Pimephales promelas and *Cyprinella leedsi* 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 1000.0.* It is used to estimate the chronic toxicity of effluents and receiving waters (freshwater aqueous samples) to the fathead minnow, *Pimephales promelas*, using < 24-hour old (or < 48 hours if shipped) organisms in a 7-day static-renewal exposure. Tests covered by this procedure are BSY-CS-FFS and BSY-CD-FFS.

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. 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 or waterbath. Tests are checked daily for survival. Survival is recorded daily. Phys-chem parameters are measured and recorded daily. The test endpoints are survival and growth of the organisms. Results are entered in the laboratory information management system (LIMS).

2.2. Test acceptability criteria: 80% or greater survival in the controls and average dry weight per surviving control organism equal to or greater than 0.25 mg.

2.3. Currently, *C. leedsi* has not been approved by EPA for NPDES compliance testing. The certification request (submitted ~1997) was inactivated in 2011 at the request of the Biology Program.

2.4. Interferences: Refer to EPA-821-R-02-013 Section 11. 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. *P. promelas* or *C. leedsi* test organisms (<48 hours old)

3.1.1. Test organisms are fed live *Artemia nauplii* at least 2 hours prior to test initiation. We currently purchase test organisms. Refer to SOP TA-04.20.

3.2. Test chambers (glass jars), 1 L, autoclaved, 4 per test concentration

3.2.1. A test with 5 test concentrations and a control, using 4 replicates per treatment would require 24 chambers.

3.2.2. A minimum of four replicates per concentration is required.

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3.2.3. The minimum test chamber size is 500 mL (recommended minimum) and the minimum volume per replicate is 250 ml (recommended minimum). A loading of 10 organisms per replicate is recommended and a minimum of 40 organisms per concentration is required.

3.2.4. We generally use 1L test chambers and 500 mL of sample. Adjust chamber size and replicate volume as necessary to accommodate available sample.

3.3. Volumetric flasks, 10 - 1000 ml (as needed)

3.3.1. One set for toxicant/sample and one for diluent.

3.4. Graduated cylinders, 100 ml, 500 ml, 1000 mL and 2000 ml (as needed)

3.4.1. For measuring amount of toxicant/sample or diluent needed for each concentration. One set for toxicant/sample and one for diluent.

3.5. Erlenmeyer flasks, 1 L, 2 L, 4 L and 6 L (as needed)

3.5.1. For mixing test dilutions.

3.6. Volumetric pipettes, 1-10 mL, graduated

3.7. Pipettor for volumetric pipettes

3.8. Magnetic stir bars and stirrer

3.8.1. For mixing test dilutions.

3.9. Carolina dish, 8" diameter

3.9.1. Transfer fish from holding container to Carolina dish for easier loading of bioassay chambers.

3.10. Droppers and glass tubing with fire polished edges, 4 mm ID, for transferring larvae

3.10.1. Nets or Nytex® screen cannot be used to transfer larvae as screen can injure them.

3.11. Dilution water - MHW (prepared per SOP TA-03.15)

3.12. Sample to be tested

3.13. Meters to measure physical-chemical parameters:

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

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

3.13.3. Temperature (Digital thermometer Calibrate per SOP TA-06.13.)

3.13.4. Conductivity/salinity (Calibrate per SOP TA-06.25.)

3.14. Hach® kits to measure:

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

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

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

3.14.4. Ammonia (Measure per SOP TA-06.33.)

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3.15. Incubator should maintain selected test temperature (25±1 °C recommended) and a photoperiod of 16-hour light: 8-hour 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.

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

3.17. Plastic cups, 30 ml, with parameter tray

3.17.1. For aliquots of concentrations for parameters

3.18. Fine mesh fish net

3.18.1. To aid in euthanizing fish at test termination.

3.19. Ice bath

3.19.1. Use 8'' Carolina dish with ice and cold water. For euthanizing fish during test termination.

3.20. Waste bucket

3.21. Live *Artemia nauplii* (Prepare per SOP TA-01.03.)

3.22. Plastic transfer pipet

3.22.1. Used for feeding

3.23. Drying oven

3.23.1. Capable of sustaining 100° C for eight hours or 60° C for 24 hours. Refer to SOP TA-06.21.

4. SAMPLE COLLECTION, PRESERVATION, AND HANDLING

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

4.1.1. 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 to test initiation (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).

