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 whole 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 by 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 (phys-chem) parameters are measured and samples are adjusted as needed to meet permit or method test requirements. Sample is filtered through a 60µm screen to remove debris and/or indigenous organisms. Sample dilutions and control waters are placed into test chambers. Test organisms are collected, fed and held for observation prior to loading into test chambers. Tests are incubated in laboratory incubators during the duration of the test. Physical-chemical parameters are measured and recorded daily. Survival/mortality are recorded daily. The test endpoint is mortality of the organisms. Statistical endpoints are obtained from the EPA WET Statistics Excel spreadsheet per SOP TA-07.17. Results are entered in 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. Fish are fed live *Artemia nauplii* at least 2 hours prior to test initiation. 10 fish are used per concentration replicate, unless the

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test method is modified to double the minimum number of replicates to reduce the number of organisms per replicate.

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

3.3. Synthetic moderately hard water (MHW)

3.3.1. Prepare in advance per SOP TA-03.15.

3.4. 30 mL disposable plastic cups with parameter tray

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. 500 mL, 1000 mL, and /or 2000 mL Graduated cylinders

3.6.1. Used for mixing test concentrations.

3.7. 1000 mL, 2000 mL and 4000 mL Erlenmeyer flasks

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 chambers. Also used to hold purchased organisms for several hours prior to test initiation.

3.9. Glass pipet with rubber bulb

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

3.10. Meters to measure physical/chemical parameters:

3.10.1. pH (Calibrate per SOP TA-06.37)

3.10.2. Dissolved Oxygen (DO) (Calibrate per SOP TA-06.39)

3.10.3. Conductivity (Calibrate per SOP TA-06.36)

3.10.4. Digital thermometer (Calibration check per SOP TA-06.13)

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

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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 or SOP TA-06.35)

3.12. Thermo Scientific Incubator (Incubator #1, #5 or #7)

3.12.1. Incubator should maintain selected test temperature (20 ± 1°C or 25 ± 1 °C recommended) and a photoperiod of 16 h light: 8 h 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.

3.12.2. 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.

4.1.1. 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 hours from time of collection (Time of last sample collect for grab samples or the end of the 24-hour collection period for composite samples) to test initiation. 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. These samples typically arrive on Tuesday, Thursday and Saturday in 4L amber jugs. The remainder of each sample is stored in the Biology Walk-in cooler for solution renewal the following day or days.

4.2. See SOP TA-06.14 for Bioassay Sample Log-in procedure

5. TEST INITIATION

5.1. Place appropriate number of clean test chambers onto laboratory cart. Label each chamber 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 chamber ID, and use waterproof marker to identify.

5.1.1. Minimum of two chambers/20 organisms per concentration including controls for effluent samples and a minimum of four chambers/40 organisms per concentration including controls for receiving water samples. Test chambers can be dried in a drying oven for eight hours at 100 ± 5°C rather than being autoclaved.

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5.1.2. Minimum test chamber size is 250 mL and minimum test volume is 200 mL. The FDEP Laboratory generally uses 1 L chambers and 500 mL of sample per chamber. The FDEP Laboratory uses four chambers per concentration for screening tests and 2 chambers per concentration for definitive tests. Test volumes may be reduced as needed based on sample volume on hand.

5.2. Calibrate meters in accordance with their respective SOP and record calibration readings in LIMS. 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 per SOP TA-06.22. Record the result in LIMS TOX_Chlorine 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. If TRC ≥0.1 mg/L, and non-chlorine toxicity is to be measured, dechlorinate a subsample (volume needed to initiate the test) of the sample using sodium thiosulfate per SOP TA.9.18. Setup a concurrent screening test using the 100% sample that has not been dechlorinated and a control. Record chlorine result, amount of sodium thiosulfate used and final chlorine result after dechlorination in the logbook and in the designated section in LIMS

5.4. Fill a 30 mL disposable, plastic cup with sample and take an initial pH measurement per SOP TA-06.37. Record value the Bioassay Sample log-in logbook and LIMS TOX_pH.

5.4.1. If the pH is outside the acceptable range of 6.0–9.0 SU, then an additional test with a pH-adjusted subsample will be run. Adjust pH of sample per SOP TA-08.06. The test with the non-adjusted sample is then analyzed as a screening test, regardless of whether the pH-adjusted aliquot is used for a screening or definitive test.

5.5. In the same sample cup, take an initial DO measurement per SOP TA-06.39. Record value in Bioassay Sample Log-in logbook and LIMS TOX_DO logbook.

5.5.1. If the DO is ≤ 4.0 mg/L or ≥ 9.0 mg/L, aerate the entire sample at a rate of ~100 bubbles per minute to as near saturation as possible prior to test initiation. Record aeration (initial and final DO. aeration rate and duration) in the appropriate bench sheet. 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 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. No action is required if DO increases after test initiation.

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5.6. In the same sample cup, take an initial conductivity measurement. Record value in the Bioassay Sample Log-in Logbook and LIMS TOX_COND_SAL. Conductivity measurements 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 chambers 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 screen* toxicity test. Diluent is synonymous with the control water.

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

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

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	1000 mL	0 mL	1000 mL
6.25%	937.5 mL	62.5 mL	1000 mL
12.5%	875 mL	125 mL	1000 mL
25%	750 mL	250 mL	1000 mL
50%	500 mL	500 mL	1000 mL
100%	0 mL	1000 mL	1000 mL

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 chambers for the 50% concentration.

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5.10. ***For the 25% Concentration**:* Add 1000 mL of the diluent to the 1000 mL of 50% concentration remaining in the flask, mix and dispense 500 mL to each of the two 25% concentration chambers.

