Americamysis (formerly *Mysidopsis) bahia* Acute Toxicity Testing Methods

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 2007.0. It is used to estimate the acute toxicity of effluents and receiving waters to the opossum shrimp, *Americamysis bahia* (formerly known as *Mysidopsis bahia*), using 1 (one) to 5 (five) day old organisms in a 48-hour static exposure or in a 96-hour static renewal exposure. Tests covered by this procedure are BSY-AS-MMY and BSY-AD-MMY.

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 fed and held for observation prior to loading into test vessels. Organisms are loaded into test vessels. Tests are incubated in laboratory incubators or waterbath. Tests are checked at ~24 hour intervals for mortality. Survival/mortality is recorded daily. Phys-chem parameters are measured daily. The test endpoint is mortality of the organisms. Results are entered into the laboratory data management system.

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; 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. *A. bahia* test organisms (1–5 days old, 24-hr range) acclimated to test salinity

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

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

3.3. Natural Seawater (NSW) or Artificial Saltwater (ASW)

3.3.1. NSW or ASW (refer to SOP TA-03.02) diluted to the salinity of the sample is used as the control. In a test where salts are added to the sample to accommodate the salinity requirements of the test organisms, NSW or ASW diluted to the original sample salinity and then adjusted with salts to the test salinity is used as a 'salt' control. (This is in addition to the control). Salt adjustments made per SOP TA-07_13. *A minimum of 1 hour, preferably 2 hours, should pass prior to loading organisms in salted solutions.*

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3.4. Aged hypersaline brine (HSB)

3.4.1. Used to adjust ASW to match higher sample salinities without adding salt crystals. Aged, hypersaline brine is made in advance of testing per SOP TA-03.09. HSB can also be made per EPA-821-R-02-012 Section 7.3.7.

3.5. Volumetric pipets, 10 mL, with Pipetter/bulb

3.6. Sample to be tested

3.7. Graduated cylinders, 250 ml, 500 ml and 1 L (as needed)

3.8. Erlenmeyer flasks, 1-L, 2 L and 4 L (as needed)

3.9. 8" Carolina dish

3.9.1. Transfer mysids from generator to Carolina dish for easier loading of test chambers. Also used to hold purchased organisms.

3.10. 5cm by 5cm spectra® mesh screen having openings ≤ 200 µm

3.10.1. Used to transfer mysids, without damage, from Carolina dish to test chambers.

3.11. Meters to measure physical-chemical parameters:

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

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

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

3.11.4. Salinity/Conductivity (Calibrate per SOP TA-06.25.)

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

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

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

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

3.13. Denver Instruments® Model 225 pH/ion meter with ammonia probe

3.13.1. Calibrate per SOP TA-06.17.

3.14. Precision Scientific Model 818 Incubator

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

3.15. Cups, 30 mL plastic w/ parameter tray

3.15.1. Subsamples from the test chambers are poured into corresponding vials and parameters are measured from the subsamples. This is done to reduce

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disturbance of the test organisms, and to minimize risk of possible contamination.

3.16. Live *Artemia* nauplii

3.16.1. Prepare per SOP TA-01.03.

4. SAMPLE COLLECTION, PRESERVATION, AND HANDLING

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

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

4.3 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, B, C, etc.), test concentration, and facility. Place tape across front of chamber, beneath jar #, 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 105 °C in place of autoclaving.

5.1.2. Minimum test chamber size is 250 mL and minimum test volume is 200 mL. We generally use 0.5-L chambers and 300 mL of sample with a loading of 5 organisms per chamber. We reduce test volumes and number of test chambers when we have inadequate sample for "regular" testing.

5.2. Calibrate meters and record calibration readings. Calibrate meters per respective SOPs. 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. Fill a disposable, plastic cup with sample and take an 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. See SOP TA 08.06 for pH adjustment.

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

5.4.1. If the DO is $\leq$ 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 the DO begins to fall, set up gentle aeration (<100

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bubbles/min) to ALL test solutions for the duration of test using micropipettes and the aeration manifold.

5.5. In same plastic cup containing the sample, measure the conductivity and temperature of the sample. Conductivity readings can be rounded to the nearest 5 µmhos or 0.1 mmhos.

5.5.1. Check the conversion table to determine salinity or read the salinity directly from the Mettler SevenMulti ® meter. If the conductivity is below 2,000 µmhos/cm or 1 ppt, then *A. bahia* is probably not the appropriate test organism for this effluent sample. Check facility NPDES permit requirements. Occasionally saltwater species are used in addition to freshwater species at the request of district personnel.

5.5.2. If the salinity is <6 ppt, and no ion imbalance is suspected, increase sample salinity up to 6 ppt with sea salts. If the salinity < 6 ppt and ion imbalance is suspected add an additional 6 ppt to the original apparent salinity of the sample. If the salinity is > 6, then no adjustment is necessary. Salinity adjustments are made per SOP TA-07.13. Salinity can be 7‰ if necessary. If a permit requires a specific salinity or if district personnel request a specific salinity, the sample, control, and salt control can be run at the requested salinity. When salt adjustments are made to the sample, an additional salt control must be prepared by diluting NSW or ASW to the sample's original salinity and adding artificial sea salts to match the salinity of the salt-adjusted sample. This salt-control is tested with the control and the salt-adjusted sample.