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

5. TEST INITIATION

5.1. Place clean test chambers onto laboratory cart. Label each chamber to identify the replicate (A–D), test concentration, and facility sampled.

5.1.1. Place tape across front of chamber, beneath jar #, and use waterproof marker to identify. Four chambers for each set of control replicates needed for each sample tested, plus four chambers per concentration for each sample tested.

5.2. Calibrate meters and record calibration readings.

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5.2.1. Calibrate meters per respective SOPs. Record calibration readings in *Daily Physical-Chemistry Calibration Log* Book.

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

5.3.1. 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 sample pH adjustment.

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

5.4.1. If DO is < 4.0 mg/L or $\geq$ 9.0 mg/L, aerate the entire sample to near 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 (<100 bubbles/min) to ALL test replicates for duration of test using micropipettes and the aeration manifolds. No action is required if DO increases after test initiation.

In the same plastic cup containing the sample, measure the conductivity and temperature of the sample. Check the conversion table to determine salinity or read salinity directly from the Mettler SevenMulti ® meter.

5.4.2. If conductivity is above 2,000 µmhos/cm or the salinity is more than 1 ppt, then freshwater organisms are probably not the appropriate test organisms for this effluent sample. Check with FDEP District personnel and also check the Facility's NPDES permit for bioassay requirements.

5.5. Measure total residual chlorine (TRC) in the sample. Check the field data sheet supplied by sampler for field TRC and atypical field measurements.

5.5.1. Measure TRC per SOP TA-06.22. 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 additional chlorinated (100% sample ONLY) replicates.

5.6. Measure the desired volumes of diluent and sample into a 6,000-mL Erlenmeyer flask using two graduated cylinders. Add the final amount of each solution with a 10-mL pipette when nearing the desired volume, so as not to overshoot. Place flask on stir table and stir until homogenized. Measure 2,000 mL of the mixture back into a 2,000-mL cylinder. Transfer 500 mL from the 2000 mL cylinder to a 500 mL cylinder. Empty the 500 mL cylinder into test chambers A through D for that particular concentration. Repeat for each test concentration.

5.6.1. For each sample tested, use four chambers per concentration in a dilution series chosen based on previous knowledge of toxicity level. Choose a minimum of 5 concentrations plus a control.

5.6.2. An example of sample volumes below for four 500 mL replicates of each concentration (0.5 dilution) of a *chronic* toxicity test:

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Concentration	Diluent	Sample	Total Volume
Control	2000 mL	0 mL	2000 mL
6.25%	2000 mL	2000 mL of 12.5%	4000mL
12.50%	2000 mL	2000 mL of 25%	4000 mL
25%	2000 mL	2000 mL of 50%	4000 mL
50%	2000 mL	2000 mL of 100%	4000 mL
l00%	0 mL	2000 mL of 100%	2000 mL

5.7. Perform QA standard reference toxicant (SRT) tests monthly (or concurrent with bioassay if bioassays performed less frequently; a concurrent SRT with one bioassay can be used as the SRT for a series of tests as long as the time interval does not exceed 30 days prior to the start of a bioassay test), using potassium chloride (other toxicants can be used) for the SRT.

An example of sample volumes for a *chronic* SRT test with potassium chloride as the toxicant:

Concentration	Diluent	Sample	Total Volume
Control	2000 mL	0 mL	2000 mL
0.0625 g/L	2000 mL	2000 mL of 0.125 g/L	4000mL
0.125 g/L	2000 mL	2000 mL of 0.25 g/L	4000 mL
0.25 g/L	2000 mL	2000 mL of 0.5 g/L	4000 mL
0.5 g/L	2000 mL	2000 mL of 1 g/L	4000 mL
1 g/L	0 mL	2000 mL of 1 g/:	2000 mL

5.8. Pour a subsample from all “A” replicates into the 30 ml cups in the parameter tray. Measure pH, DO, temperature, and conductivity of the solutions from the subsamples. Record all readings. Record any comments pertaining to the condition or appearance of the sample. On the following seven days, rotate through all of the replicates and take parameters before and after renewing the sample.

