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Freshwater Toxicity Testing Procedures

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Marine and Estuarine Toxicity Testing Procedures

Guidance on Toxicity Identification Evaluations

Subject: Preparation of Synthetic Water

Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan		09-01-19
Quality Assurance Officer	Jim Sumner		09-01-19

Document Revision History

Effective Date	Revision number	Review Type	Evaluators	Revisions
12-01-00	0	Internal	Jim Sumner (ETS)	Original document
07-20-09	1	External (TVA, Environmental Standard, Inc.) Internal	William Rogers (TVA) Cynthia Russell (TVA) Rick Sherrard (TVA) Rock Vitale (Environmental Standards, Inc.) Jim Sumner (ETS)	• Document revision history initiated. • Procedure and Exhibits AT1.1 and AT1.2 amended to include documentation of synthetic water pH. • Table AT1.1 was revised to distinguish between approximate and required analytical ranges of synthetic water.
06-01-11	2	Internal	Jim Sumner (ETS)	• Procedure and Exhibits AT1.1 and AT1.2 amended. • Analytical requirements revised for requirements of each new lot of chemicals purchased. • Updated references.
06-20-12	3	External (TVA) Internal	William Rogers (TVA) Donald Snodgrass (TVA) Rick Sherrard (TVA) Jim Sumner (ETS)	• Procedure and Exhibits AT1.1 amended to include the approximate resistivity limit. Included course of action if the resistivity limit is exceeded.
11-01-14	4	Internal	Jim Sumner (ETS)	• Provided clarification on the procedure for preparing salt synthetic water.
01-01-18	5	Internal	Jim Sumner (ETS)	• Updated procedure to include NELAP requirements. • Additional guidance included in SOP. • Updated procedure for hard synthetic water used for the culturing of fathead minnows.
09-01-19	6	Internal	Jim Sumner (ETS)	• Removed preparation of SSW. • Removed use of Milli-Q® water. • Updated logs.

Subject: Preparation of Synthetic Water

Scope and Application

To provide chemically defined water for conducting toxicity tests and maintaining cultures.

Summary of Method

Standard, synthetic dilution water is prepared with deionized water and reagent grade chemicals. The source water for the deionized water systems is tap water (as described in SOP-G8).

Holding Time

All synthetic water must be discarded **14-days** after the preparation date (date chemicals are added to the deionized water).

Quality Control

Minimum requirements: Synthetic water must meet minimum requirements, as indicated in Table AT1.1 to be used for culturing and testing.

Table AT1.1: Approximate and required ranges for chemical analyses of synthetic water.

Parameter	**Moderately hard synthetic water (MHSW)**	**Hard synthetic water (HSW)**	**Salt synthetic water (SaltSW)**
pH (S.U.)	**Required range = 6.5 – 8.5** Approximate range = 7.4 – 7.8	Approximate range = 7.6 – 8.0	**Required range = 6.5 – 8.5**
Conductance (µmhos/cm)	Approximate average = 310	Approximate average = 700	Not applicable
Alkalinity (mg/L $CaCO_3$)	**Required range = 57 – 64**	Approximate average = 110 – 120	Approximate range = 80 – 120
Hardness (mg/L $CaCO_3$)	**Required range = 80 – 100**	Approximate average = 160 – 180	Not applicable
Salinity (ppt)	Not applicable	Not applicable	**Required range = 23.0 – 26.0** Approximate range = 24.0 – 25.0

Approximate ranges and averages are for guidance only. Analyses of synthetic water must be within the required ranges prior to use.

Subject: Preparation of Synthetic Water

Synthetic water quality:
Analyses are performed on Moderately Hard Synthetic Water (MHSW) with each new lot of chemicals purchased. Chemicals are purchased so that a single lot will be used for at least 1 year. USEPA recommends < 50 ng/L total organochlorine pesticides plus PCBs, < 1 µg/L total metal each of Al, As, Cr, Co, Cu, Fe, Pb, Ni, Zn and < 100 ng/L total metal each of Cd, Hg, Ag. Pesticide concentrations should not exceed USEPA's Ambient Water Quality chronic criteria where available. Analytical detection limits may be above these established criteria; however, the lowest available detection limits for each analyte are performed. Analytical test results are maintained in the laboratory's QC files.

Analyses are performed on the Salt Synthetic Water (Salt SW) with each new lot of salt purchased. Salt is purchased so that a single lot will be used for at least 1 year. USEPA recommends < 1 µg/L total metal each of Al, As, Cr, Co, Cu, Fe, Pb, Ni, Zn and < 100 ng/L total metal each of Cd, Hg, Ag. Matrix interferences associated with salt synthetic water results in detection limits above these criteria for many of the analytes; however, the lowest available detection limit for each analyte is performed. Marinemix® contains trace elements found in natural sea water, which may result in metal concentrations higher than the USEPA recommendation. If analytical results are above the USEPA recommendation, the overall health (survival and growth) and sensitivity of the test organisms through reference toxicant testing is used to assess the acceptability of the sea salt. Analytical test results are maintained in the laboratory's QC files.

Equipment and Materials

57-L Nalgene® tank with spigot
Sodium carbonate ($NaHCO_3$)
Calcium sulfate dihydrate ($CaSO_4 \cdot 2H_2O$)
Magnesium sulfate ($MgSO_4$)
Potassium chloride (KCl)
Crystal Sea® Marinemix – Bioassay Laboratory Formula
Calibrated top-loading balance (e.g. Fisher Scientific ACCU-224)
Weigh boats
Deionized water
10% nitric acid solution
Aeration system with pump, multiple aeration sites, tubing, and air stones
Equipment and Materials as required by SOPs C3, C4, C5, C6 and C7
Synthetic Water Preparation Log
MHSW, Alkalinity and Hardness Quick Check Log

Subject: Preparation of Synthetic Water

Procedure

A. Preparation of Synthetic Water (Freshwater).

1. Moderately Hard Synthetic Water (MHSW) is used for culturing and conducting toxicity tests using the following freshwater species: *Ceriodaphnia dubia*, *Chironomus dilutus*, *Daphnia magna*, *Daphnia pulex* and *Hyalella azteca*. *Pimephales promelas* are cultured in Hard Synthetic Water (HSW) and MHSW is used for conducting toxicity tests with this species.

2. Clean the appropriate cylindrical tank with hot tap water, scouring pads and bristle brushes. Scrub the tank until clean.

3. Immediately rinse the tank with hot tap water and then with deionized water.

4. Rinse with a 10% nitric acid solution. After rinsing with nitric acid, rinse the tank repeatedly with deionized water (at least 3 times).

5. Fill the tank with approximately 50 L of deionized water to prepare HSW or MHSW.

6. Place an aeration tube with aeration stone in the tank such that the aeration stone rests on the bottom of the tank.

7. Add the required amounts of $NaHCO_3$, $CaSO_4 \cdot 2H_2O$, $MgSO_4$ and KCl according to Table AT1.2. These dry chemicals may be added directly to the top of the deionized water in the tank.

8. The synthetic water must aerate overnight before use.

Table AT1.2: Guide to preparing synthetic water.

Chemical	**Moderately hard synthetic water (MHSW)** (Total volume = 50 L)	**Hard synthetic water (HSW)** (Total volume = 50 L)
Sodium bicarbonate ($NaHCO_3$)	**5.20 g**	**10.50 g**
Calcium sulfate dihydrate ($CaSO_4 \cdot 2H_2O$)	**3.30 g**	**6.00 g**
Magnesium sulfate ($MgSO_4$)	**3.30 g**	**6.00 g**
Potassium chloride (KCl)	**0.20 g**	Potassium chloride is not added due to the high conductivity of this water.

9. Cover the tank with the lid.

10. Record the following information on the Synthetic Water Preparation Log (Exhibit AT1.1). This log is used to document MHSW and SaltSW only.
    - Analyst initials
    - Water type (MHSW)
    - Date of preparation (date that chemicals are added)
    - Date of expiration (14 days from preparation date)
    - Record chemical (CHM) numbers used

Note: The synthetic water must continuously aerate.

B. Water quality measurements.

1. Before using newly prepared MHSW, measure the pH, alkalinity and hardness of the synthetic water.

   a. Analyze pH according to SOP-C3 and record the measurement on the Synthetic Water Preparation Log. Synthetic water pH must be within the required range, as indicated in Table AT1.1. Synthetic water pH outside this required range suggests that alkalinity and hardness may not be within the required limits. If synthetic water pH is outside the required range continue to step b and c below and adjust the alkalinity and hardness of the synthetic water. Once the alkalinity and hardness have been documented to meet the required ranges, re-analyze the pH of the synthetic water.

   b. Alkalinity must be measured according to SOP-C6 or the following "Quick Check Procedure" (modification to SOP-C6).
      - Calibrate the pH meter according to SOP-C3.
      - Pour 100 mL of the synthetic water to be checked into a 150-mL beaker.
      - Place the pH probe in the synthetic water and titrate to 4.50 S.U. while stirring the synthetic water.
      - Fill a 10-mL pipette with 0.020N H_2SO_4 to the 10 mL mark.
      - Determine the total number of mL used to reach 4.50 S.U. and multiply by 10, this is the alkalinity of the synthetic water. Record the begin mL, final mL and total mL titrated multiplied by 10 in the MHSW, Alkalinity and Hardness Quick Check Log (Exhibit AT1.2). Record the alkalinity measured in the Synthetic Water Preparation Log.
      - Determine if the alkalinity is within the required range for the type of synthetic water, according to Table AT1.1.
      - If the alkalinity is out of range, additional $NaHCO_3$ or deionized water may be added to bring the synthetic water into range. Record the amount of $NaHCO_3$ or deionized water added on the Synthetic Water Preparation Log.

   c. Hardness must be measured according to SOP-C7 or the following "Quick Check Procedure" (modification to SOP-C7).
      - Pour 50 mL of the synthetic water to be checked into a 150-mL beaker.
      - Add 2 mL of water hardness buffer and a small amount of Eriochrome Black T indicator to the synthetic water.
      - Fill a 10-mL pipette with EDTA to the 10 mL mark.
      - While stirring the synthetic water, titrate to a blue color.

- Determine the total number of mL used to reach a blue color and multiply by 20, this is the hardness of the synthetic water. Record the begin mL, final mL and total mL titrated multiplied by 20 in the MHSW, Alkalinity and Hardness Quick Check Logbook. Record the hardness measured in the Synthetic Water Preparation Log.
- Determine if the hardness is within the required range for the type of synthetic water, according to Table AT1.1.
- If the hardness is out of range, additional $CaSO_4 \cdot 2H_2O$ and $MgSO_4$ (equal proportions) or deionized water may be added to bring the synthetic water into range. Record the amount of $CaSO_4 \cdot 2H_2O$ and $MgSO_4$ or deionized water added on the Synthetic Water Preparation Log.

d. Alkalinity and hardness following SOPs C6 and C7 must be performed on each batch of MHSW. Toxicity tests that used batches of synthetic water that do not meet acceptance limits identified in Table AT1.1 may be invalidated.

C. Preparation of Salt Synthetic Water.

1. Salt Synthetic Water (Salt SW) is used for culturing and conducting toxicity tests using saltwater species (i.e. *Americamysis bahia*, *Cyprinodon variegatus* and *Menidia beryllina*).

2. Clean the appropriate cylindrical tank with hot tap water, scouring pads, and bristle brushes. Scrub the carboy until clean.

3. Immediately rinse the tank with hot tap water and then with deionized water.

4. Rinse with a 10% nitric acid solution. After rinsing with nitric acid, rinse the tank repeatedly with deionized water (at least 3 times).

5. Add approximately 1250 mL Crystal Sea Marinemix® sea salt (Bioassay Grade) to the vat.

6. Fill the tank with approximately 50 L deionized water while stirring with a large paddle (4-ft long).

7. Place an aeration tube with aeration stone in the tank such that the aeration stone rests on the bottom of the tank.

8. Measure the salinity (SOP-C5) of the water by placing the probe in tank.

9. Continue to add sea salt or deionized water until the desired salinity is obtained (5 to 32 ± 2 ppt, as required by the test species and NPDES permit requirements, typically 25 ppt).

10. Cover the tank with a lid.

11. The synthetic water must aerate overnight before use. Recheck the salinity before use and adjust accordingly.

12. Record the following information on the Synthetic Water Preparation Log (Exhibit AT1.1).
    - Analyst initials
    - Water type (Salt SW)
    - Date of preparation (date that salt is added)
    - Date of expiration (14 days from preparation date)
    - Record chemical (CHM) number used
    - Salinity

Note: The synthetic water must continuously aerate.

Safety and Hazardous Waste Management

Safety glasses, gloves and lab coats should be worn at all times.

Review Policy-P6: General Safety Policy and Policy-P9: Radiation Protection Policy for additional safety requirements.

References

USEPA. October 2002. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, 5th ed. EPA-821-R-02-012. US Environmental Protection Agency, Cincinnati, OH.

USEPA. October 2002. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, 4th ed. EPA-821-R-02-013. US Environmental Protection Agency, Cincinnati, OH.

USEPA. October 2002. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms, 3rd ed. EPA-821-R-02-014. US Environmental Protection Agency, Cincinnati, OH.

USEPA. March 2000. Methods for Measuring the Toxicity and Bioaccumulation of Sediment-associated Contaminants with Freshwater Invertebrates, 2nd ed. EPA-600-R-99-064. US Environmental Protection Agency, Cincinnati, OH.

USEPA. 2009. National Recommended Water Quality Criteria. US Environmental Protection Agency, Cincinnati, OH (or most current criteria).

Shimek, Ronald L. 2002. Toxicity of Some Freshly Mixed Artificial Sea Waters. Reef Keeping Online.

TNI Standard. Management and Technical Requirements for Laboratories Performing Environmental Analysis. EL-V1-ISO-2016-Rev2.0. The NELAC Institute, PO Box 2439, Weatherford, TX 76086.

Exhibits

Exhibit AT1.1: Synthetic Water Preparation Log.
Exhibit AT1.2: MHSW, Alkalinity and Hardness Quick Check Log.

Subject: Preparation of Synthetic Water

Exhibit AT1.1: Synthetic Water Preparation Log.

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Synthetic Water Preparation Log

Analyst	Water type (MHSW or SaltSW)	Preparation date (Batch)	Expiration date (14-days from preparation)	MHSW CHM # $NaHCO_3$	MHSW CHM # $CaSO_4 \cdot 2H_2O$	MHSW CHM # $MgSO_4$	MHSW CHM # KCl	MHSW Quick Check results pH (S.U.) Required Limits: = 6.5 – 8.5 Approximate Range: = 7.4 – 7.8*	MHSW Quick Check results Alkalinity (mg $CaCO_3$/L) Required Limits: = 57 – 64	MHSW Quick Check results Hardness (mg $CaCO_3$/L) Required Limits: = 80 – 100	SaltSW CHM # Marinemix®	SaltSW Salinity (ppt) Required Limits: = 24 – 26

*Approximate ranges and limits are for guidance only.

SOP AT1-Revision 6–Exhibit AT1.1

Subject: Preparation of Synthetic Water

Exhibit AT1.2: MHSW, Alkalinity and Hardness Quick Check Log.

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MHSW Alkalinity and Hardness Quick Check

Analyst	Date	MHSW Batch	Alkalinity* (mg $CaCO_3$/L) (sample volume = 100 mL) Acceptance Criteria = 57 – 64			Hardness* (mg $CaCO_3$/L) (sample volume = 50 mL) Acceptance Criteria = 80 – 100		
			Begin mL	End mL	Total mL X 10	Begin mL	End mL	Total mL X 20

*Total hardness and alkalinity results included on this form are for in-house quality control and are not for reporting purposes.

SOP AT1-Revision 6–Exhibit AT1.2

Subject: Maintenance of *Raphidocelis subcapitata* (formerly *Selenastrum capricornutum*) Cultures

Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan		09-01-19
Quality Assurance Officer	Jim Sumner		09-01-19

Document Revision History

Effective Date	Revision number	Review Type	Evaluators	Revisions
12-01-00	0	Internal	Jim Sumner (ETS)	Original document
07-10-10	1	External (NC DENR) Internal	Lance Ferrell (NC DENR) Jim Sumner (ETS)	• Exhibit AT2.3 revised to include calculations for determining the North Carolina cell concentration.
06-01-11	2	Internal	Jim Sumner (ETS)	• Updated references.
11-01-14	3	Internal	Jim Sumner (ETS)	• Provided clarification on the procedure for receiving *Selenastrum*.
09-28-16	4	External (TVA) Internal	Rick Sherrard, Donald Snodgrass (TVA) Jim Sumner (ETS)	• Included a diagram showing the process of preparing starter and food cultures (Figure AT2.1).
01-01-18	5	Internal	Jim Sumner (ETS)	• Updated procedure to include NELAP requirements. • Updated genus and species to current taxonomic identification. • Additional guidance included in SOP.
09-01-19	6	Internal	Jim Sumner (ETS)	• Corrected typographical errors.

Scope and Application

To maintain healthy cultures of *Raphidocelis subcapitata* (formerly *Selenastrum capricornutum*), providing a consistent suitable food for *Ceriodaphnia dubia*, *Daphnia magna* and *Daphnia pulex* cultures and toxicity tests. Throughout this SOP, *Raphidocelis subcapitata* will be referred to as *Selenastrum.*

Summary of Method

This procedure describes how the laboratory starts and maintains *Selenastrum* cultures (receipt of algal slants, preparation of media, inoculating starter cultures and maintaining cultures used for daphnid food).

Subject: Maintenance of *Raphidocelis subcapitata* (formerly *Selenastrum capricornutum*) Cultures

Expiration Date

Algae slants must be discarded **1-year** after receipt.

Starter cultures may be used as a source of inoculating algae cultures for *Ceriodaphnia* or *Daphnia* food for **1 month** from the batch date at the laboratory. Unused algae starter cultures are discarded after this expiration date.

Algae food cultures may be kept as a source of food for daphnids for **1 month** from the batch date at the laboratory. Unused *Selenastrum* is discarded after this expiration date.

Quality Control

Algal slants: *Raphidocelis subcapitata* (formerly *Selenastrum capricornutum)* slants are purchased from an approved supplier (e.g., University of Texas, The Culture Collection of Algae, Austin, TX).

Algal batches: Each batch of *Selenastrum* prepared must be examined before use as *Ceriodaphnia* or *Daphnia* food to (1) ensure that algae is not contaminated (e.g., presence of microscopic organisms), (2) confirm the taxonomy of the algal species and (3) to verify of the cell count.

Toxicity checks: When new slants of algae or new algae media chemicals are purchased, a "toxicity check" must be performed. A side-by-side comparison of *Ceriodaphnia* fed the new lot to *Ceriodaphnia* fed the old lot in reference toxicant tests is used (SOP-AT14). Organism survival and reproduction and test endpoints are compared between the old and new lots. If detrimental effects are noted with the new lot, it must be discarded and another lot must be prepared.

Equipment and Materials

*Raphidocelis subcapitata (*formerly *Selenastrum capricornutum* as algal slant, concentrate, or diluted)
Refrigerator
Polypropylene bottles
Hemocytometer and cover slip
Pasteur® pipettes
Compound microscope
Test Organism Shipment Log
Selenastrum Culture Log Sheet
Algae Media Preparation and Sterility Check Logbook
Sterile pipettes
Inoculating loop

Subject: Maintenance of *Raphidocelis subcapitata* (formerly *Selenastrum capricornutum*) Cultures

Escherichia coli
Sterile test tubes
Trypticase soy broth
Incubator maintained at 35.0 ± 0.5°C
Air pump, tubing and in-line carbon filter
10-L Flasks
Lights (cool-white fluorescent bulbs)
Large glass pipettes
Siphon
Moderately hard synthetic water
Deionized water
Calibrated top-loading balance
Eppendorf pipettes
Serological pipettes
$MgCl_2 \cdot 6H_20$
$CaCl_2 \cdot 2H_20$
$NaNO_3$
$MgSO_4 \cdot 7H_20$
K_2HPO_4
$NaHCO_3$
$ZnCl_2$
$CoCl_2 \cdot 6H_20$
$Na_2MoO_4 \cdot 2H_20$
$CuCl_2 \cdot 2H_20$
Na_2SeO_4
H_3BO_3
$MnCl_2 \cdot 2H_20$
$FeCl_3 \cdot 4H_20$
$Na_2EDTA \cdot 6H_20$

Subject: Maintenance of *Raphidocelis subcapitata* (formerly *Selenastrum capricornutum*) Cultures

Procedure, Preparation of *Selenastrum*

A. Receipt of *Selenastrum*.

1. *Raphidocelis subcapitata* (refered to as *Selenastrum* in agar slants are ordered from an approved supplier (e.g., University of Texas, The Culture Collection of Algae, Austin, TX).

2. Upon arrival at the laboratory, remove the *Selenastrum* from the shipping container and record the following information on the *Raphidocelis subcapitata* Shipment Log (Exhibit AT2.1).
   - Date received at the laboratory
   - Initials of the analyst that received the shipment
   - Lot number
   - Description of initial handling (i.e algal slant vented and placed in algae culture area facing lights, date placed in algae culture area, date refrigerated, analyst initials).
   - Assign the algae slant a CHM# (SOP-G15)

3. Place the *Selenastrum* Shipment Certification in the Test Organism Shipment Log.

4. It may be necessary to grow the culture that has been inoculated on the agar slant.
   a. Loosen the screw cap on the test tube (containing the agar slant) to allow gas exchange.
   b. Place the agar slant in a glass beaker allowing the test tube to sit upright. Place the beaker containing the agar slant in the algae culture area, segregated from toxicity tests. The agar slant is maintained at ambient laboratory temperature with a photoperiod of 24-hours light and a light intensity of 360 to 440 ft-c using cool-white fluorescent bulbs. Position the beaker such that the surface of the agar faces the light.
   c. Allow the *Selenastrum* on the slant to grow for 7 days. At 7-days, place the agar slant in a refrigerator maintained at 0.0 to 6.0^{o}C to stop the algal growth.

Subject: Maintenance of *Raphidocelis subcapitata* (formerly *Selenastrum capricornutum*) Cultures

B. Algae Media Preparation.

1. Preparation of the **MACRONUTRIENTS 1A**.

   a. Carefully weigh out the following chemicals using a calibrated top-loading balance (SOP-G10):
      6.08 g of $MgCl_2 \cdot 6H_20$
      2.20 g of $CaCl_2 \cdot 2H_20$
      12.75 g $NaNO_3$
   b. Place approximately 300 mL of deionized water in a 500-mL volumetric flask.
   c. Add the chemicals to the flask and dissolve by swirling the flask.
   d. Bring to volume (**500 mL**) with deionized water.
   e. Pour the reagent into a clean polypropylene bottle.
   f. Using the Reagent Log, assign an INR number for the reagent as indicated in SOP-G15.
   g. Label the bottle with the preparation date, analyst's initials and the INR number.

2. Preparation of the **MACRONUTRIENTS 1B**.

   a. Carefully weigh out the following chemical using a calibrated top-loading balance (SOP-G10):
      14.7 g of $MgSO_4 \cdot 7H_20$
   b. Place approximately 800 mL of deionized water in a 1000-mL volumetric flask.
   c. Add the chemical to the flask and dissolve by swirling the flask.
   d. Bring to volume (**1000 mL**) with deionized water.
   e. Pour the reagent into a clean polypropylene bottle.
   f. Using the Reagent Log, assign an INR number for the reagent as indicated in SOP-G15.
   g. Label the bottle with the preparation date, analyst's initials and the INR number.

3. Preparation of the **MACRONUTRIENTS 1C**.

   a. Carefully weigh out the following chemical using a calibrated top-loading balance (SOP-G10):
      1.044 g of K_2HPO_4
   b. Place approximately 800 mL of deionized water in a 1000-mL volumetric flask.
   c. Add the chemical to the flask and dissolve by swirling the flask.
   d. Bring to volume (**1000 mL**) with deionized water.
   e. Pour the reagent into a clean polypropylene bottle.
   f. Using the Reagent Log, assign an INR number for the reagent as indicated in SOP-G15.
   g. Label the bottle with the preparation date, analyst's initials and the INR number.

4. Preparation of the **MACRONUTRIENTS 1D**.

   a. Carefully weigh out the following chemical using a calibrated top-loading balance (SOP-G10):
      7.50 g of $NaHCO_3$
   b. Place approximately 300 mL of deionized water in a 500-mL volumetric flask.
   c. Add the chemical to the flask and dissolve by swirling the flask.
   d. Bring to volume (**500 mL**) with deionized water.
   e. Pour the reagent into a clean polypropylene bottle.
   f. Using the Reagent Log, assign an INR number for the reagent as indicated in SOP-G15.
   g. Label the bottle with the preparation date, analyst's initials and the INR number.

5. Preparation of the **MICRONUTRIENTS**.

   a. Carefully weigh out the following chemical using a calibrated top-loading balance (SOP-G10) and dissolve into the respective volumes with deionized water using volumetric flasks:
      0.164 g of $ZnCl_2$ dissolve into **100 mL**
      0.0714 of $CoCl_2 \cdot 6H_20$ dissolve into **100 mL**
      0.366 of $Na_2MoO_4 \cdot 2H_20$ dissolve into **100 mL**
      0.060 of $CuCl_2 \cdot 2H_20$ dissolve into **1000 mL**
      0.1196 of Na_2SeO_4 dissolve into **100 mL**
   b. Pour each of the reagents into clean polypropylene bottles.
   c. Using the Reagent Log, assign an INR numbers for the reagents as indicated in SOP-G15.
   d. Label the bottles with the preparation date, analyst's initials and the INR numbers.

6. Preparation of the **MICRONUTRIENTS 2**.

   a. Carefully weigh out the following chemical using a calibrated top-loading balance (SOP-G10):
      0.0928 g of H_3BO_3
      0.2080 g of $MnCl_2 \cdot 4H_20$
      0.0799 g of $FeCl_3 \cdot 6H_20$
      0.1500 g of $Na_2EDTA \cdot 2H_20$
   b. Place approximately 300 mL of deionized water in a 500-mL volumetric flask.
   c. Add the chemical to the flask and dissolve by swirling the flask.
   d. Pipette 1 mL of each the following MICRONUTRIENTS (prepared in step C.5) using a calibrated Eppendorf (SOP-G11) into the 500-mL volumetric flask:
      1 mL of $ZnCl_2$ - MICRONUTRIENT
      1 mL of $CoCl_2 \cdot 6H_20$ - MICRONUTRIENT
      1 mL of $Na_2MoO_4 \cdot 2H_20$ - MICRONUTRIENT
      1 mL of $CuCl_2 \cdot 2H_20$ - MICRONUTRIENT
      1 mL of Na_2SeO_4 - MICRONUTRIENT
   e. Bring to volume (**500 mL**) with deionized water.
   f. Pour the reagent into a clean polypropylene bottle.
   g. Using the Reagent Log, assign an INR number for the reagent as indicated in SOP-G15.
   h. Label the bottle with the preparation date, analyst's initials and the INR number.

7. Preparation of the **ALGAE MEDIA**.

   a. To prepare 1000 mL of algae media, pipette each the following macronutrients and micronutrient (prepared in step C) using serological pipettes (SOP-G11) into a 1000 mL graduated cylinder:
      2 mL of MACRONUTRIENT 1A
      6 mL of MACRONUTRIENT 1B (NC DEQ Modification identified in Biological Laboratory Certification / Criteria Procedure)
      6 mL of MACRONUTRIENT 1C (NC DEQ Modification identified in Biological Laboratory Certification / Criteria Procedure)
      2 mL of MACRONUTRIENT 1D
      2 mL of MICRONUTRIENT 2
   b. Bring to volume (**1000 mL**) with deionized water.
   c. Mix the solution well and pour into 500 mL Nalgene autoclavable bottles. Loosen the screw caps and affix autoclave tape over the caps of each bottle. On the tape, label the batch of algae media with AM (for algae media), the preparation date and initials. Autoclave the algae media at 121°C (15 lbs of pressure) for 15 minutes (SOP-B1).
   d. Remove the algae media from the autoclave and allow the algae media to cool.
   e. Once the media has cooled, tighten the screw caps on the bottles and store in a cool place.
   f. Document the algae media preparation date, volume prepared, reagent numbers of the macronutrients and micronutrients and initials in the Algae Media Preparation and Sterility Check Logbook (Exhibit AT2.2).
   g. Each batch of algae media must be checked for sterility.
      - Using a sterile pipette, transfer 10 mL of double strength trypticase soy broth (SOP-B4) into two sterile test tubes. Using a new sterile pipette, transfer 10 mL of algae media into the typticase soy broth of each test tube.
      - Using a sterile inoculating loop, inoculate one of the test tubes with the *Escherichia coli*. Label this test tube as the positive control.
      - Label the second test tube (containing just trypticase soy broth and algae media) as the negative control.
      - Tighten the screw caps on the test tubes, shake the tubes to mix the solution, loosen the screw caps and incubate at 35.0 ± 0.5°C for 24 hours.
      - Record the trypticase soy broth lot and the date, time and initials that the sterility check was initiated in the Algae Media Preparation and Sterility Check Logbook.

- After 24-hours, remove the test tubes from the incubator and check for bacteria growth. Bacteria growth should occur in the positive control (trypticase soy broth becomes cloudy) and no growth should occur in the negative control (trypticase soy broth remains clear).
- If growth occurs in the negative control, try to determine the cause of bacterial contamination and re-sterilize the algae media.
- Record the presence of turbidity for each of the test tubes and the reagent number of the trypticase soy broth, date, time and initials that the sterility check was terminated in the Algae Media Preparation and Sterility Check Logbook.

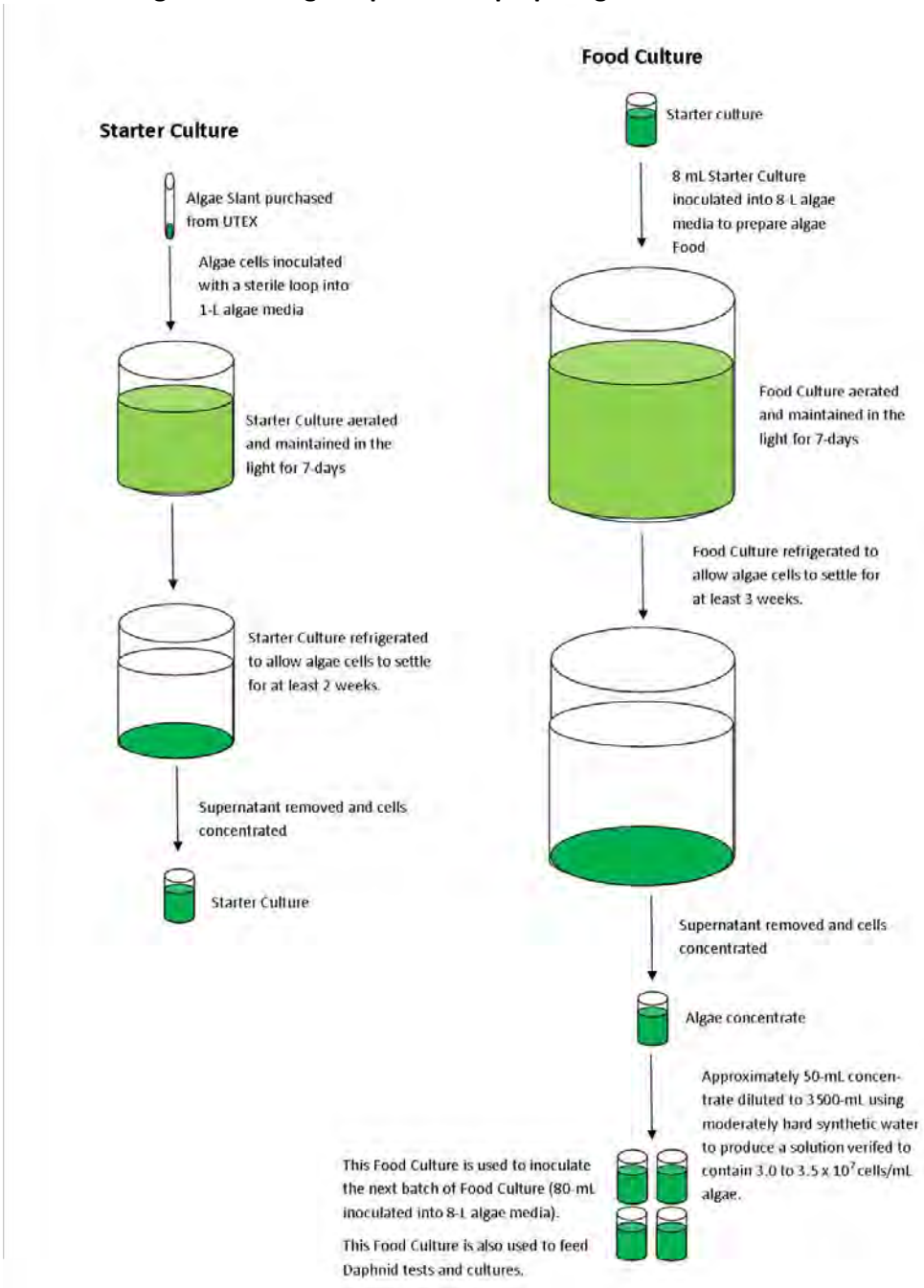
h. Once the algae media has been checked for sterility, it may be used for the preparation of algae cultures. Algae media may be kept indefinitely, as long as contamination does not occur (media becomes cloudy or bacteria growth occurs).

D. Preparation of Starter Cultures, Inoculated from Algal Slants.

Aseptic techniques should be used in preparing and maintaining algae starter cultures and care should be taken to avoid contamination by microorganisms. All activities performed on the algae cultures are documented in the *Raphidocelis subcapitata* Culture Logbook (Exhibit AT2.3). The process of preparing starter and food cultures is outlined in Figure AT2.1.

Subject: Maintenance of *Raphidocelis subcapitata* (formerly *Selenastrum capricornutum*) Cultures

Figure AT2.1: Diagram showing the process of preparing starter and food cultures.



Subject: Maintenance of *Raphidocelis subcapitata* (formerly *Selenastrum capricornutum*) Cultures

1. Algae starter cultures are segregated from toxicity tests and are maintained at ambient laboratory temperature with a photoperiod of 24-hours light and a light intensity of 360 to 440 ft-c using cool-white fluorescent bulbs. Constant, vigorous aeration is provided by placing a large pipette as close to the bottom of the flask as possible and attaching tubing with an in-line carbon filter to a standard air pump.

2. Algae starter cultures are used for inoculating algae cultures to be used for *Ceriodaphnia* or *Daphnia* food. Algae starter cultures are initiated by adding 1000 mL of algae media into a 2 L flask.

3. Using a sterile inoculating loop, remove a small quantity of algae cells from the algae agar slant and inoculate the flask containing algae media.

4. The algae starter culture is allowed to grow for 7 days. After 7 days, the aeration is removed and the algae starter culture is stored in a refrigerator. Refrigeration will prevent further algal growth and will allow the cells to settle.

5. After approximately 1-2 weeks, the supernatant is siphoned off to produce approximately 50 mL concentrated algae.

6. Label the flask indicating the Algae Starter Culture Batch.

7. Store the starter culture algae in a refrigerator maintained at 0.0 to 6.0°C. Refrigerated algae starter cultures may be kept as a source of inoculating algae cultures for *Ceriodaphnia* or *Daphnia* food for 1 month from the batch date at the laboratory. Unused algae starter cultures are discarded after this expiration date.

E. Maintenance of Algae Cultures for *Ceriodaphnia dubia* or *Daphnia* Food.

Aseptic techniques should be used in preparing and maintaining algae cultures and care should be taken to avoid contamination by microorganisms. All activities performed on the algae cultures are documented in the *Raphidocelis subcapitata* Culture Logbook (Exhibit AT2.3)

1. Algae cultures are segregated from toxicity tests and are maintained at ambient laboratory temperature with a photoperiod of 24-hours light and a light intensity of 360 to 440 ft-c using cool-white fluorescent bulbs. Constant, vigorous aeration is provided by placing a large pipette as close to the bottom of the flask as possible and attaching tubing with an in-line carbon filter to a standard air pump.

2. One large algae culture is started monthly for feeding daphnid cultures and toxicity tests. This culture is initiated by adding 8000 mL of algae media into a 10 L flask. This volume may be adjusted to produce the amount of algae needed for daphnid cultures and toxicity tests.

3. Approximately 10 mL of algal culture containing 3.0 to 3.5 x 10^7 cells/mL is used to inoculate 1000 mL of algae media. This inoculum should provide an initial cell concentration of 300,000 cells/mL. (For 8000 mL algae media, 80 mL of inoculum is used.) Algae cultures may be initiated from algae starter cultures or previous batches of algae cultures used for daphnid food.

4. The algae culture is allowed to grow for 7 days. After 7 days, the aeration is removed and the algae culture is stored in a refrigerator. Refrigeration will prevent further algal growth and will allow the cells to settle.

5. After approximately 3-4 weeks, the supernatant is siphoned off to produce approximately 50 mL concentrated algae. This algae concentrate is diluted to 1000 mL with moderately hard synthetic water (MHSW). To avoid bacteria contamination, MHSW prepared the day before is used.

F. Examination of *Selenastrum* and Cell Count Verification.

1. Using an Eppendorf pipette, dilute a 0.25 mL portion of the algae concentrate prepared above into 1 mL. Using a Pasteur pipette, obtain a small amount of this diluted algae concentrate. Place one drop in a notched slot on one side of the hemocytometer. Place the cover slip over the hemocytometer.

2. Place the hemocytometer under the microscope. Use the 40X magnification to locate the algal cells. Use the 100X magnification to examine the cells.

3. Examine the algal solution for contamination (e.g., microscopic organisms – ciliates, protozoa or other species of algae).

   a. If the solution does not appear to be contaminated, place a check in the “No” box on the culture log sheet.

   b. If microscopic organisms are observed, place a check in the “Yes” box and label the entire algal batch as “Contaminated – Do Not Use”. Notify the Laboratory Supervisor.

4. Confirm the taxonomy of the algal species.

   a. *Raphidocelis subcapitata* cells are very small (8 to 14 µm in length and 2 to 3 µm in width) and tend to have curved or twisted appearance like sickle (Figure AT2.1). The cells are normally present in solitary form.

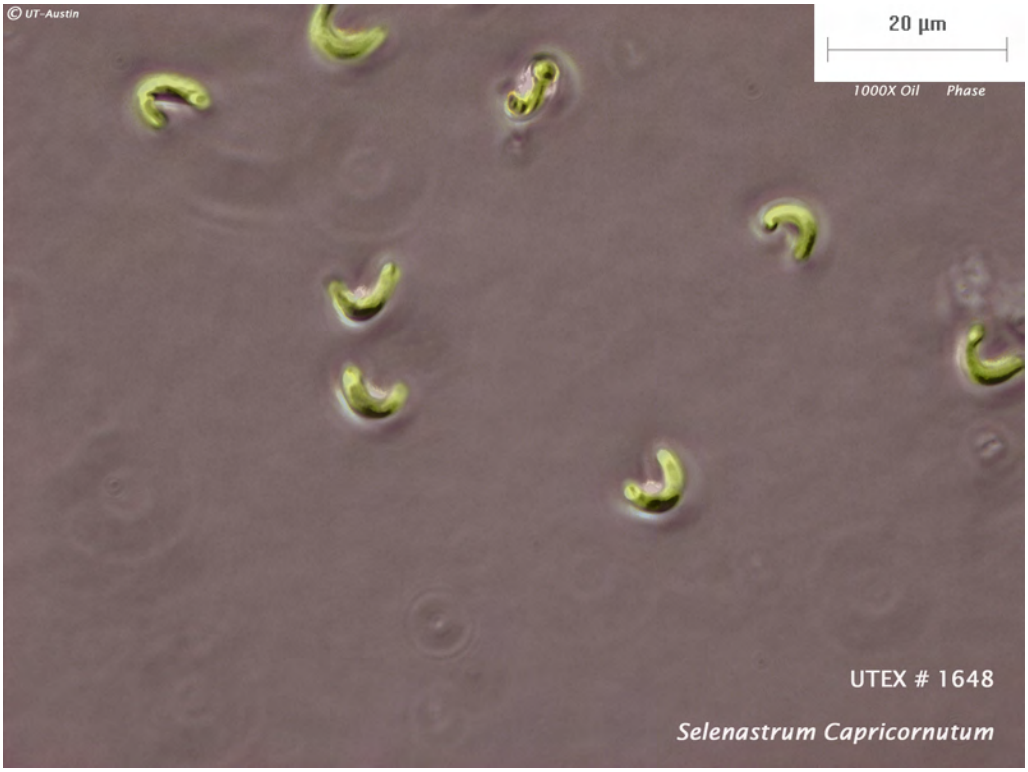


Figure AT2.1: *Selenastrum capricornutum* cells.

   b. If the algae received appear to be the correct species, place a check in the "Yes" box on the log sheet.

   c. If there is a question as to the taxonomic identification of the algal species, refer to the references cited at the beginning of this SOP. If the algal species is confirmed to not be *Raphidocelis subcapitata*, place a check in the "No" box and notify the Laboratory Supervisor.

d. Record the date the examination was performed and analyst initials on the log sheet.

5. Determine the cell count and dilute algae to correct concentration.

a. Count the number of cells contained in the grids 1 through 5, as indicated in the figure below.

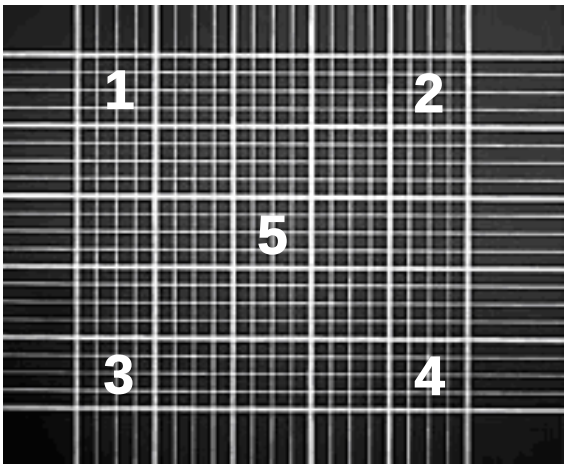


Figure AT2.2: Hemocytometer

b. Calculate the average number of cells counted on the hemocytometer.
For example, if the counts obtained for each of the grids were 123, 120, 126, 125, and 120, the average number of cells would be 122.8
[123 + 120 + 126 + 125 + 120]/5 = 122.8 cells

c. Determine the final cell count by dividing the average number of cells by 4×10^{-6}.
For example, if the average number of cells was 122.8, the final cell count would be 3.07×10^{7} cells/mL.
$122.8/[4 \times 10^{-6}] = 3.07 \times 10^{7}$ cells/mL

d. Apply the dilution factor by multiplying the final result by 5.

e. Once the corrected cell concentration is determined, the concentrated algae are diluted to a final cell concentration of 3.0 to 3.5 x 10^7 cells/mL using MHSW. Determine the necessary volume of MHSW needed to dilute the 1000 mL algae concentrate to 3.0 to 3.5 x 10^7 cells/mL, as indicated on the Culture Log Sheet.

f. Dilute the algae concentrate with MHSW and verify the cell concentration of the diluted algae, as indicated above. Assign the algae culture a batch date (the date that the algae concentrate is diluted with MHSW). Record the batch of MHSW used to dilute the algae.

g. Mix the diluted algae well and divide the algae into 900 mL aliquots in clean 1000-mL polypropylene bottles. Place a label on each *Selanastrum* bottle indicating the *Selenastrum* Batch, as shown below.

> ***Selenastrum***
> Batch: **04-01-09**

h. Place the *Selenastrum* aliquots in a refrigerator maintained at 0.0 to 6.0°C. Refrigerated *Selenastrum* may be kept as a source of food for daphnids for 1 month from the batch date at the laboratory. Unused *Selenastrum* is discarded after this expiration date.

i. A portion of this *Selenastrum* is diluted further to the North Carolina cell concentration of 1.71 x 10^7 cells/mL.

- Determine the necessary volume of moderately hard synthetic water needed to dilute 100 mL full strength algae to 1.71 x 10^7 cells/mL, as indicated on the Culture Log Sheet.

- Place diluted algal suspension in a clean 250-mL polypropylene bottle and label the bottle as shown below. This algal solution should be stored in a refrigerator at 0.0 to 6.0°C when not in use and must be discarded 1 month after it is prepared in the laboratory (1 month from the batch date).

> **NC *Selenastrum***
> Batch: **01-01-18**

j. After the algal has been verified to be *Raphidocelis subcapitata* with no apparent contamination and the cell count has been verified, the algae may be used for *Ceriodaphnia* or *Daphnia* food.

Safety and Hazardous Waste Management

Safety glasses, gloves and lab coats should be worn at all times.

Review Policy-P6: General Safety Policy and Policy-P9: Radiation Protection Policy for additional safety requirements.

Subject: Maintenance of *Raphidocelis subcapitata* (formerly *Selenastrum capricornutum*) Cultures

References

USEPA. October 2002. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, 4th ed. EPA-821-R-02-013. US Environmental Protection Agency, Cincinnati, OH.

ASTM. 2006. Standard Guide for Conducting Three-Brood, Renewal Toxicity Tests with *Ceriodaphnia dubia*. ASTM International, West Conshohocken, PA.

North Carolina Department of Environment and Natural Resources, Division of Water Quality. Biological Laboratory Certification / Criteria Procedures, Version 3.0. December 2010.

C. E. Goulden, *Ceriodaphnia* and *Daphnia* Bioassay Workshop Manual. The Academy of Natural Sciences, Philadelphia, PA.

G. W. Prescott, *Algae of the Western Great Lakes Area*, WM. C. Brown Company, 1962.

G. M. Smith, *The Fresh-Water Algae of the United States, Second Edition*, McGraw-Hill Book Company, 1950.

TNI Standard. Management and Technical Requirements for Laboratories Performing Environmental Analysis. EL-V1-ISO-2016-Rev2.0. The NELAC Institute, PO Box 2439, Weatherford, TX 76086.

Exhibits

Exhibit AT2.1: *Raphidocelis subcapitata* Shipment Log.
Exhibit AT2.2: Algae Media Preparation and Sterility Check Log Sheet.
Exhibit AT2.3: Raphidocelis subcapitata Culture Log Sheet.

Subject: Maintenance of *Raphidocelis subcapitata* (formerly *Selenastrum capricornutum*) Cultures

Exhibit AT2.1: *Raphidocelis subcapitata* Shipment Log.

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***Raphidocelis subcapita* (formerly *Selenastrum capricornutum*)**
Shipment Logsheet

Source:	University of Texas at Austin, UTEX Culture Collection of Algae Order# 1648 – *Selenastrum capricornutum*
Lot number:	
CHM#:	
Date received:	
Expiration date:	
Received by (initials)**:**	
Description of initial handling:	

SOP AT2-Revision 6–Exhibit AT2.1

Subject: Maintenance of *Raphidocelis subcapitata* (formerly *Selenastrum capricornutum*) Cultures

Exhibit AT2.2: Algae Media Preparation and Sterility Check Log Sheet.

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Algae Media Preparation and Sterility Check

Algae media preparation:

Prepared By (Initials):	
Preparation Date (Batch):	
Volume Prepared (mL):	
1A Macronutrients Reagent Number: (**2** mL/L added)	INR #
1B Macronutrients Reagent Number: (**6** mL/L added)	INR #
1C Macronutrients Reagent Number: (**6** mL/L added)	INR #
1D Macronutrients Reagent Number: (**2** mL/L added)	INR #
2 Micronutrients Reagent Number: (**2** mL/L added)	INR #

Sterility check

Trypticase Soy Broth Reagent Number:	*INR #*

Sample	Initiation			Termination			Turbid (+ or -)
	Date	Time	Analyst	Date	Time	Analyst	
Negative Control (10 mL algae media)							
Positive Control (10 mL algae media + *E. coli*)							

SOP AT2-Revision 6–Exhibit AT2.2

Subject: Maintenance of *Raphidocelis subcapitata* (formerly *Selenastrum capricornutum*) Cultures

Exhibit AT2.3: *Raphidocelis subcapitata* Culture Log Sheet.

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Raphidocelis subcapitata Culture Logsheet

Batch: _____

Culture preparation:

Algae use (stock culture or food):	
Start date (media spiked with algae and aerated):	
Algae spike source:	
Volume of algae spike:	
Cell count of algae spike (cells/mL):	
Algae media batch:	
Volume of algae media:	
Analyst (initials):	

Algae concentrate:

Date aeration removed and algae refrigerated and initials:	
Date decanted/concentrated and initials:	

Examination of algae concentrate:

Analyst (initials):	
Date performed:	
Contaminated (yes or no):	
Correct species (yes or no):	
Comments:	

Date: _____ **Analyst:** _____

Cell count of algae concentrate: Perform a 0.2 dilution factor of the algae concentrate. Pipette 1000 µL deionized water and 250 µL algae concentrate into a 1-oz medicine cup.

Number of cells on one grid	Average number of cells	Cell count (average number of cells / 4.0×10^{-5} mL)	Final cell count Correction for dilution factor (cell count X 5)
1			
2			
3			
4			
5			

Algae concentrate dilution to 3.5×10^7 cells/mL:

Total volume of algae concentrate (mL)	Cell concentration of algae concentrate (A, cells/mL)	Desired cell concentration (B, cells/mL)	Dilution factor (C = A/B)	Total volume of diluted algae concentrate (mL = C X volume of algae concentrate)
		3.5×10^7		

Verification of cell count (3.0 to 3.5×10^7 cells/mL):

Number of cells On one grid	Average number of cells	Cell count (cells/mL =average number of cells / 4.0×10^{-6})
1		
2		
3		
4		
5		

North Carolina algae dilution (1.71×10^7 cells/mL):

Volume of algae at 3.0 to 3.5 X 10^7 cells/mL (mL)	Cell concentration of algae 3.0 to 3.5 X 10^7 cells/mL (A, cells/mL)	Desired cell concentration (B, cells/mL)	Dilution factor (C = A/B)	Total volume of diluted algae (mL = C X volume of algae at 3.0 to 3.5 X 10^7 cells/mL
100		1.71 X 10^7 cells/mL		

SOP AT2-Revision 6–Exhibit AT2.3

Subject: Preparation of YWT (Yeast, Wheat Grass, Trout Chow) Mixture

Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan		09-01-19
Quality Assurance Officer	Jim Sumner		09-01-19

Document Revision History

Effective Date	Revision number	Review Type	Evaluators	Revisions
12-01-00	0	Internal	Jim Sumner (ETS)	Original document
07-10-10	1	External (NC DENR) Internal	Lance Ferrell (NC DENR) Jim Sumner (ETS)	• Section B.6.e amended to indicate that the yeast component of the YWT is added immediately after preparation and is not allowed to settle.
06-01-11	2	External (TVA) Internal	Rick Sherrard, Cindy Russell (TVA) Jim Sumner (ETS)	• The volume of YWT used per day was included in the SOP. • Updated Table AT6.1 and references. • Added procedures for performing total solids measurements of YWT.
11-01-14	3	Internal	Jim Sumner (ETS)	• Updated Table AT6.1.
01-01-18	4	Internal	Jim Sumner (ETS)	• Updated procedure to include NELAP requirements. • Updated Table AT6.1. • Additional guidance included in SOP.
09-01-19	5	Internal	Jim Sumner (ETS)	• Corrected typographical errors.

Scope and Application

To provide a consistent suitable food for *Ceriodaphnia dubia*, *Daphnia magna*, *Daphnia pulex* and *Hyalella azteca* cultures and toxicity tests.

Summary of Method

This procedure describes how the laboratory prepares yeast/wheat grass/trout chow (YWT) mixtures used for daphnid and *Hyalella* food.

Subject: Preparation of YWT (Yeast, Wheat Grass, Trout Chow) Mixture

Expiration Date

The expiration date of the consumables (yeast, wheat grass, trout chow) is provided by the manufacturer. If an expiration date is not provided, consumables are assigned a **5-year** expiration date. Consumables are discarded after this expiration date.

Frozen YWT must be discarded **3-months** after preparation.

YWT must be discarded **7-days** after being thawed.

Quality Control

YWT Batches: Each batch of YWT must be verified to contain 1.7 to 1.9 g/L solids.

For each batch of YWT prepared, the survival and reproduction of *Ceriodaphnia* fed YWT must be evaluated before it is used in toxicity tests. Side-by-side comparisons of *Ceriodaphnia* fed the new batch to *Ceriodaphnia* fed the old batch in cultures are used (SOP-AT7). Organism survival and reproduction are compared between the old and new batches. If detrimental effects are noted with the new YWT batch, it must be discarded and another batch must be prepared.

New Lots: When new lots of yeast, wheat grass or trout chow are purchased, YWT (verified to contain 1.7 to 1.9 g/L solids) must be analyzed for total organochlorine pesticides plus PCBs and metals (Ag, Al, As, Cd, Cr, Co, Cu, Fe, Hg, Pb, Ni and Zn).

USEPA recommends that YWT be verified to contain < 50 ng/L organochlorine pesticides plus PCBs, < 1 µg/L total metal each of Al, As, Cr, Co, Cu, Fe, Pb, Ni, Zn and < 100 ng/L total metal each of Cd, Hg, Ag. Pesticide concentrations should also not exceed USEPA's Ambient Water Quality chronic criteria where available.

Interferences from solids present in the YWT result in detection limits higher than concentrations cited above; however, the lowest available detection limit for each analyte is performed.

Micronutrients added by manufacturers of trout chow to promote the health of fish cultures prohibit the ability to achieve the limits established by USEPA. After correspondence with USEPA concerning with the concentration of metals in YWT, Environmental Testing Solutions (ETS) has determined that reference toxicant testing will be used to assess the suitability of new lots of YWT. In addition, metal concentrations in new YWT lots will be compared to concentrations in previous lots of YWT (Table AT6.1).

Subject: Preparation of YWT (Yeast, Wheat Grass, Trout Chow) Mixture

Table AT6.1: Concentration of metals (µg/L) contained in previous YWT lots prepared by the laboratory. Measured concentration of each analyte in YWT at 1.7 to 1.9 g/L total solids and the estimated final concentration of each analyte in 15 mL test solution at the 100 µL feeding rate are identified in the table below.

Analyte (µg/L)	YWT Lot: 06-02-16		YWT Lot: 10-23-17		YWT Lot: 11-26-18		Measured concentration in artemia nauplii mixture from previous batches			
	Measured concentration in YWT mixture	Estimated concentration at feeding rate	Measured concentration in YWT mixture	Estimated concentration at feeding rate	Measured concentration in YWT mixture	Estimated concentration at feeding rate	Mean	SD	Mean - SD	Mean + SD
Ag	0.00	0.0000	0.00	0.0000	10.00	0.0667	1.72	3.51	-1.78	5.23
Al	35	0.2333	37	0.2467	44.00	0.2933	52.78	41.09	11.68	93.87
As	4.00	0.0267	4.00	0.0267	10.00	0.0667	4.09	2.29	1.80	6.38
Cd	0.20	0.0013	0.30	0.0020	10.00	0.0667	1.32	3.26	-1.94	4.57
Cr	3.00	0.0200	2.00	0.0133	10.00	0.0667	2.82	2.84	-0.02	5.67
Co	2.00	0.0133	0.80	0.0053	10.00	0.0667	3.30	3.17	0.13	6.47
Cu	33	0.2200	33	0.2200	18.00	0.1200	28.99	11.93	17.06	40.92
Fe	320	2.1333	270	1.8000	206.00	1.3733	310.67	89.94	220.73	400.61
Hg	0.08	0.0005	0.12	0.0008	10.00	0.0667	1.17	3.31	-2.14	4.48
Pb	0.90	0.0060	0.20	0.0013	10.00	0.0667	1.58	3.18	-1.60	4.75
Ni	2.00	0.0133	2.00	0.0133	10.00	0.0667	3.83	2.49	1.34	6.32
Zn	330	2.2000	390	2.6000	318.00	2.1200	312.11	97.39	214.72	409.51
Total metal	730.18	4.87	739.42	4.93	666.00	4.44				

Complete analytical test results are maintained in the laboratory's QC files.

Toxicity checks: When new lots of yeast, wheat grass, or trout chow are purchased, a "toxicity check" must be performed before it is used. Side-by-side reference toxicant tests are used, where *Ceriodaphnia* are fed the new lot in first test and *Ceriodaphnia* are fed the old lot in the second test (SOP-AT14). Organism survival and reproduction and test endpoints are compared between the old and new lots. If detrimental effects are noted with the new trout chow lot, it must be discarded, and another lot must be ordered.

Equipment and Materials

YWT mixture
Yeast
Wheat Grass
Trout Chow
Blender
Deionized water
Calibrated top loading balance
Separatory funnels
Erlenmeyer flasks
Waterproof pens
105 µm Nitex mesh
Air pump and tubing

Subject: Preparation of YWT (Yeast, Wheat Grass, Trout Chow) Mixture

Refrigerator
Freezer
125-mL Polypropylene bottles
YWT Preparation Log Sheet

Procedure

A. Preparation of YWT.

The directions for preparing the Daphnid and *Hyalella* food are outlined below according to the day on which each task should be completed. In general, the schedule is as follows:

Day 1:	Begin trout chow digestion
Day 2-6:	Replenish deionized water to fill line on digesting trout chow
Day 6 or 7:	Prepare wheat grass and refrigerate
Day 7:	Prepare yeast. Filter trout chow and wheat grass. Combine yeast, wheat grass and trout chow. Verify the total solids and freeze YWT mixture.

Day 1:

1. Trout chow preparation.
   a. Remove the trout chow from the freezer. Using a calibrated top-loading balance, carefully weigh out 10 g of trout chow (SOP-G10).
   b. In a blender, combine 10 g trout chow and 2 L deionized water.
   c. Blend the mixture for 5 minutes and place in a 2 L separatory funnel. Using a waterproof pen, mark the water level on the funnel.
   d. Place the funnel in a fume hood. Aerate the contents of the funnel by connecting a plastic tube to an airline. Place the tube into the funnel, so that the tip is near the bottom of the funnel.
   e. Label the funnel with the initiation date and the date the trout chow will be fully digested (7 days from starting date).
   f. Record the following information on the YWT Preparation Log Sheet (Exhibit AT6.2):
      Source: (i.e. AquaMax Purina), Chemical number
      Volume prepared
      Date prepared and aerated
      Analyst (initials)

Days 2-6:

2. Trout chow maintenance.

    a. Keep the water level approximately constant by adding deionized water, as needed, each day. Record the date(s) water was added on the YWT Preparation Log Sheet.

3. Prepare bottles

    a. Obtain approximately 60 125-mL plastic bottles per batch of YWT mixture. If necessary, clean the bottles according to SOP-G1. Place a label to each bottle denoting the date that the yeast, wheat grass, and trout chow were combined (YWT batch).

Day 6 or 7:

4. Wheat grass preparation.

    a. Remove the wheat grass from the freezer. Using a calibrated top-loading balance, carefully weigh out 10 g of wheat grass (SOP-G10).
    b. In a blender, combine 10 g wheat grass and 2 L deionized water.
    c. Blend the mixture well for 5 minutes and place in a 2 L separatory funnel. Using a waterproof pen, mark the date the wheat grass was prepared.
    d. Place the funnel in a refrigerator overnight.
    e. Record the following information on the YWT Preparation Log Sheet:
        Source: (i.e. Pines), Chemical number
        Volume prepared
        Date prepared and refrigerated
        Analyst (initials)

Days 7:

5. Yeast preparation.

    a. Remove the yeast from the freezer. Using a calibrated top-loading balance, carefully weigh out 10 g of yeast (SOP-G10).
    b. In a blender, combine 10 g yeast and 2 L deionized water.
    c. Blend the mixture well for 5 minutes and place in a 2 L Erlenmeyer flask. Using a waterproof pen, mark the date the yeast was prepared.
    d. Record the following information on the YWT Preparation Log Sheet:
        Source: (i.e. Fleischmann's), Chemical number
        Volume prepared
        Date prepared and refrigerated
        Analyst (initials)

6. Combine the yeast, wheat grass and trout chow.

    a. Remove the aeration tubing from the trout chow and allow the contents to settle for 1 hour.
    b. After 1 hour, remove the contents from the bottom of the separatory funnel by opening the stopcock. Filter the remaining supernatant through a fine mesh screen (e.g. Nitex 105 µm mesh). Discard the particulate fraction that was retained on the screen.
    c. Remove the wheat grass from the refrigerator.
    d. Remove the contents from the bottom of the separatory funnel by opening the stopcock. Filter the remaining supernatant through a fine mesh screen (e.g. Nitex 105 µm mesh). Discard the particulate fraction that was retained on the screen.
    e. Combine equal portions of filtered trout chow, filtered wheat grass and yeast into a 4 L Erlenmeyer flask. The yeast component of this mixture is added immediately after it is prepared and is not allowed to settle. This mixture is referred to as "YWT". Place the flask into a refrigerator.
    f. Record the following information on the log sheet.
        Date combined
        Analyst initials
        Assign the YWT mixture a batch date (the batch date is the date the YWT components were combined.

7. Determine total solids of YWT mixture and freeze 100 mL portions.

    a. Determine the total solids of the YWT mixture.
        i. Using a Sharpie® marker, write a unique identification number on an aluminum pan and place into the drying oven (103 - 105°C). The pan must remain in the drying oven at least one hour.
        ii. Using tongs, remove the pan from the oven and place in a desiccator. The pan should remain in the desiccator at least 30 minutes.
        iii. Using tongs, remove the pan from the dessicator.
        iv. Using a calibrated top-loading balance (SOP-G10), weigh the pan and record the dish identification number and the initial weight of the pan on the benchsheet.
        v. Using tongs, remove the pan from the balance.
        vi. Thoroughly mix the YWT and pipette 10 mL of the YWT into the pan.
        vii. Place the pan into the drying oven. The pan containing YWT must remain in the oven until dry.
        viii. Once dry, remove from the oven and place into a dessiccator.
        ix. The sample must remain in the dessiccator at least 30 minutes.
        x. Using tongs, remove the pan from the dessiccator and obtain a final weight.
        xi. Record the final weight on the benchsheet and calculate the total solids of the YWT mixture (as identified on the benchsheet).
    b. Total solids are expressed in g/L. YWT must have total solids of 1.7 to 1.9 g/L. If the total solids are above this range, the mixture must be diluted and total solids reanalyzed. If the final weight is < 1.7 g/L or > 1.9 g/L contact the Laboratory Supervisor.
    c. Record the total solids calculations on the YWT Preparation Log Sheet.
    d. Once the total solids have been verified to be 1.7 to 1.9 g/L, the YWT can be separated into 100 mL aliquots in labeled 125 polypropylene bottles. Mix the YWT mixture well between each aliquot poured.
    e. Place the 100 mL aliquots in a freezer. Place a label on each YWT bottle indicating the YWT Batch or date prepared (an example is shown below).

> **YWT Batch: 04-01-09**
> Date thawed: _____
> Initials: _____

8. Frozen YWT may be kept as a source of food for daphnids and *Hyallela* for 3 months. Unused YWT is discarded after this expiration date.

9. YWT must be thawed thoroughly before use and stored in a refrigerator maintained at 0.0 to 6.0°C. YWT, which has been thawed, must be discarded after 7 days. When YWT is removed from the freezer and thawed, the date thawed and initials must be recorded on the bottle label. The daily YWT usage in the laboratory is approximately 50 mL; therefore a 100 mL aliquot of thawed YWT would not be used beyond the 7-day expiration date.

Safety and Hazardous Waste Management

Safety glasses, gloves and lab coats should be worn at all times.

Review Policy-P6: General Safety Policy and Policy-P9: Radiation Protection Policy for additional safety requirements.

References

USEPA. October 2002. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, 4th ed, Test Method 1002.0. EPA-821-R-02-013. US Environmental Protection Agency, Cincinnati, OH.

USEPA. March 2000. Methods for Measuring the Toxicity and Bioaccumulation of Sediment-associated Contaminants with Freshwater Invertebrates, 2nd ed. EPA-600-R-99-064. US Environmental Protection Agency, Cincinnati, OH.

USEPA. 2009. National Recommended Water Quality Criteria. US Environmental Protection Agency, Cincinnati, OH (or most current criteria).

TNI Standard. Management and Technical Requirements for Laboratories Performing Environmental Analysis. EL-V1-ISO-2016-Rev2.0. The NELAC Institute, PO Box 2439, Weatherford, TX 76086.

Exhibits

Exhibit AT6.2: YWT Preparation Log Sheet.

Subject: Preparation of YWT (Yeast, Wheat Grass, Trout Chow) Mixture

Exhibit AT6.1: YWT Preparation Log Sheet.

Page _____

YWT Preparation Logsheet

Batch: _____

Trout chow digestion:

Source:	Aquatox Flakes, Zeigler Feed
Chemical number:	CHM 953
Volume prepared:	2000 mL Milli-Q to 10 g Flakes
Date prepared and aerated:	
Analyst (initials):	
Dates deionized water added to trout chow mixture:	
Date aeration removed and 110 µm filtered:	
Analyst (initials):	

Wheat grass preparation:

Source:	Amazing Grass Wheat Grass
Chemical number:	CHM 958
Volume prepared:	2000 mL Milli-Q to 10 g Wheat Grass
Date refrigerated:	
Analyst (initials):	

Yeast preparation:

Source:	Red Star Active Dry Yeast
Chemical number:	CHM 957
Volume prepared:	2000 mL Milli-Q to 10 g Yeast
Date prepared:	
Analyst (initials):	

YWT preparation:

Date combined:	
Analyst (initials):	

Total solids confirmation:

Pan identification	Initial pan weight (g)	Final dry weight pan + 10 ml YCT (g)	Initial - Final weight (g)	Total solids (g/L = weight X 100) (Acceptance criteria = 1.7 – 1.9 g/L)

SOP AT6-Revision 5–Exhibit AT6.1

Subject: Maintenance of Daphnid Cultures

Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan		03-01-20
Quality Assurance Officer	Jim Sumner		03-01-20

Document Revision History

Effective Date	Revision number	Review Type	Evaluators	Revisions
12-01-00	0	Internal	Jim Sumner (ETS)	Original document
06-01-11	1	Internal	Jim Sumner (ETS)	• Updated exhibits.
11-01-14	2	Internal	Jim Sumner (ETS)	• Updated exhibits.
09-28-16	3	External (TVA) Internal	Rick Sherrard, Donald Snodgrass (TVA) Jim Sumner (ETS)	• Provided clarification that under normal circumstances, brood boards are maintained for less than 10 days.
01-01-18	4	Internal	Jim Sumner (ETS)	• Updated procedure to include NELAP requirements. • Additional guidance included in SOP.
09-01-19	5	Internal	Jim Sumner (ETS)	• Corrected typographical errors.
03-01-20	6	External (TVA) Internal	Rick Sherrard, (TVA) Jim Sumner (ETS)	• Provided clarification that *Ceriodaphnia* cultures are initiated from third broods.

Subject: Maintenance of Daphnid Cultures

Scope and Application

To maintain healthy cultures of *Ceriodaphnia dubia* and *Daphnia*.

Summary of Method

This procedure describes how the laboratory initiates and maintains individual cultures as well as backup mass cultures of *Ceriodaphnia dubia* and *Daphnia*.

Quality Control

It is important to use only healthy, productive organisms in cultures and tests. If a brood board had 20% or greater mortality or the average reproduction was < 20.0 offspring/surviving female, it must not be used to establish new cultures or a source of neonates for testing.