5.5.3. If the salinity is between 6 and 20 ppt, then add well water to the Carolina dish holding the mysids to begin their acclimation. The mysids are cultured and held in 20 ppt ASW. Decrease the salinity in the holding dish toward the sample salinity by adding well water to acclimate.

5.5.4. If the salinity is above 20 ppt, then add hypersaline brine to the Carolina dish holding the mysids to begin their acclimation. The mysids are cultured and held in 20 ppt ASW. Increase the salinity in the holding dish toward the sample salinity by adding hypersaline brine to acclimate.

5.5.5. The EPA document recommends that mysids should not be subjected to a change of more than 3-ppt salinity in 12 hours. If the salinity is known on the day prior to sample receipt, then begin acclimation that day at the EPA recommended rate and continue through the next morning if needed. If the salinity is not known until the time of sample receipt, then acclimate mysids as closely as possible to the EPA rate. Set up the test and load the mysids prior to the end of the workday. Mysids can be acclimated from 20‰ by ± 5‰ initially, then after two hours adjust by ± 3‰ every two hours until the test salinity is matched. However, it is better to know the required test salinity and make adjustments as needed three to four days in advance.

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

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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.

5.7. ***Screening Test:*** Measure 500 mL of NSW or ASW of the appropriate salinity into each of the control replicate chambers. Measure 500-mL aliquots of the 100% sample into the 100% test concentration chambers.

Example sample volume per 1L test chamber for *acute screening* toxicity test: Diluent is synonymous with control water.

Concentration	Diluent	Sample	Total Volume
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 control water 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.

5.9. The sample volumes below are an example for four 500 mL-replicates of each concentration for an *acute definitive* toxicity test: Diluent is synonymous with control water. Adjust volumes and replicates as needed; ensure that minimum volumes and minimum number of replicate requirements are met.

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

5.10. ***For the 50% Concentration:*** Measure and pour 2000 mL of diluent and 2000 mL of 100% sample in the 4000-mL flask. Swirl to mix. Then, using the 500-mL graduated cylinder measure and dispense 500 mL of the solution into each of the four replicate chambers for the 50% concentration.

5.11. ***For the 25% Concentration:*** Add 2000 mL of the diluent to the 50% concentration remaining in the flask (should be 2000 mL) mix and dispense 500 mL to each of the four 25% concentration chambers.

5.12. ***For the 12.5% Concentration:*** Add 2000 mL of the diluent to the 25% concentration remaining in the flask (should be 2000 mL) mix and dispense 500 mL to each of the four 12.5% concentration chambers.

5.13. ***For the 6.25% Concentration****:* Add 2000 mL of the diluent to the 12.5% concentration remaining in the flask (should be 2000 mL) mix and dispense 500 mL to each of the four 6.25% concentration chambers.

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5.14. Perform QA standard reference toxicant (SRT) tests monthly, using cadmium chloride (toxicant can be changed as needed) for the SRT.

5.14.1. An example of sample volumes for *acute definitive* cadmium SRT test, using the toxicant made per SOP TA-09.20: Concentrations and toxicants can be modified as needed. Diluent is synonymous with control water. Volumes rounded.

Concentration	Diluent	SRT	Total Volume
Control	2000 mL	0 mL	2000 mL
20 µg/L	1000 mL	2000 mL of 30 µg/L	6000 mL
30 µg/L	2000 mL	4000 mL of 45µg/L	6000 mL
45 µg/L	2000 mL	4000 mL of 67µg/L	6000 mL
67 µg/L	2000 mL	4000 mL of 100µg/L	6000 mL
100 µg/L	6000 mL	6 mL (100µg/ml)	6000 mL

5.15. Pour aliquots from the A replicates into 30-mL cups held within a parameter tray. Measure pH, temperature, DO, and conductivity from the subsamples. Record all readings. Record any comments pertaining to the condition or appearance of the sample.

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

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

5.16. Use the screen to load 20% of test organisms per replicate - into **each** test chamber. Repeat the loading until the desired number of mysids has been loaded into each chamber. Load 2 organisms at a time for 10 organisms/replicate and 1 organism at a time for 5 organisms/replicate.

5.17. Verify the count over a light table. A person other than the loader should verify the number of organisms per replicate. Record counts and physical-chemical readings in appropriate columns in *Acute Bioassay Bench Sheet.*

5.18. Feed test organisms (in test vessels). Feed 0.2 mL concentrated (~100 *Artemia* nauplii/mysid) each day. To avoid potential DO problems, feed half the amount twice daily, checking to determine appropriate amount of *Artemia* to add (based on *Artemia* remaining in test chamber).

