Ceriodaphnia dubia Acute Toxicity Testing Method

1. SCOPE AND APPLICATION

1.1. This method is based on EPA 821-R-02-012 Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, 5th Edition, Test Method 2002.0. It is used to estimate the acute toxicity of effluents and receiving waters (freshwater aqueous samples) to the waterflea, *Ceriodaphnia dubia* (*C. dubia*), using < 24-hour old neonates in a 48-hour static exposure or in a 96-hour static renewal exposure. Tests covered by this procedure are BSY-AS-FCD and BSY-AD-FCD.

2. SUMMARY OF METHOD

2.1. Samples are received and prepared in the laboratory. Sample physical-chemical parameters are measured and samples are manipulated as needed. Samples and controls are placed into test chambers. Physical-chemical parameters are measured on surrogate test chambers. Organisms are collected, fed and held for observation prior to loading into test vessels. Organisms are loaded into test chambers. Tests are incubated in laboratory incubators. Tests are checked at ~24-hour intervals for mortality. Mortalities are recorded daily. Physical-chemical parameters are measured at 24 hours in chambers with 100% mortality, at 48 hours in a chamber from each concentration if 48-hour test; in surrogate vessels with new test solutions, and in a test chamber from each concentration containing old solution (organisms removed) at 48 hours; at 72 hours in chambers with 100% mortality and at 96 hours in a test chamber from each concentration. The test endpoint is mortality of the organisms. Results are entered into the laboratory information management system (LIMS).

2.2. Test acceptability criteria: 90% or greater survival in the controls.

2.3. Interferences:

2.3.1. Refer to EPA-821-R-02-012 Section 9. 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.

3. EQUIPMENT AND SUPPLIES

3.1. *C. dubia* neonates (< 24 hr old)

3.1.1. Obtain neonates per SOP TA-05.01 and feed YCT and *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*) (1:1) at least 2 hours prior to test initiation per SOP TA-02.00.

3.2. Test cups, 30 ml plastic

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3.2.1. Five cups per concentration plus five cups for controls are needed for each sample tested. Cups are rinsed with DI water prior to use. Four cups are used for both effluent and receiving water samples to contain the test organisms for each test concentration; the fifth cup contains the surrogate for parameters for each concentration.

3.2.2. Minimum cup size is 30 mL. Minimum sample volume is 15 mL.

3.3. Plexiglass tray to hold cups.

3.4. Moderately Hard Water (MHWCD)

3.4.1. Prepare in advance per SOP TA-03.15.

3.5. 100 mL cylinders to pour up test concentrations.

3.6. Sample to be tested

3.6.1. For a valid test: a) sample should be held at 0-6 °C prior to test initiation, and b) sample holding time should not exceed 36 hours from time of collection (last sample time when 4 grab samples are collected over a 24-hour period, or the end of the collection period for a composite sample) to test initiation. See method manual for exceptions to the above requirement.

3.7. Flasks, 500 mL for mixing test concentrations.

3.8. Plastic transfer pipet, large bore

3.8.1. Use to transfer animals from neonate cups to test cups.

3.9. Meters to measure physical-chemical parameters:

3.9.1. pH (Calibrate per SOP TA-06.23 or TA-06.24.)

3.9.2. Dissolved Oxygen (DO) (Calibrate per SOP TA-06.04.)

3.9.3. Digital Thermometer (Calibrate per SOP TA-06.13.)

3.9.4. Conductivity (Calibrate per SOP TA-06.25.)

3.10. Hach® kits to measure physical-chemical parameters:

3.10.1. Total Residual Chlorine (TRC) (Measure per SOP TA-06.22.)

3.10.2. Alkalinity (Measure per SOP TA-06.08.)

3.10.3. Hardness (Measure per SOP TA-06.08.)

3.11. Thermo-Scientific incubator (#1, #5, #6 or #7) with temperature and lighting control.

3.11.1. Incubator should maintain selected test temperature [20±1°C or 25±1 °C (recommended)] and a photoperiod of 16h light: 8h dark. Light intensity should be 50-100 ft. candles. Test temperature must not deviate (maximum minus minimum) more than 3 °C in the test vessels for the duration of the test.

