Cyprinella leedsi and *Pimephales promelas* Acute Toxicity Testing

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 2000.0. It is used to estimate the acute toxicity of effluents and receiving waters (freshwater aqueous samples) with the bannerfin shiner, *Cyprinella leedsi* and the fathead minnow, *Pimephales promelas*, using 1 (one) to 14 (fourteen) day old organisms in a 48-hour static exposure or in a 96-hour static renewal exposure. Tests conducted using this procedure are BSY-AS-FFS and BSY-AD-FFS.

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 vessels. Test organisms are collected, fed and held for observation prior to loading into test vessels. Organisms are loaded into test vessels. Tests are incubated in laboratory incubators or water bath. Tests are checked at ~24-hour intervals for mortality. Survival/mortality is recorded daily. Physical-chemical parameters are measured daily. The test endpoint is mortality of the organisms. Results are entered into the laboratory information management system (LIMS).

2.2. Interferences: 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.

2.3. Test acceptability criteria: 90% or greater survival in the controls. Method modification allows reduction of organisms per chamber from 10 to 5 and increase in chambers/concentration from 2 to 4.

3. EQUIPMENT/SUPPLIES AND REAGENTS/STANDARDS

3.1. Young fish, *C. leedsi* or *P. promelas* (1–14 days old, 24-hr range).

3.1.1. Test organisms are obtained from vendors. Refer to SOP TA-04.20.

3.1.2. Young fish are fed live *Artemia nauplii* at least 2 hours prior to test initiation. 10 fish are used per concentration replicate unless the test method is modified to double the minimum number of replicates and to halve the number of organisms per replicate.

3.2. Test chambers (glass jars), 1 L, autoclaved

3.3. Synthetic moderately hard water (MHW)

3.3.1. Prepare in advance per SOP TA-03.15.

3.4. Cups, 30 ml plastic w/ parameter tray

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3.4.1. Used to hold aliquots collected from test solutions for measuring physical-chemical parameters. A parameter tray is prepared by placing clean vials in a pre-cut plexiglass tray. Label tape is placed in front of the vials. The tape is labeled with the appropriate concentration per vial.

3.5. Sample to be tested

3.5.1. 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 hours 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. See method manual for exceptions to the above requirement.

3.6. Graduated cylinders (500 mL, 1000 ml and/or 2000 ml)

3.6.1. Used for mixing test concentrations.

3.7. Erlenmeyer flasks (1000 ml, 2000 ml and 4000 ml)

3.7.1. Used for mixing test concentrations.

3.8. 8" Carolina dish

3.8.1. Transfer fish from the bag they were shipped in to Carolina dish for easier loading of bioassay jars. Also used to hold purchased organisms for several hours prior to test initiation.

3.9. Pipet, glass, with rubber bulb

3.9.1. For transfer of fish from Carolina dish to test jars.

3.10. Meters to measure physical-chemical parameters:

3.10.1. pH (Calibrate per SOP TA-06.23.)

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

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

3.10.4. Digital thermometer (Calibrated per SOP TA-06.13.)

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

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

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

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

3.11.4. Ammonia (Measure per SOP TA-06.33)

3.12. Thermo Scientific Incubator (Incubator # 1, #5 or # 6)

3.12.1. 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.

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3.12.2. Other incubators or the water bath can be used to regulate test temperature.

4. SAMPLE COLLECTION, PRESERVATION, AND HANDLING

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

4.2 See SOP TA-06.14 for Bioassay Sample Login Procedure

5. TEST INITIATION

5.1. Place appropriate number of clean test jars onto laboratory cart. Label each jar to identify the replicate (A, B, C, D, etc.), test concentration, and facility. Include the test species on the label when testing multiple species/samples. Place tape across front of chamber, beneath jar ID, and use waterproof marker to identify.

5.1.1. Minimum of two jars/20 organisms per concentration including controls for effluent samples and a minimum of four jars/40 organisms per concentration including controls for receiving water samples. Test jars can be dried in a drying oven for eight hours at 105 °C rather than being autoclaved.

5.1.2. Minimum test jar size is 250mL and minimum test volume is 200mL. We generally use 1-L jars and 500mL of sample per jar. We use four jars per concentration for screening tests and 2 jars per concentration for definitive tests. We reduce test volumes as needed based on sample volume on hand.