Equipment and Materials

Ceriodaphnia dubia or *Daphnia (pulex or magna)*
Temperature-controlled incubator (set to maintain test temperature = 25.0 ± 1.0°C, photoperiod of 16-hour light / 8-hours dark, light intensity = 50 – 100 ft-c)
Control water (moderately hard synthetic water)
1-oz medicine cups
1000-mL glass beakers
2000-mL finger bowls
3000-mL glass jars
Transfer pipettes
Eppendorf Repeater Pipetter
Culture holding rack
Plexiglas® slides
Thermometer
YWT mixture
Selenastrum
Moderately hard synthetic water
Light box or table
Ceriodaphnia dubia or *Daphnia* Culture and Neonate Collection Log

Subject: Maintenance of Daphnid Cultures

Procedure for *Ceriodaphnia dubia* Cultures

Note: The days stipulated in this standard operating procedure (SOP) are provided for guidance. The activities described may be performed on days different than those specified in this SOP.

A. Establish and Maintenance of Individual Cultures (Brood Boards).

1. General Information.

   a. New brood boards are typically initiated on Tuesday with neonates that are ≤ 24 hours old. Neonates used to establish new brood boards must come from third broods.

   b. Individual neonates are cultured in 15 mL of moderately hard synthetic water (MHSW) in 1-oz medicine cups. One brood board consists of 40 to 60 cups held in a holding rack.

   c. The brood boards are segregated from toxicity tests in temperature-controlled incubators maintained at 25.0 ± 1.0°C with a photoperiod of 16-hours light and 8-hours dark and a recommended light intensity of 50 to 100 ft-c.

2. Establish new brood boards on Tuesday.

   a. Prepare the appropriate number of holding racks containing 1-oz medicine cups needed to establish the new brood boards. Typically, six holding racks of 50 cups or eight holding racks of 40 cups are needed. The number of holding racks may be changed depending on the volume of toxicity tests expected the following week.

   b. Fill each cup with 15 mL of MHSW warmed to 25.0 ± 1.0 °C. Add 100 µl *Selenastrum* (cell concentration = 3.0 to 3.5 x 10^7 cells/mL) and 100 µl YWT (solids 1.7 to 1.9 g/L) to each of the cups. The MHSW may be brought to temperature by placing the holding rack in a temperature-controlled incubator.

   c. Label each holding rack with the initiation date and the board number (A through H).

   d. Obtain the “old” brood boards established from the previous Tuesday.

e. Isolate neonates which are ≤ 24 hours old from the "old" brood boards (SOP-AT8). Select 10 brood cups for use to establish the new brood board. Neonates are taken only from adults that have 8 or more young in their third brood.

f. Obtain a new brood board.

g. Obtain a cup containing 8 or more neonates (isolated in section A.2.e). Using a transfer pipette with the tip cut to > 2 mm bore size, place one neonate into each cup of the first column, taking care to release each neonate under the surface of the water. A total of 5 to 6 neonates will be used per column.

h. Obtain a second cup containing 8 or more neonates. Using a transfer pipette with the tip cut to > 2 mm bore size, place one neonate into each cup of the second column, taking care to release each neonate under the surface of the water. Continue in this manner until all cups on the board contain one neonate.

i. Place the new brood board in a temperature-controlled incubator.

j. Record the following information on the *Ceriodaphnia dubia* Culture and Neonate Collection Log (Exhibit AT7.1).

- Culture identification (initiation date and brood board letter)
- Organism age
- Date and time the organisms were born between
- Organism source
- Moderately hard synthetic water batch
- Incubator number
- YWT batch
- *Selenastrum* batch
- Date and time the brood board was initiated and analyst's initials
- Randomizing template color (indicating the random number scheme used)

k. Place a check mark in the appropriate columns to indicate that the organisms were fed, and solutions were renewed.

3. Feed the brood boards daily.

a. Remove the brood boards from the temperature-controlled incubator.

b. Add 100 µl YWT and 100 µl *Selenastrum* to each cup in the holding racks.

c. Return the brood boards to the temperature-controlled incubator and record the date and time the organisms were fed and analyst's initials on the culture log. Place a check mark in the appropriate columns to indicate that the organisms were fed.

4. Transfer the *Ceriodaphnia* to fresh MHSW on day 3 (Friday) and day 6 (Monday).

a. Prepare an appropriate number of holding racks containing 1-oz medicine cups.

b. Fill each cup with 15 mL of MHSW warmed to 25.0 ± 1.0 °C. Add 100 µl *Selenastrum* (cell concentration = 3.0 to 3.5 x 10^7 cells/mL) and 100 µl YWT to each of the cups.

c. Remove the brood boards from the temperature-controlled incubator.

d. Using a transfer pipette with the tip cut to > 2 mm bore size, transfer each adult organism from the cup containing the old MHSW to the corresponding cup containing new MHSW. Care should be taken to release each adult under the surface of the water. Label the new holding rack with the appropriate culture identification date and number.

e. Count and record in the *Ceriodaphnia dubia* Culture and Neonate Collection Log the number of neonates produced in 20% of the cups, as indicated according to the randomizing template. Record if two broods are present with a "2B".

f. For each of the remaining cups on the board, record any mortality (D), if offspring are present (+), if no offspring are present (-), and if 2 broods of offspring are present (+2B).

g. Return the brood boards to the temperature-controlled incubator after all adult organisms have been transferred and the reproduction has been counted. Discard the old brood boards.

h. Record date and time the brood board was renewed and the analyst's initials on the culture log. Check mark in the appropriate columns on the log that the brood board was fed and renewed.

Note: Under normal circumstances, brood boards are maintained for less than 10 days. Under certain circumstances, brood boards may be maintained until the adult organisms are 14 days old. At this time, the organisms are discarded.

5. Determine final (day 7) average reproduction and survival for each brood board.

   a. Remove the brood boards from the temperature-controlled incubator.

   b. Count and record in the *Ceriodaphnia dubia* Culture and Neonate Collection Log the number of neonates produced in 20% of the cups, as indicated according to the randomizing template. Determine the total number of offspring obtained from those cups and record the average number of offspring obtained from 20% of the cups, percentage of males, and percent survival for each brood board.

   c. For each of the remaining cups on the board, record any mortality (D), if offspring are present (+), if no offspring are present (-), and if 8 or more offspring are present (8+).

   d. Return the brood boards to the temperature-controlled incubator after all information has been recorded.

B. Establish and Maintenance of Backup Cultures.

Backup cultures must not be used for the collection of neonates for use in a toxicity test. Backup cultures are only used if the health of individual cultures is compromised and are needed to initiate new individual cultures.

1. Establish new backup cultures on Tuesday.

   a. Isolate neonates to initiate the backup cultures from the brood boards as indicated in section A.2.e.

   b. Label clean 1500-mL glass beakers with the initiation date. Typically six backup cultures are maintained.

   c. Add approximately 1400 mL of MHSW warmed to 25.0 ± 1.0°C to each beaker. Add 20 mL YWT and 20 mL *Selenastrum* (cell concentration = 3.0 to 3.5 x 10^7 cells/mL) to each beaker. Note: These are designated as "new" cultures.

   d. Using a transfer pipette with the tip cut to > 2 mm bore size, transfer 60 to 65 organisms (a few at a time) from the neonates isolated in section A.2.e from the brood boards. Care should be taken to release each neonate under the surface of the water. Repeat this procedure for the remaining backup culture.

e. Place the cultures in a temperature-controlled incubator. The cultures must be maintained at 25.0 ± 1.0°C with a photoperiod of 16-hours light and 8-hours dark and a light intensity of 50 to 100 ft-c. Backup cultures should be maintained in a separate incubator from the individual cultures (brood boards).

2. Using a large glass rod, stir the backup cultures on day 2 (Wednesday) and day 3 (Thursday).

3. Change out the backup cultures on day 3 (Friday) and day 6 (Monday).

a. Obtain the backup cultures from the temperature-controlled incubator.

b. Pour one of the backup cultures into a 2-L finger bowl. Wipe out the 1500-mL beaker with a paper towel and rinse the beaker with deionized water. Add fresh MHSW (1400 mL warmed to 25.0 ± 1.0°C) to the beaker. Add 20 mL YWT and 20 mL *Selenastrum* to each beaker.

c. Using a transfer pipette with the tip cut to > 2 mm bore size, transfer the adult organisms (a few at a time) from the finger bowl to the beaker containing fresh MHSW. Care should be taken to release each adult under the surface of the water. Repeat this procedure for the remaining backup cultures.

d. Return the backup cultures to the temperature-controlled incubator.

Note: Backup cultures are maintained until the adult organisms are 14 days old. At this time, the organisms are discarded.

Procedure for *Daphnia* Cultures (*Daphnia magna* and *Daphnia pulex*)

Note: The days stipulated in this standard operating procedure (SOP) are provided for guidance. The activities described may be performed on days different than those specified in this SOP.

A. Establish and Maintain Cultures.

1. Establish new cultures every 3 weeks from the previous cultures.

a. Isolate neonates that are < 24-hours old from the old culture.

b. Label clean 3000-mL glass jars with the initiation date. Typically four cultures are maintained.

c. Add approximately 2000 mL of MHSW warmed to 25.0 ± 1.0°C to each beaker. Add 25 mL YWT and 25 mL *Selenastrum* (cell concentration = 3.0 to 3.5 x 10^7 cells/mL) to each beaker. Note: These are designated as "new" cultures.

d. Using a transfer pipette with the tip cut to > 5 mm bore size, transfer 20 organisms (a few at a time) from the neonates isolated above. Care should be taken to release each neonate under the surface of the water. Repeat this procedure for the remaining cultures.

e. Place the cultures in a temperature-controlled incubator. The cultures must be maintained at 25.0 ± 1.0°C with a photoperiod of 16-hours light and 8-hours dark and a light intensity of 50 to 100 ft-c.

2. Change out the backup cultures on Monday, Wednesday, and Friday.

a. Obtain the cultures from the temperature-controlled incubator.

b. Pour one of the cultures into a 2-L finger bowl. Wipe out the 3000-mL beaker with a paper towel and rinse the beaker with deionized water. Add fresh MHSW (2000 mL warmed to 25.0 ± 1.0°C) to the jar. Add 25 mL YWT and 25 mL *Selenastrum* to each jar.

c. Using a transfer pipette with the tip cut to > 5 mm bore size, transfer the adult organisms (a few at a time) from the finger bowl to the beaker containing fresh MHSW. Care should be taken to release each adult under the surface of the water. Repeat this procedure for the remaining cultures.

d. Return the cultures to the temperature-controlled incubator.

Note: Cultures are maintained until the adult organisms are 14 days old. At this time, the organisms are discarded.

Safety and Hazardous Waste Management

Safety glasses, gloves and lab coats should always be worn.

Review Policy-P6: General Safety Policy and Policy-P9: Radiation Protection Policy for additional safety requirements.

Subject: Maintenance of Daphnid Cultures

References

USEPA. October 2002. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, 5th ed. EPA-821-R-02-012. US Environmental Protection Agency, Cincinnati, OH.

USEPA. October 2002. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, 4th ed. EPA-821-R-02-013. US Environmental Protection Agency, Cincinnati, OH.

TNI Standard. Management and Technical Requirements for Laboratories Performing Environmental Analysis. EL-V1-ISO-2016-Rev2.0. The NELAC Institute, PO Box 2439, Weatherford, TX 76086.

Exhibits

Exhibit AT7.1: *Ceriodaphnia dubia* Culture Log.
Exhibit AT7.2: *Daphnia magna* Culture Log.

Subject: Maintenance of Daphnid Cultures

Exhibit AT7.1: *Ceriodaphnia du*bia Culture and Neonate Collection Log.

***Ceriodaphnia dubia* Culture and Neonate Collection Log**

Culture: _____

Test organism information:		***Culture information:***	
Organism age:	< 24-hours old	Incubator number:	4
Date and times organisms were born between:		YWT batch:	
Organism source:		*Selenastrum* batch:	

Day	Date	Time	Analyst	MHSW batch	Activity (√)		
					*Fed (100 µL *Selenastrum* and 100 µL YWT)	Renewed	Cleared
0 (initiation)							
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							
11							
12							
13							
14							

*Organisms fed using HandyStep repeat pipettor SN 17E59354.

Comments:

SOP AT7-Revision 6– Exhibit AT7.1

Subject: Maintenance of Daphnid Cultures

ETS

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***Ceriodaphnia dubia* Culture and Neonate Collection Log**

Culture board: _____ – A Randomizing template: YELLOW

Codes: (-) Alive, no reproduction. (+) Alive with reproduction. (**N**) Number of offspring. (**M**) Male. (**D**) Dead. (**D+**) Dead with reproduction. (**2B**) 2 broods present. (**S**) Sick. (**K**) Killed. (**U**) Unhealthy and discarded. (**8+**) ≥ 8 offspring.

	Change-out		Isolation and Collection of Neonates										Total Number of Offspring
Date													
Time													
Analyst													
Replicate													
1													
2													
3													
4													
5													
6													
7													
8													
9													
10													
11													
12													
13													
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32													
33													
34													
35													
36													
37													
38													
39													
40													
Collection for Test (√)													

Culture Information on Day 7:		Acceptance criteria
% of Male Adults on culture board:		≤ 20%
% Mortality on culture board:		≤ 20%
Mean Offspring/Female:		> 20.0 offspring/female

SOP AT7-Revision 6– Exhibit AT7.1

Subject: Maintenance of Daphnid Cultures

***Ceriodaphnia dubia* Culture and Neonate Collection Log**

Culture board: _____ – B Randomizing template: YELLOW

Codes: (-) Alive, no reproduction. (+) Alive with reproduction. (*N*) Number of offspring. (M) Male. (D) Dead. (D+) Dead with reproduction. (2B) 2 broods present. (S) Sick. (K) Killed. (U) Unhealthy and discarded. (8+) ≥ 8 offspring.

	Change-out		Isolation and Collection of Neonates										Total Number of Offspring
Date													
Time													
Analyst													
Replicate													
1													
2													
3													
4													
5													
6													
7													
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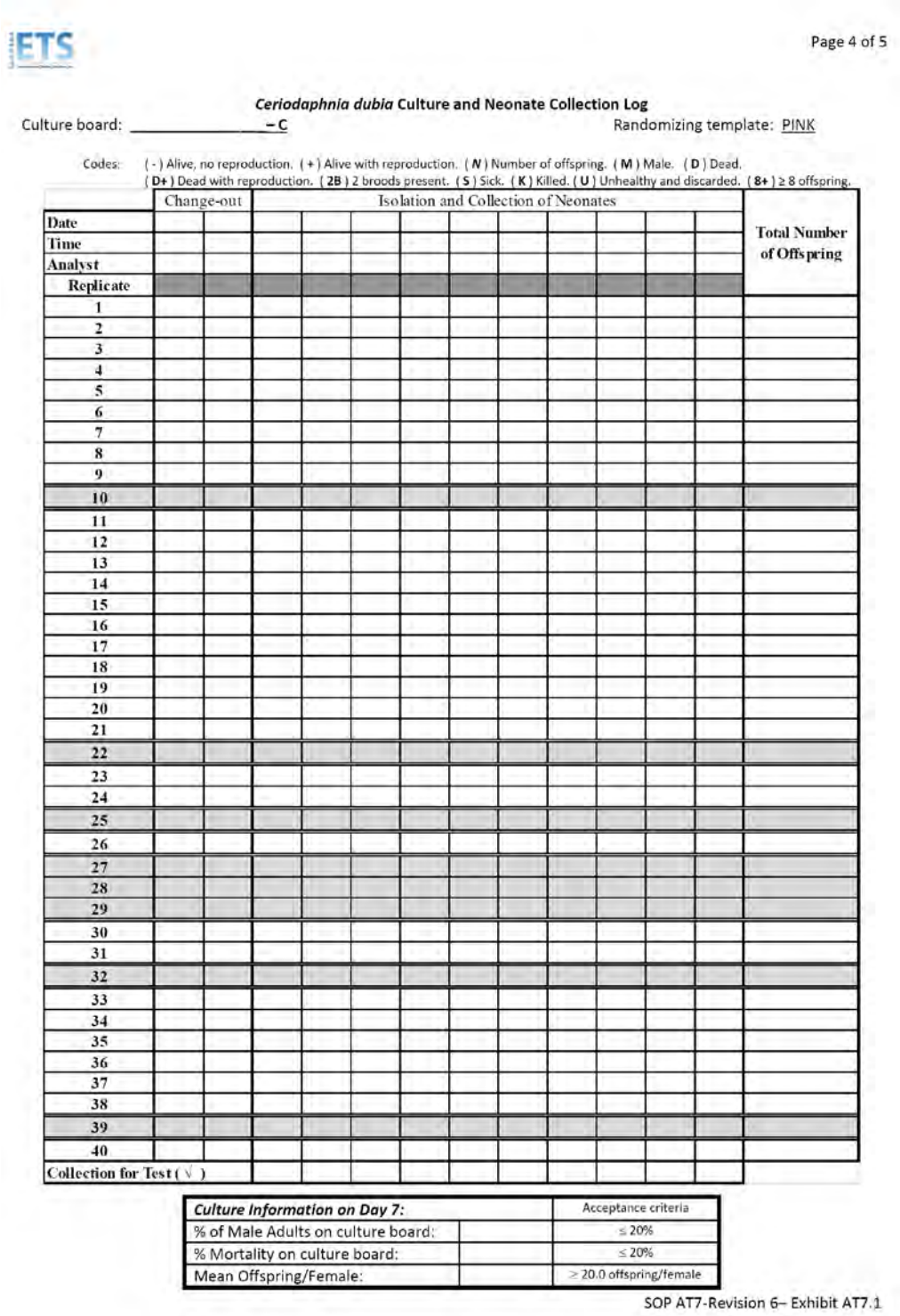
32													
33													
34													
35													
36													
37													
38													
39													
40													
Collection for Test (√)													

Culture Information on Day 7:		Acceptance criteria
% of Male Adults on culture board:		≤ 20%
% Mortality on culture board:		≤ 20%
Mean Offspring/Female:		≥ 20.0 offspring/female

SOP AT7-Revision 6– Exhibit AT7.1

Subject: Maintenance of Daphnid Cultures



ETS

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***Ceriodaphnia dubia* Culture and Neonate Collection Log**

Culture board: _____ – C

Randomizing template: PINK

Codes: (-) Alive, no reproduction. (+) Alive with reproduction. (**N**) Number of offspring. (**M**) Male. (**D**) Dead. (**D+**) Dead with reproduction. (**2B**) 2 broods present. (**S**) Sick. (**K**) Killed. (**U**) Unhealthy and discarded. (**8+**) ≥ 8 offspring.

	Change-out		Isolation and Collection of Neonates										Total Number of Offspring
Date													
Time													
Analyst													
Replicate													
1													
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3													
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33													
34													
35													
36													
37													
38													
39													
40													
Collection for Test (√)													

Culture Information on Day 7:		Acceptance criteria
% of Male Adults on culture board:		≤ 20%
% Mortality on culture board:		≤ 20%
Mean Offspring/Female:		> 20.0 offspring/female

SOP AT7-Revision 6– Exhibit AT7.1

Subject: Maintenance of Daphnid Cultures

ETS

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***Ceriodaphnia dubia* Culture and Neonate Collection Log**

Culture board: _____ – D

Randomizing template: PINK

Codes: (-) Alive, no reproduction. (+) Alive with reproduction. (***N***) Number of offspring. (**M**) Male. (**D**) Dead. (**D+**) Dead with reproduction. (**2B**) 2 broods present. (**S**) Sick. (**K**) Killed. (**U**) Unhealthy and discarded. (**8+**) ≥ 8 offspring.

	Change-out		Isolation and Collection of Neonates										Total Number of Offspring
Date													
Time													
Analyst													
Replicate													
1													
2													
3													
4													
5													
6													
7													
8													
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36													
37													
38													
39													
40													
Collection for Test (√)													

Culture Information on Day 7:		Acceptance criteria
% of Male Adults on culture board:		≤ 20%
% Mortality on culture board:		≤ 20%
Mean Offspring/Female:		≥ 20.0 offspring/female

SOP AT7-Revision 6– Exhibit AT7.1

Subject: Maintenance of Daphnid Cultures

Exhibit AT7.2: *Daphnia* Culture Log.

ETS

Page 1 of 1

Daphnia magna Culture Log

Culture: _____

Test organism information:		***Culture information:***	
Organism age:	< 24-hours old	Incubator number:	4
Date and times organisms were born between:		YWT batch:	
Organism source:		*Selenastrum* batch:	

Day	Date	Time	Analyst	MHSW batch	Activity			
					F = Fed S = Stirred Culture to Resuspend Algae	Renewed (√)	Collected Neonates for Testing (Time)	Comments
0 (initiation)								
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								
13								
14								
15								
16								
17								
18								
19								
20								
21								

Comments:

SOP AT7-Revision 6– Exhibit AT7.2

Subject: *Ceriodaphnia dubia* Neonate Collection

Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan		03-01-20
Quality Assurance Officer	Jim Sumner		03-01-20

Document Revision History

Effective Date	Revision number	Review Type	Evaluators	Revisions
12-01-00	0	Internal	Jim Sumner (ETS)	Original document
06-01-11	1	Internal	Jim Sumner (ETS)	• Updated exhibits.
11-01-14	2	Internal	Jim Sumner (ETS)	• Updated exhibits.
01-01-18	3	Internal	Jim Sumner (ETS)	• Updated procedure to include NELAP requirements. • Additional guidance included in SOP.
09-01-19	4	Internal	Jim Sumner (ETS)	• Corrected typographical errors.
03-01-20	5	Internal	Jim Sumner (ETS)	• Exhibit removed, due to being provided in SOP AT7 as Exhibit AT7.1

Scope and Application

To provide known-age neonates for toxicity testing.

Summary of Method

This procedure describes how to isolate and collect known-age neonates for use in toxicity tests.

Quality Control

It is important that the age of organisms used in tests can be traced to the culture source.

Test organism age requirements: Neonates used to initiate acute and chronic toxicity tests must be ≤ 24 hours old. In addition, all neonates used to initiate chronic toxicity tests must be within 8 hours of the same age.

Culture source requirements: Neonates used to initiate toxicity tests must be obtained from adults that have produced at least 8 neonates in their brood.

Subject: *Ceriodaphnia dubia* Neonate Collection

Neonates used to initiate toxicity tests must come from brood boards with ≤ 20.0% mortality with an average reproduction of >20.0 offspring per surviving female.

Neonates used to initiate toxicity tests must come from third or subsequent broods and must not be from adults > 14 days old.

Equipment and Materials

Ceriodaphnia dubia brood boards
Temperature-controlled incubator (set to maintain test temperature = 25.0 ± 1.0°C, photoperiod of 16-hour light / 8-hours dark, light intensity = 50 – 100 ft-c)
Transfer pipettes
Sharpie's® of various colors
Light box or table
Ceriodaphnia dubia Culture and Neonate Collection Log

Procedure

A. Isolate and collect known-age neonates for a test.

1. Remove the brood board from the temperature-controlled incubator.

2. Place the brood board on a light table. Check each of the cups for reproduction. Using a Sharpie®, mark the cups containing 8 or more neonates with a specific color. Continue marking the remaining available brood boards. Record the date and time the brood boards were marked in the *Ceriodaphnia dubia* Culture and Neonate Collection Log (Exhibit AT8.1). Record the cups containing 8 or more neonates with an 8+ for each brood board in the collection log. Identify the time and color used to mark the cups on the brood board.

   Note: The brood boards may need to be cleared of offspring initially. If so, record the date and time the brood boards were cleared in the collection log.

3. If necessary, check the brood boards at 2 to 3-hour intervals until an adequate number of known-age organisms are obtained for toxicity tests. During each marking, use a different color Sharpie® to identify the cups containing 8 or more neonates. For each brood board, record the date and time the brood was marked as well as the cups containing 8 or more neonates (with an 8+) in the collection log. Identify the time and color used to mark the cups on the brood board.

Subject: *Ceriodaphnia dubia* Neonate Collection

Safety and Hazardous Waste Management

Safety glasses, gloves and lab coats should always be worn.

Review Policy-P6: General Safety Policy and Policy-P9: Radiation Protection Policy for additional safety requirements.

References

USEPA. October 2002. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, 5th ed. EPA-821-R-02-012. US Environmental Protection Agency, Cincinnati, OH.

USEPA. October 2002. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, 4th ed. EPA-821-R-02-013. US Environmental Protection Agency, Cincinnati, OH.

TNI Standard. Management and Technical Requirements for Laboratories Performing Environmental Analysis. EL-V1-ISO-2016-Rev2.0. The NELAC Institute, PO Box 2439, Weatherford, TX 76086.

Exhibits

Exhibit AT8.1: *Ceriodaphnia dubia* Culture and Neonate Collection Log
(provided in SOP AT7 as Exhibit AT7.1.

Subject: Daphnid Acute Toxicity Test, EPA 2002.0 and EPA 2021.0

Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan		03-01-20
Quality Assurance Officer	Jim Sumner		03-01-20

Document Revision History

Effective Date	Revision number	Review Type	Evaluators	Revisions
12-01-00	0	Internal	Jim Sumner (ETS)	Original document
06-01-11	1	Internal	Jim Sumner (ETS)	• Updated exhibits and references. • Statistical analyses and data review moved to QAP-Q12.
07-01-12	2	External (NC DENR) Internal	Lance Ferrell (NC DENR) Jim Sumner (ETS)	• The measurement of pH, DO and conductivity of each new, full-strength, undiluted sample was added. • The light intensity was amended to reflect that it is a recommended range as specified in the EPA manuals.
11-01-14	3	Internal	Jim Sumner (ETS)	• Updated exhibits during document review.
07-01-18	4	Internal	Jim Sumner (ETS)	• Updated procedure to include NELAP requirements. • Additional guidance included in SOP.
09-01-19	5	Internal	Jim Sumner (ETS)	• The use of SSW for NC testing was removed.
03-01-20	6	External (TVA) Internal	Rick Sherrard (TVA) Jim Sumner (ETS)	• Updated bench sheet (Exhibits AT9.2 and AT9.3) to include reporting limits, method numbers, meters and serial numbers used for chemical analyses.

Scope and Application

To measure the acute toxicity of water samples to Daphnids (*Ceriodaphnia dubia* or *Daphnia magna*) during 24, 48 or 96-hour exposure period.

Summary of Method

The acute toxicity test generally involves the exposure of test organisms to up to five effluent concentrations and a control water. The test duration ranges from 24 to 96 hours. At the end of each 24-hour period, the number of living organisms is counted in each effluent concentration and control water.

A summary of the Daphnid acute method is provided in Exhibit AT9.1.

Subject: Daphnid Acute Toxicity Test, EPA 2002.0 and EPA 2021.0

Quality Control

Acceptance Criteria: The test acceptance criterion is ≥ 90% survival in the control. If the control survival is < 90%, the test must be invalidated.

Equipment and Materials

Ceriodaphnia dubia or *Daphnia magna*
Temperature-controlled incubator (set to maintain test temperature = 25.0 ± 1.0°C, photoperiod of 16-hour light / 8-hours dark, light intensity = 50 – 100 ft-c)
Control / Dilution water (synthetic water)
1-oz medicine cups or 150 ml glass beakers
Graduated cylinders
500-ml plastic Solo® cups
Serological, fixed, or adjustable volume pipettes (e.g., Eppendorf)
Transfer pipettes
Pasteur® pipettes
Eppendorf Repeater Pipetter
Acute test holding rack
Plexiglas® slides
Thermometer
YWT mixture
Selenastrum capricornutum
Glass finger bowl
Light box or table
Dissection microscope
Disposable gloves
Acute Toxicity Test or Pass/Fail Acute Toxicity Test Bench Sheet

Procedure

A. Test Preparation.

1. Prepare the Acute Toxicity Test Bench Sheet (for multiple concentration tests, Exhibit AT9.3) or Pass/Fail Acute Toxicity Test Bench Sheet (for Pass/Fail acute tests, Exhibit AT9.2). Record the following information on the bench sheet:
    - Client/facility name
    - Permit number
    - County
    - Outfall/Pipe number

- ETS project and sample number
- Control/Dilution water type and batch
- Test concentrations and dilution preparation information (sample, dilution and total volumes)

B. Test Initiation.

1. Prepare the test concentrations according to SOP-G5.

   a. The control/dilution water is moderately hard synthetic water (MHSW, SOP-AT1). MHSW must have an alkalinity of 57 – 64 mg $CaCO_3$/L, hardness of 80 – 100 mg $CaCO_3$/L, and an initial pH of 6.5 – 8.5 S.U. (guidance range = 7.4 – 7.8 S.U.).

   b. Measure and record the pH (SOP-C3), dissolved oxygen (SOP-C2) and conductivity (SOP-C4) of each concentration tested and control. Ensure that the dissolved oxygen is within the acceptable range (4.0 to 9.0 mg/L), aerate the sample if necessary, according to SOP-G5. Measure and record the pH (SOP-C3), dissolved oxygen (SOP-C2), conductivity (SOP-C4), total residual chlorine (SOP-C8), total alkalinity (SOP-C6), total hardness (SOP-C7) and sample characteristics of each new, full-strength, undiluted sample. The alkalinity and hardness of full-strength, undiluted samples for North Carolina tests are not required.

   c. Obtain an acute test holding rack, which is marked for the randomization of the test cups (Exhibit AT9.4). Place the medicine cups in the holding rack and record the holding rack color on the bench sheet.

   d. Pour 30 mL of control water into each of the four replicate control cups.

   e. Pour 30 mL of each test concentration into each of the four replicate medicine cups according to the randomization scheme.

   f. Maintain the test temperature (25.0 ± 1.0°C) of the test concentrations. This may be accomplished by placing the holding rack into a temperature-controlled incubator.

2. Isolate and collect known-age neonates per instructions in SOP-AT8. Neonates must be less than 24-hours old.

   a. Record the source, age, dates and times the organisms were born between on the acute bench sheet. Feed the neonates 100 µl YWT and 100 µl *Selenastrum* a

minimum of 2 hours prior to test initiation to a maximum of 5 hours prior to test initiation. Record the date and time the organisms were fed on the bench sheet.

Note: Neonates are not fed during 24 or 48-hour acute tests.

3. Transfer the neonates to the randomly placed test cups in the holding rack.

   a. Measure and record the temperature in one of the test cups for each concentration and control. The temperature must be 25.0 ± 1.0°C before the test organisms are placed in the test cups. Warm the test cups in a temperature-controlled incubator, if necessary, until the desired test temperature is obtained. Measure and record the temperature of an arbitrary cup containing neonates to be used in the toxicity test.

   b. After the neonates have fed for a minimum of 2 hours to a maximum of 5 hours prior to test initiation, pool the neonates into a glass finger bowl. Once pooled, transfer 5 neonates (10 neonates for a Pass/Fail acute) into each test cup using a transfer pipette with the tip cut to > 2 mm bore size. Care should be taken to release each neonate under the surface of the water. The volume of water transferred to the medicine cups should be minimized to avoid diluting the test concentrations. The average transfer volume for each analyst must be determined yearly. For an example average transfer volume log sheet, refer to Exhibit AT9.5.

   c. Transfer the neonates, beginning with the first test cup in the first row on the acute test holding rack. Continue in this manner (placing 5 neonates in the test cups from left to right in the first row and then the second row) until all the test cups contain 5 neonates. For Pass/Fail acute tests, 10 neonates are placed in each cup.

   d. Save approximately 25 mL of transfer water to be measured for pH (SOP-C3). Measure and record the pH of this transfer water on the acute bench sheet.

   e. Record the initiation date, time and analyst's initials on the acute bench sheet. **The acute test must be initiated within 36-hours of completion of the sampling period.**

   f. Verify that each test cup received 5 neonates by conducting a repeat count. Remove excess neonates or add neonates as necessary. Record the initial number of neonates on the bench sheet.

g. Place the acute test holding rack in a temperature-controlled incubator. The organisms must be maintained at 25.0 ± 1.0°C with a photoperiod of 16-hours light and 8-hours dark and a recommended light intensity of 50 to 100 ft-c. Cover the rack with a Plexiglas® slide. Record the incubator number and shelf used on the bench sheet.

C. Record Daily Survival.

Repeat the steps identified below daily, starting at 24-hours ± 1-hour after test initiation and continuing until test termination.

1. Measure and record the temperature in an arbitrarily selected test cup for each concentration and control.

2. Remove the holding rack from the incubator. Place the rack on a light box or table for ease of viewing.

3. Count and record (in the appropriate section) the number of neonates surviving in each replicate cup on the acute bench sheet. Dead organisms must be confirmed through a dissection microscope. The criteria that establish death are: (1) no movement and (2) no reaction to gentle prodding. Record comments, if applicable.

4. Remove any dead neonates and discard using a Pasteur® pipette.

5. Record the date, time and the analyst's initials on the bench sheet.

6. Gently decant approximately 5 ml of test solution from each replicate of each concentration and control, being careful not to decant the test organisms. The separate combined volumes of each concentration and control will be used to measure and record the pH (SOP-C3) and dissolved oxygen (SOP-C2).

7. Place the holding rack in a temperature-controlled incubator. Cover the rack with a Plexiglas® slide.

D. <u>For 96-hour Acute Tests</u>, Transfer of Test Organisms into New Test Solutions at 48-hours.

For 96-hour acute tests, organisms must be transferred to new test solutions within ± 1 hour from test initiation.

1. Feed the organisms in each test cup 100 µl YWT and 100 µL *Selenastrum* at 2-hours prior to the renewal of test solutions (at 46-hours from test initiation). Record the feeding time on the acute bench sheet.

2. Prepare fresh test water in accordance with SOP-G5. Maintain the test temperature (25.0 ± 1.0°C) of the fresh test water until needed by storing in a temperature-controlled incubator. Using the same template selected in section B.1.b, pour fresh solution into the new test cups.

3. At 48-hours, remove the holding racks from the incubator. Place the racks on a light box or table for ease of viewing.

4. Measure and record the temperature in an arbitrarily selected test cup for each concentration and control for both the new and old test solutions.

5. Using a transfer pipette with the tip cut to > 2 mm bore size, transfer each test organism to the corresponding new test cup containing the freshly prepared solution. Discard and record organisms that are missing, injured, or dead. Dead organisms must be confirmed through a dissection microscope. The criteria that establish death are: (1) no movement and (2) no reaction to gentle prodding. Record comments, if applicable.

6. Record the date and time that the test solutions were renewed and the analyst's initials on the bench sheet.

7. Place the holding rack containing the transferred organisms in a temperature-controlled incubator. Cover the rack with a Plexiglas® slide.

8. Measure and record the pH (SOP-C3) and dissolved oxygen (SOP-C2) in one of the test cups containing old test solution ("final") for each concentration and control (it may be necessary to pool the test cups of each concentration and control).

E. Test Termination.

Terminate the test after the organisms have been exposed to the test concentrations for the required time (i.e. 24, 48 or 96-hours). The test may be terminated ± 1-hour from the time it was initiated.

1. Measure and record the temperature in an arbitrarily selected test cup for each concentration and control.

2. Remove the holding rack from the incubator. Place the rack on a light box or table for ease of viewing.

3. Count and record (in the appropriate section) the number of neonates surviving in each replicate cup on the acute bench sheet. Record comments, if applicable.

4. Record the termination date, time and the analyst's initials on the bench sheet.

5. Measure and record the pH (SOP-C3) and dissolved oxygen (SOP-C2) in one of the test cups for each concentration and control.

6. Once all analyses have been completed and documented, discard the test water and neonates according to established laboratory protocol.

F. Statistical Analyses and Data Verification.

Statistical analyses and data review are performed according to QAP-Q12.

Safety and Hazardous Waste Management

Safety glasses, gloves and lab coats should always be worn.

Review Policy-P6: General Safety Policy and Policy-P9: Radiation Protection Policy for additional safety requirements.

References

USEPA. October 2002. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, 5th ed. **EPA-821-R-02-012, Method 2002.0 for *Ceriodaphnia dubia*, Method 2021.0 for *Daphnia magna***. US Environmental Protection Agency, Cincinnati, OH.

North Carolina Department of Environment and Natural Resources, Division of Water Quality. Pass/Fail Methodology for Determining Acute Toxicity in a Single Effluent, Version 3.0. December 2010.

TNI Standard. Management and Technical Requirements for Laboratories Performing Environmental Analysis. EL-V1-ISO-2016-Rev2.0. The NELAC Institute, PO Box 2439, Weatherford, TX 76086.

Subject: Daphnid Acute Toxicity Test, EPA 2002.0 and EPA 2021.0

Exhibits

Exhibit AT9.1: Summary of Test Conditions for the Daphnid Acute Toxicity Test.
Exhibit AT9.2: Pass/Fail Acute Toxicity Test Bench Sheet.
Exhibit AT9.3: Acute Toxicity Test Bench Sheet.
Exhibit AT9.4: Acute Test Holding Rack.
Exhibit AT9.5: Average Transfer Volume Log Sheet.

Subject: Daphnid Acute Toxicity Test, EPA 2002.0 and EPA 2021.0

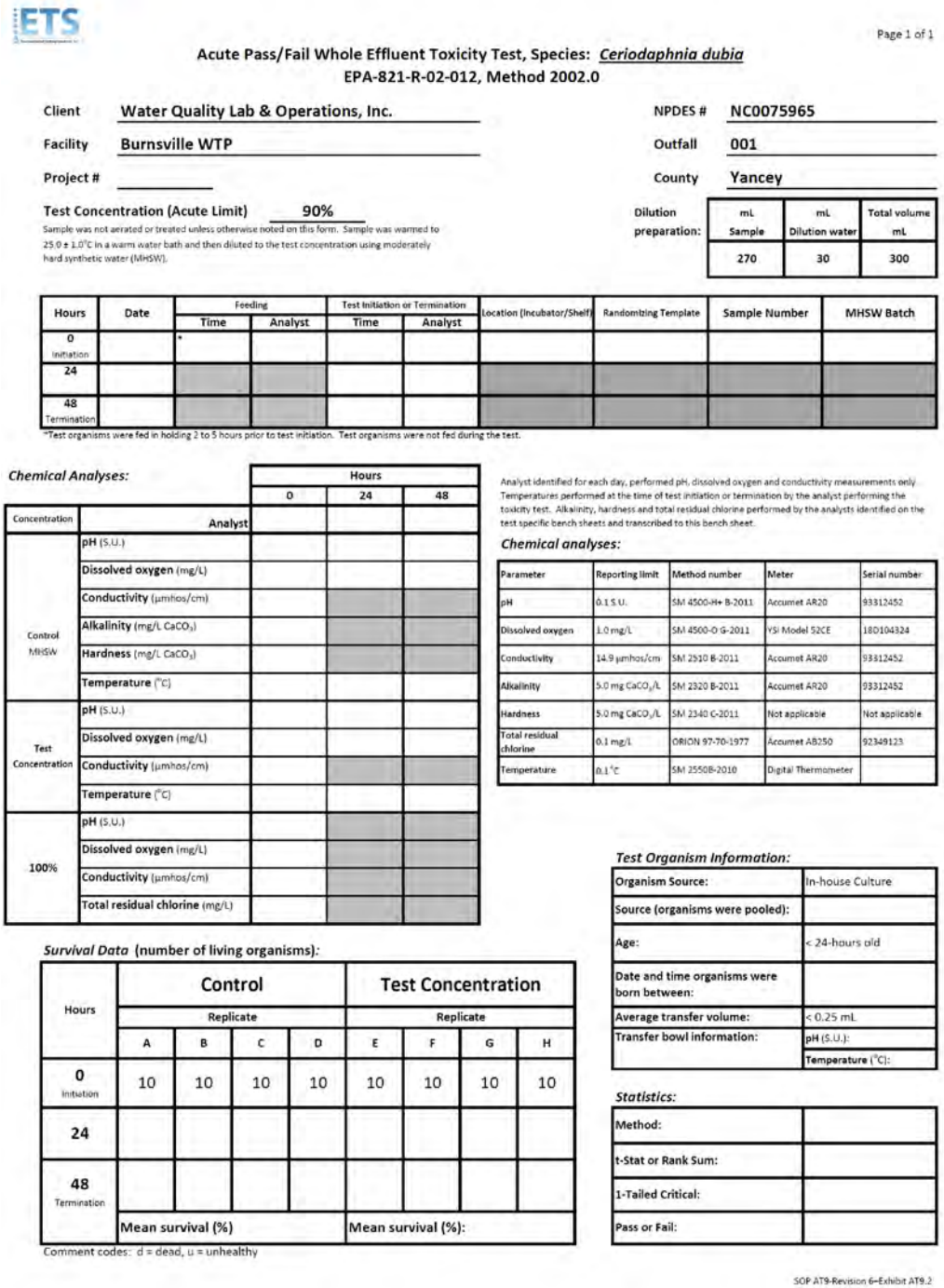
Exhibit AT9.1: Summary of Test Conditions for the Daphnid Acute Toxicity Test.

SUMMARY OF TEST CONDITIONS FOR THE DAPHNID ACUTE TOXICITY TEST

Test type:	Static non-renewal or static renewal
Test duration:	24, 48 or 96 hours
Temperature:	25.0 ± 1.0°C
Light quality:	Cool-white fluorescent
Light intensity:	50 – 100 ft-candles (recommended)
Photoperiod:	16-hours light, 8-hours dark
Test chamber size:	Multiple concentration tests: 40 mL graduated polypropylene medicine cup
Test solution volume:	Multiple concentration tests: 30 mL
Renewal of test solutions:	At 48-hours (required minimum)
Age of test organisms:	≤ 24 hours old
Number of organisms per test chamber:	Multiple concentration tests: 5 Single dilution tests: 10
Number of replicate test chambers per concentration:	4
Number of organisms per concentration:	Multiple concentration tests: 20 Single dilution tests: 40
Test concentrations:	Multiple concentration tests: 5 and a control with ≥ 0.5 dilution series (recommended) Single dilution tests: 90% or 100% and a control
Test chamber cleaning:	Dead organisms removed daily. For 96-hour tests, organisms are transferred to new medicine cups and solutions at 48-hours.
Aeration:	None
Feeding regime:	YWT and *Selenastrum* made available while holding prior to test initiation (2 to 5-hours prior to initiation). Organisms in each test cup are fed 100 µL YWT and 100 µL *Selenastrum* 2 hours prior to test solution renewal at 48-hours.
Control / Dilution water:	Moderately hard synthetic water
Sampling and sample holding:	1-gallon grab or composite sample first used within 36-hours of completion of the sampling period.
Endpoint:	Mortality
Test acceptability criterion:	≥ 90% control survival

Subject: Daphnid Acute Toxicity Test, EPA 2002.0 and EPA 2021.0

Exhibit AT9.2: Pass/Fail Acute Toxicity Test Bench Sheet.



ETS

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Acute Pass/Fail Whole Effluent Toxicity Test, Species: *Ceriodaphnia dubia*
EPA-821-R-02-012, Method 2002.0

Client: Water Quality Lab & Operations, Inc.
NPDES #: NC0075965
Facility: Burnsville WTP
Outfall: 001
Project #:
County: Yancey

Test Concentration (Acute Limit): 90%
Sample was not aerated or treated unless otherwise noted on this form. Sample was warmed to 25.0 ± 1.0°C in a warm water bath and then diluted to the test concentration using moderately hard synthetic water (MHSW).

Dilution preparation:

mL Sample	mL Dilution water	Total volume mL
270	30	300

Hours	Date	Feeding Time	Feeding Analyst	Test Initiation or Termination Time	Test Initiation or Termination Analyst	Location (Incubator/Shelf)	Randomizing Template	Sample Number	MHSW Batch
0 Initiation		*							
24									
48 Termination									

*Test organisms were fed in holding 2 to 5 hours prior to test initiation. Test organisms were not fed during the test.

Chemical Analyses:

Concentration	Analyst	Hours 0	Hours 24	Hours 48
Control MHSW	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	Conductivity (µmhos/cm)			
	Alkalinity (mg/L $CaCO_3$)			
	Hardness (mg/L $CaCO_3$)			
	Temperature (°C)			
Test Concentration	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	Conductivity (µmhos/cm)			
	Temperature (°C)			
100%	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	Conductivity (µmhos/cm)			
	Total residual chlorine (mg/L)			

Analyst identified for each day, performed pH, dissolved oxygen and conductivity measurements only. Temperatures performed at the time of test initiation or termination by the analyst performing the toxicity test. Alkalinity, hardness and total residual chlorine performed by the analysts identified on the test specific bench sheets and transcribed to this bench sheet.

Chemical analyses:

Parameter	Reporting limit	Method number	Meter	Serial number
pH	0.1 S.U.	SM 4500-H+ B-2011	Accumet AR20	93312452
Dissolved oxygen	1.0 mg/L	SM 4500-O G-2011	YSI Model 52CE	18D104324
Conductivity	14.9 µmhos/cm	SM 2510 B-2011	Accumet AR20	93312452
Alkalinity	5.0 mg $CaCO_3$/L	SM 2320 B-2011	Accumet AR20	93312452
Hardness	5.0 mg $CaCO_3$/L	SM 2340 C-2011	Not applicable	Not applicable
Total residual chlorine	0.1 mg/L	ORION 97-70-1977	Accumet AB250	92349123
Temperature	0.1 °C	SM 2550B-2010	Digital Thermometer	

Survival Data (number of living organisms):

Hours	Control Replicate A	B	C	D	Test Concentration Replicate E	F	G	H
0 Initiation	10	10	10	10	10	10	10	10
24								
48 Termination								
	Mean survival (%)				Mean survival (%):			

Comment codes: d = dead, u = unhealthy

Test Organism Information:

Organism Source:	In-house Culture
Source (organisms were pooled):	
Age:	< 24-hours old
Date and time organisms were born between:	
Average transfer volume:	< 0.25 mL
Transfer bowl information:	pH (S.U.):
	Temperature (°C):

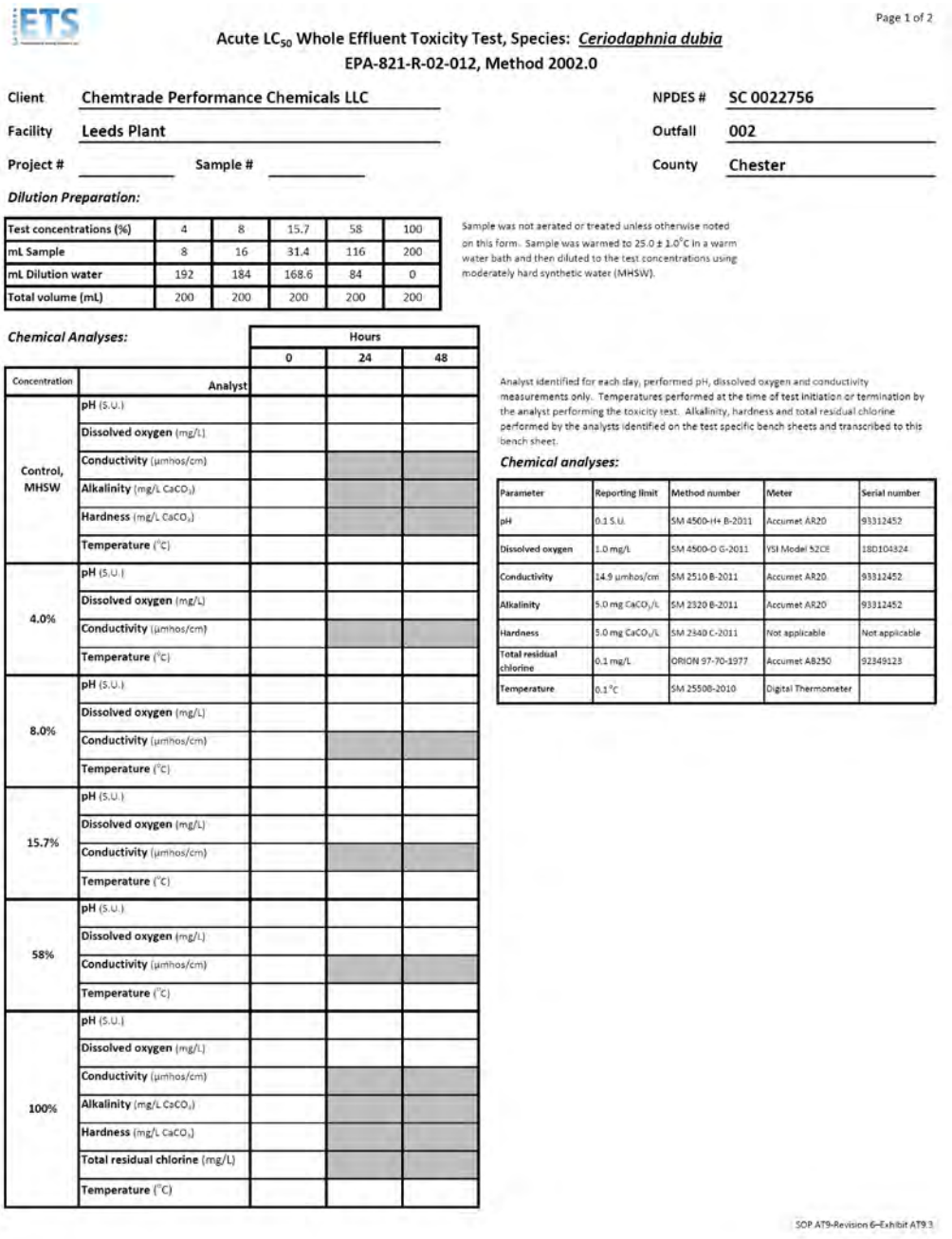
Statistics:

Method:	
t-Stat or Rank Sum:	
1-Tailed Critical:	
Pass or Fail:	

SOP AT9-Revision 6-Exhibit AT9.2

Subject: Daphnid Acute Toxicity Test, EPA 2002.0 and EPA 2021.0

Exhibit AT9.3: Acute Toxicity Test Bench Sheet.



ETS

Page 1 of 2

Acute LC_{50} Whole Effluent Toxicity Test, Species: *Ceriodaphnia dubia*
EPA-821-R-02-012, Method 2002.0

Client: Chemtrade Performance Chemicals LLC — NPDES #: SC 0022756
Facility: Leeds Plant — Outfall: 002
Project #: ____ Sample #: ____ — County: Chester

Dilution Preparation:

Test concentrations (%)	4	8	15.7	58	100
mL Sample	8	16	31.4	116	200
mL Dilution water	192	184	168.6	84	0
Total volume (mL)	200	200	200	200	200

Sample was not aerated or treated unless otherwise noted on this form. Sample was warmed to 25.0 ± 1.0°C in a warm water bath and then diluted to the test concentrations using moderately hard synthetic water (MHSW).

Chemical Analyses:

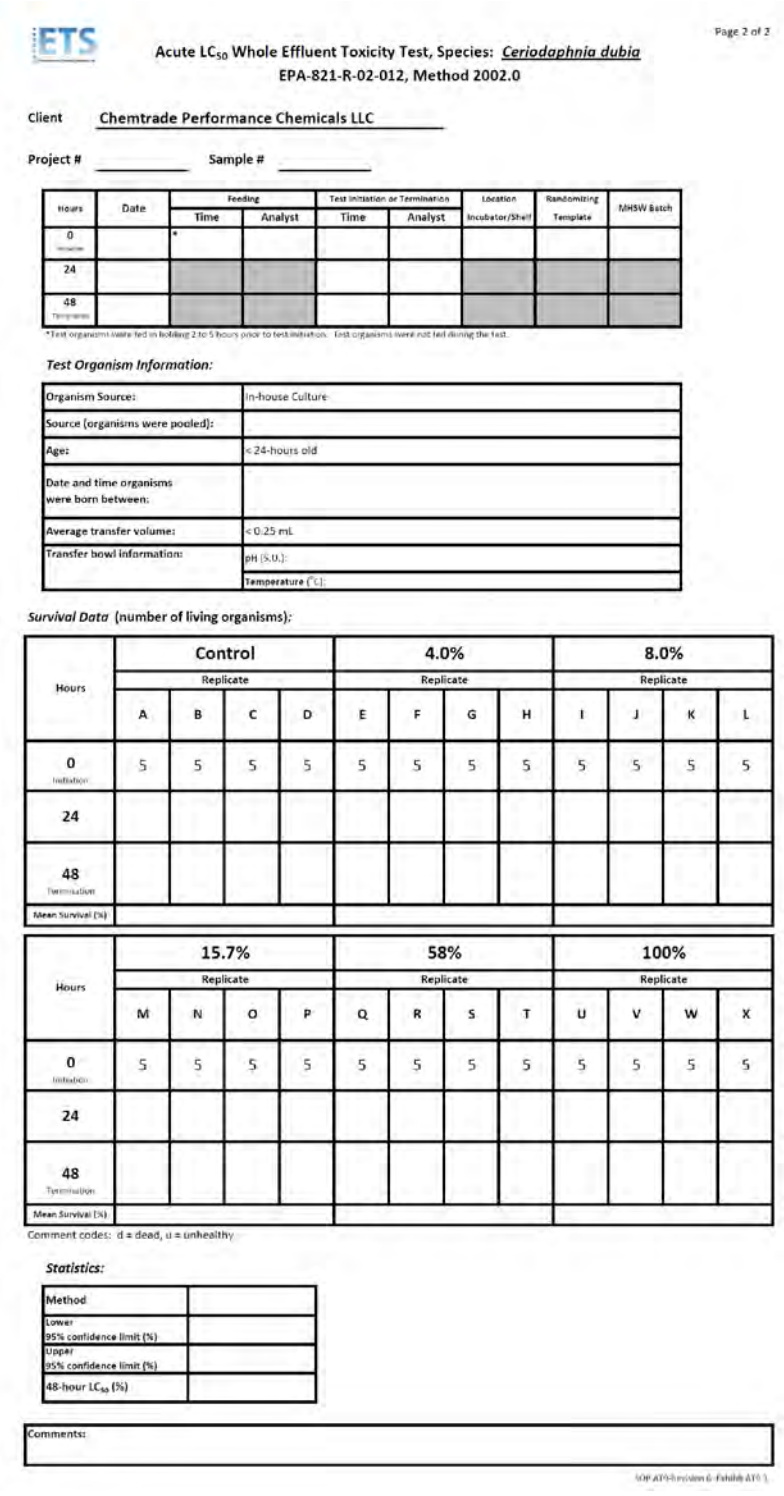
Concentration	Analyst	Hours 0	24	48
Control, MHSW	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	Conductivity (µmhos/cm)			
	Alkalinity (mg/L $CaCO_3$)			
	Hardness (mg/L $CaCO_3$)			
	Temperature (°C)			
4.0%	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	Conductivity (µmhos/cm)			
	Temperature (°C)			
8.0%	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	Conductivity (µmhos/cm)			
	Temperature (°C)			
15.7%	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	Conductivity (µmhos/cm)			
	Temperature (°C)			
58%	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	Conductivity (µmhos/cm)			
	Temperature (°C)			
100%	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	Conductivity (µmhos/cm)			
	Alkalinity (mg/L $CaCO_3$)			
	Hardness (mg/L $CaCO_3$)			
	Total residual chlorine (mg/L)			
	Temperature (°C)			

Analyst identified for each day, performed pH, dissolved oxygen and conductivity measurements only. Temperatures performed at the time of test initiation or termination by the analyst performing the toxicity test. Alkalinity, hardness and total residual chlorine performed by the analysts identified on the test specific bench sheets and transcribed to this bench sheet.

Chemical analyses:

Parameter	Reporting limit	Method number	Meter	Serial number
pH	0.1 S.U.	SM 4500-H+ B-2011	Accumet AR20	93312452
Dissolved oxygen	1.0 mg/L	SM 4500-O G-2011	YSI Model 52CE	18D104324
Conductivity	14.9 µmhos/cm	SM 2510 B-2011	Accumet AR20	93312452
Alkalinity	5.0 mg $CaCO_3$/L	SM 2320 B-2011	Accumet AR20	93312452
Hardness	5.0 mg $CaCO_3$/L	SM 2340 C-2011	Not applicable	Not applicable
Total residual chlorine	0.1 mg/L	ORION 97-70-1977	Accumet AB250	92349123
Temperature	0.1 °C	SM 2550B-2010	Digital Thermometer	

SOP AT9-Revision 6-Exhibit AT9.3



ETS

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Acute LC_{50} Whole Effluent Toxicity Test, Species: *Ceriodaphnia dubia*
EPA-821-R-02-012, Method 2002.0

Client: Chemtrade Performance Chemicals LLC

Project # _____ Sample # _____

Hours	Date	Feeding		Test Initiation or Termination		Location	Randomizing	MHSW Batch
		Time	Analyst	Time	Analyst	Incubator/Shelf	Template	
0		*						
24								
48								

*Test organisms were fed in holding 2 to 5 hours prior to test initiation. Test organisms were not fed during the test.

Test Organism Information:

Organism Source:	In-house Culture
Source (organisms were pooled):	
Age:	< 24-hours old
Date and time organisms were born between:	
Average transfer volume:	< 0.25 mL
Transfer bowl information:	pH (S.U.):
	Temperature (°C):

Survival Data (number of living organisms):

Hours	Control				4.0%				8.0%			
	Replicate				Replicate				Replicate			
	A	B	C	D	E	F	G	H	I	J	K	L
0	5	5	5	5	5	5	5	5	5	5	5	5
24												
48												
Mean Survival (%)												

Hours	15.7%				58%				100%			
	Replicate				Replicate				Replicate			
	M	N	O	P	Q	R	S	T	U	V	W	X
0	5	5	5	5	5	5	5	5	5	5	5	5
24												
48												
Mean Survival (%)												

Comment codes: d = dead, u = unhealthy

Statistics:

Method	
Lower 95% confidence limit (%)	
Upper 95% confidence limit (%)	
48-hour LC_{50} (%)	

Comments:

SOP AT9-6 review 6 - Exhibit AT9-1

Exhibit AT9.4: Acute Test Holding Rack.

Randomizing template: **RED**

Replicate #	1	2	3	4
Concentrations	6	5	4	5
	3	3	2	6
1 = Control	4	1	1	2
2 = Lowest concentration	1	2	3	1
3 - 5 = Intermediate concentrations	2	4	5	3
6 = Highest concentration	5	6	6	4

Random number seeds: 4 through 7

Subject: Daphnid Acute Toxicity Test, EPA 2002.0 and EPA 2021.0

Exhibit AT9.5: Average Transfer Volume Log Sheet.

Environmental Testing Solutions, Inc.

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Ceriodaphnia dubia Transfer Volume

Analyst: _____

Date: _____

Ambient temperature: _____

Numerically label 10 medicine cups.
Add 15 mL MHSW to each of the 10 cups.
Measure and record the weight of each cup containing MHSW.
Transfer 5 *Ceriodaphnia* to each cup, following procedures identified in SOP-AT9 for Daphnid acute toxicity tests.
Measure and record the weight of each cup containing MHSW with 5 *Ceriodaphnia.*
Determine each transfer volume, average transfer volume, and estimated volume to transfer 1 *Ceriodaphnia.*

Replicate Number	Initial Weight Medicine cup + 15 mL MHSW (g)	Final Weight Medicine cup + 15 mL MHSW + 5 *Ceriodaphnia* transferred (g)	Transfer Volume Final - Initial Weight (g = mL)
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
	Average volume to transfer 5 organisms (mL):		
	Estimated volume to transfer 1 organism (mL):		

SOP AT9-Revision 6-Exhibit AT9.5

Subject: Daphnid Acute Reference Toxicity Test, EPA 2002.0 and EPA 2021.0

Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan		03-01-20
Quality Assurance Officer	Jim Sumner		03-01-20

Document Revision History

Effective Date	Revision number	Review Type	Evaluators	Revisions
12-01-00	0	Internal	Jim Sumner (ETS)	Original document
06-01-11	1	Internal	Jim Sumner (ETS)	• Updated references and exhibits. • Updated Table AT10.1. • Included 96-hour acute reference toxicant bench sheets.
11-01-14	2	Internal	Jim Sumner (ETS)	• Updated exhibits during document review. • Removed conductivity measurement requirement of stock NaCl solution due to inaccuracy of these measurements, which are above the calibration range.
09-28-16	3	External (TVA) Internal	Rick Sherrard, Donald Snodgrass (TVA) Jim Sumner (ETS)	• Updated Table AT10.1 for test concentrations and conductivity measurement guidance values. • Deleted statement: "Verify that the conductivity measured for each test concentration is within the acceptance criteria (refer to table Table AT10.1) before proceeding with the preparation of next concentration. If the conductivity is not within the criteria, remake the test concentration and verify the conductivity."
07-01-18	4	Internal	Jim Sumner (ETS)	• Updated procedure to include NELAP requirements. • Additional guidance included in SOP.
09-01-19	5	Internal	Jim Sumner (ETS)	• Updated exhibits during document review.
03-01-20	6	External (TVA) Internal	Rick Sherrard (TVA) Jim Sumner (ETS)	• Updated bench sheet to include reporting limits, method numbers, meters and serial numbers used for chemical analyses.

Subject: Daphnid Acute Reference Toxicity Test, EPA 2002.0 and EPA 2021.0

Scope and Application

To assess the sensitivity of *Ceriodaphnia dubia* or *Daphnia* and the overall credibility of the *Ceriodaphnia dubia* or *Daphnia* acute toxicity tests. Reference toxicant data are part of the laboratory's QA/QC program to evaluate the performance of laboratory personnel and the robustness and sensitivity of the test organisms.

Summary of Method

The acute reference toxicity test generally involves the exposure of test organisms to five sodium chloride concentrations and control water for a 48-hour or 96-hour exposure period. At the end of each 24-hour period, the number of living organisms is counted in each sodium chloride concentration and control water. The median lethal concentration (LC_{50}) of sodium chloride is determined and compared to previous reference toxicant tests.

Quality Control

Acceptance Criteria: The test acceptance criterion is ≥ 90% survival in the control. If the control survival is < 90%, the test must be invalidated.

Frequency of Testing:
A *Ceriodaphnia dubia* acute reference toxicant test must be performed so that all acute whole effluent toxicity tests are conducted within 1 week of a reference toxicant test. At a minimum, acute reference toxicant tests must be performed on a quarterly basis in order to maintain certification requirements.

A *Daphnia* acute reference toxicant test must be performed such that all acute whole effluent toxicity tests are conducted within 1 week of a reference toxicant test.

Subject: Daphnid Acute Reference Toxicity Test, EPA 2002.0 and EPA 2021.0

Equipment and Materials

Ceriodaphnia dubia or *Daphnia*
Temperature-controlled incubator (set to maintain test temperature = 25.0 ± 1.0°C, photoperiod of 16-hour light / 8-hours dark, light intensity = 50 – 100 ft-c)
Control / Dilution water (synthetic water)
1-oz medicine cups or 150 mL glass beakers
Graduated cylinders
500-mL plastic Solo® cups
Serological, fixed, or adjustable volume pipettes (e.g., Eppendorf)
Transfer pipettes
Pasteur® pipettes
Eppendorf Repeater Pipetter
Acute test holding rack
Plexiglas® slides
Thermometer
YWT mixture
Selenastrum capricornutum
Glass finger bowl
Light box or table
Dissection microscope
Disposable gloves
Ceriodaphnia dubia or *Daphnia magna* Acute Reference Toxicity Test Bench Sheet

Procedure

A. Test Preparation.

1. Prepare the 48-hour or 96-hour *Ceriodaphnia dubia* or *Daphnia* Acute Reference Toxicity Test Bench Sheet (see Exhibit AT10.1). Record the test number on the bench sheet.

2. Obtain an acute test holding rack, which is marked for the randomization of the test cups. Place the medicine cups in the holding rack and record the holding rack color on the bench sheet.

B. Preparation of the Stock Solution.

1. Using a calibrated top-loading balance, carefully weigh out 50 g of NaCl (SOP-G10). Place approximately 400 mL of deionized water in a 500-mL volumetric flask. Add the

NaCl to the flask, dissolve the NaCl by swirling the flask, bring to volume with deionized water. Label the volumetric flask with the concentration (100 g/L), analyst's initials, preparation date and stock standard number (INSS, SOP-G15). Record the INSS number of the NaCl stock solution on the bench sheet.

C. Preparation of the Test Concentrations.

1. Prepare all test concentrations using graduated cylinders and serological pipettes. The precision of conductivity measurements is increased when smaller volume graduated cylinders and/or serological pipettes are used to prepare the test concentrations. For this reference toxicant test, stock solution volumes should be measured using a 10-mL serological pipette and the total volumes should be measured using a 250-mL graduated cylinder.

2. Beginning with the lowest concentration, add approximately 50 mL of moderately hard synthetic water to a 250-mL graduated cylinder, add the required volume of stock solution using a 10-mL serological pipette (refer to Table AT10.1), bring to volume (200 mL) with moderately hard synthetic water. Mix the solution well by pouring the solution into the test 500-mL plastic Solo® cup and swirling the solution in the cup.

3. Pour 30 mL of test solution into each of the replicate test cups for that concentration according to the randomization scheme of the holding rack. The remaining volume should be saved for chemical analyses.

4. Measure the conductivity (SOP-C4), pH (SOP-C3), and dissolved oxygen (SOP-C2) of each test concentration and the control and record on the bench sheet. Refer to Table AT10.1 for guidance values of conductivity measurements.

5. Rinse the graduated cylinder well with deionized water and repeat steps C.2 through C.4 for preparing the next test concentration. Record the batch date of the moderately hard synthetic water used to prepare the dilutions on the bench sheet.

Table AT10.1: Test concentration, stock volumes, moderately hard synthetic water volumes, final volumes, and conductivity measurements guidance values for the *Ceriodaphnia dubia* and *Daphnia* NaCl acute reference toxicant tests.

Ceriodaphnia dubia:

Test Concentration (mg NaCl/L)	Volume of Stock Required (mL)	Volume of Moderately hard synthetic water (mL)	Final Volume (mL)	Conductivity Guidance Values (µmhos/cm)
1750	3.5	196.5	200	3200 - 3600
2000	4.0	196.0	200	3600 - 4000
2250	4.5	195.5	200	4000 - 4500
2500	5.0	195.0	200	4500 - 4900
2750	5.5	194.5	200	4900 - 5600

Daphnia:

Test Concentration (mg NaCl/L)	Volume of Stock Required (mL)	Volume of Moderately hard synthetic water (mL)	Final Volume (mL)	Conductivity Guidance Values (µmhos/cm)
2000	4.0	196.0	200	Not determined.
3000	6.0	194.0	200	Not determined.
4000	8.0	192.0	200	Not determined.
5000	10	190.0	200	Not determined.
6000	12	188.0	200	Not determined.

6. Once all test concentrations have been prepared, follow the procedure described in SOP-AT9 for conducting Daphnid Acute Toxicity Tests.

D. Control Charts and Outlier Test Results.

Refer to QAP-Q5 for how control charts are prepared and procedures for interpreting outlier tests. Refer to Exhibit AT10.2 for an example control chart.

Safety and Hazardous Waste Management

Safety glasses, gloves and lab coats should always be worn .

Review Policy-P6: General Safety Policy and Policy-P9: Radiation Protection Policy for additional safety requirements.

Subject: Daphnid Acute Reference Toxicity Test, EPA 2002.0 and EPA 2021.0

References

USEPA. October 2002. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, 5th ed. **EPA-821-R-02-012, Method 2002.0 for *Ceriodaphnia dubia*, Method 2021.0 for *Daphnia magna***. US Environmental Protection Agency, Cincinnati, OH.

USEPA. 2000. Understanding and Accounting for Method Variability in Whole Effluent Toxicity Applications Under the National Pollutant Discharge Elimination Program. EPA-833-R-00-003. US Environmental Protection Agency, Cincinnati, OH.

USEPA. 2001. Final Report: Inter-laboratory Variability Study of EPA Short-term Chronic and Acute Whole Effluent Toxicity Test Methods, Volumes 1 and 2 - Appendix. EPA-821-B-01-004 and EPA-821-B-01-005. US Environmental Protection Agency, Cincinnati, OH.

North Carolina Department of Environment and Natural Resources, Division of Water Quality. Biological Laboratory Certification / Criteria Procedures, Version 3.0. December 2010.

TNI Standard. Management and Technical Requirements for Laboratories Performing Environmental Analysis. EL-V1-ISO-2016-Rev2.0. The NELAC Institute, PO Box 2439, Weatherford, TX 76086.