3.12. Hach Kit for total ammonia

3.12.1. See SOP TA-06.33.

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4. SAMPLE COLLECTION, PRESERVATION, AND HANDLING

4.1. See SOP FS 1000 for sample collection and preservation.

5. TEST INITIATION

5.1. The day before the test, place new 30-mL test cups in Plexiglas tray. Label each column of trays to identify the test concentration and facility sampled. Fill each cup with deionized (DI) water.

5.1.1. Place tape across front of tray and use waterproof marker to designate columns. Five cups for each set of control replicates are needed for each sample tested, plus five cups per concentration for each sample tested. DI water is usually placed in cups to detect leaks and to rinse the cups. Cups can be rinsed three times with DI water just prior to use rather than soaking in DI water overnight.

5.2. The day of the test, measure total residual chlorine (TRC) in the sample. Check the field data sheet supplied by the sampler for field TRC and atypical field measurements.

5.2.1. Measure TRC. If TRC >0.1 mg/L and non-chlorine toxicity is to be measured, dechlorinate a portion of the sample by the sodium thiosulfate method per SOP TA-09.18 and test dechlorinated replicates of all chosen test concentrations concurrently with chlorinated (100% original sample containing residual chlorine ONLY) replicates.

5.3. Measure the alkalinity, hardness, and total ammonia of the sample.

5.3.1. Measurements are made per SOP TA-06.08 and TA-06.33, respectively.

5.4. Calibrate meters per respective SOP. Record calibration readings in the *Daily Physical Chemistry Calibration Log Book*. Check meters and record values at the end of day.

5.5. Fill a spare cup with sample and take an initial pH measurement.

5.5.1. If the pH is outside the 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 is then analyzed as a screening test, regardless of whether the pH-adjusted aliquot is used for a screening or definitive test.

5.6. In the same spare cup with the sample, take an initial D.O. measurement.

5.6.1. If the D.O. is $\leq$ 4.0 mg/L, aerate at the rate of 100 bubbles per minute to $\geq$ 6.0 mg/L if practical. If the D.O. is $\geq$ 9.0 mg/L, then aerate the entire sample to < 9.0 mg/L (in extenuating circumstances such as organisms about to age out DO >9.0 mg/L is acceptable). Samples with a potential D.O. problem generally show a downward trend in D.O. during the first few hours of the test. Monitor the D.O. closely. Record aeration (initial and final DO, aeration rate and duration) on the appropriate bench sheet.

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5.7. In same spare cup with the sample, take an initial conductivity measurement. Measurements can be rounded to nearest 5 µmhos or 0.1 mmhos.

5.7.1. If the conductivity is above 2,000 µmhos/cm, then discern whether the reading is a result of salinity or high mineral content (i.e., what is the source of the sample?). If the reading results from salinity, then *C. dubia* is not the appropriate test organism for this effluent sample.

5.7.2. This rationale does **NOT** apply to the SRT test.

5.8. ***For Screening Tests:*** Empty DI water from the pre-rinsed cups prior to filling with MHWCD and sample test concentrations. Measure 20 mL of MHWCD into each of the control replicate cups. Measure 20 mL aliquots of the 100% sample into each of the five 100% sample concentration cups.

5.8.1. Include five cups for each set of control and test replicates are needed for each sample tested. The following table shows suggested sample volumes for each cup of the acute screening toxicity test. Diluent is synonymous with the control water.

Concentration	Diluent	Sample	Total Volume
Control	20 mL	0 mL	20 mL
100 %	0 mL	20 mL	20 mL

5.9. ***For Definitive tests (0.5 dilution factor):*** Empty DI water from the soaking cups prior to filling with MHWCD and sample test concentrations. Measure 20 mL of MHWCD into each of the five control replicate cups. Measure 20 mL aliquots of the 100% sample into each of the five 100% sample concentration cups.

5.9.1. Include five cups per concentration in a dilution series for each sample tested. We generally use a 0.5 dilution for definitive tests. The following table shows sample volumes used when mixing a solution for an acute definitive toxicity test.

Concentration	Diluent	Sample	Total Volume
Control	100 mL	0 mL	100 mL
6.25 %	100 mL	100 mL of 12.5% sample	200 mL
12.5%	100 mL	100 mL of 25% sample	200 mL
25 %	100 mL	100 mL of 50% sample	200 mL
50%	100 mL	100 mL of 100%sample	200 mL
l00%	0 mL	100 mL	100 mL

5.10. ***For the 50% concentration*****:** Measure and pour 100 mL of diluent (MHWCD) and 100 mL of 100% sample in the 500-mL flask and swirl to mix. Then dispense 20 mL of the solution into each of the five cups labeled for the 50% concentration.