5.2. Calibrate meters and record calibration readings. Calibrate meters per respective SOP. Record calibration readings in *Daily Physical Chemistry Calibration Log* Book. Meters are generally calibrated daily when tests are in progress, otherwise calibrate as needed.

5.3. Measure the total residual chlorine (TRC) in the sample. Record the result in the TRC logbook and on the bioassay bench sheets. Check the field data sheet supplied by the sampler for field TRC and atypical field measurements.

5.3.1. Measure TRC per SOP TA-06.22. If TRC > 0.1 mg/L and non-chlorine toxicity is to be measured, dechlorinate an aliquot of the sample by the sodium thiosulfate method per SOP TA-09.18 and test dechlorinated replicates of all chosen test concentrations concurrently with additional chlorinated (100% sample ONLY) replicates.

5.4. Fill a disposable, plastic cup with sample and take an initial pH measurement.

5.4.1. 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. Refer to SOP TA-08.06 for pH adjustment.

5.5. In the same plastic cup containing the sample, take an initial DO measurement.

5.5.1. If the DO is ≤ 4.0 mg/L or ≥ 9.0 mg/L, aerate the entire sample to as near saturation as possible prior to test initiation. Samples with a potential DO problem generally show a downward trend in DO during the first few hours

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of the test. Monitor the DO closely. If the DO begins to fall, set up gentle aeration (<100 bubbles/min) to ALL test solutions for the duration of test using micropipettes and the aeration manifold.

5.6. In same plastic cup containing the sample, take an initial conductivity measurement. Conductivity readings can be rounded to the nearest 5 µmhos or 0.1 mmhos.

5.6.1. If the conductivity is above 2,000 µmhos/cm, then try to 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. leedsi* or *P. promelas* are likely not the appropriate test species for this effluent sample. This rationale does **NOT** apply to SRT tests.

5.7. ***Screening Test:*** Measure and pour 500 mL of well water into each of the control replicate test chambers. Measure and pour 500-mL aliquots of the 100% sample into the 100% test concentration test chambers.

5.7.1. Minimum of two jars for each set of control and test replicates needed for each sample tested. Test method can be modified to double the test chambers and decrease the number of organisms per replicate. The following table shows suggested sample volumes (per replicate in a 1L test chamber) for *acute screening* toxicity test. Diluent is synonymous with the control water.

Concentration	Diluent Volume	Sample	Total
Control	500mL	0mL	500mL
100%	0mL	500mL	500mL

5.8. ***Definitive Test (0.5 dilution):*** Measure and pour 500 mL of MHW into each of the control replicate jars. Measure and pour 500 mL aliquots of the 100% sample into each of the 100% sample concentration jars.

5.8.1. An example of sample volumes using two replicates and 500-mL solution (per replicate in a 1L test chamber) for an *acute definitive* toxicity test. Diluent is synonymous with control water.

Concentration	Diluent	Sample	Total Volume
Control	1000mL	0mL	1000mL
6.25%	937.5mL	62.5	1000mL
12.5%	875mL	125mL	1000mL
25%	750mL	250mL	1000mL
50%	500mL	500mL	1000mL
100%	0mL	1000mL	1000mL

5.9. ***For the 50% Concentration:*** Measure and pour 1000 mL of diluent (MHW) and 1000 mL of 100% sample in the 2000-mL flask. Swirl to mix. Then, using the 500-mL graduated cylinder measure and dispense 500 mL of the solution into each of the two replicate jars for the 50% concentration.

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5.10. ***For the 25% Concentration:*** Add 1000 mL of the diluent to the 50% concentration remaining in the flask (should be 1000 mL) mix and dispense 500 mL to each of the two 25% concentration jars.

5.11. ***For the 12.5% Concentration:*** Add 1000 mL of the diluent to the 25% concentration remaining in the flask (should be 1000 mL) mix and dispense 500 mL to each of the two 12.5% concentration jars.

5.12. ***For the 6.25% Concentration:*** Add 1000 mL of the diluent to the 12.5% concentration remaining in the flask (should be 1000 mL) mix and dispense 500 mL to each of the two 6.25% concentration jars.