Exhibits

Exhibit AT10.1: Example *Ceriodaphnia dubia* Acute Reference Toxicity Test Bench Sheet.
Exhibit AT10.2: Example *Ceriodaphnia dubia* Acute Reference Toxicant Control Chart.

Subject: Daphnid Acute Reference Toxicity Test, EPA 2002.0 and EPA 2021.0

Exhibit AT10.1: Example *Ceriodaphnia dubia* Acute Reference Toxicity Test Bench Sheet.

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Acute LC_{50} Whole Effluent Toxicity Test, Species: *Ceriodaphnia dubia*
EPA-821-R-02-012, Method 2002.0

Ceriodaphnia dubia Sodium Chloride Acute Reference Toxicant Test

CdNaClAC # _____

Dilution Preparation:

Test concentrations (mg/L NaCl)	1750	2000	2250	2500	2750
mL Stock solution	3.5	4.0	4.5	5.0	5.5
mL Dilution water (MHSW)	196.5	196.0	195.5	195.0	194.5
Total volume (mL)	200	200	200	200	200

A stock solution was prepared by diluting 10 g NaCl into 100 mL deionized water. This 100,000 mg/L NaCl stock solution was used to prepare the concentrations evaluated for toxicity.

Stock solution INSS #: _____

Chemical Analyses:

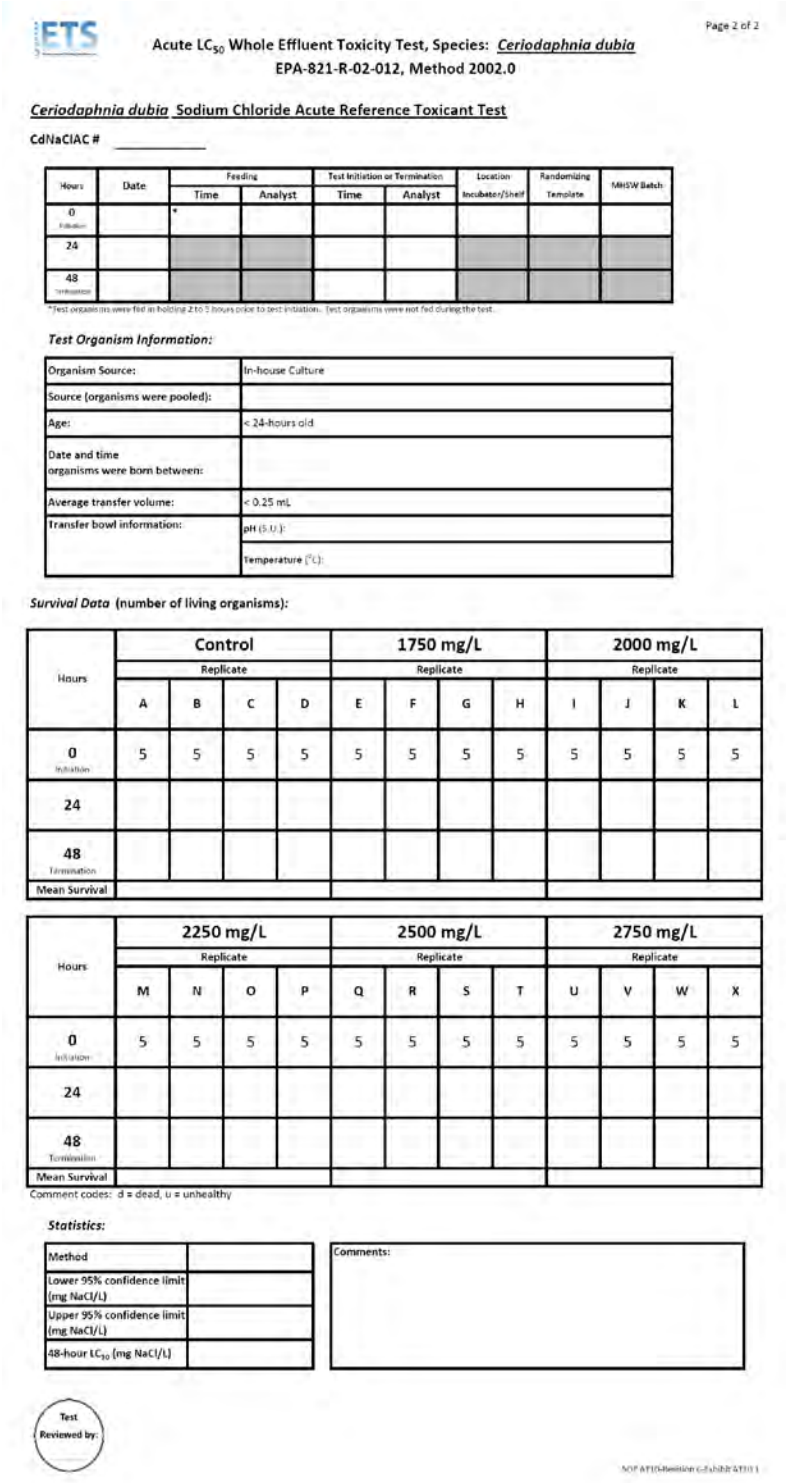
Concentration	Analyst	Hours 0	24	48
Control, MHSW	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	Conductivity (µmhos/cm)			
	Alkalinity (mg/L $CaCO_3$)			
	Hardness (mg/L $CaCO_3$)			
	Temperature (°C)			
1750 mg/L	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	Conductivity (µmhos/cm)			
	Temperature (°C)			
2000 mg/L	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	Conductivity (µmhos/cm)			
	Temperature (°C)			
2250 mg/L	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	Conductivity (µmhos/cm)			
	Temperature (°C)			
2500 mg/L	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	Conductivity (µmhos/cm)			
	Temperature (°C)			
2750 mg/L	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	Conductivity (µmhos/cm)			
	Temperature (°C)			

Analyst identified for each day, performed pH, dissolved oxygen and conductivity measurements only. Temperatures performed at the time of test initiation or termination by the analyst performing the toxicity test. Alkalinity and hardness performed by the analysts identified on the test specific bench sheets and transcribed to this bench sheet.

Chemical analyses:

Parameter	Reporting limit	Method number	Meter	Serial number
pH	0.1 S.U.	SM 4500-H+ B-2011	Accumet AR20	93312452
Dissolved oxygen	1.0 mg/L	SM 4500-O G-2011	YSI Model 52CE	18D104324
Conductivity	14.9 µmhos/cm	SM 2510 B-2011	Accumet AR20	93312452
Alkalinity	5.0 mg $CaCO_3$/L	SM 2320 B-2011	Accumet AR20	93312452
Hardness	5.0 mg $CaCO_3$/L	SM 2340 C-2011	Not applicable	Not applicable
Temperature	0.1 °C	SM 2550B-2010	Digital Thermometer	

SOP AT10-Revision 6-Exhibit AT10.1



ETS

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Acute LC_{50} Whole Effluent Toxicity Test, Species: *Ceriodaphnia dubia*
EPA-821-R-02-012, Method 2002.0

Ceriodaphnia dubia Sodium Chloride Acute Reference Toxicant Test

CdNaClAC # _____

Hours	Date	Feeding		Test Initiation or Termination		Location	Randomizing	MHSW Batch
		Time	Analyst	Time	Analyst	Incubator/Shelf	Template	
0 Initiation		*						
24								
48 Termination								

*Test organisms were fed in holding 2 to 3 hours prior to test initiation. Test organisms were not fed during the test.

Test Organism Information:

Organism Source:	In-house Culture
Source (organisms were pooled):	
Age:	< 24-hours old
Date and time organisms were born between:	
Average transfer volume:	< 0.25 mL
Transfer bowl information:	pH (S.U.):
	Temperature (°C):

Survival Data **(number of living organisms):**

Hours	Control				1750 mg/L				2000 mg/L			
	Replicate				Replicate				Replicate			
	A	B	C	D	E	F	G	H	I	J	K	L
0 Initiation	5	5	5	5	5	5	5	5	5	5	5	5
24												
48 Termination												
Mean Survival												

Hours	2250 mg/L				2500 mg/L				2750 mg/L			
	Replicate				Replicate				Replicate			
	M	N	O	P	Q	R	S	T	U	V	W	X
0 Initiation	5	5	5	5	5	5	5	5	5	5	5	5
24												
48 Termination												
Mean Survival												

Comment codes: d = dead, u = unhealthy

Statistics:

Method	
Lower 95% confidence limit (mg NaCl/L)	
Upper 95% confidence limit (mg NaCl/L)	
48-hour LC_{50} (mg NaCl/L)	

Comments:

Test Reviewed by:

SOP AT10 Revision 6 Exhibit AT10-1

Exhibit AT10.2: Example *Ceriodaphnia dubia* Acute Reference Toxicant Control Chart.

Ceriodaphnia dubia
Acute Reference Toxicant Control Chart
Source: In-house Culture

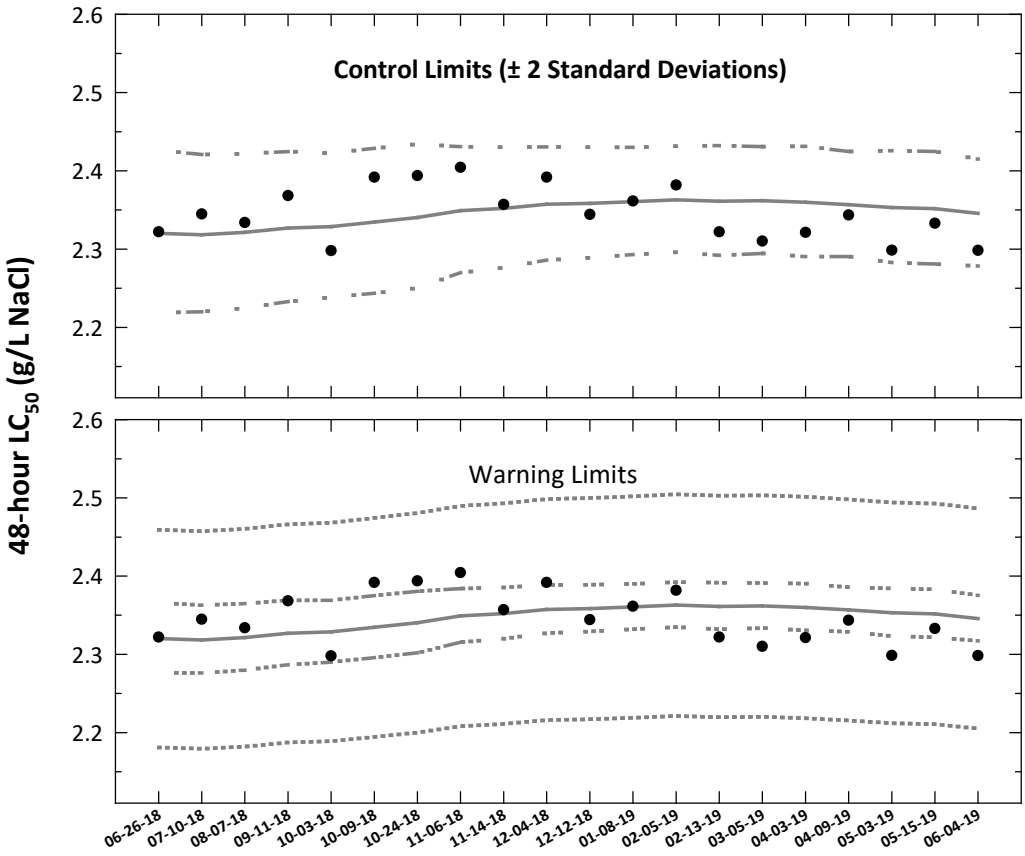
Test number	Test date	48-hour LC_{50} ToxCal Determination (g/L NaCl)	Log_{10} Conversion			Anti-logarithmic Values (g/L NaCl)						
			48-hour LC_{50}	CT	S	CT	Control Limits		Laboratory Calculated CV Warning Limits		10th Percentile CV Warning Limits	
							CT - 2S	CT + 2S	CT - 2CV	CT + 2CV	CT - $S_{A,10}$	CT + $S_{A,10}$
1	06-26-18	2.3220	0.3659	0.3655	0.0097	2.3202	2.2188	2.4263	2.2765	2.3660	2.1810	2.4594
2	07-10-18	2.3449	0.3701	0.3652	0.0094	2.3184	2.2201	2.4210	2.2760	2.3627	2.1793	2.4575
3	08-07-18	2.3339	0.3681	0.3657	0.0092	2.3214	2.2249	2.4221	2.2798	2.3648	2.1821	2.4607
4	09-11-18	2.3682	0.3744	0.3668	0.0089	2.3270	2.2331	2.4248	2.2867	2.3690	2.1874	2.4666
5	10-03-18	2.2980	0.3613	0.3671	0.0086	2.3288	2.2385	2.4227	2.2900	2.3691	2.1890	2.4685
6	10-09-18	2.3918	0.3787	0.3682	0.0086	2.3346	2.2438	2.4290	2.2957	2.3750	2.1945	2.4747
7	10-24-18	2.3938	0.3791	0.3693	0.0085	2.3405	2.2504	2.4342	2.3020	2.3805	2.2001	2.4809
8	11-06-18	2.4044	0.3810	0.3709	0.0074	2.3492	2.2700	2.4311	2.3155	2.3840	2.2082	2.4901
9	11-14-18	2.3569	0.3723	0.3715	0.0071	2.3521	2.2764	2.4303	2.3199	2.3854	2.2110	2.4932
10	12-04-18	2.3918	0.3787	0.3724	0.0067	2.3574	2.2862	2.4308	2.3272	2.3885	2.2159	2.4988
11	12-12-18	2.3442	0.3700	0.3726	0.0065	2.3586	2.2890	2.4302	2.3291	2.3889	2.2170	2.5001
12	01-08-19	2.3614	0.3732	0.3730	0.0063	2.3606	2.2931	2.4300	2.3320	2.3900	2.2189	2.5022
13	02-05-19	2.3818	0.3769	0.3735	0.0062	2.3630	2.2962	2.4318	2.3347	2.3921	2.2212	2.5048
14	02-13-19	2.3220	0.3659	0.3731	0.0065	2.3613	2.2921	2.4325	2.3320	2.3914	2.2196	2.5029
15	03-05-19	2.3102	0.3636	0.3733	0.0063	2.3619	2.2947	2.4310	2.3334	2.3911	2.2201	2.5036
16	04-03-19	2.3212	0.3657	0.3729	0.0065	2.3600	2.2906	2.4316	2.3306	2.3903	2.2184	2.5016
17	04-09-19	2.3436	0.3699	0.3723	0.0062	2.3568	2.2907	2.4247	2.3288	2.3856	2.2154	2.4982
18	05-03-19	2.2985	0.3614	0.3717	0.0066	2.3532	2.2828	2.4259	2.3233	2.3841	2.2120	2.4944
19	05-15-19	2.3330	0.3679	0.3714	0.0066	2.3518	2.2809	2.4249	2.3217	2.3829	2.2107	2.4929
20	06-04-19	2.2984	0.3614	0.3703	0.0063	2.3458	2.2784	2.4151	2.3170	2.3753	2.2050	2.4865

Note: **48-hour LC_{50}** = 48-hour median lethal concentration. An estimate of the sodium chloride concentration which is lethal to 50% of the test organisms in 48-hours (calculated using ToxCalc).
CT = Central tendency of the LC_{50} values.
S = Standard deviation of the LC_{50} values.
Control Limits = Mean logarithmic $LC_{50} \pm 2$ standard deviations converted to anti-logarithmic values.
Warning Limits = Mean logarithmic $LC_{50} \pm 2CV$ or $S_{A,10}$ converted to anti-logarithmic values.
$S_{A,10}$ = Standard deviation corresponding to the 10th percentile of CVs reported nationally by USEPA. ($S_{A,10} = 0.06$).
CV = Coefficient of variation.

Subject: Daphnid Acute Reference Toxicity Test, EPA 2002.0 and EPA 2021.0

Environmental Testing Solutions, Inc.

Ceriodaphnia dubia
Acute Reference Toxicant Control Chart
Source: In-house Culture



- ● **48-hour LC_{50}** = median lethal concentration. An estimation of the sodium chloride concentration which is lethal to 50% of the test organisms in 48-hours (calculated using ToxCalc).
- **Central Tendency** (mean logarithmic LC_{50} converted to anti-logarithmic values)
- **Control Limits** (mean logarithmic LC_{50} ± 2 standard deviations converted to anti-logarithmic values)
- **Laboratory Warning Limits** (mean logarithmic LC_{50} ± 2 coefficent of variations converted to anti-logarithmic values)
- **USEPA Warning Limits** (mean logarithmic LC_{50} ± $S_{A.10}$ converted to anti-logarithmic values, $S_{A.10}$ = 10th percentile of CVs reported nationally by USEPA)

Entered And Reviewed by Jim Sumner

Subject: *Ceriodaphnia dubia* Chronic Toxicity Test, EPA 1002.0

Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan		03-01-20
Quality Assurance Officer	Jim Sumner		03-01-20

Document Revision History

Effective Date	Revision number	Review Type	Evaluators	Revisions
12-01-00	0	Internal	Jim Sumner (ETS)	Original document
06-01-11	1	Internal	Jim Sumner (ETS)	• Updated references and exhibits. • Statistical analyses and data review moved to QAP-Q12.
07-01-12	2	External (NC DENR) Internal	Lance Ferrell (NC DENR) Jim Sumner (ETS)	• The measurement of pH, DO and conductivity of each new, full-strength, undiluted sample was added. • The light intensity was amended to reflect that it is a recommended range as specified in the EPA manuals.
07-01-13	3	External (NC DENR) **Internal**	Lance Ferrell (NC DENR) Jim Sumner (ETS)	• Added North Carolina acceptance and termination criteria: Testing in support North Carolina NPDES permits and reference testing must meet the criteria identified in Table AT11.1. In addition North Carolina testing must be terminated before 7 days + 2 hours from test initiation.
11-01-14	4	Internal	Jim Sumner (ETS)	• Updated exhibits during document review. • Changed renewal time recommendation to ± 2-hours from test initiation. Provided additional guidance in the procedure for the renewal of test solutions. • Removed KY acceptability criteria which follows EPA requirements. • Added minimum guidance criteria for PMSD to Table AT11.1.
09-01-19	5	Internal	Jim Sumner (ETS)	• Updated procedure to include NELAP requirements. • Additional guidance included in SOP.
03-01-20	6	External (TVA) Internal	Rick Sherrard (TVA) Jim Sumner (ETS)	• Updated bench sheet to include reporting limits, method numbers, meters and serial numbers used for chemical analyses.

Subject: *Ceriodaphnia dubia* Chronic Toxicity Test, EPA 1002.0

Scope and Application

To measure the chronic toxicity of water samples to the Daphnid, *Ceriodaphnia dubia*, using less than 24-hour old neonates during a three-brood (7-day), static renewal test.

Summary of Method

The chronic toxicity test generally involves the exposure of test organisms to up to five effluent concentrations and a control water. The test duration is 7-days. Test solutions are renewed daily and observations of survival and the number of offspring produced is determined.

A summary of the *Ceriodaphnia dubia* chronic method is provided in Exhibit AT11.1.

Quality Control

Acceptance Criteria: The test acceptance criteria are indicated in the table below. In general, the most stringent acceptability criteria are used by the laboratory. If acceptability criteria are not met, the test must be invalidated.

Table AT11.1: *Ceriodaphnia dubia* chronic toxicity test acceptability criteria.

Test Acceptability Criteria	USEPA	North Carolina	TVA
Control survival	≥ 80%	≥ 80%	≥ 80%
Average number of offspring per surviving female in the control	≥ 15.0	≥ 15.0	≥ 15.0
Control reproduction coefficient of variation	< 42%	< 40%	< 42%
Percentage of surviving adults having 3rd broods in the control	≥ 60%	≥ 80%	≥ 60%
Percentage of male adults in the control	≤ 20%	≤ 20%	0% for entire test
Guidance percent minimum significant difference (PMSD)	13 – 47%	No criteria	13 – 47%

Subject: *Ceriodaphnia dubia* Chronic Toxicity Test, EPA 1002.0

Equipment and Materials

Ceriodaphnia dubia
Temperature-controlled incubator (set to maintain test temperature = 25.0 ± 1.0°C, photoperiod of 16-hour light / 8-hours dark, light intensity = 50 – 100 ft-c)
Control / Dilution water (moderately hard synthetic water)
1-oz medicine cups
500-mL plastic Solo® cups
Graduated cylinders
Serological, fixed, or adjustable volume pipettes (e.g., Eppendorf)
Pasteur® pipettes
Transfer pipettes
Eppendorf Repeater Pipetter
Chronic test holding rack
Plexiglas® slides
Thermometer
YWT mixture
Selenastrum capricornutum
Light box or table
Dissection microscope (if necessary)
Disposable gloves
Chronic Toxicity Test Bench Sheet

Procedure

A. Test Preparation.

1. Prepare the Chronic Toxicity Test Bench Sheet (an example is provided in Exhibit AT11.2). Record the following information on the bench sheet:
    - Client/facility name
    - Permit number
    - County
    - Outfall/Pipe number
    - ETS project and sample number
    - Control/Dilution water type and batch
    - Test concentrations and dilution preparation information (sample, dilution and total volumes)

B. Test Initiation.

1. Prepare the test concentrations according to SOP-G5.

   a. The control/dilution water is moderately hard synthetic water (MHSW, SOP-AT1). MHSW must have an alkalinity of 57 – 64 mg $CaCO_3$/L, hardness of 80 – 100 mg $CaCO_3$/L, and an initial pH of 6.5 – 8.5 S.U. (guidance range = 7.4 – 7.8 S.U.).

   b. Measure and record the pH (SOP-C3), dissolved oxygen (SOP-C2) and conductivity (SOP-C4) of each concentration tested and control. Ensure that the dissolved oxygen is within the acceptable range (4.0 to 9.0 mg/L), aerate the sample if necessary, according to SOP-G5. Measure and record the pH (SOP-C3), dissolved oxygen (SOP-C2), conductivity (SOP-C4), total residual chlorine (SOP-C8), total alkalinity (SOP-C6), total hardness (SOP-C7) and sample characteristics of each new, full-strength, undiluted sample. Measure and record the alkalinity (SOP-C6) and hardness (SOP-C7) of the control/dilution water.

   c. Obtain a chronic test holding rack, which is marked for the randomization of the test cups (Exhibit AT11.3). Place the medicine cups in the holding rack and record the holding rack color on the bench sheet.

   d. Pour 15 mL of control water into each of the ten replicate control cups according to the randomization scheme.

   e. Pour 15 mL of each test concentration into each of the ten replicate medicine cups according to the randomization scheme. The remaining volume should be saved for chemical analyses (as indicated in B.1.b).

   f. Using an Eppendorf Repeater Pipetter, add 100 µL *Selenastrum capricornutum* and 100 µL YWT mixture to each cup. The cell density of the *Selenastrum* mixture must be between 3.0 to 3.5 x 10^7 cells/mL (SOP-AT2) and total solids concentration of the YWT mixture must be between 1.7 to 1.9 g/L (SOP-AT6). Record the batches of *Selenastrum* and YWT on the chronic bench sheet.

   g. Maintain the test temperature (25.0 ± 1.0°C) of the test concentrations. This may be accomplished by placing the holding rack into a temperature-controlled incubator.

2. Isolate and collect known-age neonates per instructions in SOP-AT8. Neonates must be less than 24-hours old and all within 8 hours of the same age.

   a. Select 10 brood cups for use in the toxicity test. Neonates are taken only from adults that have 8 or more young in their third or subsequent broods (as described in SOP-AT8). These adults can be used as brood stock until they are 14 days old.

   b. Record the brood board source and cups used for each replicate, age, and dates and times the organisms were born between on the chronic bench sheet.

3. Transfer the neonates to the randomly placed test cups in the holding rack by blocked parentage.

   a. Measure and record the temperature in one of the test cups for each concentration and control. The temperature must be 25.0 ± 1.0°C before the test organisms are placed in the test cups. Warm the test cups in a temperature-controlled incubator, if necessary, until the desired test temperature is obtained. Measure and record the temperature of an arbitrary cup containing neonates to be used in the toxicity test.

   c. Using the first brood cup, transfer one neonate into each test cup from top to bottom in the first column of the holding rack using a transfer pipette with the tip cut to > 2 mm bore size. Care should be taken to release each neonate under the surface of the water. Neonates should be transferred gently in a manner that will not expose the organisms to the air. The volume of water transferred to the medicine cups should be minimized to avoid diluting the test concentrations.

   d. Using the second brood cup, transfer one neonate into each of the six test cups in the second column of the holding rack. Continue this process until all 60 test cups contain one neonate.

   e. Save one of the cups that contained neonates used for the toxicity test. Measure and record the pH (SOP-C3) of this transfer water on the chronic bench sheet.

   f. Record the initiation date, time and analyst's initials on the chronic bench sheet. **The test must be initiated within 36-hours of completion of the first sampling period.**

g. Verify that each test cup received one neonate by conducting a repeat count. Remove excess neonates or add neonates as necessary.

h. Place the chronic test holding rack in a temperature-controlled incubator. The organisms must be maintained at 25.0 ± 1.0°C with a photoperiod of 16-hours light and 8-hours dark and a recommended light intensity of 50 to 100 ft-c. Cover the rack with a Plexiglas® slide. Record the incubator number and shelf used on the bench sheet.

C. Perform 24-hour Daily Renewal.

Repeat this process each day during the test period. The test should be renewed within ± 2 hours from test initiation. **When new samples are used for test solution renewal, the test must be renewed within 36-hours of completion of the first sampling period for each new sample.**

1. Prepare fresh test concentrations each day (following procedures outlined in section B).

2. Using an un-randomized test holding rack. Place medicine cups in the holding rack and pour 15 mL of control water into each of the ten replicate control cups located in the first row of the holding rack.

3. Pour 15 mL of each test concentration into each of their respective ten replicate medicine cups, where the lowest concentration is in the second row of the holding rack and the highest concentration is in the last row (sixth row) of the holding rack. The remaining volumes of each concentration should be saved for chemical analyses (as indicated in B.1.b).

4. Using an Eppendorf Repeater Pipetter, add 100 µL *Selenastrum capricornutum* and 100 µL YWT mixture to each cup. The cell density of the *Selenastrum* mixture must be between 3.0 to 3.5 x 10^7 cells/mL (SOP-AT2) and total solids concentration of the YWT mixture must be between 1.7 to 1.9 g/L (SOP-AT6). Record the batches of *Selenastrum* and YWT on the chronic bench sheet.

5. Maintain the test temperature (25.0 ± 1.0°C) of the test concentrations. This may be accomplished by placing the holding rack into a temperature-controlled incubator.

6. Remove the holding rack containing the test organisms from the incubator. Measure and record the temperature in an arbitrarily selected test cup of each test concentration and control.

7. Remove the holding rack containing the fresh solutions from the incubator and place on a light box or table for ease of viewing. Measure and record the temperature in an arbitrarily selected test cup of each test concentration and control. The temperature must be 25.0 ± 1.0°C before the test organisms are transferred into the new solutions. Warm the test cups in a temperature-controlled incubator, if necessary, until the desired test temperature is obtained.

8. Beginning with the control cups, remove the cups containing the test organisms from the randomized holding rack and place in order on the light table in front of the holding rack containing the fresh solutions. Using a transfer pipette with the tip cut to > 2 mm bore size, individually transfer each organism to the new test cups containing fresh solutions in the un-randomized board. Care should be taken to release each neonate under the surface of the water. Neonates should be transferred gently in a manner that will not expose the organisms to the air. The volume of water transferred to the medicine cups should be minimized to avoid diluting the test concentrations.

9. Discard and record organisms that are missing, injured or dead. Dead organisms must be confirmed through a dissection microscope. The criteria that establish death are: (1) no movement and (2) no reaction to gentle prodding. Record comments, if applicable.

10. Count and record (in the appropriate section) the number of live young in each cup on the chronic bench sheet. Any animal not producing young should be examined under a dissection microscope to determine if it is a male.

11. Placed the new cups now containing the transferred organisms into the randomized holding rack according to the randomization scheme.

12. Continue this process of transferring test organisms beginning with the lowest concentration to the highest concentration until all the organisms have been transferred.

13. Record the date and time that the test solutions were renewed and the analyst's initials on the bench sheet.

14. Place the holding rack in a temperature-controlled incubator on the same shelf and location selected when the test was initiated. Cover the rack with a Plexiglas® slide.

15. Measure and record the pH (SOP-C3) and dissolved oxygen (SOP-C2) in one of the test cups containing old test solution ("final") for each concentration and control.

D. Test Termination.

Terminate the test after 60% of the control organisms have produced their third brood (typically on day 6 or 7). The test must be terminated within ± 2 hour from test initiation and may not exceed 8 days + 1-hour. Testing in support North Carolina NPDES permits and reference testing must meet the criteria identified in Table AT11.1. In addition, North Carolina testing must be terminated before 7 days + 2 hours from test initiation.

1. Remove the holding rack containing the test organisms from the incubator. Place the rack on a light box or table for ease of viewing. Measure and record the temperature in an arbitrarily selected test cup for each concentration and control.

2. Count and record (in the appropriate section) the number of live young in each cup and record the survival of the adult test organism on the chronic bench sheet.

3. Record the date and time the test was terminated and the analyst's initials on the bench sheet.

4. Measure and record the pH (SOP-C3) and dissolved oxygen (SOP-C2) in one of the test cups for each concentration and control.

5. Once all analyses have been completed and documented, discard the test water and organisms according to established laboratory protocol.

E. Statistical Analyses and Data Verification.

Statistical analyses and data review are performed according to QAP-Q12.

Safety and Hazardous Waste Management

Safety glasses, gloves and lab coats should always be worn.

Review Policy-P6: General Safety Policy and Policy-P9: Radiation Protection Policy for additional safety requirements.

Subject: *Ceriodaphnia dubia* Chronic Toxicity Test, EPA 1002.0

References

USEPA. October 2002. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, 4th ed. **EPA-821-R-02-013, Method 1002.0**. US Environmental Protection Agency, Cincinnati, OH.

TNI Standard. Management and Technical Requirements for Laboratories Performing Environmental Analysis. EL-V1-ISO-2016-Rev2.0. The NELAC Institute, PO Box 2439, Weatherford, TX 76086.

Exhibits

Exhibit AT11.1: Summary of Test Conditions for the *Ceriodaphnia dubia* Chronic Toxicity Test.
Exhibit AT11.2: Example *Ceriodaphnia dubia* Chronic Toxicity Test Bench Sheet.
Exhibit AT11.3: Example Chronic Test Holding Rack.

Subject: *Ceriodaphnia dubia* Chronic Toxicity Test, EPA 1002.0

Exhibit AT11.1: Summary of Test Conditions for the *Ceriodaphnia dubia* Chronic Toxicity Test.

SUMMARY OF TEST CONDITIONS FOR THE *CERIODAPHNIA DUBIA* CHRONIC TOXICITY TEST

Test type:	Static renewal
Test duration:	Until 60% or more of surviving control females have three broods and ± 1 hour from test initiation (not to exceed 8-days + 1-hour).
Temperature:	25.0 ± 1.0°C
Light quality:	Cool-white fluorescent
Light intensity:	50 – 100 ft-candles (recommended)
Photoperiod:	16-hours light, 8-hours dark
Test chamber size:	40 mL graduated polypropylene medicine cup
Test solution volume:	15 mL
Renewal of test solutions:	Daily
Age of test organisms:	< 24-hours old, all released within an 8-hour period.
Number of organisms per test chamber:	1 assigned using blocking by known parentage
Number of replicate test chambers per concentration:	10
Number of organisms per concentration:	10
Test concentrations:	Multiple concentration tests: 5 and a control with ≥ 0.5 dilution series (recommended) Single dilution tests: 100% and a control
Test chamber cleaning:	Use new medicine cups daily.
Aeration:	None
Feeding regime:	100 µL YWT and 100 µL *Selenastrum* per test cup daily.
Control / Dilution water:	Moderately hard synthetic water
Sampling and sample holding:	3-gallon grab or composite samples collected on days one, three and five. Each sample must first be used within 36-hours of completion of each sampling period.
Endpoint:	Survival and reproduction
Test acceptability criterion:	≥ 80% control survival, control reproduction ≥ 15 offspring/surviving female with 60% of surviving control females producing three broods

Subject: *Ceriodaphnia dubia* Chronic Toxicity Test, EPA 1002.0

Exhibit AT11.2: Example *Ceriodaphnia dubia* Chronic Toxicity Test Bench Sheet.

Page 1 of 7

Chronic Whole Effluent Toxicity Test (EPA-821-R-02-013 Method 1002.0)
Species: *Ceriodaphnia dubia*

Client: Tennessee Valley Authority, Watts Bar Nuclear Plant **County:** Rhea
NPDES #: TN 0020168 **Outfall #:** 101
Project #: _____

Dilution preparation:

Dilution prep (%)	0.7	1.4	2.8	5.6	11.2	Sample was not aerated or treated unless otherwise noted on this form. Sample was warmed to 25.0 ± 1.0°C in a warm water bath and then diluted to the test concentrations with moderately hard synthetic water (MHSW).
Effluent volume (mL)	14	28	56	112	224	
Diluent volume (mL)	1986	1972	1944	1888	1776	
Total volume (mL)	2000	2000	2000	2000	2000	

Test organism source:

Organism age:	< 24-hours old									
Date and times organisms were born between:										
Culture board:										
Replicate number:	1	2	3	4	5	6	7	8	9	10
Culture board cup number:										
Transfer vessel information:	pH (S.U.): Temperature (°C):									
Average transfer volume (mL):	< 0.25 mL									

Test randomization and location:

Randomizing template color:	
Incubator number and shelf location:	

Daily renewal:

Day	Date	Test initiation and feeding, renewal and feeding, or termination time	*Feeding Batches Selenastrum	*Feeding Batches YWT	MHSW batch used	Sample numbers used Outfall 101	Sample numbers used Intake	Analyst
0								
1								
2								
3								
4								
5								
6								
7								

*Organisms fed daily 100 µL *Selenastrum* and 100 µL YWT per replicate using HandyStep repeat pipettor SN 17E59354

Chemical analyses:

Parameter	Reporting Limit	Method number	Meter	Serial number
pH	0.1 S.U.	SM 4500-H+ B-2011	Accumet AR20	93312452
Dissolved Oxygen (D.O.)	1.0 mg/L	SM 4500-O G-2011	YSI Model 52CE	18D104324
Conductivity	14.9 µmhos/cm	SM 2510 B-2011	Accumet AR20	93312452
Alkalinity	5.0 mg $CaCO_3$/L	SM 2320 B-2011	Accumet AR20	93312452
Hardness	5.0 mg $CaCO_3$/L	SM 2340 C-2011	Not applicable	Not applicable
Chlorine, Total Residual	0.1 mg/L	ORION 97-70-1977	Accumet AB250	92349123
Temperature	0.1 °C	SM 2550B-2010	Digital Thermometer	

Control information:	Control-1	Control-2	Acceptance criteria	***Summary of test endpoints:***	
% of Male Adults:			≤ 20%	**7-day LC_{50} (%)**	
% Adults having 3rd Broods:			≥ 60% surviving adults	**NOEC (%)**	
% Mortality:			≤ 20%	**LOEC (%)**	
Mean Offspring/Female:			≥ 15.0 offspring/female	**ChV (%)**	
% CV:			< 42.0 %	**IC_{25} (%)**	

SOP AT11-Revision 6–Exhibit AT11.2

Subject: *Ceriodaphnia dubia* Chronic Toxicity Test, EPA 1002.0

Species: *Ceriodaphnia dubia*
Client: TVA / Watts Bar Nuclear Plant, Outfall 101 **Date:** _____

CONTROL-1 ***Survival and Reproduction Data***

Day		Replicate number									
		1	2	3	4	5	6	7	8	9	10
1	Young produced										
	Adult mortality										
2	Young produced										
	Adult mortality										
3	Young produced										
	Adult mortality										
4	Young produced										
	Adult mortality										
5	Young produced										
	Adult mortality										
6	Young produced										
	Adult mortality										
7	Young produced										
Total young produced											
Final Adult Mortality											
X for 3rd Broods											

Note: Adult mortality (L = live, D = dead), SB = split brood (single brood split between two days), CO = carry over (offspring carried over with adult during transfer).

Concentration:	
% Mortality:	
Mean Offspring/Female:	

CONC: 0.7% ***Survival and Reproduction Data***

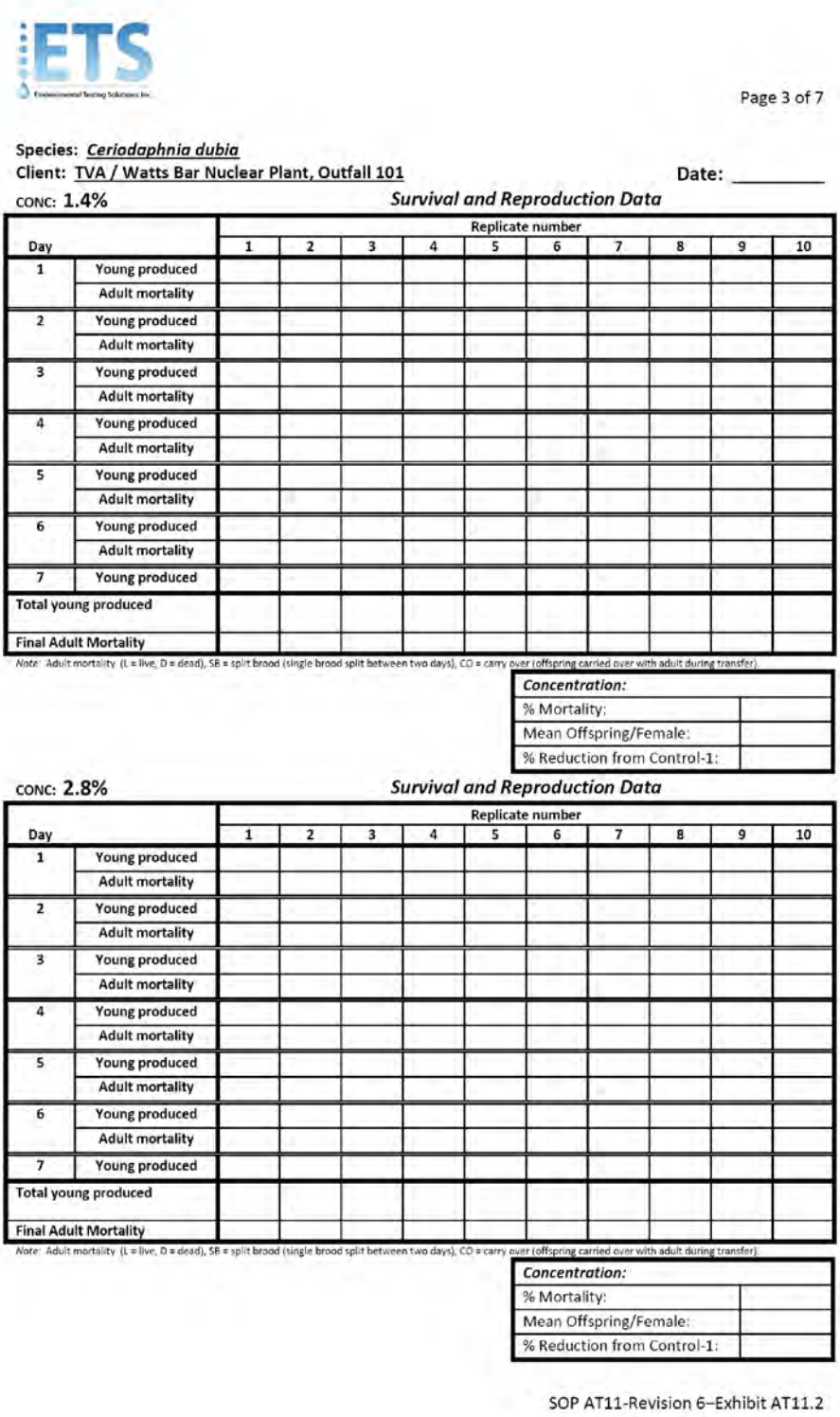
Day		Replicate number									
		1	2	3	4	5	6	7	8	9	10
1	Young produced										
	Adult mortality										
2	Young produced										
	Adult mortality										
3	Young produced										
	Adult mortality										
4	Young produced										
	Adult mortality										
5	Young produced										
	Adult mortality										
6	Young produced										
	Adult mortality										
7	Young produced										
Total young produced											
Final Adult Mortality											

Note: Adult mortality (L = live, D = dead), SB = split brood (single brood split between two days), CO = carry over (offspring carried over with adult during transfer).

Concentration:	
% Mortality:	
Mean Offspring/Female:	
% Reduction from Control-1:	

SOP AT11-Revision 6–Exhibit AT11.2

Subject: *Ceriodaphnia dubia* Chronic Toxicity Test, EPA 1002.0



ETS

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Species: *Ceriodaphnia dubia*
Client: TVA / Watts Bar Nuclear Plant, Outfall 101 **Date:** _____

CONC: **1.4%** ***Survival and Reproduction Data***

Day		Replicate number 1	2	3	4	5	6	7	8	9	10
1	Young produced										
	Adult mortality										
2	Young produced										
	Adult mortality										
3	Young produced										
	Adult mortality										
4	Young produced										
	Adult mortality										
5	Young produced										
	Adult mortality										
6	Young produced										
	Adult mortality										
7	Young produced										
Total young produced											
Final Adult Mortality											

Note: Adult mortality (L = live, D = dead), SB = split brood (single brood split between two days), CO = carry over (offspring carried over with adult during transfer).

Concentration:	
% Mortality:	
Mean Offspring/Female:	
% Reduction from Control-1:	

CONC: **2.8%** ***Survival and Reproduction Data***

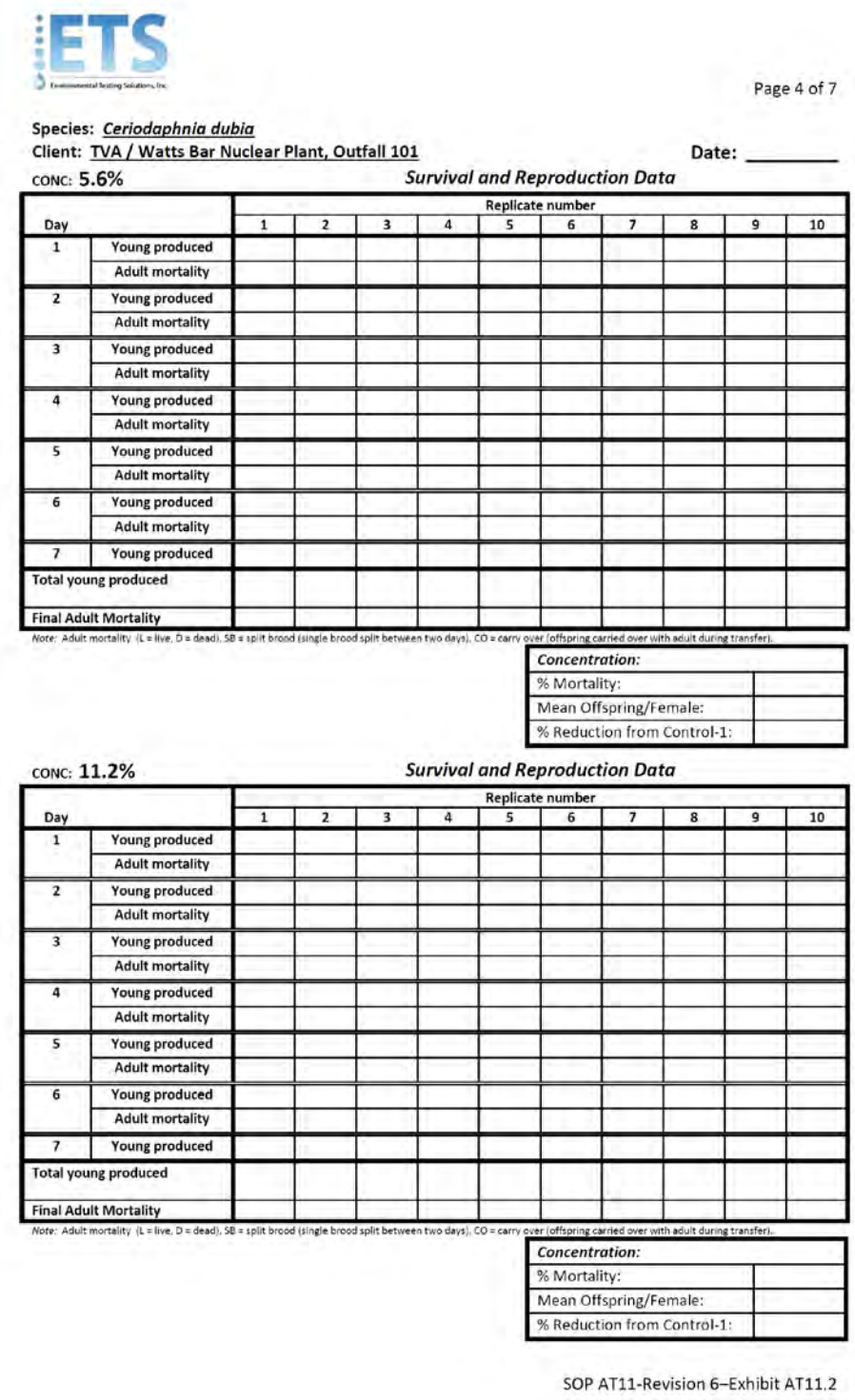
Day		Replicate number 1	2	3	4	5	6	7	8	9	10
1	Young produced										
	Adult mortality										
2	Young produced										
	Adult mortality										
3	Young produced										
	Adult mortality										
4	Young produced										
	Adult mortality										
5	Young produced										
	Adult mortality										
6	Young produced										
	Adult mortality										
7	Young produced										
Total young produced											
Final Adult Mortality											

Note: Adult mortality (L = live, D = dead), SB = split brood (single brood split between two days), CO = carry over (offspring carried over with adult during transfer).

Concentration:	
% Mortality:	
Mean Offspring/Female:	
% Reduction from Control-1:	

SOP AT11-Revision 6–Exhibit AT11.2

Subject: *Ceriodaphnia dubia* Chronic Toxicity Test, EPA 1002.0



ETS

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Species: *Ceriodaphnia dubia*

Client: TVA / Watts Bar Nuclear Plant, Outfall 101 **Date:** _____

CONC: **5.6%** ***Survival and Reproduction Data***

Day		Replicate number 1	2	3	4	5	6	7	8	9	10
1	Young produced										
	Adult mortality										
2	Young produced										
	Adult mortality										
3	Young produced										
	Adult mortality										
4	Young produced										
	Adult mortality										
5	Young produced										
	Adult mortality										
6	Young produced										
	Adult mortality										
7	Young produced										
Total young produced											
Final Adult Mortality											

Note: Adult mortality (L = live, D = dead), SB = split brood (single brood split between two days), CO = carry over (offspring carried over with adult during transfer).

Concentration:	
% Mortality:	
Mean Offspring/Female:	
% Reduction from Control-1:	

CONC: **11.2%** ***Survival and Reproduction Data***

Day		Replicate number 1	2	3	4	5	6	7	8	9	10
1	Young produced										
	Adult mortality										
2	Young produced										
	Adult mortality										
3	Young produced										
	Adult mortality										
4	Young produced										
	Adult mortality										
5	Young produced										
	Adult mortality										
6	Young produced										
	Adult mortality										
7	Young produced										
Total young produced											
Final Adult Mortality											

Note: Adult mortality (L = live, D = dead), SB = split brood (single brood split between two days), CO = carry over (offspring carried over with adult during transfer).

Concentration:	
% Mortality:	
Mean Offspring/Female:	
% Reduction from Control-1:	

SOP AT11-Revision 6–Exhibit AT11.2

Subject: *Ceriodaphnia dubia* Chronic Toxicity Test, EPA 1002.0

Species: *Ceriodaphnia dubia*
Client: TVA / Watts Bar Nuclear Plant, Outfall 101 **Date:** _____

CONTROL-2 ***Survival and Reproduction Data***

Day		Replicate number									
		1	2	3	4	5	6	7	8	9	10
1	Young produced										
	Adult mortality										
2	Young produced										
	Adult mortality										
3	Young produced										
	Adult mortality										
4	Young produced										
	Adult mortality										
5	Young produced										
	Adult mortality										
6	Young produced										
	Adult mortality										
7	Young produced										
Total young produced											
Final Adult Mortality											
X for 3rd Broods											

Note: Adult mortality (L = live, D = dead), SB = split brood (single brood split between two days), CO = carry over (offspring carried over with adult during transfer).

Concentration:	
% Mortality:	
Mean Offspring/Female:	

CONC: 100% Intake ***Survival and Reproduction Data***

Day		Replicate number									
		1	2	3	4	5	6	7	8	9	10
1	Young produced										
	Adult mortality										
2	Young produced										
	Adult mortality										
3	Young produced										
	Adult mortality										
4	Young produced										
	Adult mortality										
5	Young produced										
	Adult mortality										
6	Young produced										
	Adult mortality										
7	Young produced										
Total young produced											
Final Adult Mortality											

Note: Adult mortality (L = live, D = dead), SB = split brood (single brood split between two days), CO = carry over (offspring carried over with adult during transfer).

Concentration:	
% Mortality:	
Mean Offspring/Female:	
% Reduction from Control-2:	

SOP AT11-Revision 6–Exhibit AT11.2

Subject: *Ceriodaphnia dubia* Chronic Toxicity Test, EPA 1002.0

Species: ***Ceriodaphnia dubia*** **Date:** _____

Client: **TVA / Watts Bar Nuclear Plant, Outfall 101**

Daily Chemistry:

Temperatures performed at the time of test initiation, renewal or termination by the analyst identified in the Daily Renewal Information table located on Page 1. Alkalinity, hardness and chlorine (total residual) performed by the analyst identified on the bench sheet specific for each analysis and transcribed to this bench sheet.

		Day (Analyst identified for each day, performed pH, D.O. and conductivity measurements only.)					
		0		1		2	
	Analyst						
Concentration	Parameter						
CONTROL, MHSW	pH (S.U.)						
	Dissolved oxygen (mg/L)						
	Conductivity (µmhos/cm)						
	Alkalinity (mg $CaCO_3$/L)						
	Hardness (mg $CaCO_3$/L)						
	Temperature (°C)						
0.7%	pH (S.U.)						
	Dissolved oxygen (mg/L)						
	Conductivity (µmhos/cm)						
	Temperature (°C)						
1.4%	pH (S.U.)						
	Dissolved oxygen (mg/L)						
	Conductivity (µmhos/cm)						
	Temperature (°C)						
2.8%	pH (S.U.)						
	Dissolved oxygen (mg/L)						
	Conductivity (µmhos/cm)						
	Temperature (°C)						
5.6%	pH (S.U.)						
	Dissolved oxygen (mg/L)						
	Conductivity (µmhos/cm)						
	Temperature (°C)						
11.2%	pH (S.U.)						
	Dissolved oxygen (mg/L)						
	Conductivity (µmhos/cm)						
	Temperature (°C)						
100%	pH (S.U.)						
	Dissolved oxygen (mg/L)						
	Conductivity (µmhos/cm)						
	Alkalinity (mg $CaCO_3$/L)						
	Hardness (mg $CaCO_3$/L)						
	Chlorine (mg/L)						
	*Temperature (°C)						
100% Intake	pH (S.U.)						
	Dissolved oxygen (mg/L)						
	Conductivity (µmhos/cm)						
	Alkalinity (mg $CaCO_3$/L)						
	Hardness (mg $CaCO_3$/L)						
	Chlorine (mg/L)						
	Temperature (°C)						
		Initial	Final	Initial	Final	Initial	Final

SOP AT11-Revision 6–Exhibit AT11.2

Subject: *Ceriodaphnia dubia* Chronic Toxicity Test, EPA 1002.0

Species: ***Ceriodaphnia dubia***
Client: TVA / Watts Bar Nuclear Plant, Outfall 101 **Date:** _____

		Day (Analyst identified for each day, performed pH, D.O. and conductivity measurements only.)							
		3		4		5		6	
	Analyst								
Concentration	Parameter								
CONTROL, MHSW	pH (S.U.)								
	Dissolved oxygen (mg/L)								
	Conductivity (µmhos/cm)								
	Alkalinity (mg $CaCO_3$/L)								
	Hardness (mg $CaCO_3$/L)								
	Temperature (°C)								
0.7%	pH (S.U.)								
	Dissolved oxygen (mg/L)								
	Conductivity (µmhos/cm)								
	Temperature (°C)								
1.4%	pH (S.U.)								
	Dissolved oxygen (mg/L)								
	Conductivity (µmhos/cm)								
	Temperature (°C)								
2.8%	pH (S.U.)								
	Dissolved oxygen (mg/L)								
	Conductivity (µmhos/cm)								
	Temperature (°C)								
5.6%	pH (S.U.)								
	Dissolved oxygen (mg/L)								
	Conductivity (µmhos/cm)								
	Temperature (°C)								
11.2%	pH (S.U.)								
	Dissolved oxygen (mg/L)								
	Conductivity (µmhos/cm)								
	Temperature (°C)								
100%	pH (S.U.)								
	Dissolved oxygen (mg/L)								
	Conductivity (µmhos/cm)								
	Alkalinity (mg $CaCO_3$/L)								
	Hardness (mg $CaCO_3$/L)								
	Chlorine (mg/L)								
	*Temperature (°C)								
100% Intake	pH (S.U.)								
	Dissolved oxygen (mg/L)								
	Conductivity (µmhos/cm)								
	Alkalinity (mg $CaCO_3$/L)								
	Hardness (mg $CaCO_3$/L)								
	Chlorine (mg/L)								
	Temperature (°C)								
		Initial	Final	Initial	Final	Initial	Final	Initial	Final

SOP AT11-Revision 6–Exhibit AT11.2

Subject: *Ceriodaphnia dubia* Chronic Toxicity Test, EPA 1002.0

Exhibit AT11.3: Example Chronic Test Holding Rack.

Randomizing template: **RED**

Replicate #	1	2	3	4	5	6	7	8	9	10
Concentrations	**6**	**5**	**4**	**5**	**6**	**3**	**3**	**4**	**6**	**4**
	3	**3**	**2**	**6**	**4**	**2**	**5**	**2**	**5**	**2**
1 = Control	**4**	**1**	**1**	**2**	**2**	**1**	**2**	**6**	**2**	**5**
2 = Lowest concentration	**1**	**2**	**3**	**1**	**5**	**5**	**4**	**3**	**4**	**1**
3 - 5 = Intermediate concentrations	**2**	**4**	**5**	**3**	**1**	**6**	**6**	**1**	**3**	**3**
6 = Highest concentration	**5**	**6**	**6**	**4**	**3**	**4**	**1**	**5**	**1**	**6**

Random number seeds: 4 through 13

Subject: North Carolina *Ceriodaphnia dubia* Pass/Fail Chronic Toxicity Test, EPA 1002.0

Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan		09-01-19
Quality Assurance Officer	Jim Sumner		09-01-19

Document Revision History

Effective Date	Revision number	Review Type	Evaluators	Revisions
12-01-00	0	Internal	Jim Sumner (ETS)	Original document
06-01-11	1	Internal	Jim Sumner (ETS)	• Updated references and exhibits. • Procedure updated to reflect changes in NC procedure modifications. • Statistical analyses and data review moved to QAP-Q12.
07-01-12	2	External (NC DENR) Internal	Lance Ferrell (NC DENR) Jim Sumner (ETS)	• YWT solids typographical error was corrected to be 1.7 to 1.9 g/L. • The measurement of pH, DO and conductivity of each new, full-strength, undiluted sample was added. • The light intensity was amended to reflect that it is a recommended range as specified in the EPA manuals.
07-01-13	3	Internal	Jim Sumner (ETS)	• Updated exhibits during document review. • Provided additional guidance in the procedure for the renewal of test solutions.
09-01-19	4	Internal	Jim Sumner (ETS)	• Updated procedure to include NELAP requirements. • Additional guidance included in SOP. • Changed control/dilution water from SSW to MHSW.

Scope and Application

To measure the chronic toxicity of water samples to the Daphnid, *Ceriodaphnia dubia*, using less than 24-hour old neonates during a three-brood (7-day), static renewal test.

Summary of Method

The chronic toxicity test generally involves the exposure of test organisms to a single effluent concentration and a control water. The test duration is 7-days. With testing initiated on Wednesday, test solutions are renewed on Friday and Monday. Observations of survival and the number of offspring produced is determined.

A summary of the North Carolina *Ceriodaphnia dubia* pass/fail chronic method is provided in Exhibit AT12.1.

Subject: North Carolina *Ceriodaphnia dubia* Pass/Fail Chronic Toxicity Test, EPA 1002.0

Quality Control

Acceptance Criteria: The test acceptance criteria are indicated in the table below. If acceptability criteria are not met, the test must be invalidated.

Table AT12.1: North Carolina *Ceriodaphnia dubia* pass/fail chronic toxicity test acceptability criteria.

Test Acceptability Criteria	North Carolina
Control survival	≥ 80%
Average number of offspring per surviving female in the control	≥ 15.0
Control reproduction coefficient of variation	< 40%
Percentage of surviving adults having 3rd broods in the control	≥ 80%
Percentage of male adults in the control	≤ 20%
Guidance percent minimum significant difference (PMSD)	No criteria

Equipment and Materials

Ceriodaphnia dubia
Temperature-controlled incubator (set to maintain test temperature = 25.0 ± 1.0°C, photoperiod of 16-hour light / 8-hours dark, light intensity = 50 – 100 ft-c)
Control / Dilution water (moderately hard synthetic water)
1-oz medicine cups
500-mL plastic Solo® cups
Graduated cylinders
Serological, fixed, or adjustable volume pipettes (e.g., Eppendorf)
Pasteur® pipettes

Transfer pipettes
Eppendorf Repeater Pipetter
Chronic test holding rack
Plexiglas® slides
Thermometer
YWT mixture
Selenastrum capricornutum (cell concentration = 1.71 x 10^7 cells/ml)
Light box or table
Dissection microscope (if necessary)
Disposable gloves
North Carolina *Ceriodaphnia* Pass/Fail Chronic Toxicity Test Bench Sheet, Control Bench Sheet

Procedure

A. Test Preparation.

1. Prepare the North Carolina *Ceriodaphnia* Pass/Fail Chronic Toxicity Test Bench Sheet and Control Bench Sheet (an example is provided in Exhibit AT12.2 and Exhibit AT12.3). Record the following information on the bench sheet:
    - Client/facility name
    - Permit number
    - County
    - Outfall/Pipe number
    - ETS project and sample number
    - Control/Dilution water type and batch
    - Test concentrations and dilution preparation information (sample, dilution and total volumes)

B. Test Initiation on Day 0 (Wednesday).

1. Prepare the test concentrations according to SOP-G5.

    a. The control/dilution water is moderately hard synthetic water (MHSW, SOP-AT1). MHSW must have an alkalinity of 57 – 64 mg $CaCO_3$/L, hardness of 80 – 100 mg $CaCO_3$/L, and an initial pH of 6.5 – 8.5 S.U. (guidance range = 7.4 – 7.8 S.U.).

    b. Measure and record the pH (SOP-C3), dissolved oxygen (SOP-C2) and conductivity (SOP-C4) of each concentration tested and control. Ensure that the

dissolved oxygen is within the acceptable range (5.0 to 9.0 mg/L), aerate the sample if necessary, according to SOP-G5. Measure and record the pH (SOP-C3), dissolved oxygen (SOP-C2), conductivity (SOP-C4), total residual chlorine (SOP-C8) and sample characteristics of each new, full-strength, undiluted sample. Measure and record the alkalinity (SOP-C6) and hardness (SOP-C7) of the control/dilution water.

c. Obtain a chronic test holding rack, which is marked for the randomization of the test cups (Exhibit AT12.4). Place the medicine cups in the holding rack and record the holding rack color on the bench sheet.

d. Pour 15 mL of control water into each of the twelve replicate control cups according to the randomization scheme.

e. Pour 15 mL of each test concentration into each of the twelve replicate medicine cups according to the randomization scheme. The remaining volume should be saved for chemical analyses (as indicated in B.1.b).

f. Using an Eppendorf Repeater Pipetter, add 50 µL *Selenastrum capricornutum* and 50 µL YWT mixture to each cup. The cell density of the *Selenastrum* mixture must be 1.71 x 10^7 cells/mL (SOP-AT2) and total solids concentration of the YWT mixture must be between 1.7 to 1.9 g/L (SOP-AT6). Record the batches of *Selenastrum* and YWT on the chronic bench sheet.

g. Maintain the test temperature (25.0 ± 1.0°C) of the test concentrations. This may be accomplished by placing the holding rack into a temperature-controlled incubator.

2. Isolate and collect known-age neonates per instructions in SOP-AT8. Neonates must be less than 24-hours old and all within 8 hours of the same age.

a. Select 12 brood cups for use in the toxicity test. Neonates are taken only from adults that have 8 or more young in their third or subsequent broods (as described in SOP-AT8). These adults can be used as brood stock until they are 14 days old.

b. Record the brood board source and cups used for each replicate, age, and dates and times the organisms were born between on the chronic bench sheet.

3. Transfer the neonates to the randomly placed test cups in the holding rack by blocked parentage.

   a. Measure and record the temperature in one of the test cups for each concentration and control. The temperature must be 25.0 ± 1.0°C before the test organisms are placed in the test cups. Warm the test cups in a temperature-controlled incubator, if necessary, until the desired test temperature is obtained. Measure and record the temperature of an arbitrary cup containing neonates to be used in the toxicity test.

   c. Using the first brood cup, transfer one neonate into each test cup from top to bottom in the first column of the holding rack using a transfer pipette with the tip cut to > 2 mm bore size. Care should be taken to release each neonate under the surface of the water. Neonates should be transferred gently in a manner that will not expose the organisms to the air. The volume of water transferred to the medicine cups should be minimized to avoid diluting the test concentrations.

   d. Using the second brood cup, transfer one neonate into each of the six test cups in the second column of the holding rack. Continue this process until all test cups contain one neonate.

   e. Save one of the cups that contained neonates used for the toxicity test. Measure and record the pH (SOP-C3) of this transfer water on the chronic bench sheet.

   f. Record the initiation date, time and analyst's initials on the chronic bench sheet. **The test must be initiated within 36-hours of completion of the first sampling period.**

   g. Verify that each test cup received one neonate by conducting a repeat count. Remove excess neonates or add neonates as necessary.

   h. Place the chronic test holding rack in a temperature-controlled incubator. The organisms must be maintained at 25.0 ± 1.0°C with a photoperiod of 16-hours light and 8-hours dark and a recommended light intensity of 50 to 100 ft-c. Cover the rack with a Plexiglas® slide. Record the incubator number and shelf used on the bench sheet.

C. Feed Test Organisms on Day 1 (Thursday)

1. Remove the holding rack containing the test organisms from the incubator.

2. Using an Eppendorf Repeater Pipetter, add 50 µL *Selenastrum capricornutum* and 50 µL YWT mixture to each cup. The cell density of the *Selenastrum* mixture must be 1.71 x 10^7 cells/mL (SOP-AT2) and total solids of the YWT mixture must be 1.7 to 1.9 g/L (SOP-AT6). Record the time the organisms were fed and analyst initials on the bench sheet.

3. Place the holding rack in a temperature-controlled incubator. Cover the rack with a Plexiglas® slide.

D. Perform Renewal on Day 2 (Friday)

1. Prepare fresh test concentrations each day (following procedures outlined in section B).

2. Using an un-randomized test holding rack. Place medicine cups in the holding rack and pour 15 mL of control water into each of the twelve replicate control cups located in the first row of the holding rack.

3. Pour 15 mL of the site/facility concentration into each of their respective twelve replicate medicine cups according to the position number used at test initiation (section B.1.c). The remaining volumes of each concentration should be saved for chemical analyses (as indicated in B.1.b).

4. Using an Eppendorf Repeater Pipetter, add 50 µL *Selenastrum capricornutum* and 50 µL YWT mixture to each cup. The cell density of the *Selenastrum* mixture must be 1.71 x 10^7 cells/mL (SOP-AT2) and total solids concentration of the YWT mixture must be between 1.7 to 1.9 g/L (SOP-AT6). Record the batches of *Selenastrum* and YWT on the chronic bench sheet.

5. Maintain the test temperature (25.0 ± 1.0°C) of the test concentrations. This may be accomplished by placing the holding rack into a temperature-controlled incubator.

6. Remove the holding rack containing the test organisms from the incubator. Measure and record the temperature in an arbitrarily selected test cup of each site/facility concentration and control.

7. Remove the holding rack containing the fresh solutions from the incubator and place on a light box or table for ease of viewing. Measure and record the temperature in an arbitrarily selected test cup of test site/facility concentration and control. The temperature must be 25.0 ± 1.0°C before the test organisms are transferred into the new solutions. Warm the test cups in a temperature-controlled incubator, if necessary, until the desired test temperature is obtained.

8. Beginning with the control cups, remove the cups containing the test organisms from the randomized holding rack and place in order on the light table in front of the holding rack containing the fresh solutions. Using a transfer pipette with the tip cut to > 2 mm bore size, individually transfer each organism to the new test cups containing fresh solutions in the un-randomized board. Care should be taken to release each neonate under the surface of the water. Neonates should be transferred gently in a manner that will not expose the organisms to the air. The volume of water transferred to the medicine cups should be minimized to avoid diluting the test concentrations.

9. Discard and record organisms that are missing, injured or dead. Dead organisms must be confirmed through a dissection microscope. The criteria that establish death are: (1) no movement and (2) no reaction to gentle prodding. Record comments, if applicable.

10. Count and record (in the appropriate section) the number of live young in each cup on the chronic bench sheet. Any animal not producing young should be examined under a dissection microscope to determine if it is a male. Record the presence of 1 or 2 broods (in the appropriate section) on the chronic bench sheet.

11. Placed the new cups now containing the transferred organisms into the randomized holding rack according to the randomization scheme.

12. Continue this process of transferring test organisms beginning with the first site/facility concentration to the last site/facility concentration until all organisms have been transferred.

13. Record the date and time that the test solutions were renewed and the analyst's initials on the bench sheet. **The test must be renewed within 36-hours of completion of the second sampling period.**

14. Place the holding rack in a temperature-controlled incubator. Cover the rack with a Plexiglas® slide.

15. Measure and record the pH (SOP-C3) and dissolved oxygen (SOP-C2) in one of the test cups containing old test solution ("final") for each concentration and control.

E. Feed Test Organisms on Day 3 (Saturday) and Day 4 (Sunday)

Follow procedures outlined in section C.

F. Perform Renewal on Day 5 (Monday)

The test must be renewed, using the second sample, not more than 72-hours from the Friday renewal.

Follow procedures outlined in section D.

G. Feed Test Organisms on Day 6 (Tuesday)

Follow procedures outlined in section C.

H. Test Termination on Day 7 (Wednesday)

Terminate the test after 80% of the control organisms have produced their third brood. The test must be terminated before 7 days + 2 hours from test initiation.

1. Remove the holding rack containing the test organisms from the incubator. Place the rack on a light box or table for ease of viewing. Measure and record the temperature in an arbitrarily selected test cup for each site/facility concentration and control.

2. Count and record (in the appropriate section) the number of live young in each cup and record the survival of the adult test organism on the chronic bench sheet. Record the presence of 1 or 2 broods (in the appropriate section) on the chronic bench sheet.

3. Record the date and time the test was terminated and the analyst's initials on the bench sheet.

4. Measure and record the pH (SOP-C3) and dissolved oxygen (SOP-C2) in one of the test cups for each concentration and control.

