9.8. If a problem occurs during the sample processing or testing, the following steps are performed:

9.8.1. Lab supervisor is notified.

9.8.2. Client is notified (client makes decision for additional testing).

9.8.3. Section administrator is notified.

9.8.4. A non-conformance report (NCR) is submitted describing the problem and the corrective action.

9.8.4.1. 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 Bureau 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 FDEP Laboratory Quality Manual for sample disposal http://www.dep.state.fl.us/labs/library/index.htm

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

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

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-07.16, Using ToxCalc for Statistical Analysis of Chronic Bioassays

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

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

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

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

12. TABLES, DIAGRAMS

Table 1: Example of alternative sample volumes when mixing a solution for a *chronic definitive* 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

Table 2: Example of sample volumes for *chronic definitive* NaCl SRT test, (0.5 dilution) using the toxicant made per SOP TA-09.01.

Concentration	Diluent	Sample	Total Volume
Control	250 mL	0 mL	250 mL
0.187 ppt	250 mL	250 mL of 0.375 ppt	500 mL

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Concentration	Diluent	Sample	Total Volume
0.375 ppt	250 mL	250 mL of 0.75 ppt	500 mL
0.75 ppt	250 mL	250 mL of 1.5 ppt	500 mL
1.5 ppt	250 mL	250 mL of 3.0 ppt	500 mL
3.0 ppt	0 mL	250 mL	250 mL

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.

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.

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