5.8.1. Conductivity readings can be rounded to the nearest 5 µmhos or 0.1 mmhos. Readings for pH, DO, and temperature are read to one decimal place. Take measurements from lowest to highest concentration.

5.8.2. Record all readings in the appropriate columns on the *Chronic Bioassay Bench Sheet.*

5.9. Using a wide bore pipette, load **two** (20% of test organisms per replicate) fish into each test chamber. Repeat the loading process until **ten** fish have been loaded into each replicate. Verify count over a light table. Record counts in appropriate columns on the chronic bioassay bench sheets in laboratory notebook.

5.9.1. (10 fish per replicate) x (4 replicates per test concentration) = 40 fish per concentration tested

5.9.2. A person other than the loader should verify the number of fish in each replicate.

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5.9.3. Record counts and physical-chemical readings in appropriate columns in the *Chronic Bioassay Bench Sheet.*

5.10. Use a plastic transfer pipet to feed fish 0.2 mL *Artemia nauplii* (700-1000 artemia) from concentrate once in the morning prior to renewals, and again after the renewals. Look at the fish on the light table to determine the number of drops to feed. Adjust volume fed based on surviving organisms to avoid water quality problems.

5.10.1. Feed once on Day 1 and then twice on succeeding days. *Artemia nauplii* concentrate is prepared by pouring *Artemia nauplii* onto a 20-µm screen, rinsing artemia with DI water, then rinsing artemia from screen with minimal DI water (10 -20 ml) into a vial.

5.11. Place chambers (use randomization template and maintain initial pattern for test duration) in incubator and cover with clear Plexiglas. Covers prevent accidental contamination and minimize evaporation.

5.12. Measure alkalinity, hardness, and total ammonia of sample and control water.

5.12.1. Measure per SOP TA-06.08 and TA-06.33, respectively.

6. INTERIM READINGS

6.1. Every 24 hours, pour subsamples of the appropriate replicates into 30 mL cups. Measure pH, temperature, DO, and conductivity in the subsamples before renewing the test solutions and record readings. Count and record the number of fish in each replicate. Clean out all dead *Artemia nauplii* at the bottom of each jar going up in concentration. Carefully siphon most, but not all, of the solution in each chamber through a fine mesh net attached to a plastic hose into the waste bucket. Try to keep the fish within the chamber. Replace the contents of each chamber with respective fresh concentrations of the sample.

6.1.1. All counting is done over a light table. Fish are recorded as live if they move when lightly prodded. Record counts and physical-chemical readings in appropriate columns in *Chronic Bioassay Bench Sheet.*

6.1.2. The remaining water after siphoning should measure a half-inch to an inch from the bottom of the chamber. Check the excess mesh net below the end of hose for trapped fish before siphoning the next jar. If a fish is stuck gently move the mesh around the water to remove the fish.

6.2. Pour subsamples from the appropriate replicate after renewal. Measure pH, temperature, DO, and conductivity after renewing the test solutions and record readings.

6.2.1. Subsamples are poured to minimize disturbance to the fish.

6.3. Feed fish 0.2 mL *Artemia nauplii* concentrate once in the morning two hours prior to renewals, and again after the renewals. Cover chambers when done and return to the incubator.

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7. TEST TERMINATION

7.1. On day 7 (t = 168 hour ± 6 hours), count the number of surviving fish. Remove and discard any dead fish. Pour subsamples and measure parameters of the solutions. Record all counts and readings. Record any comments pertaining to the appearance or condition of the fish. Record test termination date and time.

7.1.1. Counting is done over a light table. Physical-chemical readings are taken from the subsample to minimize disturbance to the fish.