5. Once all analyses have been completed and documented, discard the test water and organisms according to established laboratory protocol.

Subject: North Carolina *Ceriodaphnia dubia* Pass/Fail Chronic Toxicity Test, EPA 1002.0

I. Statistical Analyses and Data Verification

Statistical analyses and data review are performed according to QAP-Q12.

Safety and Hazardous Waste Management

Safety glasses, gloves and lab coats should be worn at all times.

Review Policy-P6: General Safety Policy and Policy-P9: Radiation Protection Policy for additional safety requirements.

References

USEPA. October 2002. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, 4th ed. **EPA-821-R-02-013, Method 1002.0**. US Environmental Protection Agency, Cincinnati, OH.

North Carolina Department of Environment and Natural Resources, Division of Water Quality. North Carolina *Ceriodaphnia* Chronic Whole Effluent Toxicity Procedure, Version 3.0. December 2010.

North Carolina Department of Environment and Natural Resources, Division of Water Quality. Biological Laboratory Certification / Criteria Procedures, Version 3.0. December 2010.

TNI Standard. Management and Technical Requirements for Laboratories Performing Environmental Analysis. EL-V1-ISO-2016-Rev2.0. The NELAC Institute, PO Box 2439, Weatherford, TX 76086.

Exhibits

Exhibit AT12.1: Summary of Test Conditions for the North Carolina *Ceriodaphnia dubia* Pass/Fail Chronic Toxicity Test.
Exhibit AT12.2: North Carolina *Ceriodaphnia dubia* Pass/Fail Chronic Toxicity Test Bench Sheet.
Exhibit AT12.3: Control Bench Sheet.
Exhibit AT12.4: Chronic Test Holding Rack.

Subject: North Carolina *Ceriodaphnia dubia* Pass/Fail Chronic Toxicity Test, EPA 1002.0

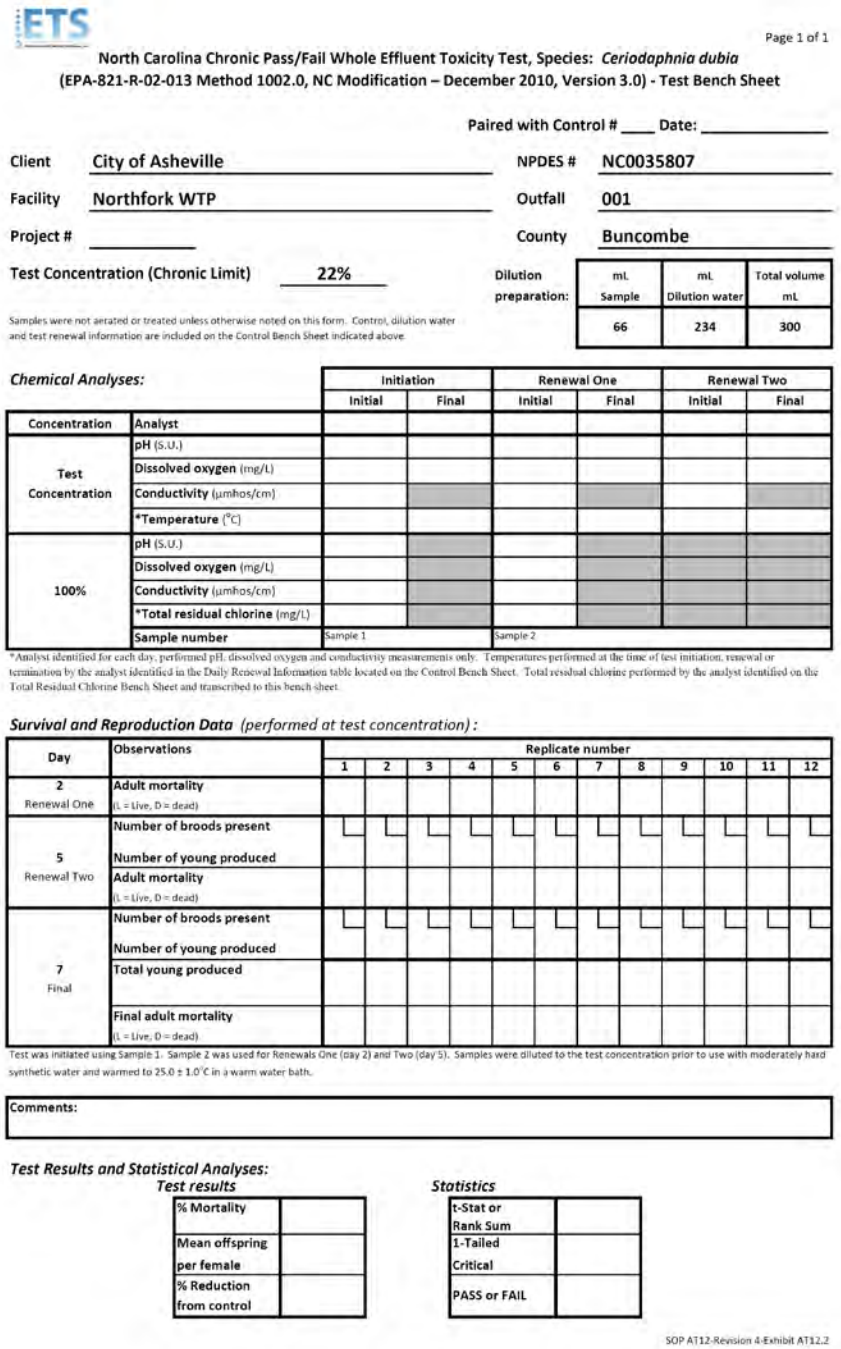
Exhibit AT12.1: Summary of Test Conditions for the North Carolina *Ceriodaphnia dubia* Pass/Fail Chronic Toxicity Test.

SUMMARY OF TEST CONDITIONS FOR THE NORTH CAROLINA *CERIODAPHNIA DUBIA* PASS/FAIL CHRONIC TOXICITY TEST

Test type:	Static renewal
Test duration:	Until 80% or more of surviving control females have three broods (maximum test duration of 7 days + 2-hours)
Temperature:	25.0 ± 1.0°C
Light quality:	Cool-white fluorescent
Light intensity:	50 – 100 ft-candles (recommended)
Photoperiod:	16-hours light, 8-hours dark
Test chamber size:	40 mL graduated polypropylene medicine cup
Test solution volume:	15 mL
Renewal of test solutions:	Renewals performed on days 2 and 5
Age of test organisms:	< 24-hours old, all released within an 8-hour period.
Number of organisms per test chamber:	1 assigned using blocking by known parentage
Number of replicate test chambers per concentration:	12
Number of organisms per concentration:	12
Test concentrations:	At chronic permit limit and a control
Test chamber cleaning:	Use new medicine cups at each renewal.
Aeration:	None
Feeding regime:	50 µL YWT and 50 µL *Selenastrum* (1.71 x 10^7 cells/ml) per test chamber daily.
Control / Dilution water:	Moderately hard synthetic water
Sampling and sample holding:	1-liter grab or composite samples collected on Tuesday and Thursday (for tests initiated on Wednesday). Each sample must be used within 36-hours of completion of each sampling period (not to exceed 72-hours from first use).
Endpoint:	Survival and reproduction
Test acceptability criterion:	≥ 80% control survival, control reproduction ≥ 15 offspring/surviving female with 80% of surviving control females producing three broods and control reproduction coefficient of variation < 40%, number of male control organisms ≤ 20%

Subject: North Carolina *Ceriodaphnia dubia* Pass/Fail Chronic Toxicity Test, EPA 1002.0

Exhibit AT12.2: North Carolina *Ceriodaphnia dubia* Pass/Fail Chronic Toxicity Test Bench Sheet.



ETS

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North Carolina Chronic Pass/Fail Whole Effluent Toxicity Test, Species: *Ceriodaphnia dubia*
(EPA-821-R-02-013 Method 1002.0, NC Modification – December 2010, Version 3.0) - Test Bench Sheet

Paired with Control # ____ Date: _____

Client: City of Asheville — NPDES #: NC0035807

Facility: Northfork WTP — Outfall: 001

Project #: _____ — County: Buncombe

Test Concentration (Chronic Limit): 22%

Dilution preparation:

mL Sample	mL Dilution water	Total volume mL
66	234	300

Samples were not aerated or treated unless otherwise noted on this form. Control, dilution water and test renewal information are included on the Control Bench Sheet indicated above.

Chemical Analyses:

		Initiation		Renewal One		Renewal Two	
		Initial	Final	Initial	Final	Initial	Final
Concentration	Analyst						
Test Concentration	pH (S.U.)						
	Dissolved oxygen (mg/L)						
	Conductivity (µmhos/cm)						
	*Temperature (°C)						
100%	pH (S.U.)						
	Dissolved oxygen (mg/L)						
	Conductivity (µmhos/cm)						
	*Total residual chlorine (mg/L)						
	Sample number	Sample 1		Sample 2			

*Analyst identified for each day, performed pH, dissolved oxygen and conductivity measurements only. Temperatures performed at the time of test initiation, renewal or termination by the analyst identified in the Daily Renewal Information table located on the Control Bench Sheet. Total residual chlorine performed by the analyst identified on the Total Residual Chlorine Bench Sheet and transcribed to this bench sheet.

Survival and Reproduction Data (performed at test concentration):

Day	Observations	Replicate number											
		1	2	3	4	5	6	7	8	9	10	11	12
2 Renewal One	Adult mortality (L = Live, D = dead)												
5 Renewal Two	Number of broods present												
	Number of young produced												
	Adult mortality (L = Live, D = dead)												
7 Final	Number of broods present												
	Number of young produced												
	Total young produced												
	Final adult mortality (L = Live, D = dead)												

Test was initiated using Sample 1. Sample 2 was used for Renewals One (day 2) and Two (day 5). Samples were diluted to the test concentration prior to use with moderately hard synthetic water and warmed to 25.0 ± 1.0°C in a warm water bath.

Comments:

Test Results and Statistical Analyses:

Test results

% Mortality	
Mean offspring per female	
% Reduction from control	

Statistics

t-Stat or Rank Sum	
1-Tailed Critical	
PASS or FAIL	

SOP AT12-Revision 4-Exhibit AT12.2

Subject: North Carolina *Ceriodaphnia dubia* Pass/Fail Chronic Toxicity Test, EPA 1002.0

Exhibit AT12.3: Control Bench Sheet.

ETS

Page 1 of 1

North Carolina Chronic Pass/Fail Whole Effluent Toxicity Test, Species: *Ceriodaphnia dubia*

(EPA-821-R-02-013 Method 1002.0, NC Modification – December 2010, Version 3.0) - Control Bench Sheet

Control #: ____ Date: **07-17-19**

Test Grouping Information:

Facility	Project #
6	
5	
4	
3	
2	
1 - Control	

Test Organism Information:

Organism Source:	In-house Culture										
Age:	< 24-hours old										
Source (culture board):											
Replicate #	1	2	3	4	5	6	7	8	9	10	11	12
Culture board cup #												
Date and time organisms were born between:												
Average transfer volume:	< 0.25 mL											
Transfer bowl information:	pH (S.U.):											
	Temperature (°C):											

Daily Renewal Information:

Day	Date	Test Initiation, renewal, feeding or termination		MHSW Batch	Selenastrum Batch	YWT Batch	Location Incubator/Shelf	Randomizing Template
		Time	Analyst					
0	07-17-19	Initiation/Feeding						
1	07-18-19	Feeding						
2	07-19-19	Renewal 1/Feeding						
3	07-20-19	Feeding						
4	07-21-19	Feeding						
5	07-22-19	Renewal 2/Feeding						
6	07-23-19	Feeding						
7	07-24-19	Termination						

Chemical Analyses:

		Initiation		Renewal One		Renewal Two	
		Initial	Final	Initial	Final	Initial	Final
Concentration	Analyst						
Control, MHSW	pH (S.U.)						
	Dissolved oxygen (mg/L)						
	Conductivity (µmhos/cm)						
	*Alkalinity (mg/L $CaCO_3$)						
	*Hardness (mg/L $CaCO_3$)						
	*Temperature (°C)						

*Analyst identified for each day performed pH, dissolved oxygen and conductivity measurements only. Temperatures performed at the time of test initiation, renewal or termination by the analyst identified in the Daily Renewal Information table. Alkalinity and hardness performed by the analysts indentified on the test bench sheets and transcribed to this bench sheet.

Survival and Reproduction Data:

Day	Observations	Replicate number											
		1	2	3	4	5	6	7	8	9	10	11	12
2 Renewal One	Adult mortality (L = Live, D = dead)												
5 Renewal Two	Number of broods present												
	Number of young produced												
	Adult mortality (L = Live, D = dead)												
7 Final	Number of broods present												
	Number of young produced												
	Total young produced												
	Final adult mortality (L = Live, D = dead)												
	X for 3rd Broods												

Control Acceptance Criteria:

% of Male Adults (≤ 20%)	
% Adults having 3rd Broods (≥ 80%)	
% Mortality (≤ 20%)	

Mean Offspring/Female (≥ 15 offspring/surviving female)	
% CV (< 40%)	

SOP AT12-Revision 4-Exhibit AT12.3

Subject: North Carolina *Ceriodaphnia dubia* Pass/Fail Chronic Toxicity Test, EPA 1002.0

Exhibit AT12.4: Chronic Test Holding Rack (Exhibit AT11.3 from SOP AT11).

Randomizing template: **RED**

Replicate #	1	2	3	4	5	6	7	8	9	10
Concentrations	6	5	4	5	6	3	3	4	6	4
	3	3	2	6	4	2	5	2	5	2
1 = Control	4	1	1	2	2	1	2	6	2	5
2 = Lowest concentration	1	2	3	1	5	5	4	3	4	1
3 - 5 = Intermediate concentrations	2	4	5	3	1	6	6	1	3	3
6 = Highest concentration	5	6	6	4	3	4	1	5	1	6

Random number seeds: 4 through 13

SOP AT11-Revision 5-Exhibit AT11.3

Subject: North Carolina *Ceriodaphnia dubia* Phase II Chronic Toxicity Test, EPA 1002.0

Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan		09-01-19
Quality Assurance Officer	Jim Sumner		09-01-19

Document Revision History

Effective Date	Revision number	Review Type	Evaluators	Revisions
12-01-00	0	Internal	Jim Sumner (ETS)	Original document
06-01-11	1	Internal	Jim Sumner (ETS)	• Updated references and exhibits. • Procedure updated to reflect changes in NC procedure modifications. • Statistical analyses and data review moved to QAP-Q12.
07-01-12	2	External (NC DENR) Internal	Lance Ferrell (NC DENR) Jim Sumner (ETS)	• YWT solids typographical error was corrected to be 1.7 to 1.9 g/L. • The measurement of pH, DO and conductivity of each new, full-strength, undiluted sample was added. • The light intensity was amended to reflect that it is a recommended range as specified in the EPA manuals.
07-01-13	3	Internal	Jim Sumner (ETS)	• Updated exhibits during document review. • Provided additional guidance in the procedure for the renewal of test solutions. • Changed the test concentration series to 0.25, 0.5, 1, 2, 4 times chronic permit limit and a control in the test conditions summary table.
09-01-19	4	Internal	Jim Sumner (ETS)	• Updated procedure to include NELAP requirements. • Additional guidance included in SOP. • Changed control/dilution water from SSW to MHSW.

Scope and Application

To measure the chronic toxicity of water samples to the Daphnid, *Ceriodaphnia dubia*, using less than 24-hour old neonates during a three-brood (7-day), static renewal test.

Subject: North Carolina *Ceriodaphnia dubia* Phase II Chronic Toxicity Test, EPA 1002.0

Summary of Method

The chronic toxicity test generally involves the exposure of test organisms to five effluent concentrations and a control water. The test duration is 7-days. With testing initiated on Wednesday, test solutions are renewed on Friday and Monday. Observations of survival and the number of offspring produced is determined.

A summary of the North Carolina *Ceriodaphnia dubia* pass/fail chronic method is provided in Exhibit AT13.1.

Quality Control

Acceptance Criteria: The test acceptance criteria are indicated in the table below. If acceptability criteria are not met, the test must be invalidated.

Table AT13.1: North Carolina *Ceriodaphnia dubia* pass/fail chronic toxicity test acceptability criteria.

Test Acceptability Criteria	North Carolina
Control survival	≥ 80%
Average number of offspring per surviving female in the control	≥ 15.0
Control reproduction coefficient of variation	< 40%
Percentage of surviving adults having 3rd broods in the control	≥ 80%
Percentage of male adults in the control	≤ 20%
Guidance percent minimum significant difference (PMSD)	No criteria

Subject: North Carolina *Ceriodaphnia dubia* Phase II Chronic Toxicity Test, EPA 1002.0

Equipment and Materials

Ceriodaphnia dubia
Temperature-controlled incubator (set to maintain test temperature = 25.0 ± 1.0°C, photoperiod of 16-hour light / 8-hours dark, light intensity = 50 – 100 ft-c)
Control / Dilution water (moderately hard synthetic water)
1-oz medicine cups
500-mL plastic Solo® cups
Graduated cylinders
Serological, fixed, or adjustable volume pipettes (e.g., Eppendorf)
Pasteur® pipettes
Transfer pipettes
Eppendorf Repeater Pipetter
Chronic test holding rack
Plexiglas® slides
Thermometer
YWT mixture
Selenastrum capricornutum (cell concentration = 1.71 x 10^7 cells/ml)
Light box or table
Dissection microscope (if necessary)
Disposable gloves
North Carolina *Ceriodaphnia* Pass/Fail Chronic Toxicity Test Bench Sheet, Control Bench Sheet

Procedure

A. Test Preparation.

1. Prepare the North Carolina *Ceriodaphnia* Phase II Chronic Toxicity Test Bench Sheet (an example is provided in Exhibit AT13.2). Record the following information on the bench sheet:
    - Client/facility name
    - Permit number
    - County
    - Outfall/Pipe number
    - ETS project and sample number
    - Control/Dilution water type and batch

- Test concentrations and dilution preparation information (sample, dilution and total volumes)

B. Test Initiation on Day 0 (Wednesday).

1. Prepare the test concentrations according to SOP-G5.

   a. The control/dilution water is moderately hard synthetic water (MHSW, SOP-AT1). MHSW must have an alkalinity of 57 – 64 mg $CaCO_3$/L, hardness of 80 – 100 mg $CaCO_3$/L, and an initial pH of 6.5 – 8.5 S.U. (guidance range = 7.4 – 7.8 S.U.).

   b. Measure and record the pH (SOP-C3), dissolved oxygen (SOP-C2) and conductivity (SOP-C4) of each concentration tested and control. Ensure that the dissolved oxygen is within the acceptable range (5.0 to 9.0 mg/L), aerate the sample if necessary, according to SOP-G5. Measure and record the pH (SOP-C3), dissolved oxygen (SOP-C2), conductivity (SOP-C4), total residual chlorine (SOP-C8) and sample characteristics of each new, full-strength, undiluted sample. Measure and record the alkalinity (SOP-C6) and hardness (SOP-C7) of the control/dilution water.

   c. Obtain a chronic test holding rack, which is marked for the randomization of the test cups (Exhibit AT13.3). Place the medicine cups in the holding rack and record the holding rack color on the bench sheet.

   d. Pour 15 mL of control water into each of the ten replicate control cups according to the randomization scheme.

   e. Pour 15 mL of each test concentration into each of the ten replicate medicine cups according to the randomization scheme. The remaining volume should be saved for chemical analyses (as indicated in B.1.b).

   f. Using an Eppendorf Repeater Pipetter, add 50 µL *Selenastrum capricornutum* and 50 µL YWT mixture to each cup. The cell density of the *Selenastrum* mixture must be 1.71 x 10^7 cells/mL (SOP-AT2) and total solids concentration of the YWT mixture must be between 1.7 to 1.9 g/L (SOP-AT6). Record the batches of *Selenastrum* and YWT on the chronic bench sheet.

g. Maintain the test temperature (25.0 ± 1.0°C) of the test concentrations. This may be accomplished by placing the holding rack into a temperature-controlled incubator.

2. Isolate and collect known-age neonates per instructions in SOP-AT8. Neonates must be less than 24-hours old and all within 8 hours of the same age.

   a. Select 10 brood cups for use in the toxicity test. Neonates are taken only from adults that have 8 or more young in their third or subsequent broods (as described in SOP-AT8). These adults can be used as brood stock until they are 14 days old.

   b. Record the brood board source and cups used for each replicate, age, and dates and times the organisms were born between on the chronic bench sheet.

3. Transfer the neonates to the randomly placed test cups in the holding rack by blocked parentage.

   a. Measure and record the temperature in one of the test cups for each concentration and control. The temperature must be 25.0 ± 1.0°C before the test organisms are placed in the test cups. Warm the test cups in a temperature-controlled incubator, if necessary, until the desired test temperature is obtained. Measure and record the temperature of an arbitrary cup containing neonates to be used in the toxicity test.

   c. Using the first brood cup, transfer one neonate into each test cup from top to bottom in the first column of the holding rack using a transfer pipette with the tip cut to > 2 mm bore size. Care should be taken to release each neonate under the surface of the water. Neonates should be transferred gently in a manner that will not expose the organisms to the air. The volume of water transferred to the medicine cups should be minimized to avoid diluting the test concentrations.

   d. Using the second brood cup, transfer one neonate into each of the six test cups in the second column of the holding rack. Continue this process until all test cups contain one neonate.

e. Save one of the cups that contained neonates used for the toxicity test. Measure and record the pH (SOP-C3) of this transfer water on the chronic bench sheet.

f. Record the initiation date, time and analyst's initials on the chronic bench sheet. **The test must be initiated within 36-hours of completion of the first sampling period.**

g. Verify that each test cup received one neonate by conducting a repeat count. Remove excess neonates or add neonates as necessary.

h. Place the chronic test holding rack in a temperature-controlled incubator. The organisms must be maintained at 25.0 ± 1.0°C with a photoperiod of 16-hours light and 8-hours dark and a recommended light intensity of 50 to 100 ft-c. Cover the rack with a Plexiglas® slide. Record the incubator number and shelf used on the bench sheet.

C. Feed Test Organisms on Day 1 (Thursday)

1. Remove the holding rack containing the test organisms from the incubator.

2. Using an Eppendorf Repeater Pipetter, add 50 µL *Selenastrum capricornutum* and 50 µL YWT mixture to each cup. The cell density of the *Selenastrum* mixture must be 1.71 x 10^7 cells/mL (SOP-AT2) and total solids of the YWT mixture must be 1.7 to 1.9 g/L (SOP-AT6). Record the time the organisms were fed and analyst initials on the bench sheet.

3. Place the holding rack in a temperature-controlled incubator. Cover the rack with a Plexiglas® slide.

D. Perform Renewal on Day 2 (Friday)

1. Prepare fresh test concentrations each day (following procedures outlined in section B).

2. Using an un-randomized test holding rack. Place medicine cups in the holding rack and pour 15 mL of control water into each of the ten replicate control cups located in the first row of the holding rack.

3. Pour 15 mL of the site/facility concentration into each of their respective ten replicate medicine cups according to the position number used at test initiation (section B.1.c). The remaining volumes of each concentration should be saved for chemical analyses (as indicated in B.1.b).

4. Using an Eppendorf Repeater Pipetter, add 50 µL *Selenastrum capricornutum* and 50 µL YWT mixture to each cup. The cell density of the *Selenastrum* mixture must be 1.71 x 10^7 cells/mL (SOP-AT2) and total solids concentration of the YWT mixture must be between 1.7 to 1.9 g/L (SOP-AT6). Record the batches of *Selenastrum* and YWT on the chronic bench sheet.

5. Maintain the test temperature (25.0 ± 1.0°C) of the test concentrations. This may be accomplished by placing the holding rack into a temperature-controlled incubator.

6. Remove the holding rack containing the test organisms from the incubator. Measure and record the temperature in an arbitrarily selected test cup of each site/facility concentration and control.

7. Remove the holding rack containing the fresh solutions from the incubator and place on a light box or table for ease of viewing. Measure and record the temperature in an arbitrarily selected test cup of test site/facility concentration and control. The temperature must be 25.0 ± 1.0°C before the test organisms are transferred into the new solutions. Warm the test cups in a temperature-controlled incubator, if necessary, until the desired test temperature is obtained.

8. Beginning with the control cups, remove the cups containing the test organisms from the randomized holding rack and place in order on the light table in front of the holding rack containing the fresh solutions. Using a transfer pipette with the tip cut to > 2 mm bore size, individually transfer each organism to the new test cups containing fresh solutions in the un-randomized board. Care should be taken to release each neonate under the surface of the water. Neonates should be transferred gently in a manner that will not expose the organisms to the air. The volume of water transferred to the medicine cups should be minimized to avoid diluting the test concentrations.

9. Discard and record organisms that are missing, injured or dead. Dead organisms must be confirmed through a dissection microscope. The criteria that establish death are: (1) no movement and (2) no reaction to gentle prodding. Record comments, if applicable.

10. Count and record (in the appropriate section) the number of live young in each cup on the chronic bench sheet. Any animal not producing young should be examined under a dissection microscope to determine if it is a male. Record the presence of 1 or 2 broods (in the appropriate section) on the chronic bench sheet.

11. Placed the new cups now containing the transferred organisms into the randomized holding rack according to the randomization scheme.

12. Continue this process of transferring test organisms beginning with the first site/facility concentration to the last site/facility concentration until all organisms have been transferred.

13. Record the date and time that the test solutions were renewed and the analyst's initials on the bench sheet. **The test must be renewed within 36-hours of completion of the second sampling period.**

14. Place the holding rack in a temperature-controlled incubator. Cover the rack with a Plexiglas® slide.

15. Measure and record the pH (SOP-C3) and dissolved oxygen (SOP-C2) in one of the test cups containing old test solution ("final") for each concentration and control.

E. Feed Test Organisms on Day 3 (Saturday) and Day 4 (Sunday)

Follow procedures outlined in section C.

F. Perform Renewal on Day 5 (Monday)

The test must be renewed, using the second sample, not more than 72-hours from the Friday renewal.

Follow procedures outlined in section D.

G. Feed Test Organisms on Day 6 (Tuesday)

Follow procedures outlined in section C.

H. Test Termination on Day 7 (Wednesday)

Terminate the test after 80% of the control organisms have produced their third brood. The test must be terminated before 7 days + 2 hours from test initiation.

1. Remove the holding rack containing the test organisms from the incubator. Place the rack on a light box or table for ease of viewing. Measure and record the temperature in an arbitrarily selected test cup for each site/facility concentration and control.

2. Count and record (in the appropriate section) the number of live young in each cup and record the survival of the adult test organism on the chronic bench sheet. Record the presence of 1 or 2 broods (in the appropriate section) on the chronic bench sheet.

3. Record the date and time the test was terminated and the analyst's initials on the bench sheet.

4. Measure and record the pH (SOP-C3) and dissolved oxygen (SOP-C2) in one of the test cups for each concentration and control.

5. Once all analyses have been completed and documented, discard the test water and organisms according to established laboratory protocol.

I. Statistical Analyses and Data Verification

Statistical analyses and data review are performed according to QAP-Q12.

Safety and Hazardous Waste Management

Safety glasses, gloves and lab coats should be worn at all times.

Review Policy-P6: General Safety Policy and Policy-P9: Radiation Protection Policy for additional safety requirements.

References

USEPA. October 2002. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, 4th ed. **EPA-821-R-02-013, Method 1002.0**. US Environmental Protection Agency, Cincinnati, OH.

North Carolina Department of Environment and Natural Resources, Division of Water Quality. North Carolina Phase II Chronic Whole Effluent Toxicity Test Procedure, Version 3.0. December 2010.

Subject: North Carolina *Ceriodaphnia dubia* Phase II Chronic Toxicity Test, EPA 1002.0

North Carolina Department of Environment and Natural Resources, Division of Water Quality. Biological Laboratory Certification / Criteria Procedures, Version 3.0. December 2010.

TNI Standard. Management and Technical Requirements for Laboratories Performing Environmental Analysis. EL-V1-ISO-2016-Rev2.0. The NELAC Institute, PO Box 2439, Weatherford, TX 76086.

Exhibits

Exhibit AT13.1: Summary of Test Conditions for the North Carolina *Ceriodaphnia dubia* Phase II Chronic Toxicity Test.
Exhibit AT13.2: North Carolina *Ceriodaphnia dubia* Phase II Chronic Toxicity Test Bench Sheet.
Exhibit AT13.3: Chronic Test Holding Rack.

Subject: North Carolina *Ceriodaphnia dubia* Phase II Chronic Toxicity Test, EPA 1002.0

Exhibit AT13.1: Summary of Test Conditions for the North Carolina *Ceriodaphnia dubia* Phase II Chronic Toxicity Test.

SUMMARY OF TEST CONDITIONS FOR THE NORTH CAROLINA *CERIODAPHNIA DUBIA* PHASE II CHRONIC TOXICITY TEST

Test type:	Static renewal
Test duration:	Until 80% or more of surviving control females have three broods (maximum test duration of 7 days + 2-hours)
Temperature:	25.0 ± 1.0°C
Light quality:	Cool-white fluorescent
Light intensity:	50 – 100 ft-candles (recommended)
Photoperiod:	16-hours light, 8-hours dark
Test chamber size:	40 mL graduated polypropylene medicine cup
Test solution volume:	15 mL
Renewal of test solutions:	Renewals performed on days 2 and 5
Age of test organisms:	< 24-hours old, all released within an 8-hour period.
Number of organisms per test chamber:	1 assigned using blocking by known parentage
Number of replicate test chambers per concentration:	10
Number of organisms per concentration:	10
Test concentrations:	At 0.25, 0.5, 1, 2, 4 times chronic permit limit and a control
Test chamber cleaning:	Use new medicine cups at each renewal.
Aeration:	None
Feeding regime:	50 µL YWT and 50 µL *Selenastrum* (1.71 x 10^7 cells/ml) per test chamber daily.
Control / Dilution water:	Moderately hard synthetic water
Sampling and sample holding:	1-gallon grab or composite samples collected on Tuesday and Thursday (for tests initiated on Wednesday). Each sample must be used within 36-hours of completion of each sampling period (not to exceed 72-hours from first use).
Endpoint:	Survival and reproduction
Test acceptability criterion:	≥ 80% control survival, control reproduction ≥ 15 offspring/surviving female with 80% of surviving control females producing three broods and control reproduction coefficient of variation < 40%, number of male control organisms ≤ 20%

Subject: North Carolina *Ceriodaphnia dubia* Phase II Chronic Toxicity Test, EPA 1002.0

Exhibit AT13.2: North Carolina *Ceriodaphnia dubia* Phase II Chronic Toxicity Test Bench Sheet.

ETS

Page 1 of 4

North Carolina Chronic Phase II Whole Effluent Toxicity Test, Species: *Ceriodaphnia dubia*
(EPA-821-R-02-013 Method 1002.0, NC Modification – December 2010, Version 3.0)

Client: Duke Energy Progress — NPDES #: NC0000396

Facility: Asheville Ash Pond — Outfall: 001

Project #: _____ — County: Buncombe

Chronic Limit: 1.9%

Dilution Preparation:

Test concentrations (%)	0.45	0.90	1.8	3.6	7.2
mL Sample	0.9	1.8	3.6	7.2	14.4
mL Dilution water	199.1	198.2	196.4	192.8	185.6
Total volume (mL)	200	200	200	200	200

Sample was not aerated or treated unless otherwise noted on this form. Sample was warmed to 25.0 ± 1.0°C in a warm water bath and then diluted to the test concentrations with moderately hard synthetic water.

Daily Renewal Information

Day	Date	Test Initiation, renewal, feeding or termination		Sample Number	MHSW Batch	Selenastrum Batch	YWT Batch	Location Incubator/Shelf	Randomizing Template
		Time	Analyst						
0	04-10-19	Initiation/Feeding		Sample 1					
1	04-11-19	Feeding							
2	04-12-19	Renewal 1/Feeding		Sample 2					
3	04-13-19	Feeding							
4	04-14-19	Feeding							
5	04-15-19	Renewal 2/Feeding		Sample 2					
6	04-16-19	Feeding							
7	04-17-19	Termination							

Test was initiated using Sample 1. Sample 2 was used for Renewals One (day 2) and Two (day 5).

Test Organism Information:

Organism Source:	In-house Culture									
Age:	< 24-hours old									
Source (culture board):										
Replicate #	1	2	3	4	5	6	7	8	9	##
Culture board cup #										
Date and time organisms were born between:										
Average transfer volume:	< 0.25 mL									
Transfer bowl information:	pH (S.U.):									
	Temperature (°C):									

Final Results - Summary of Test Endpoints:

7-day LC_{50} (%)	
NOEC (%)	
LOEC (%)	
ChV (%)	
IC_{25} (%)	

SOP AT13-Revision 4-Exhibit AT13.2

Subject: North Carolina *Ceriodaphnia dubia* Phase II Chronic Toxicity Test, EPA 1002.0

North Carolina Chronic Phase II Whole Effluent Toxicity Test, Species: *Ceriodaphnia dubia*
(EPA-821-R-02-013 Method 1002.0, NC Modification – December 2010, Version 3.0)

Facility Asheville Ash Pond

Project # _____

Survival and Reproduction Data :

Control

Day	Observations	Replicate number 1	2	3	4	5	6	7	8	9	10
2 Renewal One	Adult mortality (L = Live, D = dead)										
5 Renewal Two	Number of broods present										
	Number of young produced										
	Adult mortality (L = Live, D = dead)										
7 Final	Number of broods present										
	Number of young produced										
	Total young produced										
	Final adult mortality (L = Live, D = dead)										
	X for 3rd Broods										

Survival		Mean offspring per female	

Test concentration: 0.45%

Day	Observations	Replicate number 1	2	3	4	5	6	7	8	9	10
2 Renewal One	Adult mortality (L = Live, D = dead)										
5 Renewal Two	Number of broods present										
	Number of young produced										
	Adult mortality (L = Live, D = dead)										
7 Final	Number of broods present										
	Number of young produced										
	Total young produced										
	Final adult mortality (L = Live, D = dead)										

Survival		Mean offspring per female		% Reduction from Control	

Test concentration: 0.90%

Day	Observations	Replicate number 1	2	3	4	5	6	7	8	9	10
2 Renewal One	Adult mortality (L = Live, D = dead)										
5 Renewal Two	Number of broods present										
	Number of young produced										
	Adult mortality (L = Live, D = dead)										
7 Final	Number of broods present										
	Number of young produced										
	Total young produced										
	Final adult mortality (L = Live, D = dead)										

Survival		Mean offspring per female		% Reduction from Control	

SOP AT13-Revision 4-Exhibit AT13.2

Subject: North Carolina *Ceriodaphnia dubia* Phase II Chronic Toxicity Test, EPA 1002.0

ETS

North Carolina Chronic Phase II Whole Effluent Toxicity Test, Species: *Ceriodaphnia dubia*
(EPA-821-R-02-013 Method 1002.0, NC Modification – December 2010, Version 3.0)

Facility Asheville Ash Pond

Project # _____

Survival and Reproduction Data :

Test concentration: 1.8%

Day	Observations	Replicate number 1	2	3	4	5	6	7	8	9	10
2 Renewal One	Adult mortality (L = Live, D = dead)										
5 Renewal Two	Number of broods present										
	Number of young produced										
	Adult mortality (L = Live, D = dead)										
7 Final	Number of broods present										
	Number of young produced										
	Total young produced										
	Final adult mortality (L = Live, D = dead)										

Survival		Mean offspring per female		% Reduction from Control	

Test concentration: 3.6%

Day	Observations	Replicate number 1	2	3	4	5	6	7	8	9	10
2 Renewal One	Adult mortality (L = Live, D = dead)										
5 Renewal Two	Number of broods present										
	Number of young produced										
	Adult mortality (L = Live, D = dead)										
7 Final	Number of broods present										
	Number of young produced										
	Total young produced										
	Final adult mortality (L = Live, D = dead)										

Survival		Mean offspring per female		% Reduction from Control	

Test concentration: 7.2%

Day	Observations	Replicate number 1	2	3	4	5	6	7	8	9	10
2 Renewal One	Adult mortality (L = Live, D = dead)										
5 Renewal Two	Number of broods present										
	Number of young produced										
	Adult mortality (L = Live, D = dead)										
7 Final	Number of broods present										
	Number of young produced										
	Total young produced										
	Final adult mortality (L = Live, D = dead)										

Survival		Mean offspring per female		% Reduction from Control	

SOP AT13-Revision 4-Exhibit AT13.2

Subject: North Carolina *Ceriodaphnia dubia* Phase II Chronic Toxicity Test, EPA 1002.0

ETS

North Carolina Chronic Phase II Whole Effluent Toxicity Test, Species: *Ceriodaphnia dubia*
(EPA-821-R-02-013 Method 1002.0, NC Modification – December 2010, Version 3.0)

Facility Asheville Ash Pond

Project # _____

Chemical Analyses:

		Initiation		Renewal One		Renewal Two	
		Initial	Final	Initial	Final	Initial	Final
Concentration	Analyst						
Control MHSW	pH (S.U.)						
	Dissolved oxygen (mg/L)						
	Conductivity (µmhos/cm)						
	*Alkalinity (mg/L $CaCO_3$)						
	*Hardness (mg/L $CaCO_3$)						
	*Temperature (°C)						
0.45%	pH (S.U.)						
	Dissolved oxygen (mg/L)						
	Conductivity (µmhos/cm)						
	*Temperature (°C)						
0.90%	pH (S.U.)						
	Dissolved oxygen (mg/L)						
	Conductivity (µmhos/cm)						
	*Temperature (°C)						
1.8%	pH (S.U.)						
	Dissolved oxygen (mg/L)						
	Conductivity (µmhos/cm)						
	*Temperature (°C)						
3.6%	pH (S.U.)						
	Dissolved oxygen (mg/L)						
	Conductivity (µmhos/cm)						
	*Temperature (°C)						
7.2%	pH (S.U.)						
	Dissolved oxygen (mg/L)						
	Conductivity (µmhos/cm)						
	*Temperature (°C)						
100%	pH (S.U.)						
	Dissolved oxygen (mg/L)						
	Conductivity (µmhos/cm)						
	*Total residual chlorine (mg/L)						
	*Temperature (°C)						

*Analyst identified for each day performed pH, dissolved oxygen and conductivity measurements only. Temperatures performed at the time of test initiation, renewal or termination by the analyst identified in the Daily Renewal Information table. Alkalinity, hardness and total residual chlorine performed by the analysts identified on the test specific bench sheets and transcribed to this bench sheet.

SOP AT13-Revision 4-Exhibit AT13.2

Subject: North Carolina *Ceriodaphnia dubia* Phase II Chronic Toxicity Test, EPA 1002.0

Exhibit AT13.3: Chronic Test Holding Rack (Exhibit AT11.3 from SOP AT11).

Randomizing template: **RED**

Replicate #	1	2	3	4	5	6	7	8	9	10
Concentrations	6	5	4	5	6	3	3	4	6	4
	3	3	2	6	4	2	5	2	5	2
1 = Control	4	1	1	2	2	1	2	6	2	5
2 = Lowest concentration	1	2	3	1	5	5	4	3	4	1
3 - 5 = Intermediate concentrations	2	4	5	3	1	6	6	1	3	3
6 = Highest concentration	5	6	6	4	3	4	1	5	1	6

Random number seeds: 4 through 13

SOP AT11-Revision 5-Exhibit AT11.3

Subject: *Ceriodaphnia dubia* Chronic Reference Toxicity Test, EPA 1002.0

Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan		03-01-20
Quality Assurance Officer	Jim Sumner		03-01-20

Document Revision History

Effective Date	Revision number	Review Type	Evaluators	Revisions
12-01-00	0	Internal	Jim Sumner (ETS)	Original document
06-01-11	1	Internal	Jim Sumner (ETS)	• Updated references and exhibits. • Updated Table AT14.2.
11-01-14	2	Internal	Jim Sumner (ETS)	• Updated exhibits during document review. • Removed conductivity measurement requirement of stock NaCl solution due to inaccuracy of these measurements, which are above the calibration range.
09-28-16	3	External (TVA) Internal	Rick Sherrard, Donald Snodgrass (TVA) Jim Sumner (ETS)	• Updated Table AT14.2 for conductivity measurement guidance values. • Deleted statement: "Verify that the conductivity measured for each test concentration is within the acceptance criteria (refer to table Table AT14.2) before proceeding with the preparation of next concentration. If the conductivity is not within the criteria, remake the test concentration and verify the conductivity."
07-01-18	4	Internal	Jim Sumner (ETS)	• Updated procedure to include NELAP requirements. • Additional guidance included in SOP.
09-01-19	5	Internal	Jim Sumner (ETS)	• Updated references and exhibits. • Additional guidance included in SOP.
03-01-20	6	External (TVA) Internal	Rick Sherrard (TVA) Jim Sumner (ETS)	• Updated bench sheet to include reporting limits, method numbers, meters and serial numbers used for chemical analyses.

Subject: *Ceriodaphnia dubia* Chronic Reference Toxicity Test, EPA 1002.0

Scope and Application

To assess the sensitivity of *Ceriodaphnia dubia* and the overall credibility of *Ceriodaphnia dubia* chronic toxicity tests. Reference toxicant data are part of the laboratory's QA/QC program to evaluate the performance of laboratory personnel and the robustness and sensitivity of the test organisms.

Summary of Method

The chronic reference toxicity test generally involves the exposure of test organisms to five sodium chloride concentrations and control water for a 7-day exposure period. At the end of each 24-hour period, the number of living organisms and number of offspring is counted in each sodium chloride concentration and control water. The 25% inhibition concentration (IC_{25}) of sodium chloride is determined and compared to previous reference toxicant tests.

Quality Control

Acceptance Criteria: The test acceptance criteria are indicated in the table below. In general, the most stringent acceptability criteria are used by the laboratory. If acceptability criteria are not met, the test must be invalidated.

Table AT14.1: *Ceriodaphnia dubia* chronic toxicity test acceptability criteria.

Test Acceptability Criteria	USEPA	North Carolina	TVA
Control survival	≥ 80%	≥ 80%	≥ 80%
Average number of offspring per surviving female in the control	≥ 15.0	≥ 15.0	≥ 15.0
Control reproduction coefficient of variation	< 42%	< 40%	< 42%
Percentage of surviving adults having 3rd broods in the control	≥ 60%	≥ 80%	≥ 60%
Percentage of male adults in the control	≤ 20%	≤ 20%	0% for entire test
Guidance percent minimum significant difference (PMSD)	13 – 47%	No criteria	13 – 47%

Subject: *Ceriodaphnia dubia* Chronic Reference Toxicity Test, EPA 1002.0

Frequency of Testing: A *Ceriodaphnia dubia* chronic reference toxicant test must be performed monthly. At a minimum, chronic reference toxicant tests must be performed on a quarterly basis in order to maintain certification requirements.

Equipment and Materials

Ceriodaphnia dubia
Temperature-controlled incubator (set to maintain test temperature = 25.0 ± 1.0°C, photoperiod of 16-hour light / 8-hours dark, light intensity = 50 – 100 ft-c)
Control / Dilution water (moderately hard synthetic water)
1-oz medicine cups
500-mL plastic Solo® cups
Graduated cylinders
Serological, fixed, or adjustable volume pipettes (e.g., Eppendorf)
Pasteur® pipettes
Transfer pipettes
Eppendorf Repeater Pipetter
Chronic test holding rack
Plexiglas® slides
Thermometer
YWT mixture
Selenastrum capricornutum
Light box or table
Dissection microscope (if necessary)
Disposable gloves
Ceriodaphnia dubia Chronic Reference Toxicity Test Bench Sheet

Procedure

A. Test Preparation.

1. Prepare the glassware.

   a. Obtain enough 2000 ml Erlenmeyer flasks for each test concentration and the control. These flasks will be used in the preparation of the test concentrations. Label each flask with the test concentration.

   b. Label the appropriate graduated cylinder.

c. Prepare the *Ceriodaphnia dubia* Chronic Reference Toxicity Test Bench Sheet (see Exhibit AT14.1). Record the *Ceriodaphnia dubia* NaCl Chronic (CdNaClCR) test number on the bench sheet.

b. Obtain a chronic test holding rack, which is marked for the randomization of the test cups. Place the medicine cups in the holding rack and record the holding rack name on the bench sheet.

B. Preparation of the Stock Solution.

1. Using a calibrated top-loading balance, carefully weigh out 50 g of NaCl (SOP-G10). Place approximately 400 mL of deionized water in a 500-mL volumetric flask. Add the NaCl to the flask, dissolve the NaCl by swirling the flask, bring to volume with deionized water. Label the volumetric flask with the concentration (100 g/L), analyst's initials, preparation date and stock standard number (INSS, SOP-G15). Record the INSS number of the NaCl stock solution on the bench sheet.

C. Preparation of the Test Concentrations.

1. Prepare all test concentrations using graduated cylinders and serological pipettes. The precision of conductivity measurements is increased when smaller volume graduated cylinders and/or serological pipettes are used to prepare the test concentrations. For this reference toxicant test, stock solution volumes should be measured using 10-mL serological pipettes and the total volumes should be measured using a 2000-mL graduated cylinder.

2. Beginning with the lowest concentration, add approximately 200 mL of moderately hard synthetic water (MHSW) to a 2000-mL graduated cylinder, add the required volume of stock solution using a 10-mL serological pipette (refer to Table AT14.2), bring to volume (1500 mL) with MHSW. Mix the solution well by pouring the solution into the respective 2000 mL Erlenmeyer flask and swirling the solution in the flask.

3. Pour 15 mL of test solution into each of the replicate test cups for that concentration according to the randomization scheme of the holding rack. Pour 40 mL of the test solution into a labeled medicine cup to be saved for chemical analyses.

4. Measure and record the conductivity (SOP-C4), pH (SOP-C3) and dissolved oxygen (SOP-C2) of each test concentration on the bench sheet. Refer to Table AT14.2 for guidance values of conductivity measurements.

5. Rinse the graduated cylinder well with deionized water and repeat steps C.2 through C.4 for preparing the next test concentration. Record the batch date of the MHSW used to prepare the dilutions on the bench sheet.

Table AT14.2: Test concentration, stock volumes, moderately hard synthetic water volumes, final volumes and conductivity measurements guidance values for the *Ceriodaphnia dubia* NaCl chronic reference toxicant tests.

Test Concentration (mg NaCl/L)	Volume of Stock Required (ml)	Volume of Moderately hard synthetic water (ml)	Final Volume (ml)	Conductivity Guidance Values (µmhos/cm)
600	9	1491	1500	1300 - 1500
800	12	1488	1500	1700 - 1900
1000	15	1485	1500	2100 - 2400
1200	18	1482	1500	2500 - 2700
1400	21	1479	1500	2800 - 3100

6. Once all test concentrations have been prepared, follow the procedure described in SOP-AT11 for conducting *Ceriodaphnia dubia* Chronic Toxicity Tests.

D. Control Charts and Outlier Test Results.

Refer to QAP-Q5 for how control charts are prepared and procedures for interpreting outlier tests. Refer to Exhibit AT14.2 for an example control chart.

Safety and Hazardous Waste Management

Safety glasses, gloves and lab coats should always be worn.

Review Policy-P6: General Safety Policy and Policy-P9: Radiation Protection Policy for additional safety requirements.

Subject: *Ceriodaphnia dubia* Chronic Reference Toxicity Test, EPA 1002.0

References

USEPA. October 2002. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, 4th ed. **EPA-821-R-02-013, Method 1002.0**. US Environmental Protection Agency, Cincinnati, OH.

USEPA. 2000. Understanding and Accounting for Method Variability in Whole Effluent Toxicity Applications Under the National Pollutant Discharge Elimination Program. EPA-833-R-00-003. US Environmental Protection Agency, Cincinnati, OH.

USEPA. 2001. Final Report: Inter-laboratory Variability Study of EPA Short-term Chronic and Acute Whole Effluent Toxicity Test Methods, Volumes 1 and 2 - Appendix. EPA-821-B-01-004 and EPA-821-B-01-005. US Environmental Protection Agency, Cincinnati, OH.

North Carolina Department of Environment and Natural Resources, Division of Water Quality. Biological Laboratory Certification / Criteria Procedures, Version 3.0. December 2010.

TNI Standard. Management and Technical Requirements for Laboratories Performing Environmental Analysis. EL-V1-ISO-2016-Rev2.0. The NELAC Institute, PO Box 2439, Weatherford, TX 76086.

Exhibits

Exhibit AT14.1: Example *Ceriodaphnia dubia* Chronic Reference Toxicity Test Bench Sheet.
Exhibit AT14.2: Example *Ceriodaphnia dubia* Acute Reference Toxicant Control Chart.

Subject: *Ceriodaphnia dubia* Chronic Reference Toxicity Test, EPA 1002.0

Exhibit AT14.1: Example *Ceriodaphnia dubia* Chronic Reference Toxicity Test Bench Sheet.

Sodium Chloride Chronic Reference Toxicant Test (EPA-821-R-02-013 Method 1002.0)
Species: *Ceriodaphnia dubia*

CdNaClCR #: 241

Dilution preparation information:						***Comments:***
NaCl Stock INSS number:		INSS				
Stock preparation:		100 g NaCl/L: Dissolve 50 g NaCl in 500 mL deionized water.				
Dilution prep (mg/L)	600	800	1000	1200	1400	
Stock volume (mL)	9	12	15	18	21	
Diluent volume (mL)	1491	1488	1485	1482	1479	
Total volume (mL)	1500	1500	1500	1500	1500	

Test organism source:

Organism age:	< 24-hours old									
Date and times organisms were born between:										
Culture board:										
Replicate number:	1	2	3	4	5	6	7	8	9	10
Culture board cup number:										
Transfer vessel information:	pH (S.U.): Temperature (°C):									
Average transfer volume (mL):	< 0.25 mL									

Test randomization and location:

Randomizing template color:	
Incubator number and shelf location:	

Daily renewal:

Day	Date	Test initiation and feeding, renewal and feeding, or termination time	*Feeding Batches *Selenastrum*	*Feeding Batches YWT	MHSW batch used	Analyst
0						
1						
2						
3						
4						
5						
6						
7						

*Organisms fed daily 100 µL *Selenastrum* and 100 µL YWT per replicate using HandyStep repeat pipettor SN 17E59354

Chemical analyses:

Parameter	Reporting Limit	Method number	Meter	Serial number
pH	0.1 S.U.	SM 4500-H+ B-2011	Accumet AR20	93312452
Dissolved Oxygen (D.O.)	1.0 mg/L	SM 4500-O G-2011	YSI Model 52CE	18D104324
Conductivity	14.9 µmhos/cm	SM 2510 B-2011	Accumet AR20	93312452
Alkalinity	5.0 mg $CaCO_3$/L	SM 2320 B-2011	Accumet AR20	93312452
Hardness	5.0 mg $CaCO_3$/L	SM 2340 C-2011	Not applicable	Not applicable
Temperature	0.1°C	SM 2550B-2010	Digital Thermometer	

Control information:		Acceptance criteria	***Summary of test endpoints:***	
% of Male Adults:		≤ 20%	**7-day LC_{50}** (mg/L NaCl)	
% Adults having 3rd Broods:		≥ 80%	**NOEC** (mg/L NaCl)	
% Mortality:		≤ 20%	**LOEC** (mg/L NaCl)	
Mean Offspring/Female:		≥ 15.0 offspring/female	**ChV** (mg/L NaCl)	
% CV:		< 40.0 %	**IC_{25}** (mg/L NaCl)	

SOP AT14-Revision 6–Exhibit AT14.1

Subject: *Ceriodaphnia dubia* Chronic Reference Toxicity Test, EPA 1002.0

Species: *Ceriodaphnia dubia* CdNaClCR #: 241

CONTROL *Survival and Reproduction Data*

Day		Replicate number									
		1	2	3	4	5	6	7	8	9	10
1	Young produced										
	Adult mortality										
2	Young produced										
	Adult mortality										
3	Young produced										
	Adult mortality										
4	Young produced										
	Adult mortality										
5	Young produced										
	Adult mortality										
6	Young produced										
	Adult mortality										
7	Young produced										
Total young produced											
Final Adult Mortality											
X for 3rd Broods											

Note: Adult mortality (L = live, D = dead), SB = split brood (single brood split between two days), CO = carry over (offspring carried over with adult during transfer).

Concentration:	
% Mortality:	
Mean Offspring/Female:	

600 mg NaCl/L *Survival and Reproduction Data*

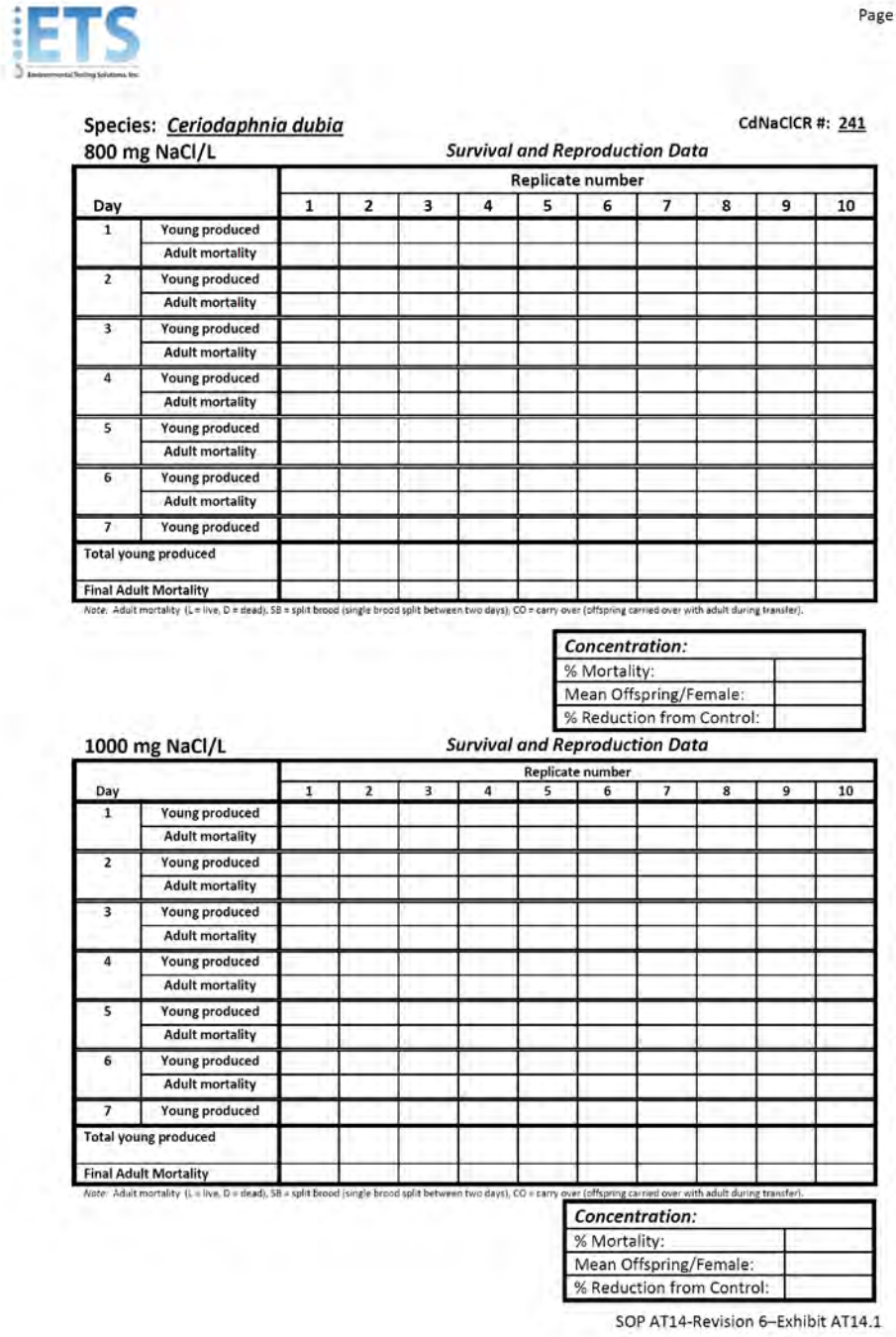
Day		Replicate number									
		1	2	3	4	5	6	7	8	9	10
1	Young produced										
	Adult mortality										
2	Young produced										
	Adult mortality										
3	Young produced										
	Adult mortality										
4	Young produced										
	Adult mortality										
5	Young produced										
	Adult mortality										
6	Young produced										
	Adult mortality										
7	Young produced										
Total young produced											
Final Adult Mortality											

Note: Adult mortality (L = live, D = dead), SB = split brood (single brood split between two days), CO = carry over (offspring carried over with adult during transfer).

Concentration:	
% Mortality:	
Mean Offspring/Female:	
% Reduction from Control:	

SOP AT14-Revision 6–Exhibit AT14.1

Subject: *Ceriodaphnia dubia* Chronic Reference Toxicity Test, EPA 1002.0



ETS

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Species: *Ceriodaphnia dubia* CdNaClCR #: 241

800 mg NaCl/L

Survival and Reproduction Data

Day		Replicate number									
		1	2	3	4	5	6	7	8	9	10
1	Young produced										
	Adult mortality										
2	Young produced										
	Adult mortality										
3	Young produced										
	Adult mortality										
4	Young produced										
	Adult mortality										
5	Young produced										
	Adult mortality										
6	Young produced										
	Adult mortality										
7	Young produced										
Total young produced											
Final Adult Mortality											

Note: Adult mortality (L = live, D = dead), SB = split brood (single brood split between two days), CO = carry over (offspring carried over with adult during transfer).

Concentration:	
% Mortality:	
Mean Offspring/Female:	
% Reduction from Control:	

1000 mg NaCl/L

Survival and Reproduction Data

Day		Replicate number									
		1	2	3	4	5	6	7	8	9	10
1	Young produced										
	Adult mortality										
2	Young produced										
	Adult mortality										
3	Young produced										
	Adult mortality										
4	Young produced										
	Adult mortality										
5	Young produced										
	Adult mortality										
6	Young produced										
	Adult mortality										
7	Young produced										
Total young produced											
Final Adult Mortality											

Note: Adult mortality (L = live, D = dead), SB = split brood (single brood split between two days), CO = carry over (offspring carried over with adult during transfer).

Concentration:	
% Mortality:	
Mean Offspring/Female:	
% Reduction from Control:	

SOP AT14-Revision 6–Exhibit AT14.1

Subject: *Ceriodaphnia dubia* Chronic Reference Toxicity Test, EPA 1002.0

Species: ***Ceriodaphnia dubia*** **CdNaClCR #: 241**

1200 mg NaCl/L ***Survival and Reproduction Data***

Day		Replicate number									
		1	2	3	4	5	6	7	8	9	10
1	Young produced										
	Adult mortality										
2	Young produced										
	Adult mortality										
3	Young produced										
	Adult mortality										
4	Young produced										
	Adult mortality										
5	Young produced										
	Adult mortality										
6	Young produced										
	Adult mortality										
7	Young produced										
Total young produced											
Final Adult Mortality											

Note: Adult mortality (L = live, D = dead), SB = split brood (single brood split between two days), CO = carry over (offspring carried over with adult during transfer).

Concentration:	
% Mortality:	
Mean Offspring/Female:	
% Reduction from Control:	

1400 mg NaCl/L ***Survival and Reproduction Data***

Day		Replicate number									
		1	2	3	4	5	6	7	8	9	10
1	Young produced										
	Adult mortality										
2	Young produced										
	Adult mortality										
3	Young produced										
	Adult mortality										
4	Young produced										
	Adult mortality										
5	Young produced										
	Adult mortality										
6	Young produced										
	Adult mortality										
7	Young produced										
Total young produced											
Final Adult Mortality											

Note: Adult mortality (L = live, D = dead), SB = split brood (single brood split between two days), CO = carry over (offspring carried over with adult during transfer).

Concentration:	
% Mortality:	
Mean Offspring/Female:	
% Reduction from Control:	

SOP AT14-Revision 6–Exhibit AT14.1

Species: *Ceriodaphnia dubia* **CdNaClCR #: 241**

Daily Chemistry:

Temperatures performed at the time of test initiation, renewal or termination by the analyst identified in the Daily Renewal Information table located on Page 1. Alkalinity and hardness performed by the analyst identified on the bench sheet specific for each analysis and transcribed to this bench sheet.

		Day (Analyst identified for each day, performed pH, D.O. and conductivity measurements only.)					
		0		1		2	
	Analyst						
Concentration	Parameter						
CONTROL, MHSW	pH (S.U.)						
	Dissolved oxygen (mg/L)						
	Conductivity (µmhos/cm)						
	Alkalinity (mg $CaCO_3$/L)						
	Hardness (mg $CaCO_3$/L)						
	Temperature (°C)						
600 mg NaCl/L	pH (S.U.)						
	Dissolved oxygen (mg/L)						
	Conductivity (µmhos/cm)						
	Temperature (°C)						
800 mg NaCl/L	pH (S.U.)						
	Dissolved oxygen (mg/L)						
	Conductivity (µmhos/cm)						
	Temperature (°C)						
1000 mg NaCl/L	pH (S.U.)						
	Dissolved oxygen (mg/L)						
	Conductivity (µmhos/cm)						
	Temperature (°C)						
1200 mg NaCl/L	pH (S.U.)						
	Dissolved oxygen (mg/L)						
	Conductivity (µmhos/cm)						
	Temperature (°C)						
1400 mg NaCl/L	pH (S.U.)						
	Dissolved oxygen (mg/L)						
	Conductivity (µmhos/cm)						
	Temperature (°C)						
		Initial	Final	Initial	Final	Initial	Final

SOP AT14-Revision 6–Exhibit AT14.1

Subject: *Ceriodaphnia dubia* Chronic Reference Toxicity Test, EPA 1002.0

Species: ***Ceriodaphnia dubia***

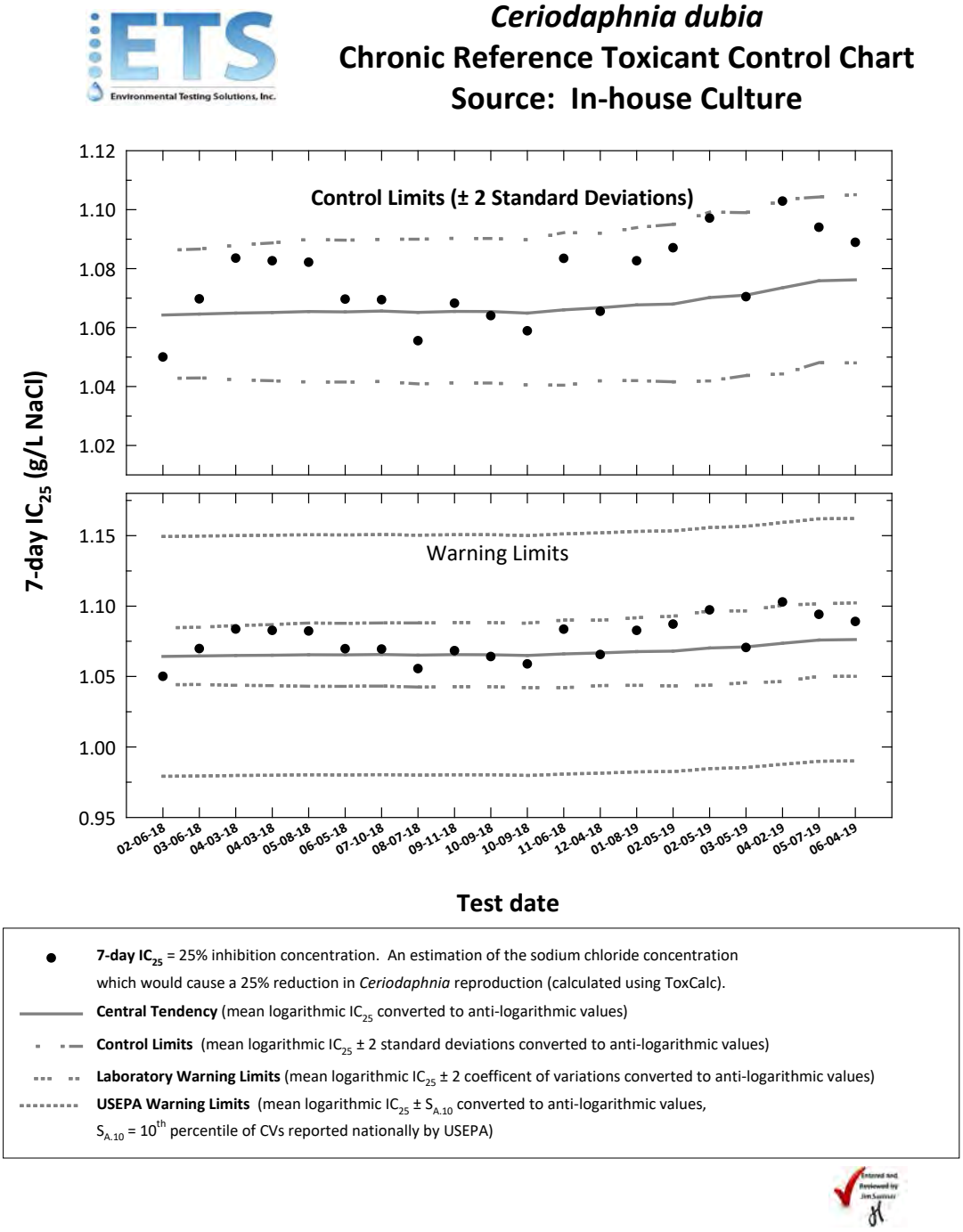
CdNaClCR #: 241

		Day (Analyst identified for each day, performed pH, D.O. and conductivity measurements only.)							
		3		4		5		6	
	Analyst								
Concentration	Parameter								
CONTROL, MHSW	pH (S.U.)								
	Dissolved oxygen (mg/L)								
	Conductivity (µmhos/cm)								
	Alkalinity (mg $CaCO_3$/L)								
	Hardness (mg $CaCO_3$/L)								
	Temperature (°C)								
600 mg NaCl/L	pH (S.U.)								
	Dissolved oxygen (mg/L)								
	Conductivity (µmhos/cm)								
	Temperature (°C)								
800 mg NaCl/L	pH (S.U.)								
	Dissolved oxygen (mg/L)								
	Conductivity (µmhos/cm)								
	Temperature (°C)								
1000 mg NaCl/L	pH (S.U.)								
	Dissolved oxygen (mg/L)								
	Conductivity (µmhos/cm)								
	Temperature (°C)								
1200 mg NaCl/L	pH (S.U.)								
	Dissolved oxygen (mg/L)								
	Conductivity (µmhos/cm)								
	Temperature (°C)								
1400 mg NaCl/L	pH (S.U.)								
	Dissolved oxygen (mg/L)								
	Conductivity (µmhos/cm)								
	Temperature (°C)								
		Initial	Final	Initial	Final	Initial	Final	Initial	Final

SOP AT14-Revision 6–Exhibit AT14.1

Subject: *Ceriodaphnia dubia* Chronic Reference Toxicity Test, EPA 1002.0

Exhibit AT14.2: Example *Ceriodaphnia dubia* Chronic Reference Toxicant Control Chart.