5.11. ***For the 25% concentration*****:** Add 100 mL of the diluent to the 50% concentration remaining in the flask (should be 100 mL) mix and dispense 20 mL to each of the five 25% concentration cups.

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5.12. ***For the 12.5% concentration*:** Add 100 mL of the diluent to the 25% concentration remaining in the flask (should be 100 mL) mix and dispense 20 mL to each of the five 12.5% concentration cups.

5.13. ***For the 6.25% concentration*:** Add 100 mL of the diluent to the 12.5% concentration remaining in the flask (should be 100 mL) mix and dispense 20 mL to each of the five 6.25% concentration cups.

5.14. Standard reference toxicant (SRT) tests will be performed monthly since the *C. dubia* are cultured in-house. Sodium chloride is used for the toxicant. Test concentrations are prepared by diluting the "NaCl in MHWCD stock solution" with MHWCD. Measure and mix the listed volumes for each concentration. Dispense ~20 mL of each concentration to each of the appropriate cups (total of 5 cups per conc.). Discard the excess. Test concentrations can be checked by measuring the conductivity of the prepared concentrations. Total volumes are greater than the actual amount needed for each concentration in case of spillage.

Example of concentrations for KCl SRT.

Concentration	Diluent	SRT solution 1 g/L KCl	Total Volume
Control	100 mL	0 mL	100 mL
0.3 g/L	70 mL	30 mL of 1 g/L sample	100 mL
0.4 g/L	60 mL	40 mL of 1 g/L sample	100 mL
0.5 g/L	50 mL	50 mL of 1 g/L sample	100 mL
0.6 g/L	40 mL	60 mL of 1 g/L sample	100 mL
0.7 g/L	30 mL	70 mL of 1 g/L sample	100 mL

Approximate Conductivity readings at 25 °C.

Test Concentration g/l KCl	~Conductivity (µmhos/cm)
Control (0)	300
0.3 g/L	870
0.4 g/L	1075
0.5 g/L	1240
0.6 g/L	1430
0.7 g/L	1600

5.15. Place one cup from each set of the 5 replicate cups (containing test solutions) onto a second tray from which physical parameters will be measured.

5.15.1. A separate tray of cups prevents buffers and standards from accidentally being splashed into the test cups.

6. COUNTING AND RECORDING

6.1. Feed test organisms at least 2 hours prior to loading.

6.2. With a Pasteur pipet (or clean plastic transfer-pipet), load one *C. dubia* neonate into each of the four test cups of the controls and each test concentration. Repeat loading the organisms one per cup until a total of five organisms are loaded into each cup.

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6.2.1. (5 *C. dubia* per replicate) x (4 replicates per test concentration) = 20 *C. dubia* per concentration tested.

6.3. An analyst other than the analyst who originally loaded the test organisms should verify that five organisms are in each cup. The verification analyst should add or remove organisms as necessary to meet the five organisms per cup requirement. Place the tray of cups into the incubator and cover the tray with clear Plexiglas. Record counts. Record date and time of test initiation.

6.3.1. Loading and verification can be done at the dissecting scope if desired; otherwise it can be done at a light table.

6.3.2. Record counts in appropriate columns on *Acute Bioassay Bench Sheet.*

6.3.3. The time and date of test initiation (organisms loaded) is recorded on the test benchsheet.

6.4. Measure the pH, temperature, DO, and conductivity of the solutions in the "parameter" cups on second tray. Record readings. Record any comments pertaining to the condition or appearance of the sample. Empty these cups into the sink and flush the drain with water. Fill with water and store for the interim readings. At test termination dispose of cups in trash can. Store tray.

6.4.1. Record all 0-hour (refers to beginning of the first 24 hour interval not actual test initiation (completion of loading) time) readings in the appropriate columns on the “1st” replicate row on the *Acute Bioassay Bench Sheet*. 0-hour and 24-hour rows are usually labeled ‘m’; 48-hr before renewal row is usually labeled ‘A’, 48-hr after renewal and 72-hour are usually labeled ‘m’; 96-hour row is usually labeled ‘A’.