5.13. Pour an aliquot from replicate A of each test concentration into the appropriately labeled parameter tray vial. Measure pH, DO, temperature and conductivity of the solution in each vial. Record readings. Record any comments pertaining to the condition or appearance of the sample.

5.13.1. Conductivity readings can be rounded to the nearest 5 µmhos or nearest 0.1 mmhos. Readings for pH, DO, and temperature shall be read to one decimal place. Due to the possibility of rapid cooling of solutions in the vials from the temperature in Room B246, test temperatures can be measured directly from the test replicate jars. The electronic thermometer is best for this measurement. It should be rinsed with DI water between each concentration.

5.13.2. Record all readings in appropriate columns on the *Acute Bioassay Bench Sheet*.

5.14. Using a glass pipet with rubber bulb, load **two** fish (20%) per replicate per test concentration then repeat until **ten (unless the method has been modified)** fish have been loaded into each test chamber. Adjust loading as necessary (add or remove test organisms). Record counts in appropriate columns on *Acute Bioassay Bench Sheet*.

5.15. Verify that the correct number of fish has been loaded within each replicate. A person other than the loader should verify the organisms per replicate. The verification person should remove malformed / injured / dead organisms and replace such with healthy organisms.

5.16. Place chambers together (in random order – use template) in the incubator and cover with clear Plexiglas. Covers prevent accidental contamination and minimize evaporation.

5.17. Measure alkalinity, hardness, and total ammonia of sample. Measurements made per SOPs TA-06.08, TA-06.09, and TA-06.33, respectively. Record measurements on *Acute Bioassay Bench Sheet*.

6. INTERIM READINGS

6.1. On day 2 (t = 24 hr and any other interim readings if test duration is >48 hr), count number of surviving fish, remove any dead fish, and record counts. Pour aliquots from one test replicate from each concentration into the 30-mL vials in the parameter

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tray. Measure pH, temperature, DO, and conductivity in the vials in the parameter tray. Record the measurements.

6.1.1. All counting is done over a light table. Fish are recorded as live if they move when lightly prodded or gill movement is seen. Record all counts and readings in appropriate columns on *Acute Bioassay Bench Sheet*.

6.1.2. The physical-chemical readings are taken from aliquots from the replicates in order to minimize disturbance to the fish and reduce the possibility of contaminating the test solutions.

6.1.3. Although not a requirement, we generally collect aliquots from the B replicates for the 24 hour parameters.

6.2. If test duration is >48 hr, feed *C. leedsi* and/or *P. promelas* 2 hr prior to renewal at 48 hours. To each chamber, add 0.2 mL concentrated *Artemia* nauplii at 48 hours (or 2 hr prior to renewal, if a renewal test is being performed).

6.3. Return the test chambers to the incubator (use initial random order template) when done and cover them with clear Plexiglas.

6.4. If test duration is >48 hr, repeat steps 6.1-6.3 at 72 hr. Although not a requirement, if a test has 2 replicates per concentration, we collect aliquots from the A replicates at 48 hours; if a test has 4 replicates per concentration, we collect aliquots from the C replicates at 48 hours.

7. TEST WITH DURATION >48 HOURS

7.1. On test day 2 (t = 48 hr), add 0.2 mL of concentrated *Artemia* nauplii at least 2 hours prior to test renewal.

7.2. Two hours after feeding, collect aliquots of "old" solution for parameters; then, use a screened siphon and carefully remove all but ~ 50 mL of "old" test solution. Remove as much debris as possible during this process. Count and record number of surviving organisms; remove dead organisms. Record counts in appropriate columns on acute bioassay bench sheets in laboratory notebook. Although not a requirement, if a test has 2 replicates per concentration, we collect aliquots of “old” solution from the A replicates at 48 hours; if a test has 4 replicates per concentration, we collect aliquots of “old” solution from the C replicates at 48 hours.

7.3. Use the 500-mL cylinder to carefully pour "new" test solutions into the appropriate test chambers.

7.3.1. New test solutions are prepared from sample received on test-day 0 that has been stored between 0-6°C (not frozen). 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 in the sample used for test renewal.