Subject: *Ceriodaphnia dubia* Chronic Reference Toxicity Test, EPA 1002.0

Ceriodaphnia dubia
Chronic Reference Toxicant Control Chart
Source: In-house Culture

Test number	Test date	7-day IC_{25} ToxCal Determination (g/L NaCl)	Log_{10} Conversion			Anti-logarithmic Values (g/L NaCl)						
							Control Limits		Laboratory Calculated CV Warning Limits		10th Percentile CV Warning Limits	
			7-day IC_{25}	CT	S	CT	CT - 2S	CT + 2S	CT - 2CV	CT + 2CV	CT - $S_{A.10}$	CT + $S_{A.10}$
1	02-06-18	1.0500	0.0212	0.0270	0.0044	1.0642	1.0427	1.0862	1.0440	1.0844	0.9791	1.1494
2	03-06-18	1.0697	0.0293	0.0272	0.0045	1.0646	1.0430	1.0867	1.0443	1.0849	0.9794	1.1498
3	04-03-18	1.0835	0.0348	0.0273	0.0047	1.0649	1.0424	1.0880	1.0437	1.0861	0.9797	1.1501
4	04-03-18	1.0827	0.0345	0.0274	0.0048	1.0651	1.0420	1.0888	1.0434	1.0869	0.9799	1.1503
5	05-08-18	1.0822	0.0343	0.0275	0.0049	1.0655	1.0415	1.0899	1.0430	1.0879	0.9802	1.1507
6	06-05-18	1.0696	0.0292	0.0275	0.0049	1.0653	1.0415	1.0897	1.0430	1.0877	0.9801	1.1506
7	07-10-18	1.0694	0.0291	0.0276	0.0049	1.0656	1.0417	1.0900	1.0432	1.0880	0.9804	1.1508
8	08-07-18	1.0555	0.0235	0.0274	0.0050	1.0652	1.0409	1.0900	1.0424	1.0879	0.9800	1.1504
9	09-11-18	1.0682	0.0287	0.0276	0.0050	1.0655	1.0413	1.0903	1.0428	1.0883	0.9803	1.1508
10	10-09-18	1.0640	0.0269	0.0275	0.0050	1.0654	1.0412	1.0902	1.0427	1.0882	0.9802	1.1507
11	10-09-18	1.0589	0.0248	0.0273	0.0050	1.0649	1.0406	1.0898	1.0421	1.0878	0.9797	1.1501
12	11-06-18	1.0835	0.0348	0.0278	0.0053	1.0660	1.0404	1.0922	1.0420	1.0900	0.9807	1.1513
13	12-04-18	1.0655	0.0275	0.0280	0.0051	1.0667	1.0420	1.0920	1.0435	1.0899	0.9814	1.1521
14	01-08-19	1.0826	0.0345	0.0284	0.0053	1.0677	1.0420	1.0940	1.0437	1.0917	0.9823	1.1531
15	02-05-19	1.0871	0.0363	0.0286	0.0054	1.0680	1.0416	1.0950	1.0433	1.0927	0.9825	1.1534
16	02-05-19	1.0971	0.0403	0.0295	0.0058	1.0702	1.0419	1.0992	1.0438	1.0966	0.9846	1.1558
17	03-05-19	1.0705	0.0296	0.0298	0.0056	1.0710	1.0438	1.0990	1.0456	1.0965	0.9853	1.1567
18	04-02-19	1.1029	0.0425	0.0308	0.0060	1.0735	1.0443	1.1035	1.0463	1.1007	0.9876	1.1594
19	05-07-19	1.0940	0.0390	0.0318	0.0057	1.0759	1.0481	1.1044	1.0501	1.1017	0.9898	1.1620
20	06-04-19	1.0889	0.0370	0.0319	0.0058	1.0762	1.0480	1.1052	1.0500	1.1024	0.9901	1.1623

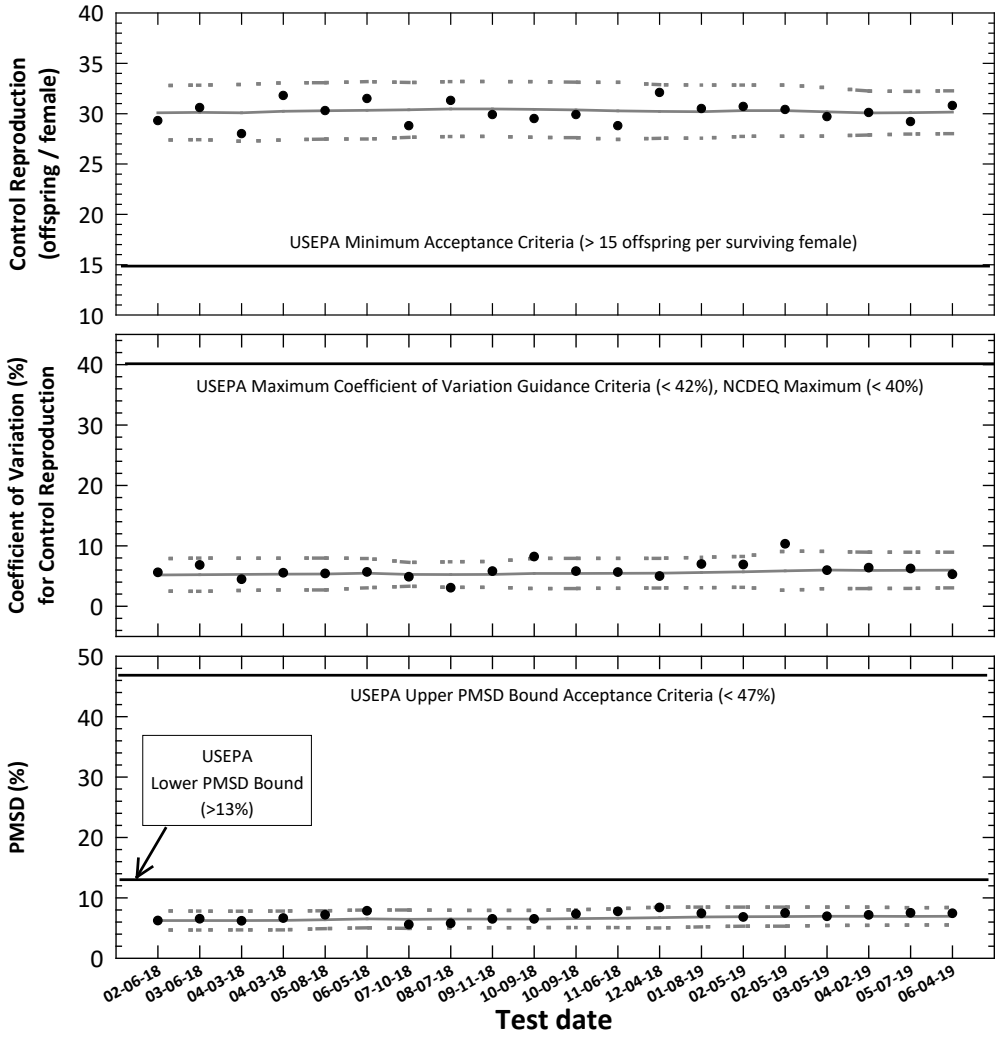
Note: **7-day IC_{25}** = 25% inhibition concentration. An estimation of the sodium chloride concentration that would cause a 25% reduction in *Ceriodaphnia* reproduction (calculated using ToxCalc).
CT = Central tendency of the IC_{25} values.
S = Standard deviation of the IC_{25} values.
Control Limits = Mean logarithmic $IC_{25} \pm 2$ standard deviations converted to anti-logarithmic values.
Warning Limits = Mean logarithmic $IC_{25} \pm 2CV$ or $S_{A.10}$ converted to anti-logarithmic values.
$S_{A.10}$ = Standard deviation corresponding to the 10th percentile of CVs reported nationally by USEPA ($S_{A.10} = 0.08$).
CV = Coefficient of variation.

Subject: *Ceriodaphnia dubia* Chronic Reference Toxicity Test, EPA 1002.0

Ceriodaphnia dubia

Chronic Reference Toxicant Testing, Test Acceptability Criteria

Organism Source: In-house Culture



- **Control Reproduction, Coefficient of Variation (CV) or Percent Minimum Significant Difference (PMSD)**
  PMSD is the percent minimum significant difference between the control and treatment that can be declared statistically significant. The lower PMSD bound represents a practical limit to the sensitivity of the test method and is not a minimum acceptance criteria.
- **Central Tendency** (mean Control Reproduction, CV or PMSD)
- **95% Confidence Interval** (mean Control Reproduction, CV or PMSD ± 2 Standard Deviations)

Entered and Reviewed by Jim Sumner

Subject: *Ceriodaphnia dubia* Chronic Reference Toxicity Test, EPA 1002.0

Ceriodaphnia dubia
Chronic Reference Toxicant Testing, Test Acceptability Criteria
Source: In-house Culture

Test number	Test date	ToxCal Determination					Control Reproduction (offspring/female)			Control Reproduction CV (%)			Test PMSD (%)		
		Control Survival	Control Reproduction Mean	Control Reproduction CV	Test MSD	Test PMSD	CT	95% Confidence Interval CT - 2S	95% Confidence Interval CT + 2S	CT	95% Confidence Interval CT - 2S	95% Confidence Interval CT + 2S	CT	95% Confidence Interval CT - 2S	95% Confidence Interval CT + 2S
		(%)	(offspring/female)	(%)		(%)									
1	02-06-18	100	29.3	5.6	1.824	6.2	30.1	27.4	32.8	5.2	2.5	7.9	6.2	4.7	7.8
2	03-06-18	100	30.6	6.8	1.995	6.5	30.1	27.4	32.8	5.2	2.4	8.0	6.2	4.7	7.8
3	04-03-18	100	28.0	4.5	1.729	6.2	30.1	27.3	32.9	5.3	2.6	7.9	6.2	4.7	7.8
4	04-03-18	100	31.8	5.5	2.108	6.6	30.2	27.4	33.1	5.3	2.7	7.9	6.3	4.7	7.8
5	05-08-18	100	30.3	5.4	2.172	7.2	30.3	27.5	33.1	5.3	2.7	8.0	6.4	4.9	7.8
6	06-05-18	100	31.5	5.6	2.469	7.8	30.3	27.5	33.2	5.5	3.0	7.9	6.5	5.0	8.0
7	07-10-18	100	28.8	4.9	1.598	5.5	30.4	27.7	33.1	5.3	3.3	7.2	6.4	4.9	8.0
8	08-07-18	100	31.3	3.0	1.806	5.8	30.5	27.7	33.2	5.2	3.1	7.4	6.5	5.0	7.9
9	09-11-18	100	29.9	5.8	1.943	6.5	30.5	27.7	33.2	5.3	3.1	7.4	6.5	5.0	7.9
10	10-09-18	100	29.5	8.2	1.912	6.5	30.4	27.7	33.2	5.4	2.9	7.9	6.5	5.1	7.9
11	10-09-18	100	29.9	5.8	2.182	7.3	30.4	27.6	33.1	5.4	2.9	7.9	6.5	5.1	8.0
12	11-06-18	100	28.8	5.6	2.231	7.7	30.3	27.4	33.1	5.4	3.0	7.9	6.6	5.1	8.2
13	12-04-18	100	32.1	5.0	2.687	8.4	30.2	27.6	32.9	5.5	3.0	7.9	6.7	5.0	8.5
14	01-08-19	100	30.5	7.0	2.266	7.4	30.2	27.6	32.8	5.6	3.0	8.1	6.8	5.2	8.5
15	02-05-19	100	30.7	6.9	2.090	6.8	30.3	27.8	32.9	5.7	3.1	8.2	6.9	5.3	8.4
16	02-05-19	100	30.4	10.3	2.273	7.5	30.3	27.8	32.9	5.9	2.6	9.1	6.9	5.3	8.5
17	03-05-19	100	29.7	5.9	2.054	6.9	30.2	27.8	32.6	6.0	2.9	9.1	6.9	5.4	8.5
18	04-02-19	100	30.1	6.4	2.152	7.1	30.1	27.9	32.3	5.9	2.9	8.9	7.0	5.4	8.5
19	05-07-19	100	29.2	6.2	2.188	7.5	30.1	28.0	32.2	5.9	2.9	8.9	6.9	5.5	8.3
20	06-04-19	100	30.8	5.3	2.287	7.4	30.2	28.0	32.3	6.0	3.0	8.9	6.9	5.5	8.4

Note:
Control Survival = USEPA minimum test acceptability criteria ≥ 80% survival.
Control Mean Reproduction = USEPA minimum test acceptability criteria ≥ 15 offspring/surviving female.
CV = Coefficient of variation for control reproduction.
USEPA maximum CV guidance criteria (90th percentile) < 42%. NCDEQ maximum CV acceptance criteria < 40%.
MSD = Minimum significant difference.
PMSD = Percent minimum significant difference.
PMSD is a measure of test precision. The PMSD is the minimum percent difference between the control and treatment that can be declared statistically significant in a whole effluent toxicity test.
Lower PMSD bound determined by USEPA (10th percentile) > 13%.
The lower PMSD bound represents a practical limit to the sensitivity of the test method and is not a minimum acceptance criteria.
Upper PMSD bound acceptance criteria determined by USEPA (90th percentile) < 47%.
CT = Central tendency of the reproduction, CV or PMSD values.
S = Standard deviation of the reproduction, CV or PMSD values.

Subject: Taxonomic Identification of *Ceriodaphnia dubia*

Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan		09-01-19
Quality Assurance Officer	Jim Sumner		09-01-19

Document Revision History

Effective Date	Revision number	Review Type	Evaluators	Revisions
12-01-00	0	Internal	Jim Sumner (ETS)	Original document
07-10-10	1	Internal	Lance Ferrell (NC DENR) Jim Sumner (ETS)	• Exhibit AT15.1 revised for the key taxonomic characteristics of *Ceriodaphnia dubia* and to provide a more efficient logsheet.
06-01-11	2	Internal	Jim Sumner (ETS)	• Updated exhibits.
11-01-14	3	External (TVA) Internal	Jim Sumner (ETS)	• Updated exhibits during document review. • Updated procedure for current slide preparation techniques.
09-01-19	4	Internal	Jim Sumner (ETS)	• Updated procedure to include NELAP requirements. • Additional guidance included in SOP.

Scope and Application

To verify the genus and species of *Ceriodaphnia dubia* cultures used by the laboratory for a source of neonates in toxicity tests.

Summary of Method

Ceriodaphnia dubia are preserved on semi-permanent mounts and the genus and species is verified. Organisms preserved for taxonomic identification are obtained from cultures used for toxicity testing.

Quality Control

The genus and species of *Ceriodaphnia dubia* is verified quarterly. Semi-permenant mounts must be maintained a minimum of 1 year.

Subject: Taxonomic Identification of *Ceriodaphnia dubia*

Equipment and Materials

Adult, *Ceriodaphnia dubia*
1-oz medicine cups
CMC-9AF Mounting Media®, manufactured by Masters Chemical Company
Clear fingernail polish
Glass slides and cover slips
Compound microscope equipped with an oil emersion lens
Pasteur® and transfer pipettes
Bulbs
Forceps
Kimwips®
Ceriodaphnia dubia Taxonomic Log Sheet

Procedure

A. Preparation.

1. Obtain adult *Ceriodaphnia dubia*, which are 7 to 14 days old, from cultures used by the laboratory as a source of neonates in toxicity tests.

2. Prepare the *Ceriodaphnia dubia* Taxonomic Identification Log Sheet (Exhibit AT15.1).

B. Preservation and Semi-Permanent Mounting of *Ceriodaphnia dubia*.

1. Using a Pasteur® pipette, transfer 1 adult *Ceriodaphnia* to a glass slide. Remove any excess water with the pipette.

2. Cover the *Ceriodaphnia* with two drops CMC-9AF Mounting Media® using a transfer pipette.

3. Using a pair of fine tipped forceps, gently position the *Ceriodaphnia* on her side so that the postabdominal claw is easily viewed.

4. Pick up a cover slip using the forceps and place one edge on the slide. Slowly lower the slip to cover the specimen. The media will spread out under the slip.

5. View the specimen under the compound microscope. The postabdominal claw should be visible and extended from the carapace. If it is not, gently tap on the slide directly over the specimen using the forceps. After each tap, view the specimen to determine if

the postabdominal claw has extended from the carapace. Once it is extended and visible, the slide is left to air dry overnight.

6. Repeat steps 1 through 5 with four additional *Ceriodaphnia*.

7. After the slides have dried. Seal the mounts by covering the edges of the cover slips with clear fingernail polish (overlapping the edge by approximately 1 cm). Label the mounted specimens with the species, source of organisms (culture date) and preservation date.

8. Once preserved and mounted, taxonomic identification of the specimens can be performed. The specimen with the most visible taxonomic features is identified and used for the taxonomic identification.

C. Taxomonic Identification.

1. Record the date the taxonomic identification was performed, analyst's initials and source of the mounted specimens on the *Ceriodaphnia dubia* Taxonomic Identification Log Sheet.

2. Place a slide under the compound microscope. Identify each of the key characteristics of *Ceriodaphnia dubia* in the mounted specimens as indicated on the log sheet. Any deviations from these characteristics should be noted. All objective lenses, including the oil immersion lens (1000X magnification), will be necessary to view all of the characters. For additional information on the taxonomic identification of *Ceriodaphnia dubia*, refer to the references sited at the beginning of this SOP.

3. If the key characteristics are not represented in the preserved specimens, preserve and mount additional organisms to confirm the identity. If necessary, an outside taxonomist should be contacted to provide guidance and confirm the discrepancies noted in the specimens.

4. These taxonomic specimens must be maintained in the laboratory for a minimum of 1 year.

Safety and Hazardous Waste Management

Safety glasses, gloves and lab coats should be worn at all times.

Review Policy-P6: General Safety Policy and Policy-P9: Radiation Protection Policy for additional safety requirements.

Subject: Taxonomic Identification of *Ceriodaphnia dubia*

References

USEPA. October 2002. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, 5th ed. EPA-821-R-02-012. US Environmental Protection Agency, Cincinnati, OH.

North Carolina Department of Environment and Natural Resources, Division of Water Quality. Biological Laboratory Certification / Criteria Procedures, Version 3.0. December 2010.

TNI Standard. Management and Technical Requirements for Laboratories Performing Environmental Analysis. EL-V1-ISO-2016-Rev2.0. The NELAC Institute, PO Box 2439, Weatherford, TX 76086.

D. B. Berner, Taxonomy of *Ceriodaphnia* (Crustacean: Cladocera) in US Environmental Protection Agency Cultures. EPA/600/4-86/032. US Environmental Protection Agency, Cincinnati, OH.

R. W. Pennak, *Fresh-Water Invertebrates of the United States, Third Edition*, John Wiley & Sons, Inc., 1989.

H. B. Ward and G. C. Wipple, *Fresh-Water Biology , Second Edition*, John Wiley & Sons, Inc., 1959.

Exhibits

Exhibit AT15.1: *Ceriodaphnia dubia* Taxonomic Identification Log Sheet.

Subject: Taxonomic Identification of *Ceriodaphnia dubia*

Exhibit AT15.1: *Ceriodaphnia dubia* Taxonomic Identification Log Sheet.

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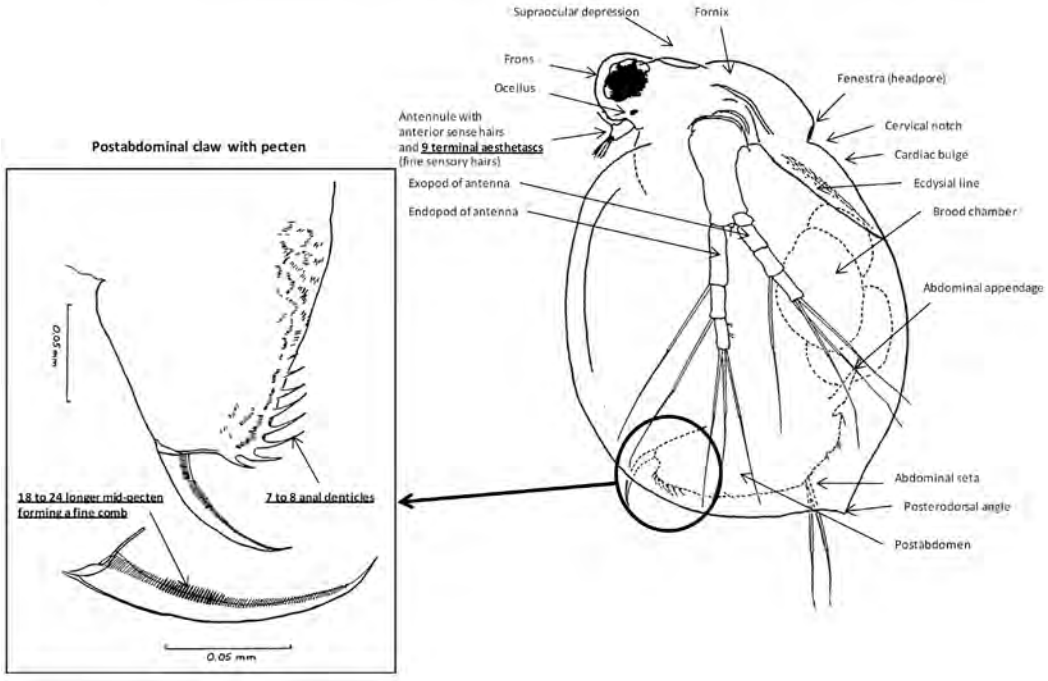
***Ceriodaphnia dubia* Taxonomic Identification Logsheet**

Date identification performed: _____ **Analyst:** _____
Culture source: _____

Key Characteristics	Present (√)	Absent (√)
9 terminal aesthetascs		
7 to 8 denticles		
18 to 24 longer mid-pecten		

Comments:



SOP AT15-Revision 4–Exhibit AT15.1

Subject: Preparation of Newly Hatched Brine Shrimp

Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan		04-25-22
Quality Assurance Officer	Jim Sumner		04-25-22

Document Revision History

Effective Date	Revision number	Review Type	Evaluators	Revisions
12-01-00	0	Internal	Jim Sumner (ETS)	Original document
06-01-11	1	Internal	Jim Sumner (ETS)	• Updated table AT16.1, exhibits, and references.
11-01-14	2	Internal	Jim Sumner (ETS)	• Updated artemia source to Brine Shrimp Direct. Since analytical testing is not performed by this supplier, the supplier certification exhibit was removed. • Updated table AT16.1. • Increased Mysid chronic feeding rate to 100 µl (from 50 µL) per feeding twice daily. • Updated exhibits during document review.
09-01-19	3	Internal	Jim Sumner (ETS)	• Updated procedure to include NELAP requirements. • Additional guidance included in SOP.
03-01-20	4	External (TVA) Internal	Rick Sherrard (TVA) Jim Sumner (ETS)	• Updated table AT16.1 to include the date that each Artemia CHM number was submitted for analytical analyses.
04-25-22	5	Internal	Jim Sumner (ETS)	• Additional feeding guidance added to SOP.

Subject: Preparation of Newly Hatched Brine Shrimp

Scope and Application

To provide food for larvae and shrimp in laboratory cultures and toxicity tests.

Summary of Method

This procedure describes how the laboratory hatches brine shrimp eggs and prepares hatched Artemia nauplii for feeding larvae and shrimp.

Quality Control

Source: Brine shrimp cysts are purchased from Brine Shrimp Direct in Ogden, UT.

New Lots: New lots of brine shrimp (prepared according to section B) must be analyzed for total organochlorine pesticides plus PCBs and metals (Ag, Al, As, Cd, Cr, Co, Cu, Fe, Hg, Pb, Ni and Zn). Chemical analyses are performed on the newly hatched artemia (diluted to the feeding rate) rather than encapsulated cysts to provide the best indication of potential problems in using the artemia as food for other organisms.

USEPA recommends that brine shrimp be verified to contain < 50 ng/L organochlorine pesticides plus PCBs, < 1 µg/L total metal each of Al, As, Cr, Co, Cu, Fe, Pb, Ni, Zn and < 100 ng/L total metal each of Cd, Hg, Ag. Pesticide concentrations should also not exceed USEPA's Ambient Water Quality chronic criteria where available.

Interferences from solids present in the artemia mixture result in detection limits higher than concentrations cited above; however, the lowest available detection limit for each analyte is performed.

Studies performed by UPEPA (March 1982) in several strains of artemia have demonstrated metal and organochlorine pesticide plus PCBs concentrations in each strain above the USEPA recommended criteria. As a result, ETS has determined that quality practices identified in section A.3 will be used to assess the suitability of new lots of artemia. In addition, metal concentrations in new artemia lots will be compared to concentrations in previous lots of artemia (Table AT16.1).

Subject: Preparation of Newly Hatched Brine Shrimp

Table AT16.1: Concentration of metals (µg/L) contained in previous artemia lots prepared by the laboratory. Measured concentration of each analyte in the artemia nauplii mixture at the feeding rate and the estimated final concentration of each analyte in 250 mL test solution at the 150 µL feeding rate are identified in the table below.

Analyte (µg/L)	Artemia Lot: CHM 914, 11-29-16		Artemia Lot: CHM 984, 12-19-17		Artemia Lot: CHM 1048, 08-27-19		Measured concentration in artemia nauplii mixture from previous batches			
	Measured concentration in artemia nauplii mixture	Estimated concentration at feeding rate	Measured concentration in artemia nauplii mixture	Estimated concentration at feeding rate	Measured concentration in artemia nauplii mixture	Estimated concentration at feeding rate	Mean	SD	Mean - SD	Mean + SD
Ag	0	0	0.07	0.000042	0	0	0.03	0.04	**0.00**	**0.07**
Al	11	0.0066	10	0.006	0	0	8.36	5.66	**2.70**	**14.03**
As	**58**	**0.0348**	**69**	**0.0414**	**81**	**0.0486**	77.09	24.76	**52.33**	**101.86**
Cd	0.02	0.000012	0	0	0	0	0.08	0.11	**-0.03**	**0.19**
Cr	0.2	0.00012	2.0	0.0012	2.0	0.0012	1.43	1.23	**0.20**	**2.66**
Co	0.7	0.00042	2.0	0.0012	1.0	0.0006	1.42	0.92	**0.50**	**2.33**
Cu	**17**	**0.0102**	**24**	**0.0144**	**22**	**0.0132**	25.82	8.00	**17.82**	**33.82**
Fe	**330**	**0.198**	**790**	**0.474**	**250**	**0.15**	462.73	216.85	**245.88**	**679.57**
Hg	0.21	0.000126	0.15	0.00009	0.44	0.000264	0.14	0.17	**-0.02**	**0.31**
Pb	0.2	0.00012	0.5	0.0003	0	0	0.25	0.15	**0.11**	**0.40**
Ni	0.4	0.00024	0	0	0	0	0.38	0.41	**-0.03**	**0.79**
Zn	**257**	**0.1542**	**309**	**0.1854**	**381**	**0.2286**	374.27	108.07	**266.21**	**482.34**
Total metal	**674.73**	**0.40**	**1206.72**	**0.72**	**737.44**	**0.44**				

Toxicity checks: When new lots of brine shrimp are purchased, a "toxicity check" must be performed before it is used. Side-by-side reference toxicant tests are used, where *Pimephales promelas* are fed the new lot in first test and *Pimephales* are fed the old lot in the second test (SOP-AT21). Organism survival and growth and test endpoints are compared between the old and new lots. If detrimental effects are noted with the new brine shrimp lot, it must be discarded, and another lot must be ordered.

Subject: Preparation of Newly Hatched Brine Shrimp

Equipment and Materials

Brine shrimp cysts
1000 mL Separatory funnels
50 mL graduated cylinder
250 mL graduated cylinder
Air line
1 mL serological pipette
400 mL plastic beakers
400 mL plastic beaker modified with a fine mesh bottom 105 µm mesh)
Filtration apparatus (vacuum pump, funnel, funnel stand, and tubing)
Membrane filters with grid marks
Forceps
Petri dishes
Dissection microscope
Aquarium pump
Heat lamp
Salt synthetic water
Transfer pipettes
1-oz medicine cups
Freezer
Separatory funnel stand with circular clamps
Artemia Shipment Log Sheet

Procedure

A. Receipt of Brine Shrimp Cysts

1. With each new lot of cysts received by the laboratory, record the following information on the *Artemia* Shipment Log Sheet (see Exhibit AT16.1).
    - Date received at the laboratory
    - Initials of the analyst that received the shipment
    - Lot number
    - Expiration date

2. Store the brine shrimp cysts in a freezer.

3. Cysts must be discarded on the expiration date recommended by the supplier. If an expiration date has not been assigned, the expiration date is 5 years from receipt or until a decrease in hatch-out is observed.

4. On each new lot, a count is performed on the concentration of *Artemia* nauplii obtained through procedures described in section C. To provide an accurate count, the nauplii are filtered onto a gridded membrane filter and then counted under a dissection microscope. Count verification procedures are described below.

   a. Assemble the filtration apparatus. Using forceps, place a 0.45 µm membrane filter, grid side up, onto the center of the funnel stand. Place the funnel on the stand.

   b. Pour approximately 100 mL moderately hard synthetic water (MHSW) into the funnel. Transfer one drop (equal to 50 µL) of *Artemia* nauplii, obtained following procedures described in section B, into the MHSW. This will help to evenly distribute the nauplii on the membrane filter.

   c. Turn on the vacuum pump to draw the water through the filtration apparatus. The nauplii will remain on the membrane filter.

   d. Rinse the sides of the funnel with deionized water to ensure that nauplii did not cling to the sides of the funnel during filtration.

   e. Turn off the vacuum pump and remove the funnel. Using forceps remove the membrane filter and place into a petri dish.

   f. Repeat procedures 4.a through e five times.

   g. Using a dissection microscope, count the number of hatched nauplii and the number of un-hatched nauplii (cysts) on each of the five membrane filters. Record the information on the Artemia Shipment Log Sheet.

   h. Calculate the average number of nauplii contained in one drop (equal to 50 µL) and the percent hatch-out and record on the log sheet.

   i. The number of nauplii contained in one drop (equal to 50 µL) must be 350 to 500. If the counts obtained are not within this range, it may be necessary to adjust the volume of MHSW used to dilute the concentrated brine. Adjustments to the dilution volume must be documented and used for preparing *Artemia* in toxicity tests using the same lot.

   j. The percent hatch-out per drop should be >80% (ideally >90%). If the percent hatch-out is below 80%, the preparation procedures must be examined to determine if temperature or salinity is inhibiting the hatch-out of the *Artemia*. If preparation procedures are not the cause, a new lot of *Artemia* must be ordered.

B. Preparation of Newly Hatched Brine Shrimp.

1. Prepare a batch of brine shrimp cysts each morning and afternoon of the toxicity test.

2. Fill a 1000-mL separatory funnel with approximately 900 mL of salt synthetic water (prepared according to SOP-AT1 with a salinity = 25 ppt).

3. Using a 1-oz medicine cup, add approximately 7.5 to 10 mL of brine shrimp cysts to the salt synthetic water.

4. Place the separatory funnel in the funnel stand.

5. Attach aeration tubing from a pump to a 1-mL serological pipette and position the tip of the pipette in the bottom of the separatory funnel.

6. Turn on the pump.

7. Position a heat lamp at the brine shrimp mixture. The lamp should be approximately 6 to 8 inches away from the brine shrimp mixture. This will ensure proper heating to allow the brine shrimp cysts to hatch.

8. Aerate the mixture for 20 to 23-hours. When the shrimp begin to hatch, the saltwater will become an orange color.

9. Turn off the lamp and pump and allow shrimp to settle for approximately 2 to 3 minutes.

10. Place a 50-ml graduated cylinder at the tip of the funnel and drain the hatched shrimp.

11. Allow the shrimp to settle in the graduated cylinder and determine the volume of concentrated shrimp. Pour the shrimp into a 300-mL plastic beaker modified with a fine mesh bottom (105 µm mesh) and rinse the brine well with MHSW to remove excess salt.

12. After the salt has been removed, rinse the shrimp into a 250 mL graduated cylinder. Bring to a total volume equal to 2 times the volume of concentrated shrimp determined above (C.11) using MHSW and pour into a clean plastic beaker.

13. The concentration of brine shrimp contained in this solution is approximately 350 to 500 nauplii per drop (50 µL). The concentration of nauplii is verified with each new lot of brine shrimp cysts obtained.

C. Feeding Requirements to Test Organisms.

1. The brine shrimp solution obtained in section B must be well mixed during feeding to maintain the correct suspension of nauplii. The transfer pipette must be held horizontal while feeding to prevent the settling of artemia during the feeding process.

2. Culture organisms in jars or tanks within the fathead minnow culture system (AT17) are fed twice daily (at the beginning of the work day prior to renewal and end of the work day following renewal, approximately 6 hours between feedings), until the organisms are used in a toxicity test, approximately 2.5 to 5.0 mL brine shrimp. This volume is dependent on the number of organisms.

3. Acute test organisms must be fed 2 to 5-hours prior to test initiation. Acute tests using *Americamysis bahia* are daily, 100 µL brine shrimp (2 drops). This volume is the equivalent to 700 to 1000 nauplii (approximately 70 to 100 nauplii per mysid daily). 96-hour acute tests are fed 2-hour prior to test solution renewal at 48-hours, 200 µL brine shrimp (4 drops). This volume is the equivalent to 1400 to 2000 nauplii (approximately 140 to 200 nauplii per organism).

4. Chronic tests using *Cyprinodon variegatus*, *Pimephales promelas* and *Menidia beryllina* are fed twice daily (at the beginning of the work day prior to renewal and end of the work day following renewal, approximately 6 hours between feedings), 150 µL brine shrimp (3 drops). This volume is the equivalent to 1050 to 1500 nauplii per feeding.

5. Chronic tests using *Americamysis bahia* are fed twice daily (at the beginning of the work day prior to renewal and end of the work day following renewal, approximately 6 hours between feedings), 100 µL brine shrimp (2 drops). This volume is the equivalent to 700 to 1000 nauplii per feeding (approximately 280 to 400 nauplii per mysid daily).

6. Enough brine shrimp should be provided to assure that some remain alive at the next feeding but not in excessive amounts which will result in the depletion of dissolved oxygen below acceptable levels (< 4.0 mg/L).

Safety and Hazardous Waste Management

Safety glasses, gloves and lab coats should always be worn.

Review Policy-P6: General Safety Policy and Policy-P9: Radiation Protection Policy for additional safety requirements.

Subject: Preparation of Newly Hatched Brine Shrimp

References

USEPA. October 2002. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, 5th ed. EPA-821-R-02-012. US Environmental Protection Agency, Cincinnati, OH.

USEPA. October 2002. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, 4th ed. EPA-821-R-02-013. US Environmental Protection Agency, Cincinnati, OH.

USEPA. October 2002. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms, 3rd ed. EPA-821-R-02-014. US Environmental Protection Agency, Cincinnati, OH.

USEPA. March 1982. International Study of Artemia VIII. Comparison of Chlorinated Hydrocarbons and Heavy Metals in Five Different Strains of Newly Hatched Artemia and a Laboratory-Reared Marine Fish. EPA-600-D-82-219. US Environmental Protection Agency, Narragansett, RI.

USEPA. 2009. National Recommended Water Quality Criteria. US Environmental Protection Agency, Cincinnati, OH (or most current criteria).

TNI Standard. Management and Technical Requirements for Laboratories Performing Environmental Analysis. EL-V1-ISO-2016-Rev2.0. The NELAC Institute, PO Box 2439, Weatherford, TX 76086.

Exhibits

Exhibit AT16.1: *Artemia* Shipment Log Sheet.

Exhibit AT16.2: *Artemia* Shipment Log Sheet.

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Artemia Shipment Log

Shipment receipt:

Source:	Brine Shrimp Direct
Lot number:	
Date received:	
Expiration date:	
Received by (initials):	
Analytical submitted:	
Reference testing performed:	

Artemia count verification:

 # contained in 1 drop or 50 µL
1 drop should contain approximately 350 to 500 nauplii

Replicate	# Hatched *Artemia*	# of Cysts	% Hatch-out
1			
2			
3			
4			
5			
Average:			

SOP AT16-Revision 4–Exhibit AT16.1

Subject: Maintenance of Fathead minnow (*Pimephales promelas*) Cultures

Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan		08-28-23
Quality Assurance Officer	Jim Sumner		08-28-23

Document Revision History

Effective Date	Revision number	Review Type	Evaluators	Revisions
04-01-09	0	Internal	Jim Sumner (ETS)	Original document
06-01-11	1	Internal	Jim Sumner (ETS)	• Updated exhibits.
11-01-14	2	Internal	Jim Sumner (ETS)	• Updated exhibits during document review.
02-01-16	3	External (TVA) Internal	Jim Sumner (ETS)	• Added a separate procedure was added for the maintenance of an in-house minnow culture.
09-01-19	4	Internal	Jim Sumner (ETS)	• Updated procedure to include NELAP requirements. • Additional guidance included in SOP. • Removed procedures for obtaining minnows from an outside supplier.
08-28-23	5	Internal	Jaydon Perez (ETS)	• Added Identification section under Fathead Minnow Stock • Removed sump procedure. • Removed Separation section under Fathead Minnow Stock • Added Dominant female section • Updated Exhibit AT17.1 *Pimephales promelas* Culture Maintenance Logs • Updated Exhibit AT17.2 *Pimephales promelas* Culture Chemistry Log • Added Hayward® Sand Filter section • Added Hayward® PowerFlo Matrix® Pump section • Added Figure AT17.1 Fathead minnow (*Pimephales promelas)* Recirculating Culture System

Scope and Application

To maintain healthy cultures of Fathead minnows (*Pimephales promelas*).

Summary of Method

This procedure describes how the laboratory maintains fathead minnow cultures as well as collects eggs and hatches and maintains larvae used for testing.

Subject: Maintenance of Fathead minnow (*Pimephales promelas*) Cultures

Quality Control

Test Organism Quality: Reference toxicant tests are performed monthly using EPA approved reference toxicants. A continuously updated control chart will show trends of changing sensitivity of culture/test organisms and can indicate problems with the cultures. Reduced weight of control organisms is another indicator of problems with the stock animals which should be monitored carefully.

Culture Water Quality: The quality of make-up water used for synthetic water preparation must be checked whenever health or performance of fish in the culture unit or fish used in testing is suspect.

All organisms brought into the culture unit should be taxonomically identified (SOP-AT22) to ensure that only *Pimephales promelas* are used. Identification of organisms brought as juveniles should be delayed until they are about 4-6 months old. Presence of an incomplete lateral line confirms the specific identification. Identification and verification of raised fish is performed a minimum of yearly.

Procedure

Fathead minnows cultured in-house is advantageous for quality test results, since the history of a given lot of eggs, larvae or juveniles is known. Culturing fathead minnows at ETS for aquatic toxicity tests provides organisms that are documented disease free, of known age and origin, have a documented genetic background, are reared in controlled physical, chemical and nutritional conditions and whose sensitivity is tracked using reference toxicants.

A. Interferences / Special Considerations

1. Pathogenic organisms may be introduced to the culture unit through improper equipment disinfection or improper isolation and treatment of outside fish brought into the culture unit. Failure to conduct routine maintenance in a timely fashion will also increase the likelihood and severity of colonization of the system by organisms introduced through food, air, water, etc. thus increasing the chances of problems resulting from these organisms.

2. Failure to bring in new fish from an outside source every few years may result in disease, reduced egg production or poor-quality embryos/larvae due to the limited gene pool.

3. Low dissolved oxygen levels and temperature fluctuations may result from malfunction or failure to clean components of the recirculating system.

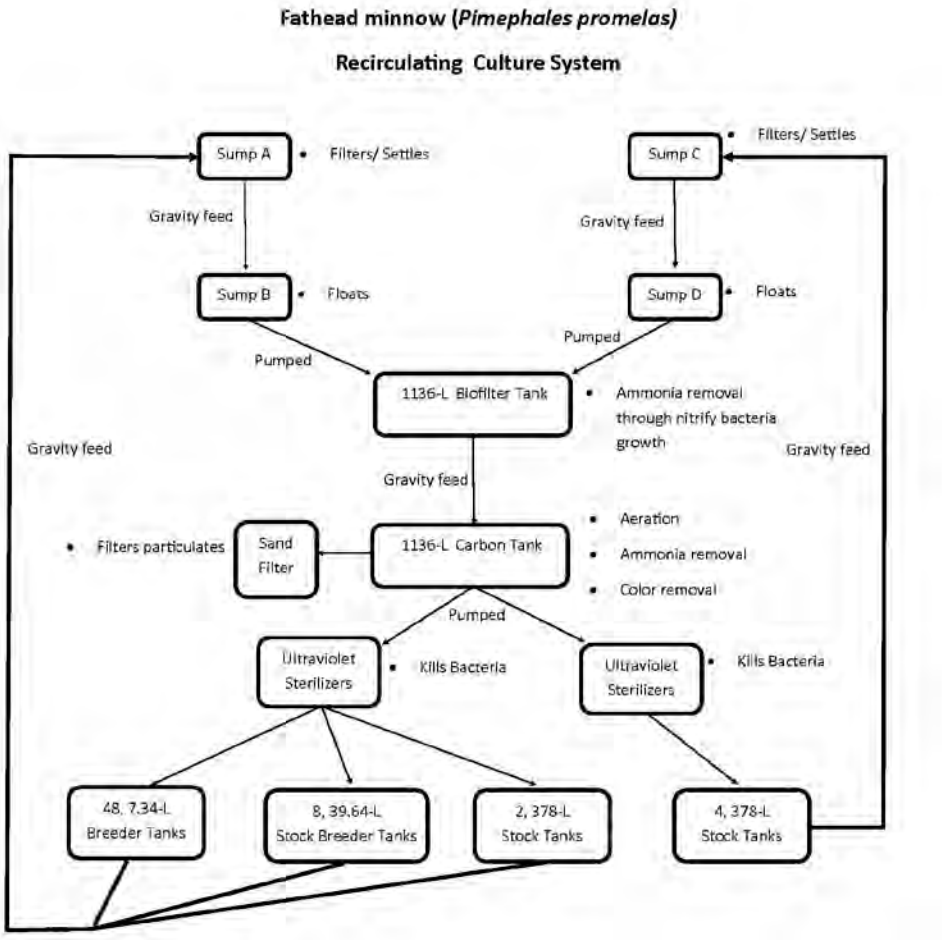
4. Low pH (< 7.0 S.U.) will result in reduced spawns and low-quality embryos. $NaHCO_3$ may be added to increase the pH back to > 7.5 S.U. when low pH occurs.

5. Fungal growth on eggs must be completely removed. Thoroughly rinsing of eggs after their removal from the system will help decrease fungal growth.

B. Recirculating Culture System

A recirculating system outlined in Figure AT17.1 is used to provide flow, remove metabolic wastes and ammonia and disinfect synthetic water used in the system.

Figure AT17.1: Fathead minnow *Pimephales promelas* Recirculating Culture System.



Subject: Maintenance of Fathead minnow (*Pimephales promelas*) Cultures

The total system volume is approximately 4500-L. Maintenance activities are recorded in the *Pimephales promelas* Culture Maintenance Log (Exhibit AT17.1). The system is comprised of the following components:

Water Supply: Culture water for fathead minnows is hard synthetic water made by adding reagent grade chemicals to deionized water (reagent water: SOP-G8, hard synthetic water: SOP AT1). 50-L Nalgene tanks serve as reservoirs for preparation and storage of culture water. On a weekly or bi-weekly basis, dissolved oxygen (SOP-C2), pH (SOP-C3), conductivity (SOP-C4), alkalinity (SOP-C6) and hardness (SOP-C7) are measured in the carbon tank and results are recorded in the *Pimephales promelas* Culture Water Chemistry Log (Exhibit AT17.2). Adjustments are made as necessary to correct any problems found (i.e. adjust thermostat, add $NaHCO_3$, increase aeration, exchange culture water.)

Sumps: The sumps receive wastewater from all culture tanks. Small particles are then removed through filter pad material attached to plexiglass stands. Filter pad material is rinsed and replaced as needed. Settled material that collects in the bottom of the sumps is also removed by siphon or net as needed. Wastewater which passes through the sump containing the filter material spills into a second sump, which is aerated. Water is pumped from the bottom of the second sump and sprayed into the top of the biofilter tank.

Hayward® High Rate Sand Filter: The sand filter uses media filter balls to remove debris from water. Water enters the filter using a Hayward PowerFlo Matrix® Pump where particles are trapped and filtered out of the system. Cleaned water is returned to the system. Accumulation of particles causes flow resistance requiring a periodic cleaning (backwashing) process.

Hayward PowerFlo Matrix® Pump: The filter pump flows water from system into filter. The pump traps large debris using a strainer basket.

Biofilter tank: The biofilter is a 300-gallon rectangular plastic tank containing plexiglass channels. The channels divert water from left to right and from top to bottom and contain plastic BioBalls. The 1.25 cm plastic BioBalls provide a high surface area for nitrifying bacteria growth. A total of 9000 BioBalls are contained in the BioFilter tank. Two independent water pumps pull water from the bottom of the biofilter and spray the water over the surface of the biofilter. BioBalls must be removed and washed periodically or when large amounts of food residue begin coming through the water delivery system. The biofilter tank is encapsulated in black plastic to prevent algal growth and for optimal bacterial growth on the BioBalls.

Carbon Tank: Water is gravity fed from the end of the biofilter tank to a second 300-gallon rectangular plastic tank. Water in this tank is vigorously aerated. In addition, two independent water pumps circulate water from the tank through two PVC columns containing activated

carbon. This provides additional ammonia removal and removes color. The carbon tank is washed, and activated carbon replaced as needed.

Ultraviolet Sterilizers: Water from the carbon tank is pumped through two UV sterilizers to provide clean water to the culture system. Two independent pumps are used to pump water from the carbon tank through the UV sterilizers. UV sterilizers operate continuously to kill potentially harmful bacteria circulating through the system. The top of the unit is transparent to view whether the internal lamp is operating. Maintenance consists of periodic replacement of the internal lamps. If the main system circulating pump is off for more than 30 minutes, the UV sterilizer should also be turned off prevent overheating and possible lamp damage.

External Pumps: Flow and pressure are provided by two external pumps and flow rate is regulated by ball valves at each culture tank.

Temperature Control: Temperature control (25.0 ± 2.0°C) is achieved through the thermostat temperature maintained in the culture room.

Culture Tanks: The system is equipped with:

- 42, 7.34-L and 8, 39.64-L plastic tanks for spawning
- 6, 7.34-L plastic tanks for raising larval minnows for testing
- 6, 1136-L fiberglass tanks for holding extra spawners and juvenile fish reared for future use as replacement spawners.

Standpipes: Each tank is fitted with an overflow standpipe which causes outflow water to be drawn from the bottom of the tanks.

Cleaning Culture Tanks: Accumulated wastes (excess food and solid metabolic material) are siphoned as needed. Tanks are removed from the system and cleaned as needed.

Spawning Substrates: Fathead minnows naturally spawn adhesive eggs on the undersides of submerged objects. PVC tiles are provided for spawning surfaces. Tiles are constructed from 7.6 cm diameter pipe cut 3 ½ inches in length and split longitudinally. The concave surface is roughened with a wire brush, sandpaper or other means to give a surface suitable for eggs to adhere.

Aeration: Air pumps operate continuously. Aeration is provided to each tank to maintain dissolved oxygen levels if water circulation is lost. Maintenance is limited to cleaning the intake air filter as needed.

Lighting: Light is provided by cool-white and/or broad-spectrum fluorescent lights. Light intensity is maintained at 50 to 100 ft-candles at the water surface. Electric timers are used to

provide a photoperiod of 16-hours light to 8-hours dark (light from 0500 to 2100). An electric timer is used for overhead lighting to simulate dawn and dusk (light from 0450 to 2110).

C. Fathead Minnow Stock

Adults Sources: Adults may be obtained from commercial sources or by rearing young in the laboratory either from brood ETS stock or from commercial sources. Sources for consideration will only be those with a history of healthy fish and preferably from a laboratory synthetic water system rather than an outside pond culture. Use of laboratory raised fish reduces the need for precautionary treatment and the risk disease or parasites contamination. New organisms must be brought in every few years to maintain genetic diversity.

Initial stock organisms were obtained from two sources I.F. Anderson Farms, Inc. in Lonoke, AR and larvae obtained from Aquatox, Inc. in Hot Springs, AR (which were raised to adulthood).

Acclimation: Fish are brought to ambient laboratory temperature at < 2°C change per hour. Make three or four, 50 percent exchanges of transport water to ETS synthetic water.

Treatment: Pond raised fish are dipped into a two percent (w:w) NaCl solution until stressed (usually < 2 minutes), then placed in fresh synthetic water for recovery. Fish are then put into an aquarium with 0.5 percent (w:w) NaCl for 24 hours and observed. Several spawning tiles placed in the aquarium serve as hiding areas for fish and reduce stress levels. Dead or moribund individuals and those exhibiting unusual behavior (erratic, sluggish) should be discarded immediately. After the initial treatment, water is exchanged in 50 percent portions every 24 hours to dilute the NaCl solution to synthetic water only. Laboratory raised fish may not be subjected to this precautionary salt treatment.

Isolation: All fish (both laboratory and pond raised) brought into the culture unit are kept isolated from the main system for 30 days. All equipment used in handling outside fish is disinfected after each use and kept separate for that use only. If no signs of disease are evident at the end of the isolation period, the fish may be moved into the system for use as spawners. Note: A glass tank is preferable for holding fish during the isolation period to allow easy observation for signs of disease. If fish are held in a fiberglass tank, they should be moved to a glass tank 3-4 days prior to integration into the system so they may be carefully observed.

Identification: At maturity, fathead minnow males are easily recognized by their relative large size, tubercles on the head, and dusky to dark color with vertical banding on the sides. Females

show no coloration, tend to have a distended abdomen if egg laden, and have a visible genital papillae when mature. Individuals are selected for spawning from these adults.

Spawning: One mature male and five mature females are placed in each spawning tank along with two spawning tiles. The male will clean one tile, entice a female to spawn, and guard eggs. The second tile is used by females for hiding. If individuals in spawning tanks do not perform satisfactorily, they should be either discarded or returned to the respective holding tank.

Adominant Males: Immature males may be mistaken for females and placed in spawning tank. The adominant male is readily identified within several days by territorial fighting with the mature male. Immediately return the young male to the holding tank.

Dominant Females: Females may develop dark vertical banding on sides in spawning tank. The dominant female is readily identified within several days by territorial fighting with the mature male and females. Immediately return the dominant female to the holding tank or remove from system.

Daily Observation: Both tiles in each spawning tank are checked daily for spawns. If eggs are present, the tile is removed and replaced with a clean tile. Either discard eggs and wash tiles or save spawns. Record all spawns in appropriate logbooks.

D. Disease and Clinical Signs

Indications that pathogenic organisms are present include abnormal behavior such as lack of feeding, reduction in feeding intensity, sluggish swimming, and reduction in number and size of spawns. External physical signs such as hemorrhage, fin erosion, and changes in normal coloration and pattern generally indicate an onset of disease.

Diagnostics: Definitive identification of pathogenic organisms and the methods to be used for eradication are best left to a trained and experienced clinician. When disease or parasites are suspected, representative samples of fish from the system can be sent to the University of North Carolina, Asheville or other acceptable facility for examination and diagnosis. Contact the staff at the facility before transporting the organisms to determine if they need live fish or what method of preservation to use, as well as to assure someone will be available to process the samples upon arrival. Several good references are available at ETS for use in preliminary or emergency evaluation of suspected problems.

Treatment: When serious disease or parasite infestations have been confirmed, the preferable course of action is to discard all affected fish and replace them with new stock. Several treatments are available that could be used on a limited basis:

- Sodium Chloride: The safest and most effective treatment for external protozoan and metazoan parasites is NaCl at a concentration of 0.5 percent (w:w) for up to 24 hours. Non-iodized table grade salt is adequate and effective. Lesser grades such as rock or agricultural grade salt may contain impurities and should not be used. Aquaria and recirculating system components should be drained as low as possible without stressing fish and refilled with fresh culture water after treatment. Water should be drained and replaced daily until conductivity returns to normal.

- Potassium Permanganate: Potassium permanganate (KMn04) is a less desirable but effective treatment for external parasites and some external bacteria. Add 1 mL/L of 1 percent KMn04 solution for 30 minutes, then neutralize with 0.01 mL/L of 0.1 N sodium thiosulfate. Drain and refill aquaria and system components as previously described for sodium chloride. Treatment should be conducted on two consecutive days without feeding. Potassium permanganate oxidizes organic material, and feeding would add organic material to the water reducing treatment effectiveness.

E. Eggs/Embryos, Hatching and Larvae Collection

Collection: Collect tiles with eggs from the spawning tanks daily. Since most of the spawning activity occurs in the mornings following turning on the lights, it is best to disturb the fish as little as possible during morning hours. Limit activity in the fish lab following the first feeding and check the tiles for eggs around noon. Record in the Culture Spawning and Hatching Log (Exhibit AT17.4). Rinse each tile thoroughly with fresh synthetic water to remove all particles of food and debris.

Incubation: Place tiles on end in clean plastic trays with enough synthetic water to cover tiles. Label each tray with the spawn date and record the date, origin and number of tiles collected in the Spawning and Hatching Log. Place two air basr into each tray between the tiles. Aerate vigorously to help prevent fungal infection.

Maintenance: Fungal infection is the most common threat to successful maturation of eggs using this method. Fungus is a light-colored, filamentous organism that gives a cottony appearance in water. At least once daily check all tiles in the trays for infected or unfertilized eggs. Remove these eggs (alive or dead) using forceps, a spatula, probe, plastic pipette, or other similar instrument without disturbing healthy eggs. Rinse eggs with synthetic water and return the tile to the tray and continue incubation.

Hatching: Embryos will develop visible eyes in 36-48 hours and will generally begin hatching after 5-6 days of incubation. Check trays for larvae as hatching time nears and collect larvae at appropriate intervals to provide organisms of specific ages to be used as described for the specific test method. Record in the Culture Spawning and Hatching Log (Exhibit AT17.4).

Larvae Collection: Remove tiles from hatching trays when approximately 90-100 percent of embryos have hatched or within 24 hours of first hatching. Larvae of the same age are pooled into a larval tank maintained in within the recirculating culture system.

Holding: Larvae are fed twice daily and may be maintained in the culture system for up to 14-days.

Health: Fungus is typically not a problem after embryos hatch. If larvae do become fungus-infested or show any other signs of disease or poor quality, destroy the entire affected lot and disinfect containers and equipment.

F. Juveniles

Every 4-6 months larvae from three or more spawns can be reared for use as replacement spawners. These fish are placed in fiberglass tanks maintained within the recirculating culture system. Place spawning tiles in all tanks with juveniles to provide cover and induce maturation as soon as possible. Feed juveniles a minimum of twice daily and maintain tanks as described for larvae or adults depending on the method of holding. Overcrowding of these fish will both contribute to water quality problems and slow growth. Recommended densities are 25-50 fish/L up to 30-days old and 5-l0 fish/L from 30-days to 3 to 4 -months old.

G. Food and Feeding

Types of Food:

- Live Brine Shrimp (Artemia nauplii) < 24-hours old are obtained by hatching Artemia cysts, according to SOP-AT16. Nauplii are used for feeding larval fathead minnows. Nauplii < 48-hours old can be fed to older fish (> 1 month old)

- Frozen Adult Brine Shrimp: Thaw frozen brine shrimp in a plastic beaker until just thawed. Use a disposable transfer pipet to dispense shrimp to adult fathead minnows. As a general guide, feed each tank of fish the amount of food that can be consumed in about 10 – 20 minutes.

- Flake Food: Tropical fish food flakes are used to supplement frozen brine shrimp in feeding of adult fathead minnows. Flake food may also be finely ground to supplement the live brine shrimp diet of juvenile fish.

- Trout Chow: Small, granular trout chow is also used to supplement frozen brine shrimp in feeding of adult fathead minnows.

- Periphyton that grows naturally in the tanks also provides a good dietary supplement for the fish.

Frequency of Feedings: Exact frequency and food volumes are adjusted based on fish age and numbers. Food should be available, but not in such excess that waste is a threat to fish health and system chemistry. Feeding activities are recording in the Culture Feeding Log (Exhibit AT17.3) General guidelines for feeding are as follows:

- Larvae: Feed larvae in culture ad libitum two or three times daily (one or two times on weekends) beginning 12-24 hours after hatching. Feeding times are recorded in the *Pimephales promelas* Culture Feeding Log (Exhibit AT17.3).

- Juveniles: Feed juveniles 2-3 times daily (1 or 2 times on weekends) ad libitum using live brine shrimp or ground flake food.

- Adults: Feed adults prepared frozen brine shrimp once and flake food or trout chow 2-3 times daily.

H. Equipment Cleaning and Disinfection

Brushes, and Miscellaneous Cleaning Equipment: Items are cleaned and disinfected after each use by one of the three methods listed below.

- Iodine: Two solutions may be used, one adjusted to pH 5.8-6.0 S.U. (bactericide), the other to pH 8.0-8.2 S.U. (viricide). Both solutions are an approximate concentration of 200 mg iodine/L (60 mL of 1 percent iodine in Section 3.5.1). Tap water is used for dilution. Equipment should be soaked at least five minutes in solution. After soaking in solution, rinse well with tap water, then synthetic water.

- Sodium Chloride Saturated Solution: Soak at least five minutes, rinse well with tap water, then synthetic water.

- Sodium Hypochlorite: Soak equipment in solution of approximately 12 g sodium hypochlorite/L for one hour followed by a tap water rinse. Neutralize residual chlorine by allowing items to dry completely after the tap water rinse.

Tiles, Beakers, Siphon Tubes, Air stones, etc.: Scrub items using a brush and hot, soapy water, and rinse well with tap water. Disinfect by soaking, as described above.

Subject: Maintenance of Fathead minnow (*Pimephales promelas*) Cultures

Safety and Hazardous Waste Management

Safety glasses, gloves and lab coats should be worn at all times.

Review Policy-P6: General Safety Policy and Policy-P9: Radiation Protection Policy for additional safety requirements.

References

USEPA. January 1987. Guidelines for the Culture of Fathead Minnows (*Pimephales promelas*) for Use in Toxicity Tests. EPA-600-3-87-001. US Environmental Protection Agency, Duluth, MN.

USEPA. December 2006. Culturing of Fathead Minnows (*Pimephales promelas*), Supplement to Training Video. EPA-833-C-06-001. US Environmental Protection Agency, Washington, DC.

Davis, H.S. 1970. Culture and Diseases of Game Fishes, University of California Press, Berkeley, CA.

USEPA. October 2002. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, 5th ed. EPA-821-R-02-012. US Environmental Protection Agency, Cincinnati, OH.

USEPA. October 2002. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, 4th ed. EPA-821-R-02-013. US Environmental Protection Agency, Cincinnati, OH.

TNI Standard. Management and Technical Requirements for Laboratories Performing Environmental Analysis. EL-V1-ISO-2016-Rev2.0. The NELAC Institute, PO Box 2439, Weatherford, TX 76086.

Exhibits

Exhibit AT17.1: *Pimephales promelas* Culture Maintenance Logs.
Exhibit AT17.2: *Pimephales promelas* Culture Water Chemistry Log.
Exhibit AT17.3: *Pimephales promelas* Culture Feeding Log.
Exhibit AT17.4: Weekly *Pimephales promelas* Spawning / Egg Collection Log.

Subject: Maintenance of Fathead minnow (*Pimephales promelas*) Cultures

Exhibit AT17.1: *Pimephales promelas* Culture Maintenance Logs.

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Pimephales promelas **Daily Culture Maintenance, Week of August 27, 2023**
(Initial Each Task Completed)

Note: Wash hands/arms prior to placing hands into tanks.

	Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
System Maintenance							
Temperature (°C), Acceptable range = 25.0 ± 2.0°C							
Check and repair: leaks, water flow / aeration in all tanks, amber alarms?, all pumps flowing							
Backwash sand filter, add DI water to system, add chemicals							
Larvae Maintenance							
If larvae are hatching: move unhatched larvae on tiles to next spawn date, record end hatch time for this spawn date in egg collection log.							
Ensure all tiles are upright, remove fugus from tiles and tiles containing unfertilized eggs in all trays. Remove tiles completely hatched and soak in bleach solution.							
If there are no empty larval tanks, remove and discard oldest larvae batch.							
Transfer hatched larvae to a new tank and place into culture system.							
Remove any dead larvae or food in all larval tanks.							
Feed larvae artemia (For 3 or more larval tanks, prepare AM and PM artemia separatory funnels.), remake artemia if necessary, record time in feeding log.							
Egg Collection and Maintenance							
Check all tanks for eggs, remove tiles with eggs, clean back of each tile, rinse with tap water and place in new tray with HSW, label with today's date (spawn date)							
Record the spawn date/time and tank ID's where eggs were collected, replace missing tiles in tanks							
Make 1 tray for next day							
Adult Minnow Maintenance							
Feed adults (AM feeding): flake food, if collecting tiles: feed frozen food to breeder tanks, record time in feeding log							
Remove unhealthy or dead minnows, replace using stock tanks 1-8, note which tank(s) and how many minnows were taken on chalk board, restock stock tanks 1-8 using black stock tanks							
Mid-Day							
Feed adults (MID-DAY feeding): flake food, record feeding time in feeding log							
Check if larvae are starting to hatch in tray with oldest spawn date, record start hatch time for this spawn date in egg collection log							
End-of-Day							
Remove any dead larvae or food in all larval tanks							
Feed larvae artemia and remake artemia, record feeding time in feeding log							
Feed adults (PM feeding): flake food, record feeding time in feeding log							
Remove unhealthy or dead minnows, replace using stock tanks 1-8, note which tank(s) and how many minnows were taken on chalk board, restock stock tanks 1-8 using black stock tanks							
DI system valve and garden hose valves off							
Temperature (°C), Acceptable range = 25.0 ± 2.0°C							
Front and metal doors locked							
Check and repair: leaks, water flow / aeration in all tanks, amber alarms?, all pumps flowing							
Comments							

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Subject: Maintenance of Fathead minnow (*Pimephales promelas*) Cultures

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***Pimephales promelas* Culture Maintenance**

	Oct 2023	Nov 2023	Dec 2023
Breeder Tanks (replace tank, air stone/weight, clean air line/white shelving and PVC drain pipes)			
Breeder tanks: A 1-6, verify 1 male to 5 females per tank			
Breeder tanks: B 1-6, verify 1 male to 5 females per tank			
Breeder tanks: C 1-6, verify 1 male to 5 females per tank			
Breeder tanks: D 1-6, verify 1 male to 5 females per tank			
Breeder tanks: E 1-6, verify 1 male to 5 females per tank			
Breeder tanks: F 1-6, verify 1 male to 5 females per tank			
Breeder tanks: G 1-3, H 1-3, verify 1 male to 5 females per tank			
Larval tanks: G 4-6 and H 4-6 (Clean Shelf)			
Stock tanks: 1-2, verify 4-5 males to 14-20 females per tank			
Stock tanks: 3-4, verify 4-5 males to 14-20 females per tank			
Stock tanks: 5-6, verify 4-5 males to 14-20 females per tank			
Stock tanks: 7-8, verify 4-5 males to 14-20 females per tank			
Black Stock Tanks (clean sides, replace standpipes/air bars and weights, clean airline tubing)			
Black stock tank: 1			
Black stock tank: 2			
Black stock tank: 3			
Black stock tank: 4			
Black stock tank: 5			
Black stock tank: 6			
Sumps			
Clean filters in sumps (remove and spray with hose to remove solids)			
Replace filters in sumps			
Clean sides of sumps and sump covers			
Vacuum solids out of sumps			
Clean screens to pump intakes			
Clean floats			
Treatment System			
Clean UV lights and verify lights are on			
Bio-Tank (clean sides/airline tubing, replace air stones/weights)			
Carbon Tank (clean sides/airline tubing, replace air stones/weights)			
Carbon tube: replace carbon, clean PVC pipe (inside and outside), clean tubing and resecure coupling to PVC pipe			
Clean water delivery gutters in bio-tank			
Flush drainpipes			
Pumps			
Rotate pump order			
Oil pumps (4-5 drops vacuum pump oil to each side)			
General Housekeeping			
Stock bathroom (toilet paper, paper towels)			
Make bleach			
Clean bathroom			
Refill soap dispensers			
Clean counters, artemia table and light table			
Clean top and bottom shelves of culture system			
Sweep and vacuum			

Comments:

Subject: Maintenance of Fathead minnow (*Pimephales promelas*) Cultures

Exhibit AT17.2: *Pimephales promelas* Culture Water Chemistry Log.

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***Pimephales promelas* Culture Water Chemistry Log**

Date	Analyst	Tank ID	Parameter					Chemical Addition based on Alkalinity/Hardness Values Obtained (Desired Alkalinity and Hardness >100 mg/L $CaCO_3$)		
								Alkalinity	Hardness	
			pH (S.U.)	Dissolved Oxygen (mg/L)	Conductivity (µmhos/cm)	Alkalinity (mg/L $CaCO_3$)	Hardness (mg/L $CaCO_3$)	Sodium bicarbonate ($NaHCO_3$) (g)	Calcium sulfate dehydrate ($CaSO_4 \cdot 2H_2O$) (g)	Magnesium sulfate ($MgSO_4$) (g)

Chemical analyses:

Parameter	Reporting Limit	Method number	Meter	Serial number
pH	0.1 S.U.	SM 4500-H+ B-2011	Accumet AR20	93312452
Dissolved Oxygen (D.O.)	1.0 mg/L	SM 4500-O G-2016	YSI Model 52CF	15D104324
Conductivity	14.9 µmhos/cm	SM 2510 B-2011	Accumet AR20	93312452
Alkalinity	5.0 mg $CaCO_3$/L	SM 2320 B-2011	Accumet AR20	93312452
Hardness	5.0 mg $CaCO_3$/L	SM 2340 C-2011	Not applicable	Not applicable

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Subject: Maintenance of Fathead minnow (*Pimephales promelas*) Cultures

Exhibit AT17.3: *Pimephales promelas* Culture Feeding Log.

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Pimephales promelas Culture Feeding Log, Month: December 2023

Day	AM Time	Food Type (check)			Analyst	Mid-day Time	Food Type (check)			Analyst	PM Time	Food Type (check)			Analyst
		Artemia nauplii	Frozen or Freeze Dried Brine Shrimp	Flake Food			Artemia nauplii	Frozen or Freeze Dried Brine Shrimp	Flake Food			Artemia nauplii	Frozen or Freeze Dried Brine Shrimp	Flake Food	
1															
2															
3															
4															
5															
6															
7															
8															
9															
10															
11															
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26															
27															
28															
29															
30															
31															

Artemia nauplii: Brine Shrimp Eggs (Brine Shrimp Direct), Frozen Brine Shrimp: Flat Packs (Brine Shrimp Direct), Freeze Dried Brine Shrimp: Cubes (Brine Shrimp Direct), Flake Food: Aquatox Fish Diet (Zeigler)

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Subject: Maintenance of Fathead minnow (*Pimephales promelas*) Cultures

Exhibit AT17.4: Weekly *Pimephales promelas* Spawning / Egg Collection Log.

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Weekly *Pimephales promelas* Spawning / Egg Collection Log

Date (Spawn date and Batch ID)	Time	Analyst	Total # Tiles	Start Hatching		End Hatching		Comments
				Date	Time	Date	Time	
09-24-23								Large stock: 1 ___, 2 ___, 3 ___, 4 ___, 5 ___, 6 ___ Removed larvae hatched prior to: _____ Analyst _____
09-25-23								Large stock: 1 ___, 2 ___, 3 ___, 4 ___, 5 ___, 6 ___ Removed larvae hatched prior to: _____ Analyst _____
09-26-23								Large stock: 1 ___, 2 ___, 3 ___, 4 ___, 5 ___, 6 ___ Removed larvae hatched prior to: _____ Analyst _____
09-27-23								Large stock: 1 ___, 2 ___, 3 ___, 4 ___, 5 ___, 6 ___ Removed larvae hatched prior to: _____ Analyst _____
09-28-23								Large stock: 1 ___, 2 ___, 3 ___, 4 ___, 5 ___, 6 ___ Removed larvae hatched prior to: _____ Analyst _____
09-29-23								Large stock: 1 ___, 2 ___, 3 ___, 4 ___, 5 ___, 6 ___ Removed larvae hatched prior to: _____ Analyst _____
09-30-23								Large stock: 1 ___, 2 ___, 3 ___, 4 ___, 5 ___, 6 ___ Removed larvae hatched prior to: _____ Analyst _____

Note: Unhatched eggs on tiles at the end hatch time are moved to the next spawn date to allow the remaining eggs to hatch. These eggs will all hatch in < 24 hours.