6.4.2. Refer to the FDEP Laboratory Quality Manual for sample disposal.

7. INTERIM READINGS

7.1. On test day 1 [t = 24 hr and any other interim readings (~24-hour intervals) if test duration is >48 hr], count number of surviving animals. Record counts.

7.1.1. Record counts in appropriate columns on acute bioassay bench sheets in laboratory notebook. DO NOT TAKE ANY METER READINGS IN THESE CUPS ON DAY 1 (t = 24 hr, or any other interim time) unless mortality in a cup = 100%.

7.2. **FOR CUPS WITH 100% MORTALITY ONLY,** measure the physical-chemical parameters and record in the appropriate spaces on the *Acute Bioassay Bench Sheet*.

7.3. If test duration is >48 hr, feed *C. dubia* 2 h prior to renewal at 48 hr.

7.3.1. To each cup, add 0.1 mL each of YCT and *Pseudokirchnerellia subcapitata* (formerly *S. capricornutum*).

7.3.2. Repeat steps 6.1 and 6.2 at ~ 24 hour intervals until test termination.

7.4. Replace tray in incubator. Cover.

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8. TESTS WITH DURATION >48 HOURS

8.1. On test day 2 (t = 48 hr), count and record number of surviving animals at least 2 hours prior to test renewal.

8.1.1. Record counts in appropriate columns on acute bioassay bench sheets in laboratory notebook. **DO NOT TAKE ANY METER READINGS IN THESE CUPS** prior to organism transfer.

8.2. Add 0.1mL of YCT and 0.1 mL of *Pseudokirchneriella subcapitata* (formerly *S. capricornutum*) to each cup containing surviving test organisms.

8.3. Prepare test cups and "new" test solutions following steps found under "Test Initiation." Measure and record DO, pH, temperature, and conductivity in the "parameter" cups containing "new" test solutions.

8.3.1. New test solutions are prepared from sample received on test day 0 that has been stored at 0-6 °C. Generally the sample is from an unopened container. Measure the same phys/chem parameters on the "new" test solution as was measured on the original sample on Day 0. If chlorine or ammonia were not detected in the original sample, it is generally not necessary to check for these components as well as alkalinity and hardness in the sample used for test renewal.

8.3.2. The "new" sample should be prepared during the two-hour feeding period. All sample manipulations (screening, pH adjustment, aeration, etc.) should be completed prior to use of the sample.

8.4. A minimum of 2 hours after feeding, use a transfer pipet to carefully transfer surviving test organisms (**adults only**-in the rare event that neonates produced by the original organisms are present at 48 hours) from cups containing "old" test solution to corresponding cups containing "new" test solution.

8.4.1. Record any additional mortality in the appropriate spaces on the bioassay bench sheets. Keep the transfer of "old" test solution to a minimum. Release test organisms under the surface of the test solution.

8.5. Measure and record DO, pH, temperature, and conductivity in cup (A) containing "old" solution (after organisms have been removed).

8.6. Perform steps 7.1 and 7.2 in the “Interim Readings” section at ~24 hr intervals until test termination.

9. TEST TERMINATION

9.1. On termination day (t = 48 hr (±~2 hr) or t = 96 hr (±~4 hr)) or whatever the test duration is), count number of surviving animals (**adults only** as it is not uncommon for the original test organisms to produce neonates by 72-96 hours). Record counts. Record any comments pertaining to the condition of the test animals. ***After** counting is completed*, measure pH, temperature, DO, and conductivity of the solutions in the cups. Record test termination date and time.

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9.1.1. Measurements for pH, temperature, DO, and conductivity are to be made (cup A) at test termination. Organisms can be present in the cups when final parameters are measured.

9.1.2. Record all counts, readings, date, and time in appropriate columns on *Acute Bioassay Bench Sheet*.

9.2. Pour contents of all test cups into sink and flush drain with water. Dispose of cups in trashcan and store tray.

9.2.1. Refer to the FDEP Laboratory Quality Manual for waste disposal procedures.

9.3. Enter test results into statistical program to obtain LC_{50}, per SOP TA-07.07 for definitive tests.

9.3.1. LC50s for screening tests are reported in LIMS as "100 L" if mortality is < 50% in the 100% sample test-solution; "100 K" if mortality in the 100% sample test-solution is > 50%; and, "100" if mortality in the 100% sample test solution is exactly 50%. The 100% percent test solution does not have to be 100% sample but does have to comply with the percent effluent designated in the facility's NPDES permit.