7.3.2. The "new" sample should be prepared during the two-hour feeding period. All sample manipulations (screening, pH adjustment, aeration, etc.) should be

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completed prior to use of the sample. Generally, two people work together performing the test renewals. One person removes the "old" solution; the other person adds "new" solution to the test chambers.

7.4. Collect aliquots of "new" solution into clean parameter vials. Measure and record DO, pH, temperature, and conductivity in each of the vials containing "new" solution.

7.5. Verify that the number of surviving organisms per replicate is the same as the survival counts prior to renewal. Record the after-renewal counts on the bench sheet.

7.6. Perform Steps 6.1-6.3 at 24 hr intervals until test termination.

8. TEST TERMINATION

8.1. On termination day (t = 48 hr (± ~2 hr) or t = 96 hr (± ~4 hr) or whatever the test duration is), count number of surviving fish. Measure parameters of the solutions in the chambers or from aliquots collected from the chambers. Record all counts and readings. Record any comments pertaining to the appearance or condition of the fish. Record test termination date and time.

8.1.1. Test may be terminated prior to the intended duration if 100% mortality has occurred in the test concentrations AND control mortality is ≤ 10%.

8.1.2. Counting is done over a light table. Physical-chemical readings (pH, DO, temperature and conductivity) can be taken directly from the test chambers.

8.1.3. Record counts, readings, date, and time in appropriate columns on *Acute Bioassay Bench Sheet*. At 96-hours we generally collect aliquots from the "A" replicates for tests with either 2 or 4 replicates.

8.2. Pour contents of all jars into the sink and flush drain with water. Place glassware on the dirty glassware cart. Refer to Laboratory Quality Manual for waste disposal procedures.

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

8.3.1. 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% 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.

8.4. Enter test results into LIMS.

8.4.1. The LIMS entry is verified by a second person prior to the result being uploaded per SOP BG-01.

9. DATA ARCHIVAL

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

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9.2. 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.

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

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

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.).

9.3. DEP Laboratory Information Management System (LIMS)

9.4. *Daily Physical Chemistry Calibration* Log

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

9.6. 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 job numbers, test chamber ID, test chamber size, test solution volume, and food type, and food batch numbers. Access scanned Field Sheets via \\fldep1\dear\Labs\BizhubScans\Receiving to obtain sampling information.

10. QUALITY CONTROL

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

10.2. Perform QA standard reference toxicant (SRT) tests monthly, using sodium chloride or potassium chloride as the toxicant.

10.3. 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..

11. SAFETY/HAZARDOUS WASTE MANAGEMENT

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

11.2. Refer to the DEP Laboratory Health and Safety Plan.

11.3. Refer to the DEP Laboratory Quality Manual for sample disposal.

11.4. Read MSDS information for reagent handling and disposal.

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12. REFERENCES:

12.1. DEP Laboratory Quality Manual, http://publicfiles.dep.state.fl.us/dear/labs/lab_qualitymanual_18.pdf

12.2. DEP Laboratory Health and Safety Plan, http://publicfiles.dep.state.fl.us/DEAR/DEAR%20Safety/FDEP%20Health%20%20Safety%20Plan%20B%20-%20June%202016.pdf

12.3. 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 2000.0.

12.4. DEP SOP FS 1000, *General Sampling*

12.5. DEP SOP BG-01, Data-Entry Verification

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

12.7. DEP SOP TA-04.20, *Receiving and Maintaining Purchased Test Organisms*

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

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

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

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

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

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

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

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

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

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

12.18. 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. Deleted *Obtain young per SOP #TA05_03* from Materials # 1 comment.

07/10/2009 Correct file name in *Documentation and Corrective Action* Step #8 comment. Updated Quality manual reference.

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05/18/2010	Added ‘CAUTION Individuals allergic to latex should not use latex gloves. A suitable substitute should be found and used.’ and deleted ‘Vinyl gloves can be substituted for individuals allergic to latex.’ in Safety Section comments. Updated the reference to the Biology Quality Manual.
04/26/2011	Changed reference to Biology Quality Manual to Bureau Quality Manual.
01/21/2014	Reformatted SOP.
02/11/2015	Formatted for 508 compliance.
01/05/2016	Updated links.
03/09/2016	Updated SOPs and references.
03/07/2017	Updated ammonia SOP information in section 3 and the reference section
02/23/2018	Updated links.

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