Egg Collection Source by Spawn Date:

Date (Spawn date and Batch ID)	Tank ID (X locations where tiles with eggs were collected)																																					
	A						B						C						D						E						F						G		H		Stock							
	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	1	2	3	1	2	3	4	5	6	7	8
09-24-23																																																
09-25-23																																																
09-26-23																																																
09-27-23																																																
09-28-23																																																
09-29-23																																																
09-30-23																																																

Comments:

SOP AT17 Revision 5-Exhibit AT17.4

Subject: *Pimephales promelas* Acute Toxicity Test, EPA 2000.0

Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan		03-01-20
Quality Assurance Officer	Jim Sumner		03-01-20

Document Revision History

Effective Date	Revision number	Review Type	Evaluators	Revisions
12-01-00	0	Internal	Jim Sumner (ETS)	Original document
06-01-11	1	Internal	Jim Sumner (ETS)	• Updated exhibits and references. • Statistical analyses and data review moved to QAP-Q12.
07-01-12	2	External (NC DENR) Internal	Lance Ferrell (NC DENR) Jim Sumner (ETS)	• The measurement of pH, DO and conductivity of each new, full-strength, undiluted sample was added. • The light intensity was amended to reflect that it is a recommended range as specified in the EPA manuals.
11-01-14	3	Internal	Jim Sumner (ETS)	• Updated exhibits during document review. • Removed loading weight determination.
09-28-16	4	External (TVA) Internal	Rick Sherrard, Donald Snodgrass (TVA) Jim Sumner (ETS)	• Updated exhibits during document review. • Updated the isolation of test larvae for using the in-house culture.
09-01-19	5	Internal	Jim Sumner (ETS)	• The use of SSW for NC testing was removed. • Updated procedure to include NELAP requirements. • Additional guidance included in SOP.
03-01-20	6	External (TVA) Internal	Rick Sherrard (TVA) Jim Sumner (ETS)	• Updated bench sheet (Exhibits AT18.2 and AT18.3) to include reporting limits, method numbers, meters and serial numbers used for chemical analyses.

Subject: *Pimephales promelas* Acute Toxicity Test, EPA 2000.0

Scope and Application

To measure the acute toxicity of water samples to Fathead minnow larvae (*Pimephales promelas*) during a 24, 48 or 96-hour exposure period.

Summary of Method

The acute toxicity test generally involves the exposure of test organisms to up to five effluent concentrations and a control water. The test duration ranges from 24 to 96 hours. At the end of each 24-hour period, the number of living organisms is counted in each effluent concentration and control water.

A summary of the Fathead minnow acute method is provided in Exhibit AT18.1.

Quality Control

Acceptance Criteria: The test acceptance criterion is ≥ 90% survival in the control. If the control survival is < 90%, the test must be invalidated.

Equipment and Materials

Fathead minnow larvae (*Pimephales promelas*)
Temperature-controlled incubator (set to maintain test temperature = 25.0 ± 1.0°C, photoperiod of 16-hour light / 8-hours dark, light intensity = 50 – 100 ft-c)
Control / Dilution water (synthetic water made with reagent grade chemicals)
500-mL plastic Solo® cups
Solo® cup lids
Graduated cylinders
Large glass jars
Serological, fixed, or adjustable volume pipettes (e.g., Eppendorf)
Transfer pipettes
Aquarium pump and tubing
Thermometer
1-oz medicine cups
Newly hatched brine shrimp
Light box or table
Forceps
Weigh boats
Calibrated top-loading balance (e.g. Fisher Scientific ACCU-224)
Disposable gloves

Subject: *Pimephales promelas* Acute Toxicity Test, EPA 2000.0

Acute Toxicity Test or Pass/Fail Acute Toxicity Test Bench Sheet
Randomization template

Procedure

A. Test Preparation.

1. Prepare the Acute Toxicity Test Bench Sheet (for multiple concentration tests, Exhibit AT18.3) or Pass/Fail Acute Toxicity Test Bench Sheet (for Pass/Fail acute tests, Exhibit AT18.2). Record the following information on the bench sheet:
    - Client/facility name
    - Permit number
    - County
    - Outfall/Pipe number
    - ETS project and sample number
    - Control/Dilution water type and batch
    - Test concentrations and dilution preparation information (sample, dilution and total volumes)

2. Prepare the plasticware.

    a. Obtain enough 500-ml plastic Solo® cups with lids for each site/sample and concentration tested, including the control. For Pass/Fail acute tests, four replicates are used for the test concentration and control. For multiple concentration acute tests, two replicates are used for each concentration and control. Label each replicate cup with the following information.
        - Facility/sample name
        - Concentration
        - Replicate number

    b. Label the appropriate graduated cylinder with the facility/sample name and concentration.

B. Test Initiation.

1. Prepare the test concentrations according to SOP-G5.

   a. The control/dilution water is moderately hard synthetic water (MHSW, SOP-AT1). MHSW must have an alkalinity of 57 – 64 mg $CaCO_3$/L, hardness of 80 – 100 mg $CaCO_3$/L, and an initial pH of 6.5 – 8.5 S.U. (guidance range = 7.4 – 7.8 S.U.).

   b. Measure and record the pH (SOP-C3), dissolved oxygen (SOP-C2) and conductivity (SOP-C4) of each concentration tested and control. Ensure that the dissolved oxygen is within the acceptable range (4.0 to 9.0 mg/L), aerate the sample if necessary, according to SOP-G5. Measure and record the pH (SOP-C3), dissolved oxygen (SOP-C2), conductivity (SOP-C4), total residual chlorine (SOP-C8), total alkalinity (SOP-C6), total hardness (SOP-C7) and sample characteristics of each new, full-strength, undiluted sample. The alkalinity and hardness of full-strength, undiluted samples for North Carolina tests are not required.

   c. Pour 250 mL of control water into each of the replicate control cups.

   d. Pour 250 mL of each test concentration into each of the replicate test cups.

   e. Obtain a randomizing template (Exhibit AT18.5). Place the tests in order according to randomizing template and record the template color on the bench sheet.

   f. Maintain the test temperature (25.0 ± 1.0°C) of the test concentrations. This may be accomplished by placing the holding rack into a temperature-controlled incubator.

2. Isolate the larvae for the test.

   a. Obtain a batch of larvae (SOP-AT17), which are 1 to 14-days old (with a maximum of 24-hour range in age). Record the spawning date, age and hatch dates and times of the organisms to be used in the test on the acute bench sheet. Feed the larvae a minimum of 2 hours prior to test initiation to a maximum of 5 hours prior to test initiation. Record the date and time the organisms were fed on the bench sheet. Transfer the larvae from the tank to a large glass finger bowl.

b. Two techniques may be used for transferring 10 organisms to each test cup from the finger bowl. The technique used is dependent on the sample being evaluated for toxicity. In both methods, larvae are transferred by plastic pipette. The end of the pipette tip should be cut to a size that will not injure or harm the larvae during transfer. Larvae should be transferred gently in a manner that will not expose the organisms to the air.

- If the transferred water may alter the toxicity of the sample (decrease toxicity), organisms should be transferred directly by pipette. This technique is predominately used by the laboratory. Organisms should be transferred in a manner that allows them to swim from the pipette into the test solutions. This will minimize the volume of transfer water introduced into the sample. Follow procedures outlined in step 3 for transferring the organisms to the randomly placed test cups. The average transfer volume by this technique for each analyst must be determined yearly. For an example transfer volume log sheet refer to Exhibit AT18.4.

- If pathogenic interferences have been identified or there is risk of cross contamination between replicates, organisms should be transferred to medicine cups (10 per cup) and then introduced into the test solutions. The final volume contained in the medicine cups after the organisms have been transferred should be between 10 and 15 ml. With a pipette, remove any food or debris that was placed in the cup during transfer. The average transfer volume by this technique for each analyst must be determined yearly. For an example transfer volume log sheet refer to Exhibit AT18.4. Continue this process until enough medicine cups containing 10 larvae each have been obtained to initiate the test. 1 medicine cup containing 10 larvae will be needed for each sample concentration and replicate. If a test consists of 5 concentrations and a control, 12 medicine cups containing 10 larvae each will be required. If a test consists of 1 concentration and a control, 8 medicine cups containing 10 larvae each will be required.

- A limit is placed on the loading (weight) of organisms per liter of test solution to minimize the depletion of dissolved oxygen, the accumulation of injurious concentrations of metabolic waste products and/or stress induced by crowding, any of which could significantly affect the test results. The loading in the test solutions must not exceed or 0.40 g live weight/L at 25°C. Through testing, ETS has determined that this loading requirement is not exceeded using *P. promelas* larvae which are 1 to 14 days old.

3. Transfer the larvae to the randomly placed test cups.

   a. Measure and record the temperature in one of the test cups for each concentration and control. The temperature must be 25.0 ± 1.0°C before the test organisms are placed in the test cups. Warm the test cups in a warm water bath or temperature-controlled incubator, if necessary, until the desired test temperature is obtained. Measure and record the temperature of an arbitrary transfer cup.

   b. Place 10 larvae in the first test cup of the first row (by pipette or medicine cup). Continue in this manner (placing the larvae in the test cups from left to right in the first row and then the second row) until all the test cups contain 10 larvae.

   c. Record the initiation date, time and analyst's initials on the acute bench sheet. **The acute test must be initiated within 36-hours of completion of the sampling period.**

   d. Save approximately 30 mL of transfer water to be measured for pH (SOP-C3). Measure and record the transfer water pH on the acute bench sheet.

   e. Verify that each cup received the required number of larvae (i.e., 10) by conducting a repeat count. Remove excess larvae or add larvae as necessary. Record the initial number of larvae on the bench sheet. Place lids on each cup.

   f. Place the test cups in order, according to the randomization template, in a temperature-controlled incubator. The organisms must be maintained at 25.0 ± 1.0°C with a photoperiod of 16-hours light and 8-hours dark and a recommended light intensity of 50 to 100 ft-c. Record the incubator number and shelf used on the bench sheet.

C. Record Daily Survival.

Repeat this process daily, starting at 24-hours ± 1-hour after test initiation and continuing until test termination.

1. Measure and record the temperature in an arbitrarily selected test cup for each concentration and control.

2. Remove the test cups from the incubator and remove the lids. Place the cups on a light box or table for ease of viewing.

3. Count and record (in the appropriate section) the number of larvae surviving in each replicate cup on the acute bench sheet. The criteria that establish death are: (1) no movement and (2) no reaction to gentle prodding. Record comments, if applicable.

4. Remove any dead larvae and discard with a transfer pipette.

5. Record the date, time and the analyst's initials on the bench sheet.

6. Carefully pour ~30 mL of test water from at least one replicate cup for each test concentration and control into labeled 1-oz medicine cups. Measure and record the pH (SOP-C3) and dissolved oxygen (SOP-C2) of this water.

7. Place the lids on the test cups and place the test cups back in order, according to the randomization template, in a temperature-controlled incubator.

D. <u>For 96-hour Acute Tests,</u> Renewal of Test Solutions at 48-hours.

For 96-hour acute tests, test solutions must be renewed within ± 1 hour from test initiation.

1. Carefully pour ~30 mL of test water from at least one replicate cup for each test concentration and control into a labeled 1-oz medicine cup. This water will be used to determine final pH and dissolved oxygen concentrations.

2. Feed the larvae in each test cup 200 µL (4-drops) of newly-hatched brine shrimp (SOP-AT16) at 2-hours prior to the renewal of test solutions (at 46-hours from test initiation). Record the feeding time and initials on the acute benchsheet.

3. Measure and record the temperature in an arbitrarily selected test replicate for each concentration and control.

4. Prepare fresh test solutions in accordance with SOP-G5. Maintain the test temperature (25.0 ± 1.0°C) of the fresh test water until needed by storing in a temperature-controlled incubator.

5. At 48-hours, remove the test cups from the incubator. Place the cups on a light box or table for ease of viewing.

6. Change the test water in all replicate cups before starting the next replicate-cup series. To change the test water, test cups are decanted.

a. Using a transfer pipette, remove any debris, dead artemia and dead larvae that may have accumulated on the bottom of the test cup. Carefully decant the water from the cup into a white plastic photographic tray until ~ 50 mL of old test water remains.

b. If any larvae are accidentally decanted with the water, retrieve them from the plastic tray, using a transfer pipette. The end of the pipette tip should be cut to a size that will not injure or harm the larvae during transfer. Larvae should be transferred gently in a manner that will not expose the organisms to the air. Return the larvae to the appropriate replicate cup. Record the number of larvae siphoned out or decanted (per replicate). Discard any dead larvae.

c. Record the following information on the acute benchsheet.

- Number of larvae surviving in each replicate cup.
- Number of dead larvae in each replicate cup (if applicable).
- Any comments (injured, sick, or larvae siphoned out).

d. Fill each replicate cup to 250 mL using fresh test solutions. Pour the test water down the side of the cup to avoid unnecessarily disturbing the larvae.

7. After all test cups have been renewed, record the renewal time and the analyst's initials on the acute bench sheet. Place the lids on the test cups and place the cups back in order, according to the randomization template, in a temperature-controlled incubator.

E. Test Termination.

Terminate the test after the organisms have been exposed to the test concentrations for the required time (i.e. 24, 48, or 96-hours). The test may be terminated ± 1-hour from the time it was initiated.

1. Measure and record the temperature in an arbitrarily selected test cup for each concentration and control.

2. Remove the test cups from the incubator and remove the lids. Place the cups on a light box or table for ease of viewing.

3. Count and record (in the appropriate section) the number of larvae surviving in each replicate cup on the acute bench sheet. Record comments, if applicable.

4. Record the termination date, time and the analyst's initials on the bench sheet.

5. Carefully pour ~30 mL of test water from at least one replicate cup for each test concentration and control into labeled 1-oz medicine cups. Measure and record the pH (SOP-C3) and dissolved oxygen (SOP-C2) of this water.

6. Once all analyses have been completed and documented, discard the test water and larvae according to established laboratory protocol.

F. Statistical Analyses and Data Verification.

Statistical analyses and data review are performed according to QAP-Q12.

Safety and Hazardous Waste Management

Safety glasses, gloves and lab coats should always be worn.

Review Policy-P6: General Safety Policy and Policy-P9: Radiation Protection Policy for additional safety requirements.

References

USEPA. October 2002. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, 5th ed. **EPA-821-R-02-012, Method 2000.0**. US Environmental Protection Agency, Cincinnati, OH.

North Carolina Department of Environment and Natural Resources, Division of Water Quality. Pass/Fail Methodology for Determining Acute Toxicity in a Single Effluent, Version 3.0. December 2010.

TNI Standard. Management and Technical Requirements for Laboratories Performing Environmental Analysis. EL-V1-ISO-2016-Rev2.0. The NELAC Institute, PO Box 2439, Weatherford, TX 76086.

Exhibits

Exhibit AT18.1: Summary of Test Conditions for the *Pimephales promelas* Acute Toxicity Test.
Exhibit AT18.2: Pass/Fail Acute Toxicity Test Bench Sheet.
Exhibit AT18.3: Acute Toxicity Test Bench Sheet.
Exhibit AT18.4: Average Transfer Volume Log Sheet.
Exhibit AT18.5: Randomization Template.

Subject: *Pimephales promelas* Acute Toxicity Test, EPA 2000.0

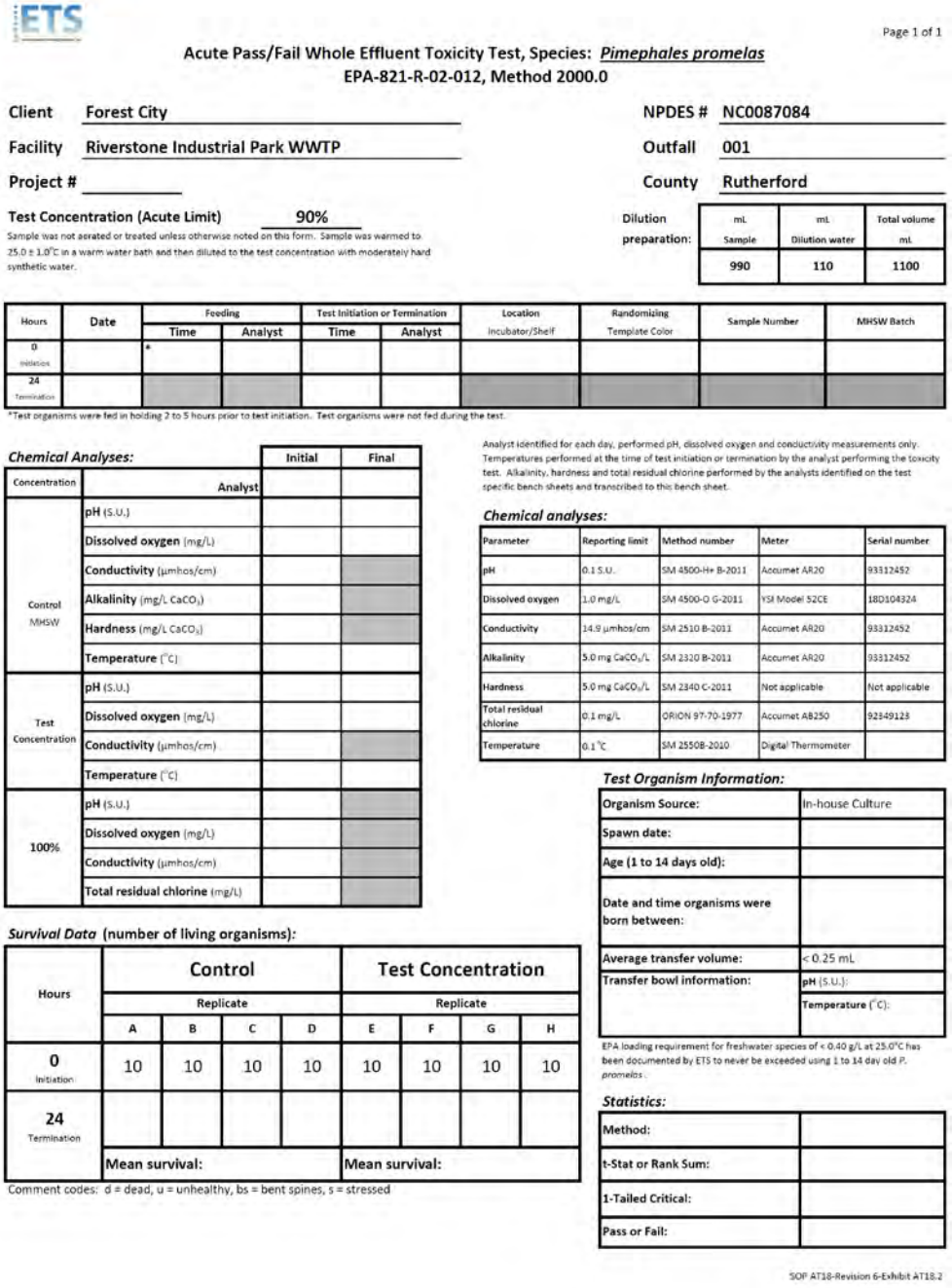
Exhibit AT18.1: Summary of Test Conditions for the *Pimephales promelas* Acute Toxicity Test.

SUMMARY OF TEST CONDITIONS FOR THE *PIMEPHALES PROMELAS* ACUTE TOXICITY TEST

Test type:	Static non-renewal or static renewal
Test duration:	24, 48, or 96 hours
Temperature:	25.0 ± 1.0°C
Light quality:	Cool-white fluorescent
Light intensity:	50 – 100 ft-candles (recommended)
Photoperiod:	16-hours light, 8-hours dark
Test chamber size:	500 mL Solo® cups
Test solution volume:	250 mL
Renewal of test solutions:	At 48-hours (required minimum)
Age of test organisms (days old):	1 to 14 days old, ≤ 24 hour range in age
Number of organisms per test chamber:	10
Number of replicate test chambers per concentration:	Multiple concentration tests: 2 Single dilution tests: 4
Number of organisms per concentration:	Multiple concentration tests: 20 Single dilution tests: 40
Test concentrations:	Multiple concentration tests: 5 and a control with ≥ 0.5 dilution series (recommended) Single dilution tests: 90% or 100% and a control
Test chamber cleaning:	Dead larvae removed daily. For 96-hour tests, test chambers are cleaned immediately before test solution renewal at 48-hours.
Aeration:	None, unless the dissolved oxygen concentration falls below 4.0 mg/L.
Feeding regime:	*Artemia nauplii* made available while holding prior to test initiation (2 to 5-hours prior to initiation). Organisms in each test cup are fed 200 µL *Artemia nauplii* 2 hours prior to test solution renewal at 48-hours.
Control / Dilution water:	Moderately hard synthetic water
Sampling and sample holding:	1-gallon grab or composite sample first used within 36-hours of completion of the sampling period.
Endpoint:	Mortality
Test acceptability criterion:	≥ 90% control survival

Subject: *Pimephales promelas* Acute Toxicity Test, EPA 2000.0

Exhibit AT18.2: Pass/Fail Acute Toxicity Test Bench Sheet.



ETS

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Acute Pass/Fail Whole Effluent Toxicity Test, Species: *Pimephales promelas*
EPA-821-R-02-012, Method 2000.0

Client: Forest City — NPDES #: NC0087084
Facility: Riverstone Industrial Park WWTP — Outfall: 001
Project #: _____ — County: Rutherford

Test Concentration (Acute Limit): 90%
Sample was not aerated or treated unless otherwise noted on this form. Sample was warmed to 25.0 ± 1.0°C in a warm water bath and then diluted to the test concentration with moderately hard synthetic water.

Dilution preparation:

mL Sample	mL Dilution water	Total volume mL
990	110	1100

Hours	Date	Feeding Time	Feeding Analyst	Test Initiation or Termination Time	Test Initiation or Termination Analyst	Location Incubator/Shelf	Randomizing Template Color	Sample Number	MHSW Batch
0 Initiation		*							
24 Termination									

*Test organisms were fed in holding 2 to 5 hours prior to test initiation. Test organisms were not fed during the test.

Chemical Analyses:

Concentration	Analyst	Initial	Final
Control MHSW	pH (S.U.)		
	Dissolved oxygen (mg/L)		
	Conductivity (µmhos/cm)		
	Alkalinity (mg/L $CaCO_3$)		
	Hardness (mg/L $CaCO_3$)		
	Temperature (°C)		
Test Concentration	pH (S.U.)		
	Dissolved oxygen (mg/L)		
	Conductivity (µmhos/cm)		
	Temperature (°C)		
100%	pH (S.U.)		
	Dissolved oxygen (mg/L)		
	Conductivity (µmhos/cm)		
	Total residual chlorine (mg/L)		

Analyst identified for each day, performed pH, dissolved oxygen and conductivity measurements only. Temperatures performed at the time of test initiation or termination by the analyst performing the toxicity test. Alkalinity, hardness and total residual chlorine performed by the analysts identified on the test specific bench sheets and transcribed to this bench sheet.

Chemical analyses:

Parameter	Reporting limit	Method number	Meter	Serial number
pH	0.1 S.U.	SM 4500-H+ B-2011	Accumet AR20	93312452
Dissolved oxygen	1.0 mg/L	SM 4500-O G-2011	YSI Model 52CE	18D104324
Conductivity	14.9 µmhos/cm	SM 2510 B-2011	Accumet AR20	93312452
Alkalinity	5.0 mg $CaCO_3$/L	SM 2320 B-2011	Accumet AR20	93312452
Hardness	5.0 mg $CaCO_3$/L	SM 2340 C-2011	Not applicable	Not applicable
Total residual chlorine	0.1 mg/L	ORION 97-70-1977	Accumet AB250	92349123
Temperature	0.1 °C	SM 2550B-2010	Digital Thermometer	

Survival Data (number of living organisms):

Hours	Control Replicate A	B	C	D	Test Concentration Replicate E	F	G	H
0 Initiation	10	10	10	10	10	10	10	10
24 Termination								
	Mean survival:				Mean survival:			

Comment codes: d = dead, u = unhealthy, bs = bent spines, s = stressed

Test Organism Information:

Organism Source:	In-house Culture
Spawn date:	
Age (1 to 14 days old):	
Date and time organisms were born between:	
Average transfer volume:	< 0.25 mL
Transfer bowl information:	pH (S.U.):
	Temperature (°C):

EPA loading requirement for freshwater species of < 0.40 g/L at 25.0°C has been documented by ETS to never be exceeded using 1 to 14 day old *P. promelas*.

Statistics:

Method:	
t-Stat or Rank Sum:	
1-Tailed Critical:	
Pass or Fail:	

SOP AT18-Revision 6-Exhibit AT18.2

Subject: *Pimephales promelas* Acute Toxicity Test, EPA 2000.0

Exhibit AT18.3: Acute Toxicity Test Bench Sheet.

ETS

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Acute LC_{50} Whole Effluent Toxicity Test, Species: *Pimephales promelas*
EPA-821-R-02-012, Method 2000.0

Client: Nutrien Aurora Phosphate — NPDES #: NC0003255
Facility: PCS Phosphate, Inc. — Outfall: 007
Project #: _____ Sample #: _____ — County: Beaufort

Dilution Preparation:

Test concentrations (%)	6.25	12.5	25	50	100
mL Sample	31.25	62.5	125	250	500
mL Dilution water	468.75	437.5	375	250	0
Total volume (mL)	500	500	500	500	500

Sample was not aerated or treated unless otherwise noted on this form. Sample was warmed to 25.0 ± 1.0°C in a warm water bath and then diluted to the test concentrations using moderately hard synthetic water (MHSW).

Chemical Analyses:

Concentration	Analyst	Hours 0	Hours 24	Hours 48
Control, MHSW	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	Conductivity (µmhos/cm)			
	Alkalinity (mg/L $CaCO_3$)			
	Hardness (mg/L $CaCO_3$)			
	Temperature (°C)			
6.25%	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	Conductivity (µmhos/cm)			
	Temperature (°C)			
12.5%	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	Conductivity (µmhos/cm)			
	Temperature (°C)			
25%	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	Conductivity (µmhos/cm)			
	Temperature (°C)			
50%	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	Conductivity (µmhos/cm)			
	Temperature (°C)			
100%	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	Conductivity (µmhos/cm)			
	Alkalinity (mg/L $CaCO_3$)			
	Hardness (mg/L $CaCO_3$)			
	Total residual chlorine (mg/L)			
	Temperature (°C)			

Analyst identified for each day, performed pH, dissolved oxygen and conductivity measurements only. Temperatures performed at the time of test initiation or termination by the analyst performing the toxicity test. Alkalinity, hardness and total residual chlorine performed by the analysts identified on the test specific bench sheets and transcribed to this bench sheet.

Chemical analyses:

Parameter	Reporting limit	Method number	Meter	Serial number
pH	0.1 S.U.	SM 4500-H+ B-2011	Accumet AR20	93312452
Dissolved oxygen	1.0 mg/L	SM 4500-O G-2011	YSI Model 52CE	18D104824
Conductivity	14.9 µmhos/cm	SM 2510 B-2011	Accumet AR20	93312452
Alkalinity	5.0 mg $CaCO_3$/L	SM 2320 B-2011	Accumet AR20	93312452
Hardness	5.0 mg $CaCO_3$/L	SM 2340 C-2011	Not applicable	Not applicable
Total residual chlorine	0.1 mg/L	ORION 97-70-1977	Accumet AB250	92349123
Temperature	0.1 °C	SM 2550B-2010	Digital Thermometer	

SOP AT18-Revision 6-Exhibit AT18.3

Subject: *Pimephales promelas* Acute Toxicity Test, EPA 2000.0

Acute LC_{50} Whole Effluent Toxicity Test, Species: *Pimephales promelas*
EPA-821-R-02-012, Method 2000.0

Client PCS Phosphate, Inc.

Project # _____ **Sample #** _____

Hours	Date	Feeding		Test Initiation or Termination		Location	Randomizing	MHSW Batch
		Time	Analyst	Time	Analyst	Incubator/Shelf	Template	
0 Initiation		*						
24								
48 Termination								

*Test organisms were fed in holding 2 to 5 hours prior to test initiation. Test organisms were not fed during the test.

Test Organism Information:

Organism source:	In-house Culture
Spawn date:	
Age (1 to 14 days old):	< 24-hours old
Date and time organisms were born between:	
Average transfer volume:	< 0.25 mL
Transfer bowl information:	**pH** (S.U.):
	Temperature (°C):

Survival Data **(number of living organisms):**

Hours	Control		6.25%		12.5%		25%		50%		100%	
	Replicate		Replicate		Replicate		Replicate		Replicate		Replicate	
	A	B	C	D	E	F	G	H	I	J	K	L
0 Initiation	10	10	10	10	10	10	10	10	10	10	10	10
24												
48 Termination												
Mean Survival												

Comment codes: d = dead, u = unhealthy, bs = bent spines, s = stressed

Statistics:

Method	
Lower 95% confidence limit (%)	
Upper 95% confidence limit (%)	
48-hour LC_{50} (%)	

Comments:

SOP AT18-Revision 6-Exhibit AT18.3

Subject: *Pimephales promelas* Acute Toxicity Test, EPA 2000.0

Exhibit AT18.4: Average Transfer Volume Log Sheet.

ETS Environmental Testing Solutions, Inc.

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Larval Fish Transfer Volume

Analyst: _____ Species: _____

Date: _____ Source / Batch: _____

Ambient temperature: _____ Wet Weight of 10 Larvae (g): _____

Estimate transfer volume, where minnows are allowed to swim from the pipette into the test vessel.

Numerically label 10 medicine cups.
Add 10 mL MHSW to each of the 10 cups.
Measure and record the weight of each cup containing MHSW.
Transfer 10 larvae to each cup, following procedures identified in SOP-AT18, AT47, or AT53 for vertebrate acute toxicity tests
Transfer the larvae in a manner that allows them to swim from the pipette into the MHSW contained in each cup.
Measure and record the weight of each cup containing MHSW with 10 larvae.
Determine each transfer volume and average transfer volume.

Replicate Number	Initial Weight Medicine cup + 10 mL MHSW (g)	Final Weight Medicine cup + 10 mL MHSW + 10 Larval Fish transferred (g)	Transfer Volume Final - Initial Weight (g = mL)
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
	Average volume to transfer 10 organisms (mL):		

Estimate transfer volume, where the minnows are transferred with MHSW into the test vessels.

Numerically label 10 medicine cups.
Measure and record the weight of each cup.
Add approximately 10 mL MHSW to each of the 10 cups.
Transfer 10 larvae to each cup, following procedures identified in SOP-AT18, AT47, or AT53 for vertebrate acute toxicity tests
Transfer the larvae in a manner that allows them to swim from the pipette into the MHSW contained in each cup.
Measure and record the weight of each cup containing MHSW with 10 larvae.
Determine each transfer volume and average transfer volume.

Replicate Number	Initial Weight Medicine cup (g)	Final Weight Medicine cup + 10 mL MHSW + 10 Larval Fish transferred (g)	Transfer Volume Final - Initial Weight (g = mL)
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
	Average volume to transfer 10 organisms (mL):		

SOP AT18-Revision 6-Exhibit AT18.4

Exhibit AT18.5: Randomization Template.

Randomizing template: **RED**

Replicate #	1	2	3	4
Concentrations	6	5	4	5
	3	3	2	6
1 = Control	4	1	1	2
2 = Lowest concentration	1	2	3	1
3 - 5 = Intermediate concentrations	2	4	5	3
6 = Highest concentration	5	6	6	4

Random number seeds: 4 through 7

Subject: *Pimephales promelas* Acute Reference Toxicity Test, EPA 2000.0

Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan		03-01-20
Quality Assurance Officer	Jim Sumner		03-01-20

Document Revision History

Effective Date	Revision number	Review Type	Evaluators	Revisions
12-01-00	0	Internal	Jim Sumner (ETS)	Original document
07-10-10	1	External (NC DENR) Internal	Lance Ferrell (NC DENR) Jim Sumner (ETS)	• Section D.5 updated for the current *Pimephales* Acute SOP, AT18. • Exhibits updated and included 96-hour acute reference toxicant benchsheets and control charts.
06-01-11	2	**Internal**	Jim Sumner (ETS)	• Updated references and exhibits. • Updated Table AT19.1.
11-01-14	3	Internal	Jim Sumner (ETS)	• Updated exhibits during document review. • Removed conductivity measurement requirement of stock KCl solution due to inaccuracy of these measurements, which are above the calibration range.
09-28-16	4	External (TVA) Internal	Rick Sherrard, Donald Snodgrass (TVA) Jim Sumner (ETS)	• Updated Table AT19.1 for conductivity measurement guidance values. • Deleted statement: "Verify that the conductivity measured for each test concentration is within the acceptance criteria (refer to table Table AT19.1) before proceeding with the preparation of next concentration. If the conductivity is not within the criteria, remake the test concentration and verify the conductivity." • Updated exhibits during document review.
09-01-19	5	Internal	Jim Sumner (ETS)	• Updated exhibits during document review. • Updated procedure to include NELAP requirements. • Additional guidance included in SOP.
03-01-20	6	External (TVA) Internal	Rick Sherrard (TVA) Jim Sumner (ETS)	• Updated bench sheet to include reporting limits, method numbers, meters and serial numbers used for chemical analyses.

Subject: *Pimephales promelas* Acute Reference Toxicity Test, EPA 2000.0

Scope and Application

To assess the sensitivity of *Pimephales promelas* and the overall credibility of the *Pimephales promelas* acute toxicity tests. Reference toxicant data are part of the laboratory's QA/QC program to evaluate the performance of laboratory personnel and the robustness and sensitivity of the test organisms.

Summary of Method

The acute reference toxicity test generally involves the exposure of test organisms to five potassium chloride concentrations and control water for a 48-hour or 96-hour exposure period. At the end of each 24-hour period, the number of living organisms is counted in each potassium chloride concentration and control water. The median lethal concentration (LC_{50}) of potassium chloride is determined and compared to previous reference toxicant tests.

Quality Control

Acceptance Criteria: The test acceptance criterion is ≥ 90% survival in the control. If the control survival is < 90%, the test must be invalidated.

Frequency of Testing:
A *Pimephales promelas* acute reference toxicant test must be performed so that all acute whole effluent toxicity tests are conducted within 1 week of a reference toxicant test. At a minimum, acute reference toxicant tests must be performed on a quarterly basis in order to maintain certification requirements.

Equipment and Materials

Fathead minnow larvae (*Pimephales promelas*)
Temperature-controlled incubator (set to maintain test temperature = 25.0 ± 1.0°C, photoperiod of 16-hour light / 8-hours dark, light intensity = 50 – 100 ft-c)
Control / Dilution water (moderately synthetic water)
Potassium chloride (KCl, reagent grade)
1000-mL volumetric flask
Deionized water
500-ml plastic Solo® cups
Solo® cup lids
500-mL graduated cylinder
1000-mL Erlenmeyer flask
Large glass finger bowls
10-mL serological pipettes

Transfer pipettes
Calibrated top-loading balance (e.g. Fisher Scientific ACCU-224)
Thermometer
1-oz disposable medicine cups
Forceps
Weigh boats
Newly hatched brine shrimp
Light box or table
Disposable gloves
Pimephales promelas Acute Reference Toxicity Test Bench Sheet
Randomization template

Procedure

A. Test Preparation.

1. Prepare the pasticware.

   a. Obtain two replicate 500-ml plastic Solo® cups with lids for each of the five KCl concentrations tested and the control. Label each replicate cup with the following information.
   - Concentration
   - Replicate number

   b. Label the appropriate graduated cylinder.

   c. Prepare the 48-hour or 96-hour *Pimephales promelas* Acute Reference Toxicity Test Bench Sheet (see Exhibit AT19.1). Record the *Pimephales promelas* KCl Acute (PpKClAC) test number on the bench sheet.

B. Preparation of the Stock Solution.

1. Using a calibrated top-loading balance, carefully weigh out 50 g of KCl (SOP-G10). Place approximately 900 mL of deionized water in a 1000-mL volumetric flask. Add the KCl to the flask, dissolve the KCl by swirling the flask; bring to volume with deionized water. Label the volumetric flask with the concentration (50 g/L), analyst's initials, preparation date and stock standard number (INSS, SOP-G15). Record the INSS number of the KCl stock solution on the bench sheet.

C. Preparation of the Test Concentrations.

1. Prepare all test concentrations using graduated cylinders and serological pipettes. The precision of conductivity measurements is increased when smaller volume graduated cylinders and/or serological pipettes are used to prepare the test concentrations. For this reference toxicant test, stock solution volumes should be measured using a 10-mL serological pipette and the total volumes should be measured using a 500-mL graduated cylinder.

2. Beginning with the lowest concentration, add approximately 100 mL of moderately hard synthetic water to a 500-mL graduated cylinder, add the required volume of stock solution using a 10-mL serological pipette (refer to Table AT19.1), bring to volume (500 mL) with moderately hard synthetic water. Mix the solution well by pouring the solution into a 1000-mL Erlenmeyer flask.

3. Pour 250 mL of test solution into each of the replicate test cups for that concentration. 30 mL should be saved for chemical analyses. Measure and record the pH (SOP-C3), dissolved oxygen (SOP-C2) and conductivity (SOP-C4) of each test solution.

4. Refer to Table AT19.1 for guidance values of conductivity measurements.

5. Rinse the graduated cylinder well with deionized water and repeat steps D.2 through D.5 for preparing the next test concentration. Record the batch date of moderately hard synthetic water used to prepare the dilutions.

Table AT19.1: Test concentration, stock volumes, moderately hard synthetic water volumes, final volumes, and conductivity guidance values for *Pimephales promelas* KCl acute reference toxicant tests.

Test Concentration (mg KCl/L)	Volume of Stock Required (mL)	Volume of Moderately Hard Synthetic Water (mL)	Final Volume (mL)	Conductivity Guidance Values (µmhos/cm)
500	5.0	495.0	500	1100 - 1300
750	7.5	492.5	500	1500 - 1700
1000	10.0	490.0	500	1900 - 2200
1250	12.5	487.5	500	2300 - 2600
1500	15.0	485.0	500	2700 - 3100

6. Once all test concentrations have been prepared, follow the procedure described in SOP-AT18 for conducting *Pimephales promelas* Acute Toxicity Tests.

D. Preparation of Control Charts.

Refer to QAP-Q5 for how control charts are prepared and procedures for interpreting outlier tests. Refer to Exhibit AT19.2 for example control charts.

Safety and Hazardous Waste Management

Safety glasses, gloves and lab coats should always be worn.

Review Policy-P6: General Safety Policy and Policy-P9: Radiation Protection Policy for additional safety requirements.

References

USEPA. October 2002. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, 5th ed. **EPA-821-R-02-012, Method 2000.0**. US Environmental Protection Agency, Cincinnati, OH.

USEPA. 2000. Understanding and Accounting for Method Variability in Whole Effluent Toxicity Applications Under the National Pollutant Discharge Elimination Program. EPA-833-R-00-003. US Environmental Protection Agency, Cincinnati, OH.

USEPA. 2001. Final Report: Inter-laboratory Variability Study of EPA Short-term Chronic and Acute Whole Effluent Toxicity Test Methods, Volumes 1 and 2 - Appendix. EPA-821-B-01-004 and EPA-821-B-01-005. US Environmental Protection Agency, Cincinnati, OH.

North Carolina Department of Environment and Natural Resources, Division of Water Quality. Biological Laboratory Certification / Criteria Procedures, Version 3.0. December 2010.

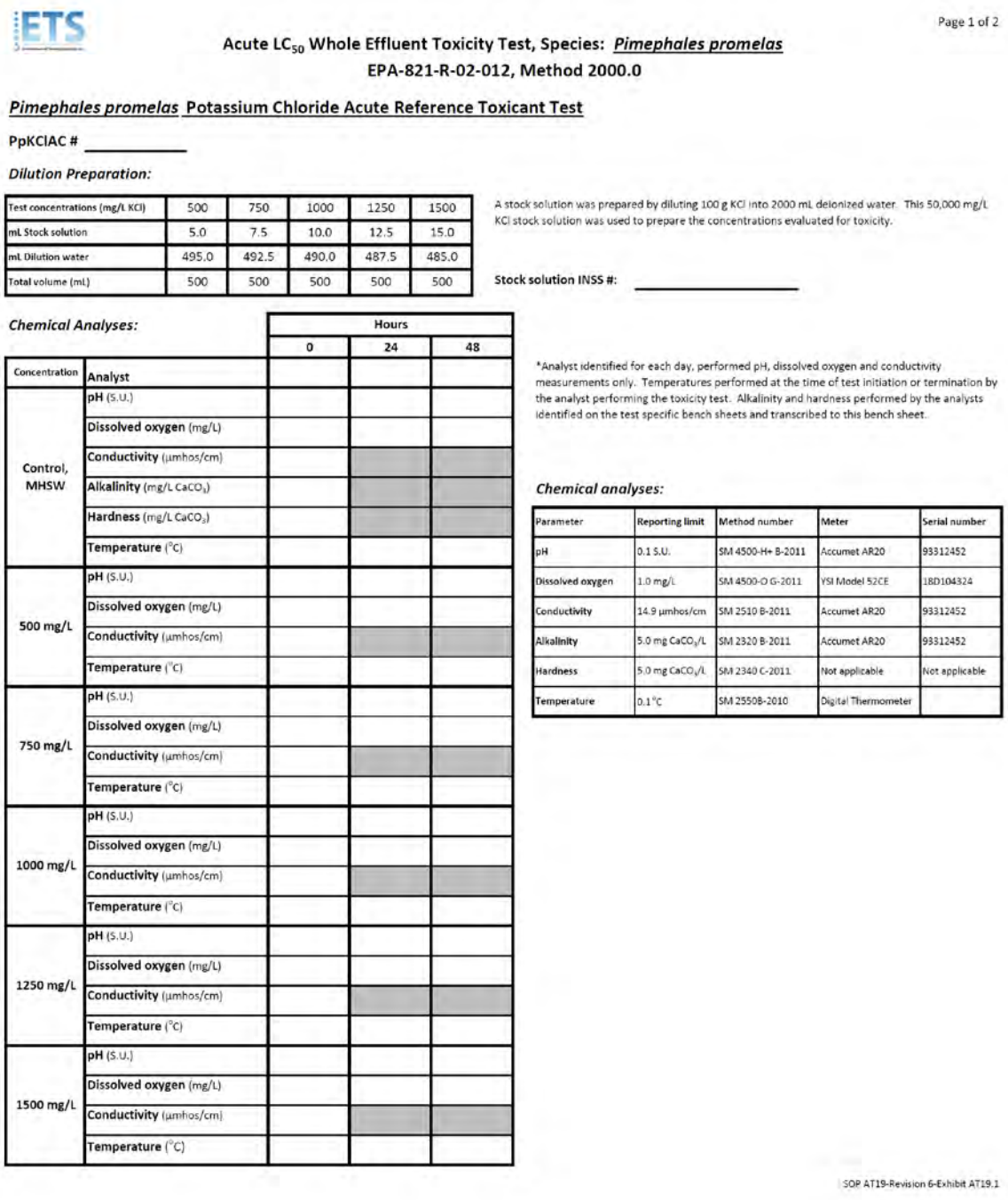
TNI Standard. Management and Technical Requirements for Laboratories Performing Environmental Analysis. EL-V1-ISO-2016-Rev2.0. The NELAC Institute, PO Box 2439, Weatherford, TX 76086.

Exhibits

Exhibit AT19.1: *Pimephales promelas* Acute Reference Toxicity Test Bench Sheet.
Exhibit AT19.2: Example *Pimephales promelas* Acute Reference Toxicant Control Chart.

Subject: *Pimephales promelas* Acute Reference Toxicity Test, EPA 2000.0

Exhibit AT19.1: *Pimephales promelas* Acute Reference Toxicity Test Bench Sheet.



ETS

Page 1 of 2

Acute LC_{50} Whole Effluent Toxicity Test, Species: *Pimephales promelas*
EPA-821-R-02-012, Method 2000.0

Pimephales promelas Potassium Chloride Acute Reference Toxicant Test

PpKClAC # _____

Dilution Preparation:

Test concentrations (mg/L KCl)	500	750	1000	1250	1500
mL Stock solution	5.0	7.5	10.0	12.5	15.0
mL Dilution water	495.0	492.5	490.0	487.5	485.0
Total volume (mL)	500	500	500	500	500

A stock solution was prepared by diluting 100 g KCl into 2000 mL deionized water. This 50,000 mg/L KCl stock solution was used to prepare the concentrations evaluated for toxicity.

Stock solution INSS #: _____

Chemical Analyses:

Concentration	Analyst	Hours 0	24	48
Control, MHSW	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	Conductivity (µmhos/cm)			
	Alkalinity (mg/L $CaCO_3$)			
	Hardness (mg/L $CaCO_3$)			
	Temperature (°C)			
500 mg/L	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	Conductivity (µmhos/cm)			
	Temperature (°C)			
750 mg/L	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	Conductivity (µmhos/cm)			
	Temperature (°C)			
1000 mg/L	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	Conductivity (µmhos/cm)			
	Temperature (°C)			
1250 mg/L	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	Conductivity (µmhos/cm)			
	Temperature (°C)			
1500 mg/L	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	Conductivity (µmhos/cm)			
	Temperature (°C)			

*Analyst identified for each day, performed pH, dissolved oxygen and conductivity measurements only. Temperatures performed at the time of test initiation or termination by the analyst performing the toxicity test. Alkalinity and hardness performed by the analysts identified on the test specific bench sheets and transcribed to this bench sheet.

Chemical analyses:

Parameter	Reporting limit	Method number	Meter	Serial number
pH	0.1 S.U.	SM 4500-H+ B-2011	Accumet AR20	93312452
Dissolved oxygen	1.0 mg/L	SM 4500-O G-2011	YSI Model 52CE	18D104324
Conductivity	14.9 µmhos/cm	SM 2510 B-2011	Accumet AR20	93312452
Alkalinity	5.0 mg $CaCO_3$/L	SM 2320 B-2011	Accumet AR20	93312452
Hardness	5.0 mg $CaCO_3$/L	SM 2340 C-2011	Not applicable	Not applicable
Temperature	0.1 °C	SM 2550B-2010	Digital Thermometer	

SOP AT19-Revision 6-Exhibit AT19.1

Subject: *Pimephales promelas* Acute Reference Toxicity Test, EPA 2000.0

Acute LC_{50} Whole Effluent Toxicity Test, Species: *Pimephales promelas*
EPA-821-R-02-012, Method 2000.0

***Pimephales promelas* Potassium Chloride Acute Reference Toxicant Test**

PpKClAC # _____

Hours	Date	Feeding		Test Initiation or Termination		Location	Randomizing	MHSW Batch
		Time	Analyst	Time	Analyst	Incubator/Shelf	Template	
0 Initiation		*						
24								
48 Termination								

*Test organisms were fed in holding 2 to 5 hours prior to test initiation. Test organisms were not fed during the test.

Test Organism Information:

Organism Source:	In-house culture
Spawning date:	
Age (1 to 14 days old):	
Hatch date and times:	
Average transfer volume:	< 0.25 mL
Transfer bowl information:	pH (S.U.):
	Temperature (°C):

EPA loading requirement for freshwater species of < 0.40 g/L at 25.0°C has been documented by ETS to never be exceeded using 1 to 14 day old *P. promelas*.

Survival Data **(number of living organisms):**

Hours	Control		500 mg/L		750 mg/L		1000 mg/L		1250 mg/L		1500 mg/L	
	Replicate		Replicate		Replicate		Replicate		Replicate		Replicate	
	A	B	C	D	E	F	G	H	I	J	K	L
0 Initiation	10	10	10	10	10	10	10	10	10	10	10	10
24												
48 Termination												
Mean Survival												

Comment codes: d = dead, u = unhealthy, bs = bent spines, s = stressed

Statistics:

Method	
Lower 95% confidence limit (mg KCl/L)	
Upper 95% confidence limit (mg KCl/L)	
48-hour LC_{50} (mg KCl/L)	

Comments:

Test Reviewed by: _____

SOP AT19-Revision 6-Exhibit AT19.1

Subject: *Pimephales promelas* Acute Reference Toxicity Test, EPA 2000.0

Exhibit AT19.2: Example of a *Pimephales promelas* Acute Reference Toxicant Control Chart.

Pimephales promelas
Acute Reference Toxicant Control Chart
Source: In-house Culture

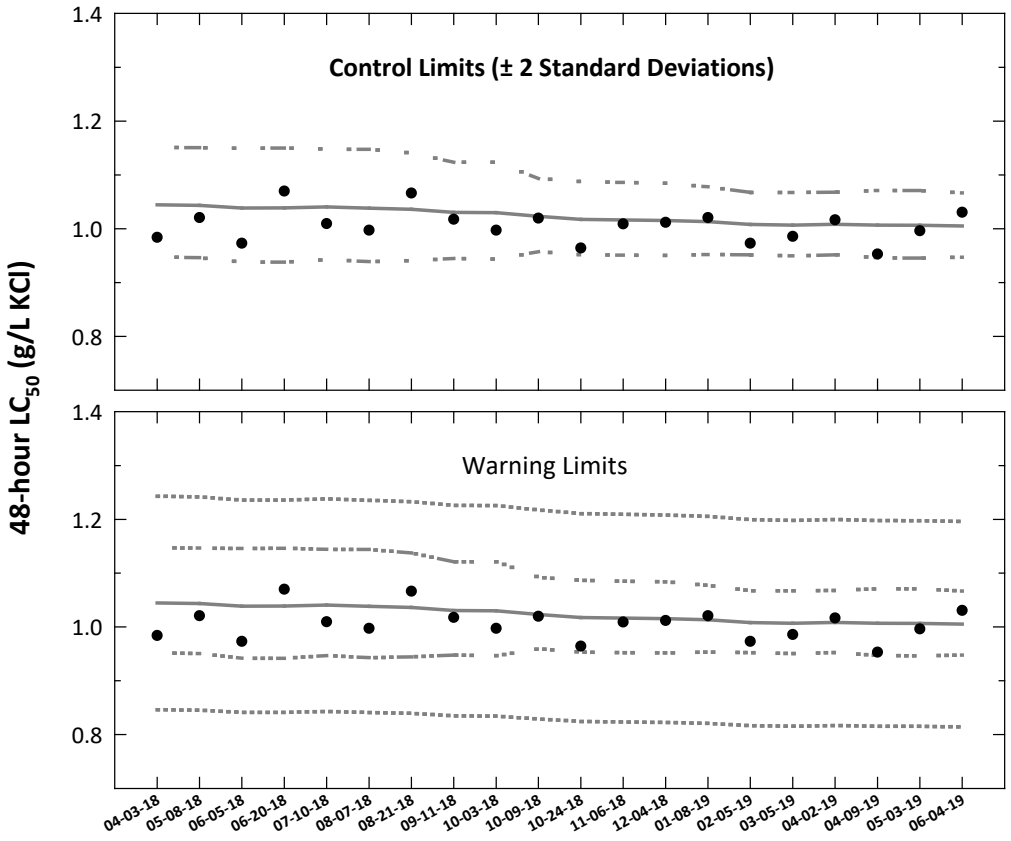
Test number	Test date	48-hour LC_{50} ToxCal Determination (g/L KCl)	Log_{10} Conversion			Anti-logarithmic Values (g/L KCl)						
			48-hour LC_{50}	CT	S	CT	Control Limits		Laboratory Calculated CV Warning Limits		75th Percentile CV Warning Limits	
							CT - 2S	CT + 2S	CT - 2CV	CT + 2CV	CT - $S_{A.75}$	CT + $S_{A.75}$
1	04-03-18	0.9839	-0.0070	0.0190	0.0211	1.0446	0.9478	1.1513	0.9520	1.1467	0.8462	1.2431
2	05-08-18	1.0205	0.0088	0.0185	0.0212	1.0435	0.9463	1.1507	0.9504	1.1462	0.8452	1.2418
3	06-05-18	0.9729	-0.0119	0.0164	0.0221	1.0385	0.9380	1.1498	0.9418	1.1456	0.8412	1.2358
4	06-20-18	1.0698	0.0293	0.0165	0.0221	1.0387	0.9380	1.1502	0.9418	1.1460	0.8413	1.2360
5	07-10-18	1.0095	0.0041	0.0173	0.0214	1.0405	0.9429	1.1482	0.9467	1.1440	0.8428	1.2382
6	08-07-18	0.9973	-0.0012	0.0163	0.0218	1.0382	0.9392	1.1477	0.9428	1.1437	0.8410	1.2355
7	08-21-18	1.0660	0.0278	0.0154	0.0209	1.0362	0.9410	1.1409	0.9444	1.1373	0.8393	1.2331
8	09-11-18	1.0174	0.0075	0.0131	0.0188	1.0305	0.9451	1.1237	0.9477	1.1209	0.8347	1.2263
9	10-03-18	0.9971	-0.0013	0.0128	0.0190	1.0300	0.9439	1.1239	0.9464	1.1212	0.8343	1.2257
10	10-09-18	1.0196	0.0084	0.0100	0.0144	1.0233	0.9575	1.0935	0.9590	1.0919	0.8288	1.2177
11	10-24-18	0.9642	-0.0158	0.0076	0.0145	1.0175	0.9520	1.0876	0.9531	1.0864	0.8242	1.2109
12	11-06-18	1.0087	0.0038	0.0071	0.0144	1.0164	0.9511	1.0861	0.9522	1.0850	0.8233	1.2095
13	12-04-18	1.0118	0.0051	0.0066	0.0143	1.0154	0.9505	1.0847	0.9515	1.0836	0.8225	1.2083
14	01-08-19	1.0205	0.0088	0.0057	0.0135	1.0132	0.9523	1.0780	0.9530	1.0771	0.8207	1.2057
15	02-05-19	0.9729	-0.0119	0.0035	0.0125	1.0081	0.9517	1.0677	0.9522	1.0672	0.8165	1.1996
16	03-05-19	0.9857	-0.0063	0.0030	0.0127	1.0069	0.9498	1.0674	0.9502	1.0670	0.8156	1.1982
17	04-02-19	1.0165	0.0071	0.0036	0.0125	1.0084	0.9519	1.0683	0.9524	1.0678	0.8168	1.2000
18	04-09-19	0.9529	-0.0210	0.0029	0.0135	1.0068	0.9461	1.0714	0.9465	1.0709	0.8155	1.1981
19	05-03-19	0.9960	-0.0017	0.0028	0.0135	1.0064	0.9456	1.0712	0.9460	1.0707	0.8152	1.1977
20	06-04-19	1.0306	0.0131	0.0023	0.0129	1.0053	0.9473	1.0667	0.9476	1.0664	0.8143	1.1963

Note: **48-hour LC_{50}** = 48-hour median lethal concentration. An estimate of the potassium chloride concentration which is lethal to 50% of the test organisms in 48-hours (calculated using ToxCalc).
CT = Central tendency of the LC_{50} values.
S = Standard deviation of the LC_{50} values.
Control Limits = Mean logarithmic $LC_{50} \pm 2$ standard deviations converted to anti-logarithmic values.
Warning Limits = Mean logarithmic $LC_{50} \pm 2CV$ or $S_{A.75}$ converted to anti-logarithmic values.
$S_{A.75}$ = Standard deviation corresponding to the 75th percentile of CVs reported nationally by USEPA. ($S_{A.75} = 0.19$).
CV = Coefficient of variation.

Subject: *Pimephales promelas* Acute Reference Toxicity Test, EPA 2000.0

Environmental Testing Solutions, Inc.

Pimephales promelas
Acute Reference Toxicant Control Chart
Source: In-house Culture



- ● **48-hour LC_{50}** = median lethal concentration. An estimation of the potassium chloride concentration which is lethal to 50% of the test organisms in 48-hours (calculated using ToxCalc).
- ——— **Central Tendency** (mean logarithmic LC_{50} converted to anti-logarithmic values)
- - - - **Control Limits** (mean logarithmic LC_{50} ± 2 standard deviations converted to anti-logarithmic values)
- - - - **Laboratory Warning Limits** (mean logarithmic LC_{50} ± 2 coefficent of variations converted to anti-logarithmic values)
- ····· **USEPA Warning Limits** (mean logarithmic LC_{50} ± $S_{A.75}$ converted to anti-logarithmic values, $S_{A.75}$ = 75th percentile of CVs reported nationally by USEPA)

Entered and Reviewed by Jim Summer

Subject: *Pimephales promelas* Chronic Toxicity Test, EPA 1000.0

Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan		03-01-20
Quality Assurance Officer	Jim Sumner		03-01-20

Document Revision History

Effective Date	Revision number	Review Type	Evaluators	Revisions
12-01-00	0	Internal	Jim Sumner (ETS)	Original document
06-01-11	1	**Internal**	Jim Sumner (ETS)	• Updated references and exhibits. • Statistical analyses and data review moved to QAP-Q12.
07-01-12	2	External (NC DENR) Internal	Lance Ferrell (NC DENR) Jim Sumner (ETS)	• The measurement of pH, DO and conductivity of each new, full-strength, undiluted sample was added. • The light intensity was amended to reflect that it is a recommended range as specified in the EPA manuals.
11-01-14	3	Internal	Jim Sumner (ETS)	• Updated exhibits during document review. • Changed renewal time recommendation to ± 2-hours from test initiation. • Removed KY acceptability criteria which follows EPA requirements. • Added minimum guidance criteria for PMSD to Table AT20.1.
09-28-16	4	External (TVA) Internal	Rick Sherrard, Donald Snodgrass (TVA) Jim Sumner (ETS)	• Updated exhibits during document review. • Updated the isolation of test larvae for using the in-house culture.
09-01-19	5	Internal	Jim Sumner (ETS)	• Updated procedure to include NELAP requirements. • Additional guidance included in SOP.
03-01-20	6	External (TVA) Internal	Rick Sherrard (TVA) Jim Sumner (ETS)	• Updated bench sheet to include reporting limits, method numbers, meters and serial numbers used for chemical analyses.

Subject: *Pimephales promelas* Chronic Toxicity Test, EPA 1000.0

Scope and Application

To measure the chronic toxicity of water samples to *Pimephales promelas*, using less than 24-hour old larvae during a 7-day, static renewal test.

Summary of Method

The chronic toxicity test generally involves the exposure of test organisms to up to five effluent concentrations and a control water. The test duration is 7-days. Test solutions are renewed daily, and observations of survival are documented. At the end of the 7-day exposure period, organisms are killed, and a dry weight is determined.

A summary of the *Pimephales promelas* chronic method is provided in Exhibit AT20.1.

Quality Control

Acceptance Criteria: The test acceptance criteria are indicated in the table below. If acceptability criteria are not met, the test must be invalidated.

Table AT20.1: *Pimephales promelas* chronic toxicity test acceptability criteria.

Test Acceptability Criteria	USEPA
Control survival	≥ 80%
Mean dry weight of surviving control larvae (mg)	≥ 0.25
Guidance control growth coefficient of variation	< 20%
Guidance percent minimum significant difference (PMSD)	12 – 30%

Subject: *Pimephales promelas* Chronic Toxicity Test, EPA 1000.0

Equipment and Materials

Fathead minnow larvae (*Pimephales promelas*)
Temperature-controlled incubator (set to maintain test temperature = 25.0 ± 1.0°C, photoperiod of 16-hour light / 8-hours dark, light intensity = 50 – 100 ft-c)
Control water (synthetic water made with reagent grade chemicals)
Microbalance accurate to 0.00001 mg (e.g. Cahn)
Class S or Class I certified weights
Microweight aluminum pans (e.g. Cahn)
Drying oven
Desiccator
Scintillation vials
Plastic tray
500-mL plastic Solo® Cups
Solo® Cup Lids
Graduated cylinders
Large glass finger bowls
Serological, fixed, or adjustable volume pipette (e.g., Eppendorf)
Transfer pipettes
Siphoning hose (rubber tubing affixed with a plastic tube fitted with a soft, angled rubber tip)
White plastic photographic tray
Fine mesh sieve
Forceps
Ice water
Aquarium pump, tubing, and air stones
Plexiglas® slides
Thermometer
1-oz medicine cups
Newly hatched brine shrimp
Light box or table
Disposable gloves
Pimephales promelas Chronic Toxicity Test Bench Sheet
Randomization template

Subject: *Pimephales promelas* Chronic Toxicity Test, EPA 1000.0

Procedure

A. Test Preparation.

1. Prepare the pasticware.

   a. Obtain four replicate 500-mL plastic Solo® cups with lids (or equivalent) for each site/sample and concentration tested, including the control. Label each replicate cup with the following information.
   - Facility/sample name
   - Concentration
   - Replicate number

   b. Label the appropriate graduated cylinder with the facility/sample name and concentration.

   c. Prepare the *Pimephales promelas* Chronic Toxicity Test Bench Sheet (Exhibit AT20.3). Record the following information on the Bench Sheet:
   - Client/facility name
   - Permit number
   - County
   - Outfall/Pipe number
   - ETS project and sample numbers
   - Control/Dilution water type and batch
   - Test concentrations and dilution preparation information (sample, dilution and total volumes)

2. Weigh the microweight pans (This step may be completed at any time before test termination on day 7).

   a. Label 20-mL glass beakers or Coors® spot plates with the facility or sample name, concentration, and replicate number.

   b. Obtain the microweight aluminum pans from the desiccator.

   c. Using forceps, place one microweight pan into each of the 20-mL glass beakers or each of the wells of the spot plates.

   d. Place the 20-mL glass beakers or spot plates in a drying oven and let the contents dry a minimum of 24-hours at 60 ± 2°C or 6-hours at 100 ± 2°C.

e. Remove the 20-mL glass beakers or spot plates from the oven and place in a desiccator a minimum of 4-hours to cool the pans before they are weighted on a calibrated microbalance.

f. Verify the accuracy of the microbalance as described in SOP-G10.

g. Using forceps, remove a microweight pan and place it on the balance pan. Allow the reading to stabilize and record the measurement on the chronic benchsheet. Record the date, beaker/spot plate color identification and analyst initials on the chronic benchsheet. Return the microweight pan to the appropriate 20-mL glass beaker or well on the spot plate.

h. Repeat Step 2.g to obtain the initial weight of each pan needed for the test. After all the initial weights are obtained, place the 20-mL glass beakers or spot plates in a desiccator until needed on day 7.

B. Test Initiation (Day 0).

1. Prepare the test concentrations according to SOP-G5.

a. The control/dilution water is moderately hard synthetic water (MHSW, SOP-AT1). MHSW must have an alkalinity of 57 – 64 mg $CaCO_3$/L, hardness of 80 – 100 mg $CaCO_3$/L, and an initial pH of 6.5 – 8.5 S.U. (guidance range = 7.4 – 7.8 S.U.).

b. Measure and record the pH (SOP-C3), dissolved oxygen [SOP-C2, ensure that the dissolved is within the acceptable range (4.0 to 9.0 mg/L), aerate the sample if necessary according to SOP-G5] and conductivity (SOP-C4) of each concentration tested and control. Measure and record the pH (SOP-C3), dissolved oxygen (SOP-C2), conductivity (SOP-C4), total residual chlorine (SOP-C8), total alkalinity (SOP-C6), total hardness (SOP-C7) and sample characteristics of each new, full-strength, undiluted sample. Measure and record the alkalinity (SOP-C6) and hardness (SOP-C7) of the control/dilution water.

c. Pour 250 mL of control water into each of the control cups.

d. Pour 250 mL of each test concentration into each of the labeled test cups.

e. Maintain the test temperature (25.0 ± 1.0°C) of the test concentrations. This may be accomplished by placing the test cups into a temperature-controlled incubator.

2. Isolate the larvae for the test.

a. Obtain a batch of larvae (SOP-AT17), which are < 24 hours old. The test organisms must come from a pool of larvae consisting of at least three separate spawnings. Please refer to Exhibit AT20.2: Weekly *Pimephales promelas* Spawning / Egg Collection Log. Record the spawning date, age and hatch dates and times of the organisms to be used in the test on the chronic bench sheet. Transfer the larvae from the tank to a large finger bowl.

b. After the larvae have acclimated to the test conditions, the larvae may be transferred by transfer pipette to the test solutions. The end of the pipette tip should be cut to a size that will not injure or harm the larvae during transfer. Larvae should be transferred gently in a manner that will not expose the organisms to the air.

c. Two techniques may be used for transferring 10 organisms to each test cup from the finger bowl. The technique used is dependent on the sample being evaluated for toxicity.

- If the transferred water may alter the toxicity of the sample (decrease toxicity), organisms should be transferred directly by pipette. This technique is predominately used by the laboratory. Organisms should be transferred in a manner that allows them to swim from the pipette into the test solutions. This will minimize the volume of transfer water introduced into the sample. Follow procedures outlined in step 3 for transferring the organisms to the randomly placed test cups. The average transfer volume by this technique for each analyst must be determined yearly. For an example transfer volume log sheet refer to Exhibit AT20.4.

- If pathogenic interferences have been identified or there is risk of cross contamination between replicates, organisms should be transferred to medicine cups (10 per cup) and then introduced into the test solutions. The final volume contained in the medicine cups after the organisms have been transferred should be between 10 and 15 ml. With a transfer pipette, remove any food or debris that was placed in the cup during transfer. The average transfer volume by this technique for each analyst must be determined yearly. For an example transfer volume log sheet refer to

Exhibit AT20.4. Continue this process until enough medicine cups containing 10 larvae each have been obtained to initiate the test. 1 medicine cup containing 10 larvae will be needed for each sample concentration and replicate. If a test consists of 5 concentrations and a control, 24 medicine cups containing 10 larvae each will be required. If a test consists of 1 concentration and a control, 8 medicine cups containing 10 larvae each will be required.

d. Save approximately 30 mL of transfer water to be measured for pH (SOP-C3). Measure and record the transfer water pH and temperature on the chronic bench sheet.

3. Transfer the larvae to the randomly placed test cups.

a. Obtain a randomization template (Exhibit AT20.5). Order the test cups according to the randomization template and record the template name on the bench sheet.

b. Measure and record the temperature in one of the test cups for each concentration and control. The temperature must be 25.0 ± 1.0°C before the test organisms are placed in the test cups. Warm the test cups in a warm water bath or temperature-controlled incubator, if necessary, until the desired test temperature is obtained. Measure and record the temperature of an arbitrary transfer cup.

c. Place 10 larvae in the first test cup of the first row (by pipette or medicine cup). Continue in this manner (placing the larvae in the test cups from left to right in the first row and then the second row) until all the test cups contain 10 larvae.

d. Record the initiation date, time and analyst's initials on the chronic bench sheet. Record the average transfer volume by the technique used on the chronic bench sheet. **The test must be initiated within 36-hours of completion of the first sampling period.**

e. Verify that each cup received the required number of larvae (i.e., 10) by conducting a repeat count. Remove excess larvae or add larvae as necessary. Record the initial number of larvae on the bench sheet. Place the lids on each cup.

f. Place the test cups in order according to the randomization template in a temperature-controlled incubator. The organisms must be maintained at 25.0 ±

1.0°C with a photoperiod of 16-hours light and 8-hours dark and a recommended light intensity of 50 to 100 ft-c. Record the incubator number used on the bench sheet.

g. Using a transfer pipette, feed the larvae in each test cup 3 drops (150 µL) newly hatched brine shrimp (1050 to1500 shrimp). To obtain the appropriate suspension of brine shrimp, refer to SOP-AT16. [Note: The test larvae are fed twice daily at a 6 ± 1-hour interval (generally at the beginning and at the end of the workday).] Record the time(s) the larvae were fed on the *Pimephales promelas* Chronic Toxicity Test Bench Sheet.

Note: Since the larvae are fed in holding prior to test initiation, the larvae may be fed only once in the test cups on the first day.