9.4. Enter test results into LIMS.

9.4.1. Results are entered into LIMS by one person and verified by a second person per SOP BG-01.

10. DATA ARCHIVAL

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

10.2. Record information in appropriate logbooks (*Acute Bioassay Bench Sheet* and *Daily Physical Chemistry Calibration Log Book*).

10.3. To correct raw data entries, place a single line through the incorrect entry, write the corrected entry near the error, date and initial the correction. Do not overwrite entries.

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

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

10.3.3. 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.).

10.4. Bioassay Sample Log-In log book. Record per SOP TA-06.14

10.5. Log sample receipt, temperature, and condition into Acute Bioassay Bench Sheets log book. Fill in information requested at top of bench sheets as completely as possible prior to test initiation. Include full name of facility, address, contact person, date and time collected, field TRC, LIMS sample and

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job numbers, test chamber ID, test chamber size, test solution volume, and food type, and food batch numbers. Access scanned Field Sheets via X:\Labs\BizhubScans\Receiving to obtain sampling information.

11. QUALITY CONTROL

11.1. Refer to the FDEP Laboratory Quality Manual for corrective action and handling out-of-control data.

11.2. Perform QA standard reference toxicant (SRT) tests monthly, using sodium chloride for the SRT.

11.3. If a problem occurs during 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, and 4) a nonconformance report (NCR) is submitted describing the problem and the corrective action.

12. SAFETY/HAZARDOUS WASTE MANAGEMENT

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

12.2. Refer to the FDEP Laboratory Health and Safety Plan.

12.3. Refer to the FDEP Laboratory Quality Manual for sample disposal.

12.4. Read MSDS information for reagent handling and disposal.

13. REFERENCES

13.1. FDEP Laboratory Quality Manual, https://floridadep.gov/dear/florida-dep-laboratory/content/dep-laboratory-quality-assurance-manual-and-sops

13.2. FDEP Laboratory Health and Safety Plan, Health and Safety Plan

13.3. EPA 821-R-02-012 Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, 5th Edition, Test Method 2002.0.

13.4. DEP SOP FS 1000, *General Sampling*

13.5. DEP SOP BG-01, *Data-Entry Verification*

13.6. DEP SOP TA-02.00, *Feeding Test-Organism Cultures*

13.7. DEP SOP TA-03.15, *Preparation of Synthetic Moderately Hard Freshwater*

13.8. DEP SOP TA-05.01, *Production of Ceriodaphnia dubia Young for Acute Toxicity Testing*

13.9. DEP SOP TA-06.04, *YSI Model 58 Dissolved Oxygen Meter Calibration and Measurement*

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13.10. DEP SOP TA-06.03, *Thermometer Accuracy Check and Use*

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

13.12. DEP SOP TA-06.14, *Bioassay Sample Log-In Procedure*

13.13. DEP SOP TA-06.33, *Measuring Ammonia using HACH® Ammonia Nitrogen Test Kits for Freshwater and Saltwater Samples*

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

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

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

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

13.18. DEP SOP TA-07.07, *Statistical Analysis of Acute Toxicity Test Results*

13.19. DEP SOP TA-09.18, *Dechlorination Procedure using Sodium Thiosulfate*

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*. Removed SOP TA-06.06 from Materials # 9 comment.
07/10/2009	Deleted (Based on *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms*, 5th Edition, EPA-821-R-21-012 Test Method 2002.0) from title. Updated Quality manual reference.
05/17/2010	Added 'CAUTION Individuals allergic to latex should not use latex gloves. A suitable substitute should be found and used. Deleted 'Vinyl gloves can be substituted for individuals allergic to latex in Safety Section comments. Updated the reference to the Biology Quality Manual. Revised "example of SRT concentrations" table in comment for Methods Step 14. Deleted '3ppt' from Step 14.
04/26/2011	Changed Biology Quality Manual reference to Bureau Quality Manual.
01/25/2014	Reformatted SOP.
02/11/2015	Formatted for 508 compliance.
01/06/2016	Updated links.
03/09/2016	Updated SOPs and references.
08/16/2016	Corrected SRT volumes for 1.25 ppth.
08/16/2017	Changed SRT from NaCl and KCL. Added SOP for HACH kit. Changed vial(s) to cup(s).
07/18/2018	Updated links. Changed incubator numbers. Added information for secondary verification when loading organisms.

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