5.19. Place chambers together but in random order in incubator and cover with clear Plexiglas. Covers prevent accidental contamination and minimize evaporation.

5.20. Measure alkalinity and total ammonia of sample following test initiation. Measurements made per SOP TA-06.08 and TA-06.17, respectively.

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6. INTERIM READINGS

6.1. On day 2 (t = 24 hr and any other interim readings if test duration is > 48 hr), count and record the number of surviving mysids. Use a transfer pipet to remove any dead test organisms and to remove dead *Artemia* nauplii. Pour aliquots (see comment) into the parameter tray and measure pH, temperature, DO, and conductivity daily. Record readings. When done, feed mysids, return jars to incubator, and cover.

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

6.1.2. Although not a requirement, we generally follow: (four replicate concentrations) Aliquots are taken from B replicates at 24 hours, C replicates at 48 hours (for 48 hour termination or “old” and “new” solutions for renewals), D replicates at 72 hours and A replicates at 96 hours. (Two replicate concentrations) Aliquots are taken from B replicates at 24 hours, A replicates at 48 hours (for 48 hour termination or “old” and “new” solutions for renewals), B replicates at 72 hours and A replicates at 96 hours. (If a replicate is not available (all dead in the replicate the previous day), the next sequential replicate is used.

6.2. If test duration is >48 hr, repeat step 6.1 at 72 hr.

7. TEST WITH DURATION > 48 HOURS

7.1. Acute bioassay test duration generally does not exceed 96 hours.

7.2. On test day 2 (t = 48 hr), add 0.1 mL of concentrated *Artemia* nauplii at least 2 hours prior to test renewal.

7.3. Collect aliquots of "old" solution for parameters; then, use a screened siphon to 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*.

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

7.4.1. 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 or measure alkalinity in the sample used for test renewal.

7.4.2. 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

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performing the test renewals. One person removes the "old" solution; the other person adds "new" solution to the test chambers.

7.5. Collect aliquots of "new" solution (from test chambers) into clean parameter vials. Measure and record DO, pH, temperature, and conductivity in each of the vials containing "new" solution.

7.6. 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.

7.7. Perform Step 6.1 at 24 hr intervals until test termination. Feed only 0.1 mL concentrated *Artemia* nauplii immediately after renewal. Resume regular feeding at 72 hours.

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 the number of surviving mysids. Measure parameters of the solutions in the chambers or in aliquots from the chambers poured into vials in the parameter tray. Record all counts and readings. Record any comments pertaining to the appearance or condition of the mysids. 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%. Test can also be terminated in the event of a control failure.

8.1.2. Counting is done over a light table. Physical-chemical readings (pH, DO, and conductivity) are taken in the appropriate replicate chamber for each concentration per Step 6.1.2.

8.1.3. Record counts, readings, date, and time in appropriate columns in *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 Bureau Quality Manual for waste disposal procedures.

8.3. Enter test results into 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% 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.

8.4. Enter test results into LIMS.

8.4.1. Test results are verified by a second person prior to data being uploaded in LIMS.

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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 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.

9.3. 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.).DEP Laboratory Information Management System (LIMS)

9.4. *Daily Physical Chemistry Calibration Log*

9.5. *Bioassay Sample Log-In log* book

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\Lab3\Receiving to obtain sampling information.

10. QUALITY CONTROL

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

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

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 FDEP Laboratory Health and Safety Plan.

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

11.4. Read MSDS information for reagent handling and disposal.

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12. REFERENCES:

12.1. FDEP Laboratory Quality Manual, http://www.dep.state.fl.us/labs/library/lab_sops.htm

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

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 2007.0.

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-03.02, *Preparation of Artificial Seawater (ASW)*

12.8. DEP SOP TA-03.09, *Hypersaline Brine Production*

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

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

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

12.12. DEP SOP TA-06.13, *Thermometer Accuracy Check and use*

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

12.14. DEP SOP TA-06.17, *Measuring Total Ammonia*

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-06.24, *Calibration and Use of Mettler Toledo SevenMulti for Measuring pH*

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

12.19. DEP SOP TA-07.07, *Statistical Analysis of Acute Toxicity Test Results* DEP SOP TA-07.13, *Salinity Adjustment of Samples: Toxicity Bioassay*

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

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

12.22 DEP SOP TA-09.20, *Cadmium Chloride Reference Toxicant Solutions*

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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_04* from Materials # 1 comment. Inserted *(refer to SOP TA-03.02)* and changed *TA-07.12* to *TA-07.13* in Materials # 3 comment.

07/10/2009 Replaced "all chambers" with "the appropriate replicate chamber for each concentration per comment for *Interim Readings* Step 1". 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 Biology Quality Manual reference to Bureau Quality Manual.

01/06/2014 Reformatted SOP.

02/11/2015 Formatted for 508 compliance.

01/06/2016 Updated links.

03/09/2016 Updated SOPs and references.

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