C. Daily Test Renewal (Days 1-6).

Repeat this process each day during the test period. The test must be renewed within ± 2 hours from test initiation. **When new samples are used for test solution renewal, the test must be renewed within 36-hours of completion of the first sampling period for each new sample.**

1. Prior to renewal of the test water in the cups, carefully pour ~30 mL of test water from at least one replicate cup for each test concentration and control into a labeled 1-oz medicine cup. This water will be used to determine final pH and dissolved oxygen concentrations.

2. Feed the larvae in the test cup 150 µL of newly-hatched brine shrimp a minimum of 2-hours prior to renewal of the test concentrations. Record the feeding time on the *Pimephales promelas* Chronic Toxicity Test Bench Sheet.

3. Measure and record the temperature in an arbitrarily selected test replicate for each concentration and control.

4. Prepare fresh test water in accordance with SOP-G5. Maintain the test temperature (25.0 ± 1.0°C) of the fresh test water until needed by storing in a temperature-controlled incubator.

5. Remove the test cups from the incubator and remove the lids. Place the cups on a light box or table for ease of viewing.

6. Change the test water in all four replicate cups before starting the next four-cup series. To change the test water, test cups may be either siphoned or decanted.

a. Siphoning method: Siphon off old water, excess shrimp and detritus from the cups using rubber tubing affixed with a plastic tube fitted with a soft, angled rubber tip. Slowly siphon the water from the cup into a white plastic photographic tray until ~ 50 mL of old test water remains. Control the flow through the tubing by holding one gloved finger over the end of the tubing.

Decanting method: Using a transfer pipette, remove any debris, dead artemia and dead larvae that may have accumulated on the bottom of the test cup. Carefully decant the water from the cup into a white plastic photographic tray until ~ 50 mL of old test water remains. This technique is predominately used by the laboratory.

b. If any larvae are accidentally siphoned off or decanted with the water, retrieve them from the plastic tray, using a transfer pipette. The end of the transfer pipette tip should be cut to a size that will not injure or harm the larvae during transfer. Larvae should be transferred gently in a manner that will not expose the organisms to the air. Return the larvae to the appropriate replicate cup. Record the number of larvae siphoned out or decanted (per replicate). Discard any dead larvae.

c. Record the following information on the chronic bench sheet.
- Number of larvae surviving in each replicate cup
- Number of dead larvae in each replicate cup (if applicable)
- Any comments (injured, sick or larvae siphoned out)

d. Fill each replicate cup to 250 mL using fresh test water. Pour the test water down the side of the cup to avoid unnecessarily disturbing the larvae.

h. After all test cups have been renewed, record the renewal time and the analyst's initials on the chronic bench sheet.

i. Place the lids on each cup. Place the test cups in order according to the randomization template in a temperature-controlled incubator.

7. At 6 ± 1-hour after the first feeding, feed the test larvae 3 drops (150 µL) of newly-hatched brine shrimp. Record the feeding time on the chronic bench sheet.

Note: Test solutions may be renewed prior to the first feeding.

D. Test Termination (Day 7, not to exceed 7 days + 2 hours).

Terminate the test after the organisms have been exposed to the test concentrations for 7 consecutive days ± 2-hours.

1. Measure and record the temperature in an arbitrarily selected test cup for each concentration and control.

2. Remove the test cups from the incubator and remove the lids. Place the cups on a light box or table for ease of viewing.

3. Carefully pour ~30 mL of test water from at least one replicate cup for each test concentration and control into a labeled 1-oz medicine cup. This water will be used to determine final pH and dissolved oxygen concentrations.

4. Obtain the appropriately labeled 20-mL glass beakers or spot plates containing pre-weighed microweight pans.

5. Fill a 600-mL beaker or equivalent with ice water and obtain a fine mesh sieve with a handle.

6. Beginning with the first replicate cup of the control.

    a. Count and record (in the appropriate section) the number of living and dead larvae in each replicate cup on the chronic bench sheet. Record comments, if applicable. Discard any dead larvae.

    b. Holding the mesh sieve over an empty beaker, pour the test water containing the larvae from one replicate cup through the sieve. The larvae will be retained on the mesh.

    c. Immediately submerge the sieve containing the larvae into the ice water. Keep the larvae in the ice water for 3 to 4 seconds.

    d. Remove the sieve from the ice water. Carefully rinse the larvae with deionized water to remove any excess food or detritus.

    e. Using forceps, remove the microweight pan from the appropriate 20-mL glass beaker or well on the spot plate. Using the forceps, transfer the larvae from the mesh to the microweight pan. In the process, to ensure the larvae are dead, sever their spinal cords with forceps. Ensure that all the larvae have been

transferred to the microweight pan. Verify against the number recorded in Step 6.a. above.

A study was performed to determine if solids are lost by this method of killing the larvae before they are placed on the microweight pans. The study determined that the amount of solids lost from larvae killed by severing the spinal cords was not significantly different than the amount of moisture lost during the weighing process (study performed using wet weights, Exhibit AT20.6).

f. Return the pan to the appropriate 20-mL glass beaker or well on the spot plate.

g. Repeat Step 6 for the remaining test cups for each test concentration (from lowest to highest).

7. Place the 20-mL glass beakers or spot plates in a drying oven and let the contents dry a minimum of 24-hours at 60 ± 2°C or 6-hours at 100 ± 2°C. Yearly laboratory studies have confirmed that drying the larvae longer than the recommended time will not alter the final dry weight.

8. Remove the 20-mL glass beakers or spot plates from the oven and place in a desiccator a minimum of 4-hours to cool the larvae before weighing them on a calibrated microbalance.

9. Measure the final pan weights.

a. Verify the accuracy of the microbalance as described in SOP-G10.

b. Using forceps, remove the microweight pan from the 20-mL glass beaker or well on the spot plate and place it on the balance pan. Allow the reading to stabilize and record the measurement on the chronic benchsheet. Return the microweight pan to the 20-mL glass beaker or well on the spot plate. Record the date the weights were measured and analyst initials on the chronic benchsheet.

c. Repeat Step 9.b. to obtain the final weight of each remaining pan. After all the final weights are obtained, return the 20-mL glass beakers or spot plates to a desiccator until the survival and weight data have been verified.

E. Statistical Analyses and Test Data Verification.

Statistical analyses and data review are performed according to QAP-Q12.

Safety and Hazardous Waste Management

Safety glasses, gloves and lab coats should always be worn.

Review Policy-P6: General Safety Policy and Policy-P9: Radiation Protection Policy for additional safety requirements.

References

USEPA. October 2002. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, 4th ed. **EPA-821-R-02-013, Method 1000.0**. US Environmental Protection Agency, Cincinnati, OH.

TNI Standard. Management and Technical Requirements for Laboratories Performing Environmental Analysis. EL-V1-ISO-2016-Rev2.0. The NELAC Institute, PO Box 2439, Weatherford, TX 76086.

Exhibits

Exhibit AT20.1: Summary of Test Conditions for the *Pimephales promelas* Chronic Toxicity Test.
Exhibit AT20.2: Weekly *Pimephales promelas* Spawning / Egg Collection Log.
Exhibit AT20.3: *Pimephales promelas* Chronic Toxicity Test Bench Sheet.
Exhibit AT20.4: Average Transfer Volume Log Sheet.
Exhibit AT20.5: Randomization Template.
Exhibit AT20.6: Determination of Solids Loss from Killing of Larvae at Test Termination.

Subject: *Pimephales promelas* Chronic Toxicity Test, EPA 1000.0

Exhibit AT20.1: Summary of Test Conditions for the *Pimephales promelas* Chronic Toxicity Test.

SUMMARY OF TEST CONDITIONS FOR THE *PIMEPHALES PROMELAS* CHRONIC TOXICITY TEST

Test type:	Static renewal
Test duration:	7-days
Temperature:	25.0 ± 1.0°C
Light quality:	Cool-white fluorescent
Light intensity:	50 – 100 ft-candles (recommended)
Photoperiod:	16-hours light, 8-hours dark
Test chamber size:	500 mL Solo® cups
Test solution volume:	250 mL
Renewal of test solutions:	Daily
Age of test organisms:	< 24-hours old.
Number of organisms per test chamber:	10
Number of replicate test chambers per concentration:	4
Number of organisms per concentration:	40
Test concentrations:	Multiple concentration tests: 5 and a control with ≥ 0.5 dilution series (recommended) Single dilution tests: 100% and a control
Test chamber cleaning:	Daily, test chambers are cleaned immediately before test solution renewal.
Aeration:	None, unless the dissolved oxygen concentration falls below 4.0 mg/L.
Feeding regime:	On days 0 through 6, organisms in each test cup are fed 150 µL *Artemia nauplii* twice daily at 6-hour intervals.
Control / Dilution water:	Moderately hard synthetic water
Sampling and sample holding:	3-gallon grab or composite samples collected on days one, three and five. Each sample must first be used within 36-hours of completion of each sampling period.
Endpoint:	Survival and growth (dry weight per initial number of larvae)
Test acceptability criterion:	≥ 80% control survival, control growth ≥ 0.25 mg/surviving larvae

Subject: *Pimephales promelas* Chronic Toxicity Test, EPA 1000.0

Exhibit AT20.2: Weekly *Pimephales promelas* Spawning / Egg Collection Log.

ETS

Page ______

Weekly *Pimephales promelas* Spawning / Egg Collection Log

Date (Spawn date and Batch ID)	Time	Analyst	Total # Tiles	Start Hatching		End Hatching		Comments
				Date	Time	Date	Time	
07-28-19								Large stock: 1 ____, 2 ____, 3 ____, 4 ____, 5 ____, 6 ____ Removed larvae hatched prior to: ________ Analyst ______
07-29-19								Large stock: 1 ____, 2 ____, 3 ____, 4 ____, 5 ____, 6 ____ Removed larvae hatched prior to: ________ Analyst ______
07-30-19								Large stock: 1 ____, 2 ____, 3 ____, 4 ____, 5 ____, 6 ____ Removed larvae hatched prior to: ________ Analyst ______
07-31-19								Large stock: 1 ____, 2 ____, 3 ____, 4 ____, 5 ____, 6 ____ Removed larvae hatched prior to: ________ Analyst ______
08-01-19								Large stock: 1 ____, 2 ____, 3 ____, 4 ____, 5 ____, 6 ____ Removed larvae hatched prior to: ________ Analyst ______
08-02-19								Large stock: 1 ____, 2 ____, 3 ____, 4 ____, 5 ____, 6 ____ Removed larvae hatched prior to: ________ Analyst ______
08-03-19								Large stock: 1 ____, 2 ____, 3 ____, 4 ____, 5 ____, 6 ____ Removed larvae hatched prior to: ________ Analyst ______

Note: Unhatched eggs on tiles at the end hatch time are moved to the next spawn date to allow the remaining eggs to hatch. These eggs will all hatch in < 24-hours.

Egg Collection Source by Spawn Date:

Date	Tank ID (X locations where tiles with eggs were collected)																																																	
(Spawn date and Batch ID)	A						B						C						D						E						F						G			H			Stock							
	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	1	2	3	1	2	3	4	5	6	7	8
07-28-19																																																		
07-29-19																																																		
07-30-19																																																		
07-31-19																																																		
08-01-19																																																		
08-02-19																																																		
08-03-19																																																		

Comments:

SOP-AT17-Revision 4–Exhibit AT17.4

Subject: *Pimephales promelas* Chronic Toxicity Test, EPA 1000.0

Exhibit AT20.3: Example *Pimephales promelas* Chronic Toxicity Test Bench Sheet.

Environmental Testing Solutions, Inc.

Chronic Whole Effluent Toxicity Test (EPA-821-R-02-013, Method 1000.0)
Species: *Pimephales promelas*

Client: Tennessee Valley Authority, Watts Bar Nuclear Plant **County:** Rhea
NPDES #: TN 0020168 **Outfall #:** 101
Project #: _____

Dilution preparation:

Dilution prep (%)	0.7	1.4	2.8	5.6	11.2	Sample was not aerated or treated unless otherwise noted on this form. Sample was warmed to 25.0 ± 1.0°C in a warm water bath and then diluted to the test concentrations with moderately hard synthetic water (MHSW).
Effluent volume (mL)	14	28	56	112	224	
Diluent volume (mL)	1986	1972	1944	1888	1776	
Total volume (mL)	2000	2000	2000	2000	2000	

Test organism information:

Organism source:	In-house culture
Age:	< 24-hours old
Spawn date:	
Hatch dates and times:	
Transfer vessel information:	pH (S.U.) = Temperature (°C) =
Average transfer volume (mL):	< 0.25 mL

Test information:

Randomizing template:	
Incubator number and shelf location:	
Artemia CHM number:	CHM1048
Drying information for weight determination:	
Date / Time in oven:	
Initial oven temperature:	
Date / Time out of oven:	
Final oven temperature:	
Total drying time:	

Daily feeding and renewal information:

Day	Date	Morning feeding		Afternoon feeding		Test initiation, renewal, or termination		Sample numbers used		MHSW batch used
		Time	Analyst	Time	Analyst	Time	Analyst	Outfall 101	Intake	
0										
1										
2										
3										
4										
5										
6										
7										

Chemical analyses:

Parameter	Reporting Limit	Method number	Meter	Serial number
pH	0.1 S.U.	SM 4500-H+ B-2011	Accumet AR20	93312452
Dissolved Oxygen (D.O.)	1.0 mg/L	SM 4500-O G-2011	YSI Model 52CE	18D104324
Conductivity	14.9 µmhos/cm	SM 2510 B-2011	Accumet AR20	93312452
Alkalinity	5.0 mg $CaCO_3$/L	SM 2320 B-2011	Accumet AR20	93312452
Hardness	5.0 mg $CaCO_3$/L	SM 2340 C-2011	Not applicable	Not applicable
Chlorine, Total Residual	0.1 mg/L	ORION 97-70-1977	Accumet AB250	92349123
Temperature	0.1 °C	SM 2550B-2010	Digital Thermometer	

Control information:		Acceptance criteria	***Summary of test endpoints:***	
% Mortality:		≤ 20%	**7-day LC_{50} (%)**	
Average weight per initial larvae:			**NOEC (%)**	
Average weight per surviving larvae:		≥ 0.25mg/larvae	**LOEC (%)**	
			ChV (%)	
			IC_{25} (%)	

Subject: *Pimephales promelas* Chronic Toxicity Test, EPA 1000.0

Species: *Pimephales promelas*
Client: TVA / Watts Bar Nuclear Plant, Outfall 101 Date: _____

Survival and Growth Data

Day	CONTROL				0.7%				1.4%			
	A	B	C	D	E	F	G	H	I	J	K	L
0	10	10	10	10	10	10	10	10	10	10	10	10
1												
2												
3												
4												
5												
6												
7												
A = Pan weight (mg) Tray color code: _____ Analyst: _____ Date: _____												
B = Pan + Larvae weight (mg) Analyst: _____ Date: _____												
C = Larvae weight (mg) = B – A Analyst: _____												
Weight per initial number of larvae (mg) = C / Initial number of larvae Analyst: _____												
Average weight per initial number of larvae (mg) / Percent reduction from control (%)												

Comment codes: c = clear, d = dead, fg = fungus, k = killed, m = missing, sk = sick, sm = unusually small, lg = unusually large, d&r = decanted and returned, w = wounded.

Comments:

SOP AT20-Revision 6–Exhibit AT20.3

Subject: *Pimephales promelas* Chronic Toxicity Test, EPA 1000.0

Species: *Pimephales promelas*
Client: TVA / Watts Bar Nuclear Plant, Outfall 101 Date: _____

Survival and Growth Data

Day	2.8%				5.6%				11.2%			
	M	N	O	P	Q	R	S	T	U	V	W	X
0	10	10	10	10	10	10	10	10	10	10	10	10
1												
2												
3												
4												
5												
6												
7												
A = Pan weight (mg) Tray color code: _____ Analyst: _____ Date: _____												
B = Pan + Larvae weight (mg) Analyst: _____ Date: _____												
C = Larvae weight (mg) = B – A Analyst: _____												
Weight per initial number of larvae (mg) = C / Initial number of larvae Analyst: _____												
Average weight per initial number of larvae (mg) / Percent reduction from control (%)												

Comment codes: c = clear, d = dead, fg = fungus, k = killed, m = missing, sk = sick, sm = unusually small, lg = unusually large, d&r = decanted and returned, w = wounded.

Comments:

SOP AT20-Revision 6–Exhibit AT20.3

Species: ***Pimephales promelas***
Client: **TVA / Watts Bar Nuclear Plant, Outfall 101** **Date:** _____

Survival and Growth Data

Day	100% Intake			
	Y	Z	AA	BB
0	10	10	10	10
1				
2				
3				
4				
5				
6				
7				
A = Pan weight (mg) Tray color code: _____ Analyst: _____ Date: _____				
B = Pan + Larvae weight (mg) Analyst: _____ Date: _____				
C = Larvae weight (mg) = B – A Analyst: _____				
Weight per initial number of larvae (mg) = C / Initial number of larvae Analyst: _____				
Average weight per initial number of larvae (mg) / Percent reduction from control (%)				

Comment codes: c = clear, d = dead, fg = fungus, k = killed, m = missing, sk = sick, sm = unusually small, lg = unusually large, d&r = decanted and returned, w = wounded.

Comments:

SOP AT20-Revision 6–Exhibit AT20.3

Subject: *Pimephales promelas* Chronic Toxicity Test, EPA 1000.0

Environmental Testing Solutions, Inc.

Species: ***Pimephales promelas*** **Date:** _____
Client: **TVA / Watts Bar Nuclear Plant, Outfall 101**

Daily Chemistry:
Temperatures performed at the time of test initiation, renewal or termination by the analyst identified in the Daily Renewal Information table located on Page 1. Alkalinity, hardness and chlorine (total residual) performed by the analyst identified on the bench sheet specific for each analysis and transcribed to this bench sheet.

		Day (Analyst identified for each day, performed pH, D.O. and conductivity measurements only.)					
		0		1		2	
	Analyst						
Concentration	Parameter						
CONTROL, MHSW	pH (S.U.)						
	Dissolved oxygen (mg/L)						
	Conductivity (µmhos/cm)						
	Alkalinity (mg $CaCO_3$/L)						
	Hardness (mg $CaCO_3$/L)						
	Temperature (°C)						
0.7%	pH (S.U.)						
	Dissolved oxygen (mg/L)						
	Conductivity (µmhos/cm)						
	Temperature (°C)						
1.4%	pH (S.U.)						
	Dissolved oxygen (mg/L)						
	Conductivity (µmhos/cm)						
	Temperature (°C)						
2.8%	pH (S.U.)						
	Dissolved oxygen (mg/L)						
	Conductivity (µmhos/cm)						
	Temperature (°C)						
5.6%	pH (S.U.)						
	Dissolved oxygen (mg/L)						
	Conductivity (µmhos/cm)						
	Temperature (°C)						
11.2%	pH (S.U.)						
	Dissolved oxygen (mg/L)						
	Conductivity (µmhos/cm)						
	Temperature (°C)						
100%	pH (S.U.)						
	Dissolved oxygen (mg/L)						
	Conductivity (µmhos/cm)						
	Alkalinity (mg $CaCO_3$/L)						
	Hardness (mg $CaCO_3$/L)						
	Chlorine (mg/L)						
	*Temperature (°C)						
100% Intake	pH (S.U.)						
	Dissolved oxygen (mg/L)						
	Conductivity (µmhos/cm)						
	Alkalinity (mg $CaCO_3$/L)						
	Hardness (mg $CaCO_3$/L)						
	Chlorine (mg/L)						
	Temperature (°C)						
		Initial	Final	Initial	Final	Initial	Final

SOP AT20-Revision 6–Exhibit AT20.3

Subject: *Pimephales promelas* Chronic Toxicity Test, EPA 1000.0

Environmental Testing Solutions, Inc.

Species: ***Pimephales promelas***
Client: **TVA / Watts Bar Nuclear Plant, Outfall 101** **Date:** _____

		Day (Analyst identified for each day, performed pH, D.O. and conductivity measurements only.)							
		3		4		5		6	
	Analyst								
Concentration	Parameter								
CONTROL, MHSW	pH (S.U.)								
	Dissolved oxygen (mg/L)								
	Conductivity (µmhos/cm)								
	Alkalinity (mg $CaCO_3$/L)								
	Hardness (mg $CaCO_3$/L)								
	Temperature (°C)								
0.7%	pH (S.U.)								
	Dissolved oxygen (mg/L)								
	Conductivity (µmhos/cm)								
	Temperature (°C)								
1.4%	pH (S.U.)								
	Dissolved oxygen (mg/L)								
	Conductivity (µmhos/cm)								
	Temperature (°C)								
2.8%	pH (S.U.)								
	Dissolved oxygen (mg/L)								
	Conductivity (µmhos/cm)								
	Temperature (°C)								
5.6%	pH (S.U.)								
	Dissolved oxygen (mg/L)								
	Conductivity (µmhos/cm)								
	Temperature (°C)								
11.2%	pH (S.U.)								
	Dissolved oxygen (mg/L)								
	Conductivity (µmhos/cm)								
	Temperature (°C)								
100%	pH (S.U.)								
	Dissolved oxygen (mg/L)								
	Conductivity (µmhos/cm)								
	Alkalinity (mg $CaCO_3$/L)								
	Hardness (mg $CaCO_3$/L)								
	Chlorine (mg/L)								
	*Temperature (°C)								
100% Intake	pH (S.U.)								
	Dissolved oxygen (mg/L)								
	Conductivity (µmhos/cm)								
	Alkalinity (mg $CaCO_3$/L)								
	Hardness (mg $CaCO_3$/L)								
	Chlorine (mg/L)								
	Temperature (°C)								
		Initial	Final	Initial	Final	Initial	Final	Initial	Final

SOP AT20-Revision 6–Exhibit AT20.3

Subject: *Pimephales promelas* Chronic Toxicity Test, EPA 1000.0

Exhibit AT20.4: Average Transfer Volume Log Sheet.

ETS Environmental Testing Solutions, Inc.

Page 1 of 1

Larval Fish Transfer Volume

Analyst:		Species:	
Date:		Source / Batch:	
Ambient temperature:		Wet Weight of 10 Larvae (g):	

Estimate transfer volume, where minnows are allowed to swim from the pipette into the test vessel.

Numerically label 10 medicine cups.
Add 10 mL MHSW to each of the 10 cups.
Measure and record the weight of each cup containing MHSW.
Transfer 10 larvae to each cup, following procedures identified in SOP-AT18, AT47, or AT53 for vertebrate acute toxicity tests
Transfer the larvae in a manner that allows them to swim from the pipette into the MHSW contained in each cup.
Measure and record the weight of each cup containing MHSW with 10 larvae.
Determine each transfer volume and average transfer volume.

Replicate Number	Initial Weight Medicine cup + 10 mL MHSW (g)	Final Weight Medicine cup + 10 mL MHSW + 10 Larval Fish transferred (g)	Transfer Volume Final - Initial Weight (g = mL)
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
	Average volume to transfer 10 organisms (mL):		

Estimate transfer volume, where the minnows are transferred with MHSW into the test vessels.

Numerically label 10 medicine cups.
Measure and record the weight of each cup.
Add approximately 10 mL MHSW to each of the 10 cups.
Transfer 10 larvae to each cup, following procedures identified in SOP-AT18, AT47, or AT53 for vertebrate acute toxicity tests
Transfer the larvae in a manner that allows them to swim from the pipette into the MHSW contained in each cup.
Measure and record the weight of each cup containing MHSW with 10 larvae.
Determine each transfer volume and average transfer volume.

Replicate Number	Initial Weight Medicine cup (g)	Final Weight Medicine cup + 10 mL MHSW + 10 Larval Fish transferred (g)	Transfer Volume Final - Initial Weight (g = mL)
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
	Average volume to transfer 10 organisms (mL):		

SOP AT18-Revision 6-Exhibit AT18.4

Exhibit AT20.5: Example Randomization Template.

Randomizing template: **BLUE**

Replicate #	1	2	3	4
Concentrations	**1**	**7**	**3**	**5**
	7	**3**	**4**	**6**
1 = Control	**4**	**2**	**6**	**1**
2 = Lowest concentration	**3**	**5**	**5**	**2**
3 - 5 = Intermediate concentrations	**6**	**4**	**2**	**4**
6 = Highest concentration	**2**	**1**	**1**	**7**
7 = Intake/Upstream	**5**	**6**	**7**	**3**

Random number seeds: 10 through 13

Exhibit AT20.6: Determination of Solids Loss from Killing of Larvae at Test Termination.

Study to determine the amount of solids lost by killing the minnows (severing the spinal cords) at test termination.

Study performed using 1 minnow per replicate.

Analyst: J. Sumner

Date: 08-23-08

Replicate	Initial Pan Weight (mg)	Pan + Larvae weight (mg)	Larvae removed, killed, and returned to pan. Pan + Larvae weight (mg)	Weight loss (mg)
1	14.53	16.14	16.06	**0.08**
2	14.98	16.87	16.80	**0.07**
3	14.60	16.13	16.05	**0.08**
4	14.73	16.53	16.46	**0.07**
5	12.55	13.79	13.70	**0.09**
6	13.73	16.15	16.05	**0.10**
7	13.89	15.30	15.21	**0.09**
8	15.65	17.40	17.30	**0.10**
9	13.19	14.35	14.27	**0.08**
10	14.14	15.52	15.45	**0.07**
11	13.34	14.11	14.04	**0.07**
12	14.95	16.96	16.86	**0.10**
13	14.09	14.92	14.84	**0.08**
14	13.02	15.06	14.96	**0.10**
15	14.15	15.79	15.70	**0.09**
16	13.01	14.36	14.28	**0.08**
17	13.55	14.57	14.51	**0.06**
18	14.20	15.68	15.60	**0.08**
19	14.21	15.57	15.49	**0.08**
20	12.85	13.90	13.82	**0.08**
			Average:	**0.08**

Method:

Pan + Larvae weight =

a. Holding the mesh sieve over an empty beaker, pour the test water containing the larvae from one replicate cup through the sieve. The larvae will be retained on the mesh.

b. Immediately submerge the sieve containing the larvae into the ice water. Keep the larvae in the ice water for 3 to 4 seconds.

c. Remove the sieve from the ice water. Carefully rinse the larvae with deionized water to remove any excess food or detritus.

d. Using forceps, carefully remove the larvae by the tail and place on the pan.

Larvae killed and re-weighed =

a. Using forceps, sever the spinal cord of the larvae on the pan.

Larvae never removed from pan.

SOP AT-20, page: 9 of 13

Subject: *Pimephales promelas* Chronic Toxicity Test, EPA 1000.0

Study to determine the amount of moisture lost during weighing.

Study performed using 1 minnow per replicate.

Analyst: J. Sumner

Date: 08-23-08

Replicate	Initial Pan Weight (mg)	Pan + Larvae weight (mg)	Pan + Larvae reweighed after 3-5 seconds. Pan + Larvae weight (mg)	Weight loss (mg)
1	13.17	14.69	14.62	**0.07**
2	14.02	15.66	15.58	**0.08**
3	14.93	17.14	17.06	**0.08**
4	14.48	16.00	15.93	**0.07**
5	14.53	15.82	15.77	**0.05**
6	14.42	17.15	17.05	**0.10**
7	14.71	17.27	17.18	**0.09**
8	14.88	16.75	16.70	**0.05**
9	13.50	16.17	16.09	**0.08**
10	14.32	16.87	16.79	**0.08**
11	14.29	16.83	16.75	**0.08**
12	14.80	16.78	16.71	**0.07**
13	15.69	18.72	18.63	**0.09**
14	14.16	16.59	16.50	**0.09**
15	14.65	16.91	16.83	**0.08**
16	13.47	15.71	15.62	**0.09**
17	13.81	16.24	16.15	**0.09**
18	15.10	17.08	16.98	**0.10**
19	14.12	16.60	16.49	**0.11**
20	13.42	17.31	17.22	**0.09**
			Average:	**0.08**

Method:

Pan + Larvae weight =

a. Holding the mesh sieve over an empty beaker, pour the test water containing the larvae from one replicate cup through the sieve. The larvae will be retained on the mesh.

b. Immediately submerge the sieve containing the larvae into the ice water. Keep the larvae in the ice water for 3 to 4 seconds.

c. Remove the sieve from the ice water. Carefully rinse the larvae with deionized water to remove any excess food or detritus.

d. Using forceps, carefully remove the larvae by the tail and place on the pan.

Larvae re-weighed = a. Pan + larvae reweighed after 3 to 5 seconds.
(length of time to kill minnow by severing spinal cord)
Larvae never removed from pan.

Subject: *Pimephales promelas* Chronic Reference Toxicity Test, EPA 1000.0

Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan		03-01-20
Quality Assurance Officer	Jim Sumner		03-01-20

Document Revision History

Effective Date	Revision number	Review Type	Evaluators	Revisions
12-01-00	0	Internal	Jim Sumner (ETS)	Original document
06-01-11	1	Internal	Jim Sumner (ETS)	• Updated references and exhibits. • Updated Table AT21.1.
11-01-14	2	Internal	Jim Sumner (ETS)	• Updated exhibits during document review. • Removed conductivity measurement requirement of stock KCl solution due to inaccuracy of these measurements, which are above the calibration range. • Changed the test concentration range to: 300, 450, 600, 750, 900 and 1050 mg/L KCl for each supplier.
09-28-16	3	External (TVA) Internal	Rick Sherrard, Donald Snodgrass (TVA) Jim Sumner (ETS)	• Updated Table AT21.1 for conductivity measurement guidance values. • Deleted statement: "Verify that the conductivity measured for each test concentration is within the acceptance criteria (refer to table Table AT21.1) before proceeding with the preparation of next concentration. If the conductivity is not within the criteria, remake the test concentration and verify the conductivity." • Updated exhibits during document review.
09-01-19	4	Internal	Jim Sumner (ETS)	• Updated references and exhibits. • Updated procedure to include NELAP requirements. • Additional guidance included in SOP.
03-01-20	5	External (TVA) Internal	Rick Sherrard (TVA) Jim Sumner (ETS)	• Updated bench sheet to include reporting limits, method numbers, meters and serial numbers used for chemical analyses.

Subject: *Pimephales promelas* Chronic Reference Toxicity Test, EPA 1000.0

Scope and Application

To assess the sensitivity of *Pimephales promelas* and the overall credibility of *Pimephales promelas* chronic toxicity tests. Reference toxicant data are part of the laboratory's QA/QC program to evaluate the performance of laboratory personnel and the robustness and sensitivity of the test organisms.

Summary of Method

The chronic reference toxicity test generally involves the exposure of test organisms to six potassium chloride concentrations and control water for a 7-day exposure period. At the end of each 24-hour period, the number of living organisms is counted in each potassium chloride concentration and control water. At the end of the exposure period, the minnows are killed, and a dry weight is determined. The 25% inhibition concentration (IC_{25}) of potassium chloride is determined and compared to previous reference toxicant tests.

Quality Control

Acceptance Criteria: The test acceptance criteria are indicated in the table below. In general, the most stringent acceptability criteria are used by the laboratory.

Table AT21.1: *Pimephales promelas* chronic toxicity test acceptability criteria.

Test Acceptability Criteria	USEPA
Control survival	≥ 80%
Mean dry weight of surviving control larvae (mg)	≥ 0.25
Guidance control growth coefficient of variation	< 20%
Guidance percent minimum significant difference (PMSD)	12 – 30%

Frequency of Testing: A *Pimephales promelas* chronic reference toxicant test must be performed monthly. At a minimum, chronic reference toxicant tests must be performed on a quarterly basis in order to maintain certification requirements.

Subject: *Pimephales promelas* Chronic Reference Toxicity Test, EPA 1000.0

Equipment and Materials

Fathead minnow larvae (*Pimephales promelas*)
Temperature-controlled incubator (set to maintain test temperature = 25.0 ± 1.0°C, photoperiod of 16-hour light / 8-hours dark, light intensity = 50 – 100 ft-c)
Control water (synthetic water made with reagent grade chemicals)
Microbalance accurate to 0.00001 mg (e.g. Cahn)
Class S or Class I certified weights
Microweight aluminum pans (e.g. Cahn)
Drying oven
Desiccator
Scintillation vials
Plastic tray
500-mL plastic Solo® Cups
Solo® Cup Lids
Graduated cylinders
1000 mL Erlenmeyer flasks
Large glass finger bowls
Serological, fixed, or adjustable volume pipette (e.g., Eppendorf)
Transfer pipettes
Siphoning hose (rubber tubing affixed with a plastic tube fitted with a soft, angled rubber tip)
White plastic photographic tray
Fine mesh sieve
Forceps
Ice water
Aquarium pump, tubing, and air stones
Plexiglas® slides
Thermometer
1-oz medicine cups
Newly hatched brine shrimp
Light box or table
Disposable gloves
Pimephales promelas Shipment Log and Organism History Information Sheet
Potassium chloride (KCl, reagent grade)
1000-mL volumetric flask
1000-mL graduated cylinder
10-mL serological pipettes
Pimephales promelas Chronic Reference Toxicity Test Bench Sheet
Randomization template

Subject: *Pimephales promelas* Chronic Reference Toxicity Test, EPA 1000.0

Procedure

A. Test Preparation.

1. Prepare the pasticware.

   a. Obtain four replicate 500-mL plastic Solo® cups with lids (or equivalent) for each concentration tested including the control. Label each replicate cup with the following information.
   - Concentration
   - Replicate number

   b. Obtain enough 1000 mL Erlenmeyer flasks for each test concentration and the control. These flasks will be used in the preparation of the test concentrations. Label each flask with the test concentration.

   c. Label the appropriate graduated cylinder.

   d. Prepare the *Pimephales promelas* Chronic Reference Toxicity Test Bench Sheet (see Exhibit AT21.1). Record the *Pimephales promelas* KCl Chronic (PpKClCR) test number on the bench sheet.

B. Preparation of the Stock Solution.

1. Using a calibrated top-loading balance, carefully weigh out 50 g of KCl (SOP-G10). Place approximately 900 mL of deionized water in a 1000-ml volumetric flask. Add the KCl to the flask. Dissolve the KCl by swirling the flask and bring to volume with deionized water. Label the volumetric flask with the concentration (50 g/L), analyst's initials, preparation date and stock standard number (INSS, SOP-G15). Record the INSS number of the KCl stock solution on the bench sheet.

C. Preparation of the Test Concentrations.

1. Prepare all test concentrations using graduated cylinders and serological pipettes. The precision of conductivity measurements is increased when smaller volume graduated cylinders and/or serological pipettes are used to prepare the test concentrations. For this reference toxicant test, stock solution volumes should be measured using a 10-mL serological pipette and the total volumes should be measured using a 1000-mL graduated cylinder.

2. Beginning with the lowest concentration, add approximately 200 mL of moderately hard synthetic water (MHSW) to a 1000-mL graduated cylinder, add the required volume of stock solution using a 10-mL serological pipette (refer to Table AT21.2), bring to volume (1000 mL) with MHSW. Mix the solution well by pouring the solution into the respective 1000 mL Erlenmeyer flask and swirling the solution in the flask.

3. Pour approximately 250 mL of test solution into each of the replicate test beakers for that concentration. Pour 30 mL of test solutions into a 1-oz medicine cup and measure and record the pH (SOP-C3) and dissolved oxygen (SOP-C2) of the test solution.

4. Measure and record the conductivity (SOP-4) of each test concentration on the bench sheet. Refer to Table AT21.2 for guidance values of conductivity measurements.

5. Rinse the graduated cylinder well with deionized water and repeat steps C.2 through C.4 for preparing the next test concentration. Record the batch date of moderately hard synthetic water used to prepare the dilutions on the bench sheet.

Table AT21.2: Test concentration, stock volumes, moderately hard synthetic water volumes, final volumes and conductivity measurements guidance values for the *Pimephales promelas* KCl chronic reference toxicant tests.

Test Concentration (mg KCl/L)	Volume of Stock Required (mL)	Volume of Moderately Hard Synthetic Water (mL)	Final Volume (mL)	Conductivity Guidance Values (µmhos/cm)
300	6	994	1000	800 - 900
450	9	991	1000	1000 - 1200
600	12	988	1000	1300 - 1400
750	15	985	1000	1500 - 1700
900	18	982	1000	1800 - 2000
1050	21	979	1000	2100 - 2300

6. Once all test concentrations have been prepared, follow the procedure described in SOP-AT20 for conducting *Pimephales promelas* Chronic Toxicity Tests.

D. Control Charts and Outlier Test Results.

Refer to QAP-Q5 for how control charts are prepared and procedures for interpreting outlier tests. Refer to Exhibit AT21.2 for an example control chart.

Subject: *Pimephales promelas* Chronic Reference Toxicity Test, EPA 1000.0

Safety and Hazardous Waste Management

Safety glasses, gloves and lab coats should always be worn.

Review Policy-P6: General Safety Policy and Policy-P9: Radiation Protection Policy for additional safety requirements.

References

USEPA. October 2002. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, 4th ed. **EPA-821-R-02-013, Method 1000.0**. US Environmental Protection Agency, Cincinnati, OH.

USEPA. 2000. Understanding and Accounting for Method Variability in Whole Effluent Toxicity Applications Under the National Pollutant Discharge Elimination Program. EPA-833-R-00-003. US Environmental Protection Agency, Cincinnati, OH.

USEPA. 2001. Final Report: Inter-laboratory Variability Study of EPA Short-term Chronic and Acute Whole Effluent Toxicity Test Methods, Volumes 1 and 2 - Appendix. EPA-821-B-01-004 and EPA-821-B-01-005. US Environmental Protection Agency, Cincinnati, OH.

North Carolina Department of Environment and Natural Resources, Division of Water Quality. Biological Laboratory Certification / Criteria Procedures, Version 3.0. December 2010.

TNI Standard. Management and Technical Requirements for Laboratories Performing Environmental Analysis. EL-V1-ISO-2016-Rev2.0. The NELAC Institute, PO Box 2439, Weatherford, TX 76086.

Exhibits

Exhibit AT21.1: *Pimephales promelas* Chronic Reference Toxicity Test Bench Sheet.
Exhibit AT21.2: Example of a *Pimephales promelas* Chronic Reference Toxicant Control Chart.

Subject: *Pimephales promelas* Chronic Reference Toxicity Test, EPA 1000.0

Exhibit AT21.1: *Pimephales promelas* Chronic Reference Toxicity Test Bench Sheet.

Potassium Chloride Chronic Reference Toxicant Test (EPA-822-R-02-013 Method 1000.0)
Species: *Pimephales promelas*

PpKClCR Test Number: **62**

Dilution preparation information:							***Comments:***
KCl Stock INSS number:			INSS				
Stock preparation:			50 g KCl/L: Dissolve 50 g KCl in 1-L deionized water.				
Dilution prep (mg/L)	300	450	600	750	900	1050	
Stock volume (mL)	12	18	24	30	36	42	
Diluent volume (mL)	1988	1982	1976	1970	1964	1958	
Total volume (mL)	2000	2000	2000	2000	2000	2000	

Test organism information:		***Test information:***	
Organism source:	In-house culture	Randomizing template:	
Age:	< 24-hours old	Incubator number and shelf location:	
Spawn date:		*Artemia* CHM number:	CHM1048
Hatch dates and times:		***Drying information for weight determination:***	
Transfer vessel information:	pH = S.U. Temperature = °C	Date / Time in oven:	
		Initial oven temperature:	
Average transfer volume:	< 0.25 mL	Date / Time out of oven:	
		Final oven temperature:	
		Total drying time:	

Daily feeding and renewal information:

Day	Date	Morning feeding		Afternoon feeding		Test initiation, renewal, or termination		MHSW batch used
		Time	Analyst	Time	Analyst	Time	Analyst	
0								
1								
2								
3								
4								
5								
6								
7								

Chemical analyses:

Parameter	Reporting Limit	Method number	Meter	Serial number
pH	0.1 S.U.	SM 4500-H+ B-2011	Accumet AR20	93312452
Dissolved Oxygen (D.O.)	1.0 mg/L	SM 4500-O G-2011	YSI Model 52CE	18D104324
Conductivity	14.9 µmhos/cm	SM 2510 B-2011	Accumet AR20	93312452
Alkalinity	5.0 mg $CaCO_3$/L	SM 2320 B-2011	Accumet AR20	93312452
Hardness	5.0 mg $CaCO_3$/L	SM 2340 C-2011	Not applicable	Not applicable
Temperature	0.1°C	SM 2550B-2010	Digital Thermometer	

Control information:		Acceptance criteria	***Summary of test endpoints:***	
% Mortality:		≤ 20%	**7-day LC_{50}** (mg/L KCl)	
Average weight per initial larvae:			**NOEC** (mg/L KCl)	
Average weight per surviving larvae:		≥ 0.25 mg/larvae	**LOEC** (mg/L KCl)	
			ChV (mg/L KCl)	
			IC_{25} (mg/L KCl)	

Subject: *Pimephales promelas* Chronic Reference Toxicity Test, EPA 1000.0

Species: ***Pimephales promelas***

PpKClCR Test Number: **62**

Survival and Growth Data

Day	Control				300 mg KCl/L				450 mg KCl/L			
	A	B	C	D	E	F	G	H	I	J	K	L
0	10	10	10	10	10	10	10	10	10	10	10	10
1												
2												
3												
4												
5												
6												
7												
A = Pan weight (mg) Tray color code:: _____ Analyst: _____ Date: _____												
B = Pan + Larvae weight (mg) Analyst: _____ Date: _____												
C = Larvae weight (mg) = B – A Analyst: _____												
Weight per initial number of larvae (mg) = C / Initial number of larvae Analyst: _____												
Average weight per initial number of larvae (mg) / Percent reduction from control (%)												

Comment codes: c = clear, d = dead, fg = fungus, k = killed, m = missing, sk = sick, sm = unusually small, lg = unusually large, d&r = decanted and returned, w = wounded.

Comments:

Species: ***Pimephales promelas***

PpKClCR Test Number: **62**

Survival and Growth Data

Day	600 mg KCl/L				750 mg KCl/L				900 mg KCl/L			
	M	N	O	P	Q	R	S	T	U	V	W	X
0	10	10	10	10	10	10	10	10	10	10	10	10
1												
2												
3												
4												
5												
6												
7												
A = Pan weight (mg) Tray color code:: _____ Analyst: _____ Date: _____												
B = Pan + Larvae weight (mg) Analyst: _____ Date: _____												
C = Larvae weight (mg) = B – A Analyst: _____												
Weight per initial number of larvae (mg) = C / Initial number of larvae Analyst: _____												
Average weight per initial number of larvae (mg) / Percent reduction from control (%)												

Comment codes: c = clear, d = dead, fg = fungus, k = killed, m = missing, sk = sick, sm = unusually small, lg = unusually large, d&r = decanted and returned, w = wounded.

Comments:

Species: *Pimephales promelas*

PpKClCR Test Number: 62

Survival and Growth Data

Day	1050 mg KCl/L			
	Y	Z	AA	BB
0	10	10	10	10
1				
2				
3				
4				
5				
6				
7				
A = Pan weight (mg) Tray color code:: _____ Analyst: _____ Date: _____				
B = Pan + Larvae weight (mg) Analyst: _____ Date: _____				
C = Larvae weight (mg) = B – A Analyst: _____				
Weight per initial number of larvae (mg) = C / Initial number of larvae Analyst: _____				
Average weight per initial number of larvae (mg) / Percent reduction from control (%)				

Comment codes: c = clear, d = dead, fg = fungus, k = killed, m = missing, sk = sick, sm = unusually small, lg = unusually large, d&r = decanted and returned, w = wounded.

Comments:

Subject: *Pimephales promelas* Chronic Reference Toxicity Test, EPA 1000.0

Species: ***Pimephales promelas***

PpKCICR Test Number: **62**

Daily Chemistry:

Temperatures performed at the time of test initiation, renewal or termination by the analyst identified in the Daily Renewal Information table located on Page 1. Alkalinity and hardness performed by the analyst identified on the bench sheet specific for each analysis and transcribed to this bench sheet.

		Day (Analyst identified for each day, performed pH, D.O. and conductivity measurements only.)					
		0		1		2	
Analyst							
Concentration	Parameter						
CONTROL, MHSW	pH (S.U.)						
	Dissolved oxygen (mg/L)						
	Conductivity (µmhos/cm)						
	Alkalinity (mg $CaCO_3$/L)						
	Hardness (mg $CaCO_3$/L)						
	Temperature (°C)						
300 mg KCl/L	pH (S.U.)						
	Dissolved oxygen (mg/L)						
	Conductivity (µmhos/cm)						
	Temperature (°C)						
450 mg KCl/L	pH (S.U.)						
	Dissolved oxygen (mg/L)						
	Conductivity (µmhos/cm)						
	Temperature (°C)						
600 mg KCl/L	pH (S.U.)						
	Dissolved oxygen (mg/L)						
	Conductivity (µmhos/cm)						
	Temperature (°C)						
750 mg KCl/L	pH (S.U.)						
	Dissolved oxygen (mg/L)						
	Conductivity (µmhos/cm)						
	Temperature (°C)						
900 mg KCl/L	pH (S.U.)						
	Dissolved oxygen (mg/L)						
	Conductivity (µmhos/cm)						
	Temperature (°C)						
1050 mg KCl/L	pH (S.U.)						
	Dissolved oxygen (mg/L)						
	Conductivity (µmhos/cm)						
	Temperature (°C)						
		Initial	Final	Initial	Final	Initial	Final

SOP AT21-Revision 5–Exhibit AT21.1

Species: *Pimephales promelas* PpKClCR Test Number: **62**

		Day (Analyst identified for each day, performed pH, D.O. and conductivity measurements only.)							
		3		4		5		6	
	Analyst								
Concentration	Parameter								
CONTROL, MHSW	pH (S.U.)								
	Dissolved oxygen (mg/L)								
	Conductivity (µmhos/cm)								
	Alkalinity (mg $CaCO_3$/L)								
	Hardness (mg $CaCO_3$/L)								
	Temperature (°C)								
300 mg KCl/L	pH (S.U.)								
	Dissolved oxygen (mg/L)								
	Conductivity (µmhos/cm)								
	Temperature (°C)								
450 mg KCl/L	pH (S.U.)								
	Dissolved oxygen (mg/L)								
	Conductivity (µmhos/cm)								
	Temperature (°C)								
600 mg KCl/L	pH (S.U.)								
	Dissolved oxygen (mg/L)								
	Conductivity (µmhos/cm)								
	Temperature (°C)								
750 mg KCl/L	pH (S.U.)								
	Dissolved oxygen (mg/L)								
	Conductivity (µmhos/cm)								
	Temperature (°C)								
900 mg KCl/L	pH (S.U.)								
	Dissolved oxygen (mg/L)								
	Conductivity (µmhos/cm)								
	Temperature (°C)								
1050 mg KCl/L	pH (S.U.)								
	Dissolved oxygen (mg/L)								
	Conductivity (µmhos/cm)								
	Temperature (°C)								
		Initial	Final	Initial	Final	Initial	Final	Initial	Final

SOP AT21-Revision 5–Exhibit AT21.1

Subject: *Pimephales promelas* Chronic Reference Toxicity Test, EPA 1000.0

Exhibit AT21.2: Example of a *Pimephales promelas* Chronic Reference Toxicant Control Chart.

Pimephales promelas
Chronic Reference Toxicant Control Chart
Source: In-house Culture

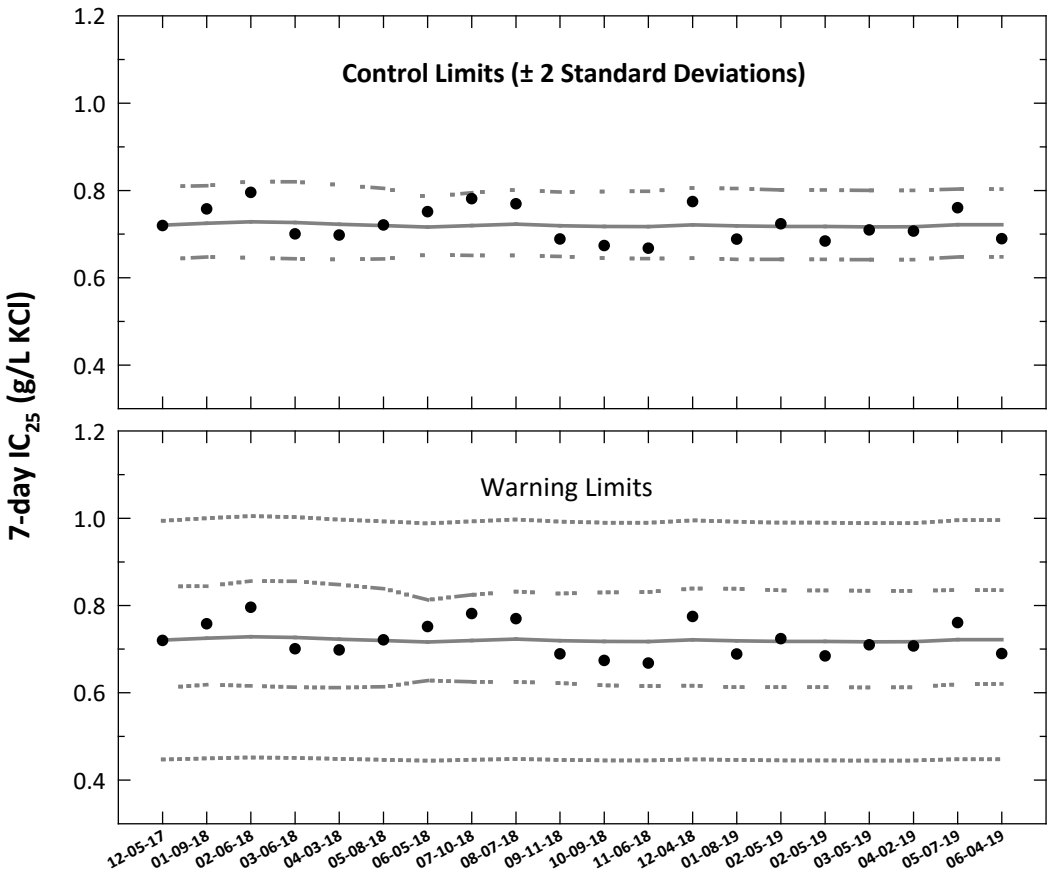
Test number	Test date	7-day IC_{25} ToxCal Determination (g/L KCl)	Log_{10} Conversion			Anti-logarithmic Values (g/L KCl)						
			7-day IC_{25}	CT	S	CT	Control Limits CT - 2S	Control Limits CT + 2S	Laboratory Calculated CV Warning Limits CT - 2CV	Laboratory Calculated CV Warning Limits CT + 2CV	75th Percentile CV Warning Limits CT - $S_{A.75}$	75th Percentile CV Warning Limits CT + $S_{A.75}$
1	12-05-17	0.7191	-0.1432	-0.1424	0.0251	0.7205	0.6418	0.8089	0.6112	0.8432	0.4467	0.9943
2	01-09-18	0.7574	-0.1207	-0.1397	0.0244	0.7249	0.6478	0.8113	0.6185	0.8441	0.4495	1.0004
3	02-06-18	0.7951	-0.0996	-0.1377	0.0260	0.7283	0.6461	0.8211	0.6154	0.8557	0.4516	1.0051
4	03-06-18	0.7002	-0.1548	-0.1387	0.0263	0.7266	0.6437	0.8201	0.6126	0.8552	0.4505	1.0027
5	04-03-18	0.6973	-0.1566	-0.1411	0.0256	0.7225	0.6423	0.8128	0.6115	0.8474	0.4480	0.9971
6	05-08-18	0.7204	-0.1424	-0.1429	0.0243	0.7196	0.6435	0.8048	0.6138	0.8380	0.4462	0.9931
7	06-05-18	0.7512	-0.1243	-0.1451	0.0200	0.7160	0.6530	0.7852	0.6280	0.8126	0.4440	0.9881
8	07-10-18	0.7808	-0.1075	-0.1429	0.0216	0.7196	0.6514	0.7950	0.6248	0.8244	0.4462	0.9931
9	08-07-18	0.7690	-0.1141	-0.1410	0.0225	0.7228	0.6518	0.8015	0.6245	0.8317	0.4481	0.9974
10	09-11-18	0.6884	-0.1621	-0.1432	0.0222	0.7191	0.6491	0.7967	0.6217	0.8270	0.4458	0.9924
11	10-09-18	0.6735	-0.1717	-0.1442	0.0231	0.7175	0.6452	0.7979	0.6167	0.8296	0.4448	0.9901
12	11-06-18	0.6673	-0.1757	-0.1444	0.0234	0.7171	0.6440	0.7986	0.6152	0.8307	0.4446	0.9896
13	12-04-18	0.7740	-0.1113	-0.1419	0.0241	0.7212	0.6454	0.8060	0.6160	0.8388	0.4472	0.9953
14	01-08-19	0.6882	-0.1623	-0.1434	0.0245	0.7188	0.6422	0.8045	0.6123	0.8380	0.4456	0.9919
15	02-05-19	0.7232	-0.1407	-0.1442	0.0240	0.7174	0.6422	0.8013	0.6126	0.8344	0.4448	0.9900
16	02-05-19	0.6837	-0.1651	-0.1442	0.0240	0.7174	0.6423	0.8013	0.6128	0.8343	0.4448	0.9901
17	03-05-19	0.7090	-0.1494	-0.1448	0.0240	0.7165	0.6416	0.8002	0.6119	0.8333	0.4442	0.9888
18	04-02-19	0.7064	-0.1509	-0.1446	0.0239	0.7167	0.6419	0.8003	0.6123	0.8333	0.4444	0.9891
19	05-07-19	0.7600	-0.1192	-0.1418	0.0234	0.7214	0.6478	0.8035	0.6193	0.8352	0.4473	0.9956
20	06-04-19	0.6892	-0.1616	-0.1417	0.0233	0.7217	0.6484	0.8032	0.6201	0.8347	0.4474	0.9959

Note:
7-day IC_{25} = 25% inhibition concentration. An estimation of the potassium chloride concentration that would cause a 25% reduction in *Pimephales* growth (calculated using ToxCalc).
CT = Central tendency of the IC_{25} values.
S = Standard deviation of the IC_{25} values.
Control Limits = Mean logarithmic IC_{25} ± 2 standard deviations converted to anti-logarithmic values.
Warning Limits = Mean logarithmic IC_{25} ± 2CV or $S_{A.75}$ converted to anti-logarithmic values.
$S_{A.75}$ = Standard deviation corresponding to the 75th percentile of CVs reported nationally by USEPA ($S_{A.75}$ = 0.38).
CV = Coefficient of variation.

Subject: *Pimephales promelas* Chronic Reference Toxicity Test, EPA 1000.0

Environmental Testing Solutions, Inc.

Pimephales promelas
Chronic Reference Toxicant Control Chart
Source: In-house Culture



- ● **7-day IC_{25}** = 25% inhibition concentration. An estimation of the potassium chloride concentration which would cause a 25% reduction in *Pimephales* growth (calculated using ToxCalc).
- ——— **Central Tendency** (mean logarithmic IC_{25} converted to anti-logarithmic values)
- - - — **Control Limits** (mean logarithmic IC_{25} ± 2 standard deviations converted to anti-logarithmic values)
- ··· ·· **Laboratory Warning Limits** (mean logarithmic IC_{25} ± 2 coefficent of variations converted to anti-logarithmic values)
- ····· **USEPA Warning Limits** (mean logarithmic IC_{25} ± $S_{A.75}$ converted to anti-logarithmic values, $S_{A.75}$ = 75th percentile of CVs reported nationally by USEPA)

Subject: *Pimephales promelas* Chronic Reference Toxicity Test, EPA 1000.0

Pimephales promelas
Chronic Reference Toxicant Testing, Test Acceptability Criteria
Source: In-house Culture

Test number	Test date	ToxCal Determination					Control Growth (mg/initial larvae)			Control Growth CV (%)			Test PMSD (%)		
		Control	Control Growth		Test			95% Confidence Interval			95% Confidence Interval			95% Confidence Interval	
		Survival (%)	Mean (mg/initial larvae)	CV (%)	MSD	PMSD (%)	CT	CT - 2S	CT + 2S	CT	CT - 2S	CT + 2S	CT	CT - 2S	CT + 2S
1	12-05-17	100	0.676	2.3	0.0607	9.0	0.647	0.545	0.750	5.2	0.4	10.0	10.6	5.0	16.1
2	01-09-18	100	0.612	8.3	0.0752	12.3	0.643	0.543	0.744	5.4	0.5	10.4	10.8	5.0	16.6
3	02-06-18	100	0.772	8.4	0.0815	10.6	0.651	0.538	0.765	5.6	0.5	10.7	10.6	5.0	16.2
4	03-06-18	100	0.810	4.7	0.0640	7.9	0.660	0.526	0.794	5.6	0.5	10.7	10.6	4.9	16.3
5	04-03-18	100	0.743	6.1	0.0836	11.3	0.668	0.534	0.802	5.8	0.8	10.7	10.7	5.1	16.3
6	05-08-18	100	0.576	4.9	0.0516	9.0	0.663	0.523	0.803	5.6	0.7	10.5	10.8	5.3	16.3
7	06-05-18	100	0.643	8.7	0.0992	15.4	0.664	0.525	0.803	5.8	0.7	10.9	11.2	4.8	17.6
8	07-10-18	100	0.678	3.9	0.0850	12.5	0.669	0.535	0.803	5.6	0.5	10.7	11.3	4.9	17.7
9	08-07-18	100	0.692	6.4	0.0813	11.7	0.675	0.547	0.802	5.3	1.0	9.7	10.9	6.0	15.8
10	09-11-18	100	0.709	8.4	0.0680	9.6	0.678	0.551	0.805	5.5	0.9	10.0	10.9	6.0	15.8
11	10-09-18	100	0.973	6.8	0.0693	7.1	0.696	0.516	0.876	5.5	1.0	10.1	10.6	5.5	15.7
12	11-06-18	100	0.666	5.3	0.0744	11.2	0.693	0.513	0.873	5.5	0.9	10.1	10.6	6.2	15.1
13	12-04-18	100	0.705	6.7	0.0876	12.4	0.698	0.524	0.872	5.7	1.3	10.1	10.7	6.2	15.3
14	01-08-19	100	0.629	5.6	0.0758	12.1	0.699	0.528	0.871	5.6	1.3	10.0	10.6	6.1	15.1
15	02-05-19	100	0.871	7.1	0.0929	10.7	0.707	0.519	0.895	5.8	1.6	10.1	10.5	6.1	14.9
16	02-05-19	100	0.856	3.9	0.0671	7.8	0.718	0.522	0.914	5.6	1.4	9.8	10.3	5.8	14.7
17	03-05-19	100	0.773	7.3	0.1141	14.8	0.725	0.532	0.919	5.7	1.4	10.0	10.6	5.8	15.4
18	04-02-19	100	0.762	9.1	0.1406	18.4	0.730	0.539	0.922	5.9	1.4	10.4	11.1	5.3	16.9
19	05-07-19	100	0.670	6.3	0.0763	11.4	0.727	0.534	0.920	6.1	1.7	10.5	11.3	5.7	16.9
20	06-04-19	100	0.694	3.8	0.0602	8.7	0.725	0.532	0.919	6.2	2.4	10.0	11.2	5.4	16.9

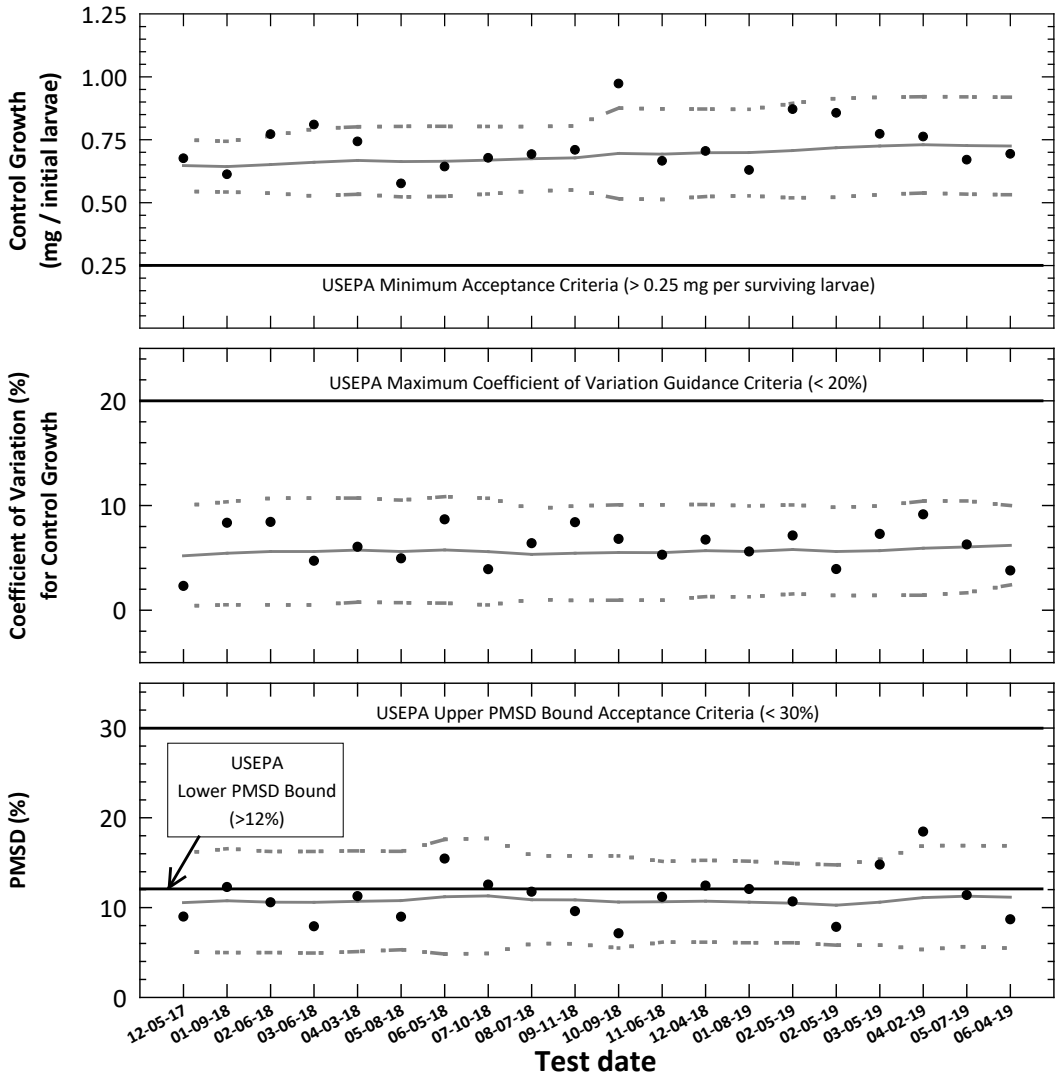
Note:
Control Survival = USEPA minimum test acceptability criteria ≥ 80% survival.
Control Mean Growth = USEPA minimum test acceptability criteria ≥ 0.25 mg/surviving larvae.
CV = Coefficient of variation for control growth.
USEPA maximum CV guidance criteria (90th percentile) < 20%
MSD = Minimum significant difference.
PMSD = Percent minimum significant difference.
PMSD is a measure of test precision. The PMSD is the minimum percent difference between the control and treatment that can be declared statistically significant in a whole effluent toxicity test.
Lower PMSD bound determined by USEPA (10th percentile) > 12%.
The lower PMSD bound represents a practical limit to the sensitivity of the test method and is not a minimum acceptance criteria.
Upper PMSD bound acceptance criteria determined by USEPA (90th percentile) < 30%.
CT = Central tendency of the growth, CV or PMSD values.
S = Standard deviation of the growth, CV or PMSD values.

Subject: *Pimephales promelas* Chronic Reference Toxicity Test, EPA 1000.0

Pimephales promelas

Chronic Reference Toxicant Testing, Test Acceptability Criteria

Organism Source: In-house Culture



- **Control Growth, Coefficient of Variation (CV) or Percent Minimum Significant Difference (PMSD)**
  PMSD is the percent minimum significant difference between the control and treatment that can be declared statistically significant. The lower PMSD bound represents a practical limit to the sensitivity of the test method and is not a minimum acceptance criteria.
- **Central Tendency** (mean Control Growth, CV or PMSD)
- **95% Confidence Interval** (mean Control Growth, CV or PMSD ± 2 Standard Deviations)

Entered and Reviewed by Jim Sumner

Subject: Taxonomic Identification of *Pimephales promelas*

Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan		09-01-19
Quality Assurance Officer	Jim Sumner		09-01-19

Document Revision History

Effective Date	Revision number	Review Type	Evaluators	Revisions
12-01-00	0	Internal	Jim Sumner (ETS)	Original document
06-01-11	1	**Internal**	Jim Sumner (ETS)	• **Updated references and exhibits.**
11-01-14	2	**Internal**	Jim Sumner (ETS)	• Updated exhibits during document review.
09-28-16	3	External (TVA) Internal	Rick Sherrard, Donald Snodgrass (TVA) Jim Sumner (ETS)	• Updated exhibits during document review. • Updated SOP for using the in-house culture.
09-01-19	4	Internal	Jim Sumner (ETS)	• Updated procedure to include NELAP requirements. • Additional guidance included in SOP.

Scope and Application

To verify the genus and species of *Pimephales promelas* breeder cultures used by the laboratory for a source of larvae in toxicity tests.

Summary of Method

Adult male and female *Pimephales promelas* are preserved in alcohol and the genus and species is verified. Organisms preserved for taxonomic identification are obtained from cultures used for toxicity testing.

Quality Control

The genus and species of *Pimephales promelas* is verified quarterly. Preserved specimens must be maintained a minimum of 1 year.

Subject: Taxonomic Identification of *Pimephales promelas*

Equipment and Materials

Adult, male fathead minnows (*Pimephales promelas*)
Ice water
Plastic beaker or equivalent
Alcohol
Glass Vials
Dissection microscope
Forceps
Disposable gloves
Pimephales promelas Taxonomic Log and Log Sheet

Procedure

A. Preparation.

1. Select two (one male and one female), adult, fathead minnows (*Pimephales promelas*) from the laboratory's breeding cultures.

2. Prepare the *Pimephales promelas* Taxonomic Identification Log Sheet (Exhibit AT22.1).

B. Preservation of Adult Fathead Minnows.

1. Remove the minnows from the breeding cultures and transfer the minnows to a plastic beaker (or equivalent).

2. Euthanize the minnows by placing them in ice water until no movement is observed.

3. Preserve the minnows by placing them in a sealed vial containing alcohol. Record the date the minnows were preserved on the vial. Once preserved, taxonomic identification of the preserved specimens can be performed.

C. Taxonomic Identification.

1. Record the date the taxonomic identification was performed, analyst's initials, the source of the preserved specimens on the *Pimephales promelas* Taxonomic Identification Log Sheet.

2. Remove the preserved specimens from the vials. Identify each of the distinguishing characteristics of *Pimephales promelas* in the preserved specimens as indicated on the

log sheet. Any deviations from these characteristics should be noted. A dissection microscope may be used for ease in viewing each of the characteristics. For additional information on the taxonomic identification of *Pimephales promelas*, refer to the references sited at the beginning of this SOP.

3. If several of the distinguishing characteristics are not represented in the preserved specimens, contact the supplier and order additional minnows to confirm the identity. If necessary, an ichthyologist at a local university should be contacted to provide guidance and confirm the discrepancies noted in the specimens.

4. Once the taxonomic identification is complete, place the specimens in the vials containing reagent alcohol. These taxonomic specimens must be maintained in the laboratory for a minimum of 1 year.

Safety and Hazardous Waste Management

Safety glasses, gloves and lab coats should be worn at all times.

Review Policy-P6: General Safety Policy and Policy-P9: Radiation Protection Policy for additional safety requirements.

References

USEPA. October 2002. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, 5th ed. EPA-821-R-02-012. US Environmental Protection Agency, Cincinnati, OH.