7.1.2. Record counts, readings, date, and time in appropriate columns in *Chronic Bioassay Bench Sheet.*

7.2. Make weigh boats two days before test termination per SOP TA-06.20.

7.3. Pour the contents of Control replicate "A" onto a fine mesh net held over the waste bucket. Immediately euthanize the fish by immersing the fish in ice water in the Carolina dish. Keep the fish immersed for two minutes after the last movement is noted in the fish.

7.4. When the fish are dead, take a pair of forceps and place each fish into the respective weigh boat.

7.5. Repeat steps 6.3-6.4 for all test concentrations and replicates.

7.6. Dry the weigh boats containing the fish for eight hours in the sediment-drying oven the day after test termination. Store the Carolina dish in the desiccator in a vacuum before and after they are dried.

7.6.1. Refer to SOP TA-06.21.

7.6.2. Dry for eight (8) hours (minimum of six hours) at 100 °C.

7.6.3. Fish can also be dried for 24 hours at 60 °C.

7.7. Remove the dish after cooling, and weigh the weigh-boats. Record the weights in the *Chronic Bioassay Bench Sheet*.

7.7.1. Refer to SOP TA-06.21.

8. STATISTICAL ANALYSIS

8.1. Complete the weight page in the *Chronic Bioassay Bench Sheet* book.

8.2. Enter test results into statistical program to obtain LC_{50}, NOEC, LOEC and IC20.

8.2.1. Refer to SOPs TA-07.07 and TA-07.16.

8.3. Enter test results into LIMS.

8.3.1. Data is verified by a second person prior to being uploaded into LIMS.

9. DATA ARCHIVAL

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

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9.2. Log sample receipt, temperature and condition into *Log In Book: Bioassay*.

9.2.1. Per SOP TA-06.14, include LIMS numbers from Chemistry.

9.3. Record information in appropriate logbooks (*Chronic Bioassay Bench Sheets* and *Daily Physical Chemistry Calibration Log Book*).

9.4. 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.

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

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

9.4.3. 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.).

9.5. DEP Laboratory Information Management System (LIMS)

9.6. Log sample receipt, temperature, and condition into *Chronic 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 X:\Labs\BizhubScans\Receiving to obtain sampling information.

10. QUALITY CONTROL

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

10.2. 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.

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

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 DEP Laboratory Quality Manual for sample disposal

11.3. Refer to the DEP Laboratory Health and Safety Plan

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11.4. Read MSDS information for reagent handling and disposal.

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 Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, 4th Edition, Test Method 1000.0

12.4. DEP SOP FS 1000, *General Sampling*

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

12.6. DEP SOP TA-01.03, *Artemia nauplii* Culture

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

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

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

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

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

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

12.13. DEP SOP TA-06.20, *Making Weigh Pans For Chronic Tests*

12.14. DEP SOP TA-06.21, *Weighing Chronic Tests,*

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

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

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

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

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

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

12.21. DEP SOP TA-08.06, *pH adjustment of Samples: Algal Assay and Toxicity Bioassay*

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

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

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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 # 11 comment. Deleted *Obtain young per SOP #TA05_03* from Materials # 1 comment.
07/13/2009	Updated Quality Manual reference.
08/14/2009	Replaced reference to SOP TA-07.08 with reference 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.
03/17/2011	Updated references to Biology Quality Manual, now Bureau Quality Manual. Changed 105ºC to 100ºC throughout the SOP.
02/27/2012	Updated reference to Bureau Quality Manual. Added correction of illegible entries to the Documentation Section.
12/20/2013	Reformatted. Deleted step 3 "Count the fish in each replicate. Remove and discard any dead fish. Record number in the *Chronic Bioassay Bench Sheets*" under TEST TERMINATION and added "Repeat steps 6.3-6.4 for all test concentrations and replicates" after step 4.
02/12/2015	formatted for 508 compliance.
01/13/2016	Updated links.
03/09/2016	Updated SOPs and References.
03/10/2017	Updated SOPs and References.
02/26/2018	Updated links and removed reference to SOP TA-06.24.

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