5.11. ***For the 12.5% Concentration**:* Add 1000 mL of the diluent to the 1000 mL of 25% concentration remaining in the flask, mix and dispense 500 mL to each of the two 12.5% concentration chambers.

5.12. ***For the 6.25% Concentration**:* Add 1000 mL of the diluent to the 1000 mL of 12.5% concentration remaining in the flask, mix and dispense 500 mL to each of the two 6.25% concentration chambers.

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 chambers. 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 located in B246) in the incubator and cover with clear Plexiglas. Order is determined by one roll of the dice located in the laboratory drawer. This same randomizer number will be used throughout the test. Covers prevent accidental contamination and minimize evaporation.

5.17. Measure alkalinity, hardness, and total ammonia of sample. Measurements made per SOPs TA-06.08, and TA-06.33/TA-06.35, respectively. Record measurements on *Acute Bioassay Bench Sheet*.

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6. INTERIM READINGS

6.1. On day 1 (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 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, the FDEP Laboratory generally collect aliquots from the B replicates for the 24 hour parameters.

6.2. If test duration is >48 hr, proceed to section 7. If the test duration is 48 hours, proceed to section 8.

7. TEST WITH DURATION >48 HOURS

7.1. On test day 2 (t = 48 hr), feed *C. leedsi* and/or *P. promelas* according to SOP TA-02.00. Add 0.2 mL of concentrated *Artemia* nauplii at least 2 hours prior to test renewal. Collect aliquots of "old" solution for parameters.

7.2. Two hours after feeding, Count and record number of surviving organisms; remove dead organisms. Record counts in appropriate columns on acute bioassay bench sheets in laboratory notebook. 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. Although not a requirement, if a test has 2 replicates per concentration, collect aliquots of “old” solution from the A replicates at 48 hours; if a test has 4 replicates per concentration, 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 and not frozen. Generally, the sample is obtained from an unopened container. Measure the same physical/chemical 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

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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 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. Feed organisms after renewal according to SOP TA-02.00. Return the test chambers to the incubator (use initial random order template) when done and cover them with clear Plexiglass.

7.7. Perform Step 6.1 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, and conductivity) are taken from a parameter vial. Temperature can be taken directly from the test chambers. At 96-hours, collect aliquots from the A replicates for tests with either 2 or 4 replicates.

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

8.2. Pour contents of all chambers 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-7.17 for definitive tests.

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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.

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 as possible to the error or 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 with a letter. Initial and date the explanation. Do not attempt to put all information (correction and comments) into a small space.

9.2.3. 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.).

9.3. FDEP Laboratory Information Management System (LIMS)

9.4. Record information in appropriate logbooks (Acute Bioassay Bench Sheet).

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, food type, and food batch numbers. Access scanned Field Sheets via \\fldep1\dear\Labs\BizhubScans\Receiving to obtain sampling information.

9.7. Enter the LC50, EC50, NOEC, LOEC, and/or IC20 and IC25 results from the EPA WET Excel spreadsheet into the appropriate logbook, appropriate computer file and LIMS results editor. A second person must verify data prior to uploading into LIMS.

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10. QUALITY CONTROL

10.1. Refer to DEP Laboratory Quality Manual for corrective action and handling out-of-control data. Refer to EPA 821-R-02-012 for test conditions and acceptability criteria.

10.2. Perform QA standard reference toxicant (SRT) tests monthly or concurrently.

10.3. Samples should be held at 0-6 degrees C prior to test initiation and between renewals (exception for hand delivered sample day of collection - sample should be cooling relative to collection temperature). Sample holding time should not exceed 36 hours from time of collection until initial use of sample.

10.3.1. Incubator maintained at the selected test temperature of 25 ± 1°C.

10.3.2. Test temperature must not deviate more than 3°C in the test cups for the duration of the test.

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

10.4. 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.

10.5. To create NCR: open LIMS and go to Laboratory/Administrative/Non-Conformance Report. Refer to https://floridadep.gov/dear/quality-assurance/content/dep-sops for sample collection and preservation, and the Laboratory Quality Manual for corrective action and handling out-of-control data.

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.

12. REFERENCES:

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

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12.2. DEP Laboratory Health and Safety Plan, https://www.floridadep.net/resources/health-safety-manual

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-01.03-4.22, *Artemia nauplii Culture*

12.7. DEP SOP TA-02.00-6.7, *Feeding Test-Organism Cultures*

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

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

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

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

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

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

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

12.15. DEP SOP TA-06.35, *Measuring Total Ammonia using the HACH Ammonia Nitrogen Pocket Colorimeter II Test Kit*

12.16. DEP SOP TA-06.36, *Calibration and Use of Fisherbrand Traceable Bench Conductivity Meter for Conductivity and Salinity*

12.17. DEP SOP TA-06.37, *Calibration and Use of Fisherbrand FE150 pH Benchtop Meter*

12.18. DEP SOP TA-06.39, *Thermo Scientific Orion Star A213 Benchtop RDO/DO Meter Calibration and DO Measurement*

12.19. DEP SOP TA-07.17, *Using EPA Whole Effluent (WET) Statistics for Acute and Chronic Bioassay Tests*

12.20. DEP SOP TA-09.01, *Sodium Chloride and Potassium Chloride Reference Toxicant Stock Solutions*

12.21. 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*.

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	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.
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 and SOPs for new meters.
02/22/2019	Updated links and SOPs for new meters.
01/02/2020	Updated links and SOPs for new meters.
01/29/2021	Updated SOP for common universal language and grammar accuracy.

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