North Carolina Department of Environment and Natural Resources, Division of Water Quality. Biological Laboratory Certification / Criteria Procedures, Version 3.0. December 2010.

TNI Standard. Management and Technical Requirements for Laboratories Performing Environmental Analysis. EL-V1-ISO-2016-Rev2.0. The NELAC Institute, PO Box 2439, Weatherford, TX 76086.

W. L. Pflieger, *The Fishes of Missouri*, Missouri Department of Conservation, 1975.

M. B. Trautman, *The Fishes of Ohio*, Ohio State University Press, 1981.

Exhibits

Exhibit AT22.1: *Pimephales promelas* Taxonomic Identification Log Sheet.

Subject: Taxonomic Identification of *Pimephales promelas*

Exhibit AT22.1: *Pimephales promelas* Taxonomic Identification Log Sheet.

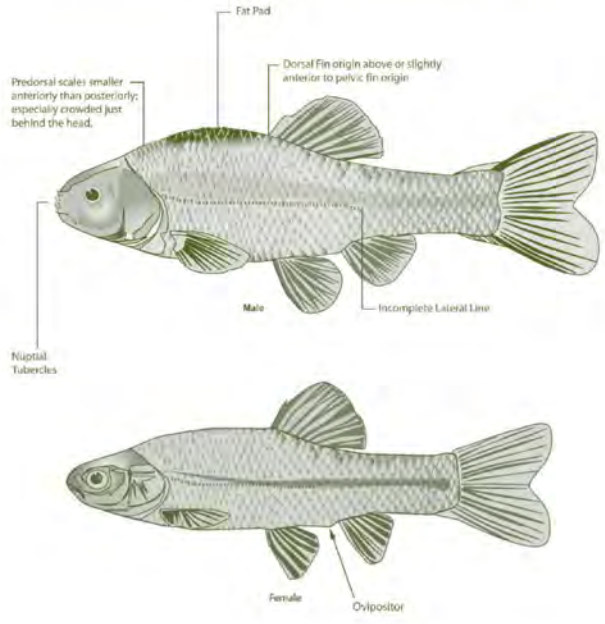
Pimephales promelas Taxonomic Identification Log Sheet

Date identification performed: _____ **Analyst:** _____

Source: In-house Culture
Specimens preserved from stock organisms in culture system.

Comments:

ILLUSTRATION OF FATHEAD MINNOW WITH ANATOMICAL IDENTIFICATIONS



Subject: Receipt, Acclimation and Maintenance of *Americamysis bahia* Cultures

Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan		05-31-22
Quality Assurance Officer	Jim Sumner		05-31-22

Document Revision History

Effective Date	Revision number	Review Type	Evaluators	Revisions
12-01-00	0	Internal	Jim Sumner (ETS)	Original document
06-01-11	1	Internal	Jim Sumner (ETS)	• Updated exhibits.
11-01-14	2	Internal	Jim Sumner (ETS)	• Updated exhibits during document review.
09-01-19	3	Internal	Jim Sumner (ETS)	• Updated procedure to include NELAP requirements. • Additional guidance included in SOP.
04-25-22	4	External (NC DWR) Internal	Lorimar Henning, Cindy Moore, Zach Thomas (NC DWR) Jim Sumner (ETS)	• Updated procedure to include additional organism age requirements.
05-31-22	5	External (NC DWR) **Internal**	Lorimar Henning, Cindy Moore, Zach Thomas (NC DWR) Jim Sumner (ETS)	• Provided clarification based on NC DWR comments.

Scope and Application

To maintain healthy cultures of *Americamysis bahia*.

Summary of Method

This procedure describes how the laboratory receives, acclimates and maintains mysid cultures purchased from an outside vendor.

Quality Control

It is important to use only healthy organisms in tests. If a batch of organisms purchased contains 10% or greater mortality, it must be discarded and not used in testing.

Subject: Receipt, Acclimation and Maintenance of *Americamysis bahia* Cultures

Equipment and Materials

Mysid shrimp (*Americamysis bahia*)
Temperature-controlled incubator (set to maintain test temperature = 25.0 ± 1.0°C for acute test organisms and 26.0 ± 1.0°C for chronic test organisms, photoperiod of 16-hour light / 8-hours dark, light intensity = 50 – 100 ft-c)
Salt synthetic water
Large glass jars
Transfer pipettes
Aquarium pump and tubing
Thermometer
1-oz medicine cups
Newly hatched brine shrimp
Light box or table
Disposable gloves
Test Organism Shipment Log and Test Organism History Information Sheet
C. variegates, M. beryllina and *A. bahia* and Culture Log

Procedure

A. Receipt of Test Mysids, Acclimation and Holding.

1. Order mysids (*Americamysis bahia*) from an approved supplier (e.g., Aquatic Indicators, Inc. – St. Augustine, FL).
   - For acute testing, tests are initiated using 1 – 5-day old Mysids which are released within 24-hours of one another. Organisms are purchased that are released between 12:00 pm Sunday and 11:30 am Monday and shipped on Monday for receipt on Tuesday at ETS. These organisms can be used for acute testing after 11:30 am Tuesday until before 12:00 pm Friday of the same week.
   - For chronic testing, tests are initiated using 7-day old Mysids which are released within 24-hours of one another. Organisms are purchased that are released between 12:00 pm Tuesday and 11:30 am Wednesday and shipped on the following Monday for receipt on Tuesday at ETS. These organisms are used for chronic testing on the day they are received.

2. Obtain the Test Organism Shipment Log and Culture Log.

3. Organisms are shipped next day air in insulated boxes and are contained in clear plastic bags. Remove the plastic bags containing the mysids from the shipping container (insulated box). Carefully transfer the water containing the mysids from each plastic bag

to large glass jars (or equivalent). Measure the temperature (SOP-C1), dissolved oxygen (SOP-C2), pH (SOP-C3) and salinity (SOP-C5) of the water in the jar. Record the following information on the Test Organism History Information Sheet provided by the supplier (Exhibit AT40.1).

- Date and time received at the laboratory
- Initials of the analyst that received the shipment
- Water temperature
- Dissolved oxygen, pH and salinity
- Appearance and health of the organisms. Unhealthy or diseased mysids (fungus present) must be discarded and may not be used for testing. Document the number of unhealthy or diseased mysids which are discarded.
- Number of dead mysids and the total number of mysids received
- Date and time the organisms were fed

4. Place the Test Organism History Information Sheet in the Test Organism Shipment Log.

5. Record the following information on the Culture Log (Exhibit AT40.2).
   - Organism source (Aquatic Indicators, Inc.)
   - Organism type (*Americamysis bahia*)
   - Organism batch (initial release date)
   - Organism age upon receipt
   - Dates organisms were released
   - Incubator number
   - Synthetic water type (Salt synthetic water is used to culture *Americamysis bahia*)

6. Remove any debris or dead mysids from the jar with a transfer pipette and replace approximately ¾ of the water with salt synthetic water. This activity should be performed daily, until the organisms are used in a toxicity test. Document in the Culture Log the date and time water is renewed. If at any time before a test is initiated the mysids appear unhealthy, diseased (fungus present) or > 10% mortality is identified; the mysids must be discarded and may not be used for testing. Document in the Culture Log the number of dead, diseased, and discarded mysids, total number of mysids and the date the entire culture is discarded.

7. Feed the mysids in the jar twice daily newly hatched brine shrimp (*Artemia nauplii*) which are < 24-hours old (SOP-AT16), until the organisms are used in a toxicity test. Test organisms are typically fed (at the beginning of the work day prior to renewal and end of the work day following renewal, approximately 6 hours between feedings), until the organisms are used in a toxicity test. Approximately 2.5 to 5.0 mL brine shrimp are

added to each jar using a suspension of artemia as discussed in SOP-AT16. This volume is dependent on the number of organisms. Organisms must be fed a minimum of 2-hour to a maximum of 5 hours prior to initiating acute tests. Enough nauplii should be provided to assure that some remain alive in the jar at the next feeding, but not in excessive amounts which will result in the depletion of dissolved oxygen below acceptable levels (< 4.0 mg/L). Document in the Culture Log the times that the organisms are fed daily. If the organisms are used for initiating tests, record in the Culture Log the tests that were initiated on that day.

8. Place the jar in a temperature-controlled incubator. Gently aerate the water using an aquarium pump and tubing. Organisms intended for acute tests should be acclimated to 25.0 ± 1.0°C and organisms intended for chronic tests should be acclimated to 26.0 ± 1.0°C such that no more than a 3 °C change in temperature occurs over a 12-hour period. It may be necessary to place the organisms in an incubator set at a lower temperature to acclimate the organisms gradually. Once acclimated, the organisms are maintained at the appropriate test temperature with a photoperiod of 16-hours light and 8-hours dark and a recommended light intensity of 50 to 100 ft-c.

B. Exhibits.

Exhibit AT40.1: Test Organism History Information Sheet.
Exhibit AT40.2: *C. variegates, M. beryllina* and *A. bahia* Culture Log.

Subject: Receipt, Acclimation and Maintenance of *Americamysis bahia* Cultures

Exhibit AT40.1: Test Organism History Information Sheet.

AQUATIC INDICATORS

Date: 5-2-22

05-03-22 1115 jh
TEMP = 24.9°C

Species:
1. M. bahia
2. M. bahia
3. M. beryllina

	Ab 05-02-22	Ab 04-27-22	Mb 04-24-22
pH s.u.	8.11	8.10	8.07
DO mg/L	9.0	8.6	9.0
SALINITY ppt	22.9	25.3	25.2
~~DEAD~~/TOTAL #	0/350+	0/600+	3/400+

Organisms appear healthy
Fed at 1120

Total Supplied:
1. 350 @ 20‰
2. 600 @ 25‰
3. 400 @ 25‰

Brood Description
1. EEA
2. EEA
3. EEA

Age:
1. "Ø" days - collected between 05-01-22 @ Noon and 05-02-22 @ 11:30AM
2. "6" days - collected between 04-26-22 @ Noon and 04-27-22 @ 11:30AM
3. 9 days - hatched between 04-23-22 @ Noon and 04-24-22 @ 11:30AM

Environmental Regime | Feeding: Zooplankton Artemia NH ✓ | Photo: L 16 D 8

P.H.: 8.1 | Temp: 25°C | Salinity: 25‰ / 20‰ see above

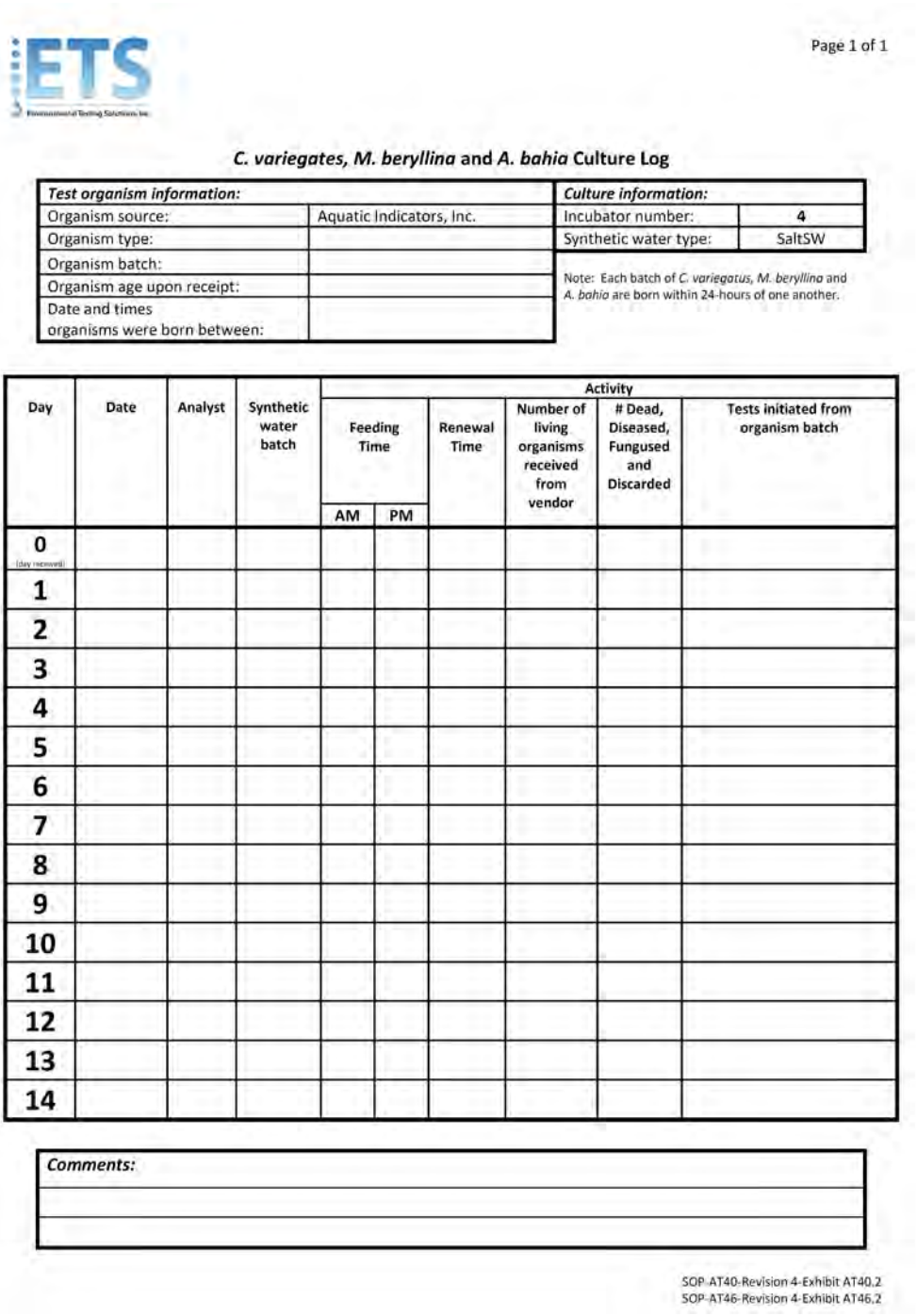
Comments: Thanks.

WD-190365 & RC-215067

P.O. BOX 632 ST. AUGUSTINE, FL 32085 (904) 829-2780

Subject: Receipt, Acclimation and Maintenance of *Americamysis bahia* Cultures

Exhibit AT40.2: *C. variegates, M. beryllina* and *A. bahia* Culture Log.



Page 1 of 1

C. variegates, M. beryllina and *A. bahia* Culture Log

Test organism information:		*Culture information:*	
Organism source:	Aquatic Indicators, Inc.	Incubator number:	4
Organism type:		Synthetic water type:	SaltSW
Organism batch:			
Organism age upon receipt:			
Date and times organisms were born between:			

Note: Each batch of *C. variegatus, M. beryllina* and *A. bahia* are born within 24-hours of one another.

Day	Date	Analyst	Synthetic water batch	Activity					
				Feeding Time		Renewal Time	Number of living organisms received from vendor	# Dead, Diseased, Fungused and Discarded	Tests initiated from organism batch
				AM	PM				
0 (day received)									
1									
2									
3									
4									
5									
6									
7									
8									
9									
10									
11									
12									
13									
14									

Comments:

SOP-AT40-Revision 4-Exhibit AT40.2
SOP-AT46-Revision 4-Exhibit AT46.2

Subject: *Americamysis bahia* Acute Toxicity Test, EPA 2007.0

Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan		09-01-19
Quality Assurance Officer	Jim Sumner		09-01-19

Document Revision History

Effective Date	Revision number	Review Type	Evaluators	Revisions
12-01-00	0	Internal	Jim Sumner (ETS)	Original document
06-01-11	1	Internal	Jim Sumner (ETS)	• Updated exhibits and references. • Statistical analyses and data review moved to QAP-Q12.
07-01-12	2	External (NC DENR) Internal	Lance Ferrell (NC DENR) Jim Sumner (ETS)	• The measurement of pH, DO, conductivity and salinity of each new, full-strength, undiluted sample was added. • The light intensity was amended to reflect that it is a recommended range as specified in the EPA manuals.
11-01-14	3	Internal	Jim Sumner (ETS)	• Updated exhibits during document review.
09-01-19	4	Internal	Jim Sumner (ETS)	• Updated procedure to include NELAP requirements. • Additional guidance included in SOP. • Included procedures for 96-hour testing.
04-25-21	4	External (NC DWR) Internal	Lorimar Henning, Cindy Moore, Zach Thomas (NC DWR) Jim Sumner (ETS)	• Updated procedure to include additional organism age requirements. • Updated bench sheet (Exhibits AT18.2 and AT18.3) to include reporting limits, method numbers, meters and serial numbers used for chemical analyses.

Scope and Application

To measure the acute toxicity of water samples to Mysid shrimp (*Americamysis bahia*) during a 24, 48 or 96-hour exposure period.

Summary of Method

The acute toxicity test generally involves the exposure of test organisms to up to five effluent concentrations and a control water. The test duration ranges from 24 to 96 hours. At the end of each 24-hour period, the number of living organisms is counted in each effluent concentration and control water.

A summary of the Mysid shrimp acute method is provided in Exhibit AT41.1.

Subject: *Americamysis bahia* Acute Toxicity Test, EPA 2007.0

Quality Control

Acceptance Criteria: The test acceptance criterion is ≥ 90% survival in the control. If the control survival is < 90%, the test must be invalidated.

Equipment and Materials

Mysid shrimp (*Americamysis bahia*)
Temperature-controlled incubator (set to maintain test temperature = 25.0 ± 1.0°C, photoperiod of 16-hour light / 8-hours dark, light intensity = 50 – 100 ft-c)
Control / Dilution water
(salt synthetic water made with Bioassay Grade 40-Fathoms Sea Salt, MarineMix®)
500-mL plastic Solo® cups
Solo® cup lids
Graduated cylinders
Large glass jars
Serological, fixed, or adjustable volume pipettes (e.g., Eppendorf)
Transfer pipettes
Aquarium pump and tubing
Thermometer
1-oz medicine cups
Newly hatched brine shrimp
Light box or table
Forceps
Weigh boats
Calibrated top-loading balance (e.g. Fisher Scientific ACCU-224)
Disposable gloves
Acute Toxicity Test or Pass/Fail Acute Toxicity Test Bench Sheet
Randomization template

Subject: *Americamysis bahia* Acute Toxicity Test, EPA 2007.0

Procedure

A. Test Preparation.

1. Prepare the Acute Toxicity Test Bench Sheet (for multiple concentration tests, Exhibit AT41.3) or Pass/Fail Acute Toxicity Test Bench Sheet (for Pass/Fail acute tests, Exhibit AT41.2). Record the following information on the bench sheet:
    - Client/facility name
    - Permit number
    - County
    - Outfall/Pipe number
    - ETS project and sample number
    - Control/Dilution water type and batch
    - Test concentrations and dilution preparation information (sample, dilution and total volumes)

2. Prepare the plasticware.

    a. Obtain enough 500-ml plastic Solo® cups with lids for each site/sample and concentration tested, including the control. For Pass/Fail acute tests, four replicates are used for the test concentration and control. For multiple concentration acute tests, two replicates are used for each concentration and control. Label each replicate cup with the following information.
        - Facility/sample name
        - Concentration
        - Replicate number

    b. Label the appropriate graduated cylinder with the facility/sample name and concentration.

B. Test Initiation.

1. Prepare the test concentrations according to SOP-G5. It may be necessary to salt-up the sample prior to making the test concentrations. Refer to SOP-G5 for the appropriate procedures for salting-up samples.

    a. The control/dilution water is salt synthetic water (SaltSW, SOP-AT1). SaltSW must have a salinity of 25.0 ± 1.0 ppt and an initial pH of 6.5 – 8.5 S.U.

b. Measure and record the pH (SOP-C3), dissolved oxygen (SOP-C2) and salinity (SOP-C5) of each concentration tested and control. Ensure that the dissolved oxygen is within the acceptable range (4.0 to 9.0 mg/L), aerate the sample if necessary, according to SOP-G5. Measure and record the pH (SOP-C3), dissolved oxygen (SOP-C2), conductivity (SOP-C4), salinity (SOP-C5) total residual chlorine (SOP-C8), total alkalinity (SOP-C6) and sample characteristics of each new, full-strength, undiluted sample. The alkalinity of full-strength, undiluted samples for North Carolina tests is not required.

c. Pour 250 mL of control water into each of the replicate control cups.

d. Pour 250 mL of each test concentration into each of the replicate test cups.

e. Obtain a randomizing template (Exhibit AT41.5). Place the tests in order according to randomizing template and record the template color on the bench sheet.

f. Maintain the test temperature (25.0 ± 1.0°C) of the test concentrations. This may be accomplished by placing the holding rack into a temperature-controlled incubator.

2. Isolate the shrimp for the test.

a. Obtain a batch of shrimp (SOP-AT40), which are 1 to 5-days old (with a maximum of 24-hour range in age). Record the source, hatch date and age of the organisms to be used in the test on the acute bench sheet. Feed the shrimp a minimum of 2 hours prior to test initiation to a maximum of 5 hours prior to test initiation. Record the date and time the organisms were fed on the bench sheet. Transfer the shrimp from the jar to a large glass finger bowl.

b. Two techniques may be used for transferring 10 organisms to each test cup from the finger bowl. The technique used is dependent on the sample being evaluated for toxicity. In both methods, shrimp are transferred by plastic pipette. The end of the pipette tip should be cut to a size that will not injure or harm the shrimp during transfer. Shrimp should be transferred gently in a manner that will not expose the organisms to the air.

- If the transferred water may alter the toxicity of the sample (decrease toxicity), organisms should be transferred directly by pipette. This technique is predominately used by the laboratory. Minimize the volume of transfer water introduced into the sample. Follow procedures outlined in

step 3 for transferring the organisms to the randomly placed test cups. The average transfer volume by this technique for each analyst must be determined yearly. For an example transfer volume log sheet refer to Exhibit AT41.4.

- If there is risk of cross contamination between replicates, organisms should be transferred to medicine cups (10 per cup) and then introduced into the test solutions. The final volume contained in the medicine cups after the organisms have been transferred should be between 10 and 15 ml. With a pipette, remove any food or debris that was placed in the cup during transfer. The average transfer volume by this technique for each analyst must be determined yearly. For an example transfer volume log sheet refer to Exhibit AT41.4. Continue this process until enough medicine cups containing 10 shrimp each have been obtained to initiate the test. 1 medicine cup containing 10 shrimp will be needed for each sample concentration and replicate. If a test consists of 5 concentrations and a control, 12 medicine cups containing 10 shrimp each will be required. If a test consists of 1 concentration and a control, 8 medicine cups containing 10 shrimp each will be required.

3. Transfer the shrimp to the randomly placed test cups.

   a. Measure and record the temperature in one of the test cups for each concentration and control. The temperature must be 25.0 ± 1.0°C before the test organisms are placed in the test cups. Warm the test cups in a warm water bath or temperature-controlled incubator, if necessary, until the desired test temperature is obtained. Measure and record the temperature of an arbitrary transfer cup.

   b. Place 10 shrimp in the first test cup of the first row (by pipette or medicine cup). Continue in this manner (placing the shrimp in the test cups from left to right in the first row and then the second row) until all the test cups contain 10 shrimp.

   c. Record the initiation date, time and analyst's initials on the acute bench sheet. **The acute test must be initiated within 36-hours of completion of the sampling period.**

   d. Save approximately 30 mL of transfer water to be measured for pH (SOP-C3). Measure and record the transfer water pH on the acute bench sheet.

e. Verify that each cup received the required number of shrimp (i.e., 10) by conducting a repeat count. Remove excess shrimp or add shrimp as necessary. Record the initial number of shrimp on the bench sheet. Place lids on each cup.

f. Place the test cups in order, according to the randomization template, in a temperature-controlled incubator. The organisms must be maintained at 25.0 ± 1.0°C with a photoperiod of 16-hours light and 8-hours dark and a recommended light intensity of 50 to 100 ft-c. Record the incubator number and shelf used on the bench sheet.

C. Record Daily Survival.

Repeat this process daily, starting at 24-hours ± 1-hour after test initiation and continuing until test termination.

1. Measure and record the temperature in an arbitrarily selected test cup for each concentration and control.

2. Remove the test cups from the incubator and remove the lids. Place the cups on a light box or table for ease of viewing.

3. Count and record (in the appropriate section) the number of shrimp surviving in each replicate cup on the acute bench sheet. The criteria that establish death are: (1) no movement and (2) no reaction to gentle prodding. Record comments, if applicable.

4. Remove any dead shrimp and discard with a transfer pipette.

5. Record the date, time and the analyst's initials on the bench sheet.

6. Carefully pour ~30 mL of test water from at least one replicate cup for each test concentration and control into labeled 1-oz medicine cups. Measure and record the pH (SOP-C3), salinity (SOP-C5) and dissolved oxygen (SOP-C2) of this water.

7. Feed the shrimp in each test cup 100 µL (2-drops) of newly-hatched brine shrimp (SOP-AT16).

8. Place the lids on the test cups and place the test cups back in order, according to the randomization template, in a temperature-controlled incubator.

D. <u>For 96-hour Acute Tests,</u> Renewal of Test Solutions at 48-hours.

For 96-hour acute tests, test solutions must be renewed within ± 1 hour from test initiation.

1. Carefully pour ~30 mL of test water from at least one replicate cup for each test concentration and control into a labeled 1-oz medicine cup. This water will be used to determine final pH, salinity and dissolved oxygen concentrations.

2. Feed the shrimp in each test cup 200 µL (4-drops) of newly-hatched brine shrimp (SOP-AT16) at 2-hours prior to the renewal of test solutions (at 46-hours from test initiation). Record the feeding time and initials on the acute benchsheet.

3. Measure and record the temperature in an arbitrarily selected test replicate for each concentration and control.

4. Prepare fresh test solutions in accordance with SOP-G5. Maintain the test temperature (25.0 ± 1.0°C) of the fresh test water until needed by storing in a temperature-controlled incubator.

5. At 48-hours, remove the test cups from the incubator. Place the cups on a light box or table for ease of viewing.

6. Change the test water in all replicate cups before starting the next replicate-cup series. To change the test water, test cups are decanted.

   a. Using a transfer pipette, remove any debris, dead artemia and dead shrimp that may have accumulated on the bottom of the test cup. Carefully decant the water from the cup into a white plastic photographic tray until ~ 50 mL of old test water remains.

   b. If any shrimp are accidentally decanted with the water, retrieve them from the plastic tray, using a transfer pipette. The end of the pipette tip should be cut to a size that will not injure or harm the shrimp during transfer. Shrimp should be transferred gently in a manner that will not expose the organisms to the air. Return the shrimp to the appropriate replicate cup. Record the number of shrimp siphoned out or decanted (per replicate). Discard any dead shrimp.

   c. Record the following information on the acute benchsheet.

      - Number of shrimp surviving in each replicate cup.
      - Number of dead shrimp in each replicate cup (if applicable).

- Any comments (injured, sick, or shrimp siphoned out).

d. Fill each replicate cup to 250 mL using fresh test solutions. Pour the test water down the side of the cup to avoid unnecessarily disturbing the shrimp.

7. After all test cups have been renewed, record the renewal time and the analyst's initials on the acute bench sheet. Place the lids on the test cups and place the cups back in order, according to the randomization template, in a temperature-controlled incubator.

E. Test Termination.

Terminate the test after the organisms have been exposed to the test concentrations for the required time (i.e. 24, 48, or 96-hours). The test may be terminated ± 1-hour from the time it was initiated.

1. Measure and record the temperature in an arbitrarily selected test cup for each concentration and control.

2. Remove the test cups from the incubator and remove the lids. Place the cups on a light box or table for ease of viewing.

3. Count and record (in the appropriate section) the number of shrimp surviving in each replicate cup on the acute bench sheet. Record comments, if applicable.

4. Record the termination date, time and the analyst's initials on the bench sheet.

5. Carefully pour ~30 mL of test water from at least one replicate cup for each test concentration and control into labeled 1-oz medicine cups. Measure and record the pH (SOP-C3) and dissolved oxygen (SOP-C2) of this water.

6. Once all analyses have been completed and documented, discard the test water and shrimp according to established laboratory protocol.

F. Statistical Analyses and Data Verification.

Statistical analyses and data review are performed according to QAP-Q12.

Safety and Hazardous Waste Management

Safety glasses, gloves and lab coats should always be worn.

Review Policy-P6: General Safety Policy and Policy-P9: Radiation Protection Policy for additional safety requirements.

References

USEPA. October 2002. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, 5th ed. **EPA-821-R-02-012, Method 2007.0**. US Environmental Protection Agency, Cincinnati, OH.

North Carolina Department of Environment and Natural Resources, Division of Water Quality. Pass/Fail Methodology for Determining Acute Toxicity in a Single Effluent, Version 3.0. December 2010.

TNI Standard. Management and Technical Requirements for Laboratories Performing Environmental Analysis. EL-V1-ISO-2016-Rev2.0. The NELAC Institute, PO Box 2439, Weatherford, TX 76086.

Exhibits

Exhibit AT41.1: Summary of Test Conditions for the *Pimephales promelas* Acute Toxicity Test.
Exhibit AT41.2: Pass/Fail Acute Toxicity Test Bench Sheet.
Exhibit AT41.3: Acute Toxicity Test Bench Sheet.
Exhibit AT41.4: Average Transfer Volume Log Sheet.
Exhibit AT41.5: Randomization Template.

Subject: *Americamysis bahia* Acute Toxicity Test, EPA 2007.0

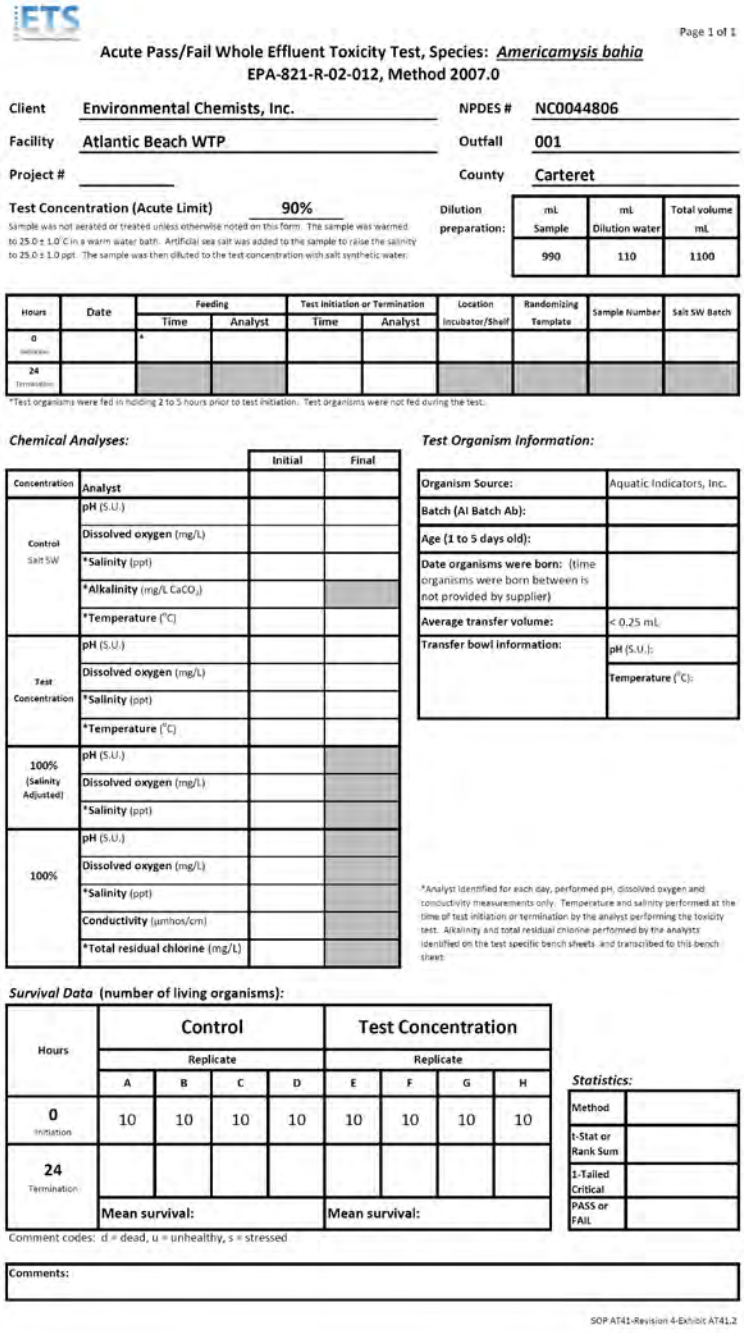
Exhibit AT41.1: Summary of Test Conditions for the *Americamysis bahia* Acute Toxicity Test.

SUMMARY OF TEST CONDITIONS FOR THE *AMERICAMYSIS BAHIA* ACUTE TOXICITY TEST

Test type:	Static non-renewal or static renewal
Test duration:	24, 48 or 96 hours
Temperature:	25.0 ± 1.0°C
Light quality:	Cool-white fluorescent
Light intensity:	50 – 100 ft-candles (recommended)
Photoperiod:	16-hours light, 8-hours dark
Test chamber size:	500 mL Solo® cups
Test solution volume:	250 mL
Renewal of test solutions:	At 48-hours (required minimum)
Age of test organisms (days old):	1 to 5 days old, ≤ 24 hour range in age
Number of organisms per test chamber:	10
Number of replicate test chambers per concentration:	Multiple concentration tests: 2 Single dilution tests: 4
Number of organisms per concentration:	Multiple concentration tests: 20 Single dilution tests: 40
Test concentrations:	Multiple concentration tests: 5 and a control with ≥ 0.5 dilution series (recommended) Single dilution tests: 90% or 100% and a control
Test chamber cleaning:	Dead shrimp removed daily. For 96-hour tests, test chambers are cleaned immediately before test solution renewal at 48-hours.
Aeration:	None, unless the dissolved oxygen concentration falls below 4.0 mg/L.
Feeding regime:	*Artemia nauplii* made available while holding prior to test initiation (2 to 5-hours prior to initiation). Organisms in each test cup are fed daily 100 µL *Artemia nauplii.* Organisms in each test cup are fed 200 µL *Artemia nauplii* 2 hours prior to test solution renewal at 48-hours.
Control / Dilution water:	Salt synthetic water
Sampling and sample holding:	1-gallon grab or composite sample first used within 36-hours of completion of the sampling period.
Endpoint:	Mortality
Test acceptability criterion:	≥ 90% control survival

Subject: *Americamysis bahia* Acute Toxicity Test, EPA 2007.0

Exhibit AT41.2: Pass/Fail Acute Toxicity Test Bench Sheet.



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Acute Pass/Fail Whole Effluent Toxicity Test, Species: *Americamysis bahia*
EPA-821-R-02-012, Method 2007.0

Client: Environmental Chemists, Inc. NPDES #: NC0044806

Facility: Atlantic Beach WTP Outfall: 001

Project #: _____ County: Carteret

Test Concentration (Acute Limit): 90%

Sample was not aerated or treated unless otherwise noted on this form. The sample was warmed to 25.0 ± 1.0°C in a warm water bath. Artificial sea salt was added to the sample to raise the salinity to 25.0 ± 1.0 ppt. The sample was then diluted to the test concentration with salt synthetic water.

Dilution preparation:

mL Sample	mL Dilution water	Total volume mL
990	110	1100

Hours	Date	Feeding Time	Feeding Analyst	Test Initiation or Termination Time	Test Initiation or Termination Analyst	Location Incubator/Shelf	Randomizing Template	Sample Number	Salt SW Batch
0 initiation		*							
24 Termination									

*Test organisms were fed in holding 2 to 5 hours prior to test initiation. Test organisms were not fed during the test.

Chemical Analyses:

Concentration		Initial	Final
Control Salt SW	Analyst		
	pH (S.U.)		
	Dissolved oxygen (mg/L)		
	*Salinity (ppt)		
	*Alkalinity (mg/L $CaCO_3$)		
	*Temperature (°C)		
Test Concentration	pH (S.U.)		
	Dissolved oxygen (mg/L)		
	*Salinity (ppt)		
	*Temperature (°C)		
100% (Salinity Adjusted)	pH (S.U.)		
	Dissolved oxygen (mg/L)		
	*Salinity (ppt)		
100%	pH (S.U.)		
	Dissolved oxygen (mg/L)		
	*Salinity (ppt)		
	Conductivity (µmhos/cm)		
	*Total residual chlorine (mg/L)		

Test Organism Information:

Organism Source:	Aquatic Indicators, Inc.
Batch (AI Batch Ab):	
Age (1 to 5 days old):	
Date organisms were born: (time organisms were born between is not provided by supplier)	
Average transfer volume:	< 0.25 mL
Transfer bowl information:	pH (S.U.):
	Temperature (°C):

*Analyst identified for each day, performed pH, dissolved oxygen and conductivity measurements only. Temperature and salinity performed at the time of test initiation or termination by the analyst performing the toxicity test. Alkalinity and total residual chlorine performed by the analysts identified on the test specific bench sheets and transcribed to this bench sheet.

Survival Data (number of living organisms):

Hours	Control Replicate A	B	C	D	Test Concentration Replicate E	F	G	H
0 Initiation	10	10	10	10	10	10	10	10
24 Termination								
	Mean survival:				Mean survival:			

Comment codes: d = dead, u = unhealthy, s = stressed

Statistics:

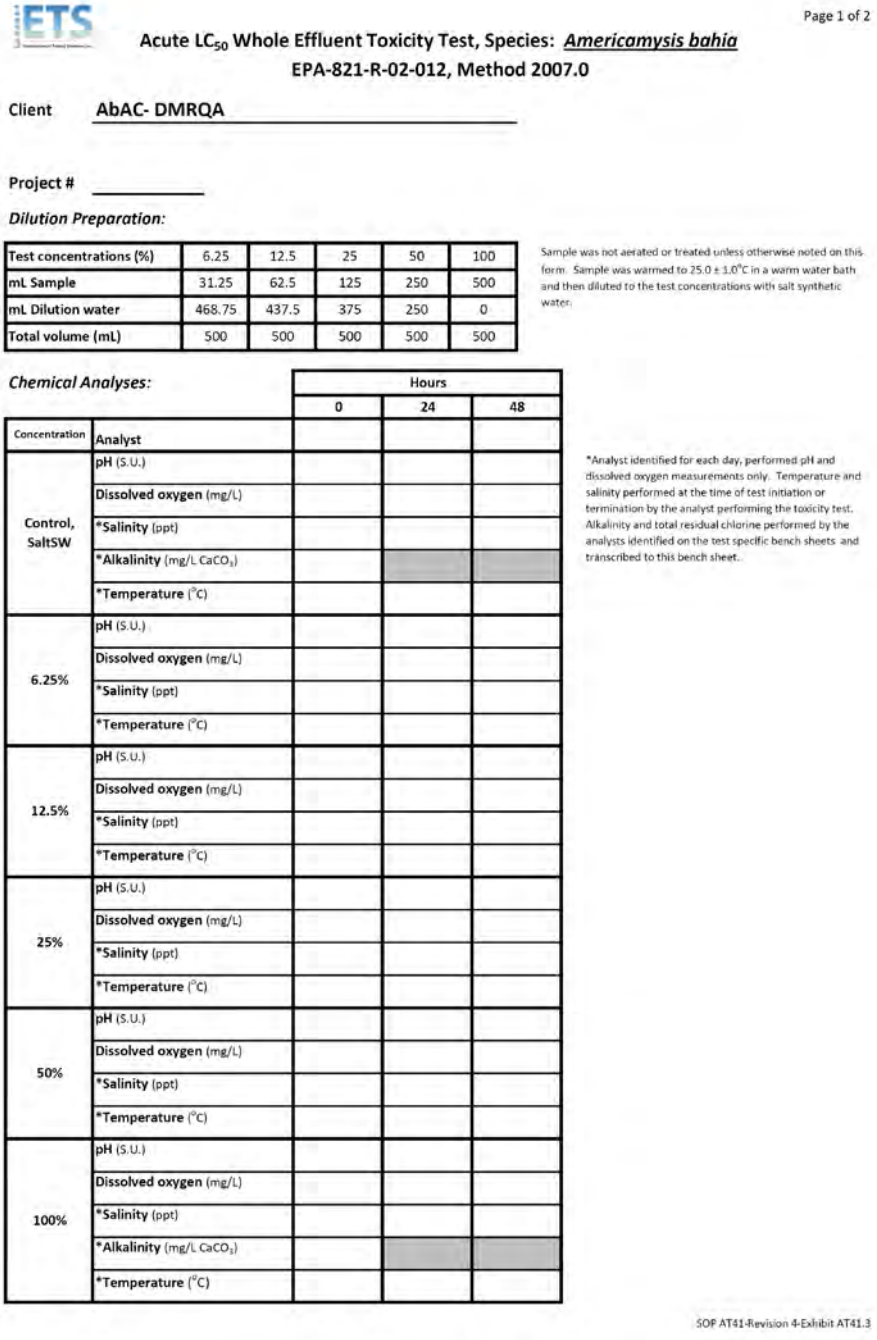
Method	
t-Stat or Rank Sum	
1-Tailed Critical	
PASS or FAIL	

Comments:

SOP AT41-Revision 4-Exhibit AT41.2

Subject: *Americamysis bahia* Acute Toxicity Test, EPA 2007.0

Exhibit AT41.3: Acute Toxicity Test Bench Sheet.



ETS

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Acute LC_{50} Whole Effluent Toxicity Test, Species: ***Americamysis bahia***
EPA-821-R-02-012, Method 2007.0

Client AbAC- DMRQA

Project # _____

Dilution Preparation:

Test concentrations (%)	6.25	12.5	25	50	100
mL Sample	31.25	62.5	125	250	500
mL Dilution water	468.75	437.5	375	250	0
Total volume (mL)	500	500	500	500	500

Sample was not aerated or treated unless otherwise noted on this form. Sample was warmed to 25.0 ± 1.0°C in a warm water bath and then diluted to the test concentrations with salt synthetic water.

Chemical Analyses:

		Hours		
		0	24	48
Concentration	Analyst			
Control, SaltSW	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	*Salinity (ppt)			
	*Alkalinity (mg/L $CaCO_3$)			
	*Temperature (°C)			
6.25%	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	*Salinity (ppt)			
	*Temperature (°C)			
12.5%	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	*Salinity (ppt)			
	*Temperature (°C)			
25%	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	*Salinity (ppt)			
	*Temperature (°C)			
50%	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	*Salinity (ppt)			
	*Temperature (°C)			
100%	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	*Salinity (ppt)			
	*Alkalinity (mg/L $CaCO_3$)			
	*Temperature (°C)			

*Analyst identified for each day, performed pH and dissolved oxygen measurements only. Temperature and salinity performed at the time of test initiation or termination by the analyst performing the toxicity test. Alkalinity and total residual chlorine performed by the analysts identified on the test specific bench sheets and transcribed to this bench sheet.

SOP AT41-Revision 4-Exhibit AT41.3

Subject: *Americamysis bahia* Acute Toxicity Test, EPA 2007.0

Acute LC_{50} Whole Effluent Toxicity Test, Species: *Americamysis bahia*
EPA-821-R-02-012, Method 2007.0

Client AbAC- DMRQA

Project # _____

Hours	Date	Feeding		Test Initiation or Termination		Location	Randomizing	Sample Number	SaltSW Batch
		Time	Analyst	Time	Analyst	Incubator/Shelf	Template		
0 Initiation		*							
24									
48 Termination									

*Test organisms were fed in holding 2 to 5 hours prior to test initiation. Test organisms were not fed during the test.

Test Organism Information:

Organism Source:	Aquatic Indicators, Inc.
Batch (AI Batch Ab):	
Age (1 to 5 days old):	
Date organisms were born: (time organisms were born between is not provided by supplier)	
Average transfer volume:	< 0.25 mL
Transfer bowl information:	**pH** (S.U.):
	Temperature (°C):

Survival Data **(number of living organisms):**

Hours	Control		6.25%		12.5%		25%		50%		100%	
	Replicate		Replicate		Replicate		Replicate		Replicate		Replicate	
	A	B	C	D	E	F	G	H	I	J	K	L
0 Initiation	10	10	10	10	10	10	10	10	10	10	10	10
24												
48 Termination												
Mean Survival												

Comment codes: d = dead, u = unhealthy, s = stressed

Statistics:

Method	
Lower 95% confidence limit (%)	
Upper 95% confidence limit (%)	
48-hour LC_{50} (%)	

Comments:

SOP AT41-Revision 4-Exhibit AT41.3

Subject: *Americamysis bahia* Acute Toxicity Test, EPA 2007.0

Exhibit AT41.4: Average Transfer Volume Log Sheet.

ETS

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Larval Fish Transfer Volume

Analyst: J. Sumner
Species: *P. promelas*
Date: 12-05-17
Source / Batch: Spawn date: 11-29-17
Ambient temperature: 24.3°C
Wet Weight of 10 Larvae (g): 0.0063 g

Estimate transfer volume, where minnows are allowed to swim from the pipette into the test vessel.

Numerically label 10 medicine cups.
Add 10 mL MHSW to each of the 10 cups.
Measure and record the weight of each cup containing MHSW.
Transfer 10 larvae to each cup, following procedures identified in SOP-AT18, AT47, or AT53 for vertebrate acute toxicity tests.
Transfer the larvae in a manner that allows them to swim from the pipette into the MHSW contained in each cup.
Measure and record the weight of each cup containing MHSW with 10 larvae.
Determine each transfer volume and average transfer volume.

Replicate Number	Initial Weight Medicine cup + 10 mL MHSW (g)	Final Weight Medicine cup + 10 mL MHSW + 10 Larval Fish transferred (g)	Transfer Volume Final - Initial Weight (g = mL)
1	11.3649	11.4693	0.1044
2	11.3957	11.4430	0.0473
3	11.4323	11.6293	0.1970
4	11.3821	11.4334	0.0513
5	11.3271	11.4008	0.0737
6	11.3224	11.4435	0.1211
7	11.4096	11.8059	0.3963
8	11.1915	11.2037	0.0122
9	11.2186	11.3718	0.1532
10	11.3001	11.3103	0.0102
		Average volume to transfer 10 organisms (mL):	0.1167

Estimate transfer volume, where the minnows are transferred with MHSW into the test vessels.

Numerically label 10 medicine cups.
Measure and record the weight of each cup.
Add approximately 10 mL MHSW to each of the 10 cups.
Transfer 10 larvae to each cup, following procedures identified in SOP-AT18, AT47, or AT53 for vertebrate acute toxicity tests.
Transfer the larvae in a manner that allows them to swim from the pipette into the MHSW contained in each cup.
Measure and record the weight of each cup containing MHSW with 10 larvae.
Determine each transfer volume and average transfer volume.

Replicate Number	Initial Weight Medicine cup (g)	Final Weight Medicine cup + 10 mL MHSW + 10 Larval Fish transferred (g)	Transfer Volume Final - Initial Weight (g = mL)
1	1.6179	11.4693	9.8514
2	1.6074	11.4430	9.8356
3	0.6279	11.6293	11.0014
4	1.5349	11.4334	9.8985
5	1.6472	11.4008	9.7536
6	1.5997	11.4435	9.8438
7	1.5972	11.8059	10.2087
8	1.5358	11.2037	9.6679
9	1.5956	11.3718	9.7762
10	1.6018	11.3103	9.7085
		Average volume to transfer 10 organisms (mL):	9.9546

SOP AT18-Revision 5-Exhibit AT18.4

Exhibit AT41.5: Randomization Template.

Randomizing template: **BLUE**

Replicate #	1	2	3	4
Concentrations	**1**	**7**	**3**	**5**
	7	**3**	**4**	**6**
1 = Control	**4**	**2**	**6**	**1**
2 = Lowest concentration	**3**	**5**	**5**	**2**
3 - 5 = Intermediate concentrations	**6**	**4**	**2**	**4**
6 = Highest concentration	**2**	**1**	**1**	**7**
7 = Intake/Upstream	**5**	**6**	**7**	**3**

Random number seeds: 10 through 13

SOP AT18-Revision 5-Exhibit AT18.5

Subject: *Americamysis bahia* Acute Reference Toxicity Test, EPA 2007.0

Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan		09-01-19
Quality Assurance Officer	Jim Sumner		09-01-19

Document Revision History

Effective Date	Revision number	Review Type	Evaluators	Revisions
12-01-00	0	Internal	Jim Sumner (ETS)	Original document
06-01-11	1	Internal	Jim Sumner (ETS)	• Updated references and exhibits.
11-01-14	2	Internal	Jim Sumner (ETS)	• Updated references and exhibits.
09-01-19	3	Internal	Jim Sumner (ETS)	• Updated exhibits during document review. • Updated procedure to include NELAP requirements. • Additional guidance included in SOP.

Subject: *Americamysis bahia* Acute Reference Toxicity Test, EPA 2007.0

Scope and Application

To assess the sensitivity of *Americamysis bahia* and the overall credibility of the *Americamysis bahia* acute toxicity tests. Reference toxicant data are part of the laboratory's QA/QC program to evaluate the performance of laboratory personnel and the robustness and sensitivity of the test organisms.

Summary of Method

The acute reference toxicity test generally involves the exposure of test organisms to five potassium chloride concentrations and control water for a 48-hour or 96-hour exposure period. At the end of each 24-hour period, the number of living organisms is counted in each potassium chloride concentration and control water. The median lethal concentration (LC_{50}) of potassium chloride is determined and compared to previous reference toxicant tests.

Quality Control

Acceptance Criteria: The test acceptance criterion is ≥ 90% survival in the control. If the control survival is < 90%, the test must be invalidated.

Frequency of Testing:
A *Americamysis bahia* acute reference toxicant test must be performed so that all acute whole effluent toxicity tests are conducted within 1 week of a reference toxicant test. In addition, an acute reference toxicant test must be performed on each batch of organisms received from an outside supplier. At a minimum, acute reference toxicant tests must be performed on a quarterly basis in order to maintain certification requirements.

Equipment and Materials

Mysid shrimp (*Americamysis bahia*)
Temperature-controlled incubator (set to maintain test temperature = 25.0 ± 1.0°C, photoperiod of 16-hour light / 8-hours dark, light intensity = 50 – 100 ft-c)
Control / Dilution water
(salt synthetic water made with Bioassay Grade 40-Fathoms Sea Salt, MarineMix®)
Potassium chloride (KCl, reagent grade)
1000-mL volumetric flask
Deionized water
500-ml plastic Solo® cups
Solo® cup lids
500-mL graduated cylinder

1000-mL Erlenmeyer flask
Large glass finger bowls
10-mL serological pipettes
Transfer pipettes
Calibrated top-loading balance (e.g. Fisher Scientific ACCU-224)
Thermometer
1-oz disposable medicine cups
Forceps
Weigh boats
Newly hatched brine shrimp
Light box or table
Disposable gloves
Americamysis bahia Acute Reference Toxicity Test Bench Sheet
Randomization template

Procedure

A. Test Preparation.

1. Prepare the pasticware.

   a. Obtain two replicate 500-ml plastic Solo® cups with lids for each of the five KCl concentrations tested and the control. Label each replicate cup with the following information.
   - Concentration
   - Replicate number

   b. Label the appropriate graduated cylinder.

   c. Prepare the 48-hour or 96-hour *Americamysis bahia* Acute Reference Toxicity Test Bench Sheet (see Exhibit AT42.1). Record the *Americamysis bahia* KCl Acute (AbKClAC) test number on the bench sheet.

B. Preparation of the Stock Solution.

1. Using a calibrated top-loading balance, carefully weigh out 50 g of KCl (SOP-G10). Place approximately 900 mL of deionized water in a 1000-mL volumetric flask. Add the KCl to the flask, dissolve the KCl by swirling the flask; bring to volume with deionized water. Label the volumetric flask with the concentration (50 g/L), analyst's initials, preparation

date and stock standard number (INSS, SOP-G15). Record the INSS number of the KCl stock solution on the bench sheet.

C. Preparation of the Test Concentrations.

1. Prepare all test concentrations using graduated cylinders and serological pipettes. The precision of conductivity measurements is increased when smaller volume graduated cylinders and/or serological pipettes are used to prepare the test concentrations. For this reference toxicant test, stock solution volumes should be measured using a 10-mL serological pipette and the total volumes should be measured using a 500-mL graduated cylinder.

2. Beginning with the lowest concentration, add approximately 100 mL of salt synthetic water to a 500-mL graduated cylinder, add the required volume of stock solution using a 10-mL serological pipette (refer to Table AT42.1), bring to volume (500 mL) with salt synthetic water. Mix the solution well by pouring the solution into a 1000-mL Erlenmeyer flask.

3. Pour 250 mL of test solution into each of the replicate test cups for that concentration. 30 mL should be saved for chemical analyses. Measure and record the pH (SOP-C3), dissolved oxygen (SOP-C2) and salinity (SOP-C5) of each test solution.

4. Rinse the graduated cylinder well with deionized water and repeat steps D.2 through D.5 for preparing the next test concentration. Record the batch date of salt synthetic water used to prepare the dilutions.

Table AT19.1: Test concentration, stock volumes, salt synthetic water volumes and final volumes for *Americamysis bahia* KCl acute reference toxicant tests.

Test Concentration (mg KCl/L)	Volume of Stock Required (mL)	Volume of Salt Synthetic Water (mL)	Final Volume (mL)
500	5.0	495.0	500
750	7.5	492.5	500
1000	10.0	490.0	500
1250	12.5	487.5	500
1500	15.0	485.0	500

5. Once all test concentrations have been prepared, follow the procedure described in SOP-AT41 for conducting *Americamysis bahia* Acute Toxicity Tests.

D. Preparation of Control Charts.

Refer to QAP-Q5 for how control charts are prepared and procedures for interpreting outlier tests. Refer to Exhibit AT42.2 for example control charts.

Safety and Hazardous Waste Management

Safety glasses, gloves and lab coats should be worn at all times.

Review Policy-P6: General Safety Policy and Policy-P9: Radiation Protection Policy for additional safety requirements.

References

USEPA. October 2002. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, 5th ed. **EPA-821-R-02-012, Method 2007.0**. US Environmental Protection Agency, Cincinnati, OH.

USEPA. 2000. Understanding and Accounting for Method Variability in Whole Effluent Toxicity Applications Under the National Pollutant Discharge Elimination Program. EPA-833-R-00-003. US Environmental Protection Agency, Cincinnati, OH.

USEPA. 2001. Final Report: Inter-laboratory Variability Study of EPA Short-term Chronic and Acute Whole Effluent Toxicity Test Methods, Volumes 1 and 2 - Appendix. EPA-821-B-01-004 and EPA-821-B-01-005. US Environmental Protection Agency, Cincinnati, OH.

North Carolina Department of Environment and Natural Resources, Division of Water Quality. Biological Laboratory Certification / Criteria Procedures, Version 3.0. December 2010.

TNI Standard. Management and Technical Requirements for Laboratories Performing Environmental Analysis. EL-V1-ISO-2016-Rev2.0. The NELAC Institute, PO Box 2439, Weatherford, TX 76086.

Exhibits

Exhibit AT42.1: *Americamysis bahia* Acute Reference Toxicity Test Bench Sheet.
Exhibit AT42.2: Example *Americamysis bahia* Acute Reference Toxicant Control Chart.

Subject: *Americamysis bahia* Acute Reference Toxicity Test, EPA 2007.0

Exhibit AT42.1: *Americamysis bahia* Acute Reference Toxicity Test Bench Sheet.

ETS

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Acute LC_{50} Whole Effluent Toxicity Test, Species: *Americamysis bahia*
EPA-821-R-02-012, Method 2007.0

Americamysis bahia Potassium Chloride Acute Reference Toxicant Test

AbKClAC # _____

Dilution Preparation:

Test concentrations (mg/L KCl)	250	375	500	750	1000
mL Stock solution	2.5	3.75	5.0	7.5	10.0
mL Dilution water	497.5	496.25	495.0	492.5	490.0
Total volume (mL)	500	500	500	500	500

A stock solution was prepared by diluting 100 g KCl into 2000 mL deionized water. This 50,000 mg/L KCl stock solution was used to prepare the concentrations evaluated for toxicity.

Stock solution INSS #: _____

Chemical Analyses:

Concentration	Analyst	Hours 0	24	48
Control, SaltSW	**pH** (S.U.)			
	Dissolved oxygen (mg/L)			
	***Salinity** (ppt)			
	***Alkalinity** (mg/L $CaCO_3$)			
	***Temperature** (°C)			
250 mg/L	**pH** (S.U.)			
	Dissolved oxygen (mg/L)			
	***Salinity** (ppt)			
	***Temperature** (°C)			
375 mg/L	**pH** (S.U.)			
	Dissolved oxygen (mg/L)			
	***Salinity** (ppt)			
	***Temperature** (°C)			
500 mg/L	**pH** (S.U.)			
	Dissolved oxygen (mg/L)			
	***Salinity** (ppt)			
	***Temperature** (°C)			
750 mg/L	**pH** (S.U.)			
	Dissolved oxygen (mg/L)			
	***Salinity** (ppt)			
	***Temperature** (°C)			
1000 mg/L	**pH** (S.U.)			
	Dissolved oxygen (mg/L)			
	***Salinity** (ppt)			
	***Temperature** (°C)			

*Analyst identified for each day, performed pH and dissolved oxygen measurements only. Temperature and salinity performed at the time of test initiation or termination by the analyst performing the toxicity test. Alkalinity performed by the analyst identified on the test specific bench sheet and transcribed to this bench sheet.

SOP AT42-Revision 3-Exhibit AT42.1

Subject: *Americamysis bahia* Acute Reference Toxicity Test, EPA 2007.0

ETS

Acute LC_{50} Whole Effluent Toxicity Test, Species: *Americamysis bahia*
EPA-821-R-02-012, Method 2007.0

Americamysis bahia Potassium Chloride Acute Reference Toxicant Test

AbKClAC # _____

Hours	Date	Feeding		Test Initiation or Termination		Location	Randomizing	SaltSW Batch
		Time	Analyst	Time	Analyst	Incubator/Shelf	Template	
0 Initiation		*						
24								
48 Termination								

*Test organisms were fed in holding 2 to 5 hours prior to test initiation. Test organisms were not fed during the test.

Test Organism Information:

Organism Source:	Aquatic Indicators, Inc.
Batch (AI Batch Ab):	
Age (1 to 5 days old):	
Date organisms were born: (time organisms were born between is not provided by supplier)	
Average transfer volume:	< 0.25 mL
Transfer bowl information:	**pH** (S.U.):
	Temperature (°C)

Survival Data (number of living organisms):

Hours	Control		250 mg/L		375 mg/L		500 mg/L		750 mg/L		1000 mg/L	
	Replicate		Replicate		Replicate		Replicate		Replicate		Replicate	
	A	B	C	D	E	F	G	H	I	J	K	L
0 Initiation	10	10	10	10	10	10	10	10	10	10	10	10
24												
48 Termination												
Mean Survival												

Comment codes: d = dead, u = unhealthy, s = stressed

Statistics:

Method	
Lower 95% confidence limit (mg KCl/L)	
Upper 95% confidence limit (mg KCl/L)	
48-hour LC_{50} (mg KCl/L)	

Comments:

Test Reviewed by: _____

SOP AT42-Revision 3-Exhibit AT42-1

Subject: *Americamysis bahia* Acute Reference Toxicity Test, EPA 2007.0

Exhibit AT42.2: Example of an *Americamysis bahia* Acute Reference Toxicant Control Chart.

Americamysis (Mysidopsis) bahia
Acute Reference Toxicant Control Chart
Source: Aquatic Indicators, Inc.

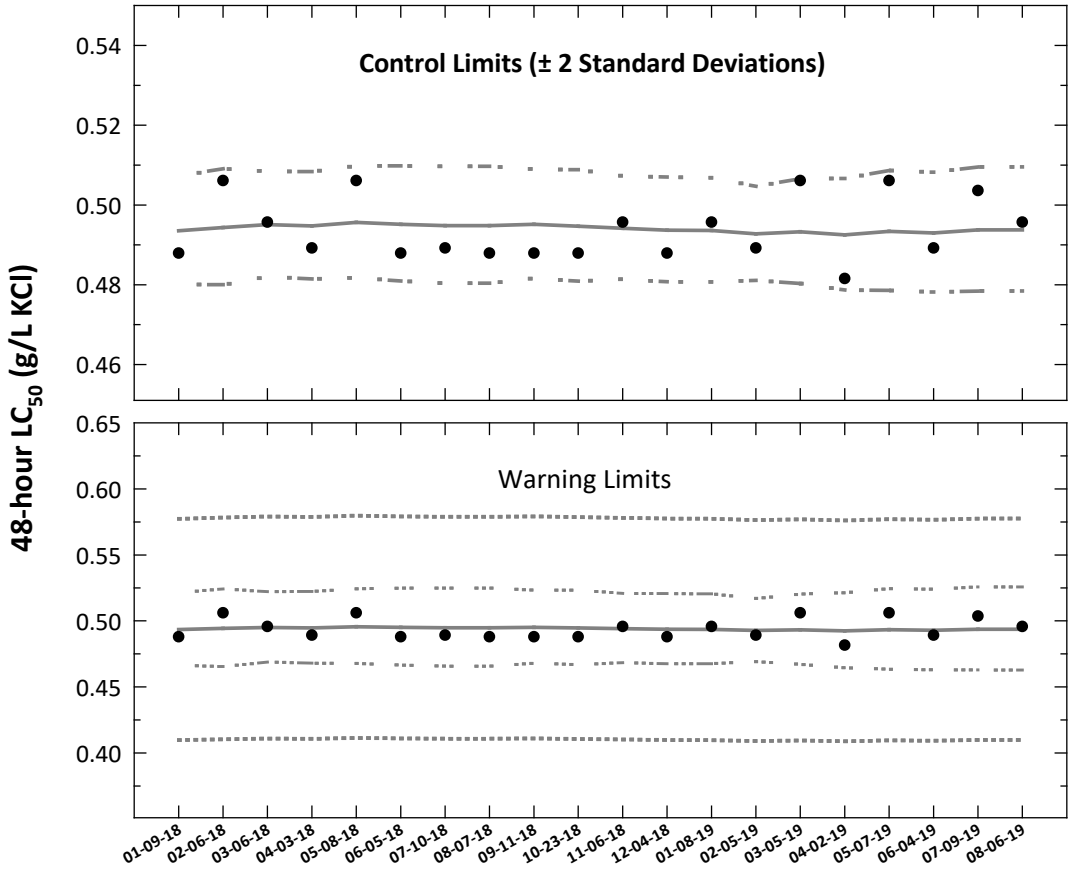
Test number	Test date	48-hour LC_{50} ToxCal Determination (g/L KCl)	Log_{10} Conversion			Anti-logarithmic Values (g/L KCl)						
			48-hour LC_{50}	CT	S	CT	Control Limits		Laboratory Calculated CV Warning Limits		10th Percentile CV Warning Limits	
							CT - 2S	CT + 2S	CT - 2CV	CT + 2CV	CT - $S_{A,10}$	CT + $S_{A,10}$
1	01-09-18	0.4879	-0.3117	-0.3067	0.0060	0.4935	0.4801	0.5073	0.4664	0.5214	0.4096	0.5774
2	02-06-18	0.5061	-0.2958	-0.3060	0.0064	0.4944	0.4800	0.5091	0.4654	0.5242	0.4103	0.5784
3	03-06-18	0.4957	-0.3048	-0.3053	0.0058	0.4951	0.4820	0.5085	0.4688	0.5221	0.4109	0.5792
4	04-03-18	0.4892	-0.3105	-0.3056	0.0059	0.4948	0.4815	0.5084	0.4679	0.5223	0.4106	0.5789
5	05-08-18	0.5061	-0.2958	-0.3048	0.0061	0.4956	0.4818	0.5099	0.4677	0.5243	0.4114	0.5799
6	06-05-18	0.4879	-0.3117	-0.3052	0.0063	0.4952	0.4810	0.5098	0.4665	0.5247	0.4110	0.5793
7	07-10-18	0.4892	-0.3105	-0.3055	0.0064	0.4948	0.4804	0.5097	0.4657	0.5249	0.4107	0.5790
8	08-07-18	0.4879	-0.3117	-0.3055	0.0064	0.4948	0.4804	0.5097	0.4657	0.5249	0.4107	0.5790
9	09-11-18	0.4879	-0.3117	-0.3052	0.0060	0.4952	0.4817	0.5090	0.4680	0.5231	0.4110	0.5793
10	10-23-18	0.4879	-0.3117	-0.3057	0.0061	0.4947	0.4809	0.5089	0.4668	0.5233	0.4106	0.5788
11	11-06-18	0.4957	-0.3048	-0.3061	0.0057	0.4942	0.4814	0.5073	0.4683	0.5207	0.4102	0.5782
12	12-04-18	0.4879	-0.3117	-0.3065	0.0058	0.4937	0.4808	0.5070	0.4675	0.5206	0.4098	0.5776
13	01-08-19	0.4957	-0.3048	-0.3066	0.0057	0.4936	0.4807	0.5068	0.4675	0.5204	0.4097	0.5775
14	02-05-19	0.4892	-0.3105	-0.3073	0.0052	0.4928	0.4811	0.5047	0.4692	0.5170	0.4090	0.5766
15	03-05-19	0.5061	-0.2958	-0.3069	0.0058	0.4933	0.4803	0.5066	0.4670	0.5203	0.4094	0.5772
16	04-02-19	0.4816	-0.3174	-0.3076	0.0062	0.4925	0.4787	0.5067	0.4645	0.5213	0.4088	0.5762
17	05-07-19	0.5061	-0.2958	-0.3068	0.0066	0.4934	0.4786	0.5087	0.4634	0.5244	0.4095	0.5773
18	06-04-19	0.4892	-0.3105	-0.3072	0.0066	0.4930	0.4782	0.5082	0.4630	0.5239	0.4092	0.5768
19	07-09-19	0.5036	-0.2979	-0.3065	0.0068	0.4938	0.4785	0.5096	0.4628	0.5258	0.4098	0.5777
20	08-06-19	0.4957	-0.3048	-0.3065	0.0068	0.4938	0.4785	0.5096	0.4628	0.5258	0.4098	0.5777

Note: **48-hour LC_{50}** = 48-hour median lethal concentration. An estimate of the potassium chloride concentration which is lethal to 50% of the test organisms in 48-hours (calculated using ToxCalc).
CT = Central tendency of the LC_{50} values.
S = Standard deviation of the LC_{50} values.
Control Limits = Mean logarithmic LC_{50} ± 2 standard deviations converted to anti-logarithmic values.
Warning Limits = Mean logarithmic LC_{50} ± 2CV or $S_{A,10}$ converted to anti-logarithmic values.
$S_{A,10}$ = Standard deviation corresponding to the 10th percentile of CVs reported nationally by USEPA. ($S_{A,10}$ = 0.17).
CV = Coefficient of variation.

Subject: *Americamysis bahia* Acute Reference Toxicity Test, EPA 2007.0

Environmental Testing Solutions, Inc.

Americamysis (Mysidopsis) bahia
Acute Reference Toxicant Control Chart
Source: Aquatic Indicators, Inc.



- ● **48-hour LC_{50}** = median lethal concentration. An estimation of the potassium chloride concentration which is lethal to 50% of the test organisms in 48-hours (calculated using ToxCalc).
- **Central Tendency** (mean logarithmic LC_{50} converted to anti-logarithmic values)
- **Control Limits** (mean logarithmic LC_{50} ± 2 standard deviations converted to anti-logarithmic values)
- **Laboratory Warning Limits** (mean logarithmic LC_{50} ± 2 coefficent of variations converted to anti-logarithmic values)
- **USEPA Warning Limits** (mean logarithmic LC_{50} ± $S_{A.10}$ converted to anti-logarithmic values, $S_{A.10}$ = 10th percentile of CVs reported nationally by USEPA)

Entered and Reviewed by Jim Sumner

Subject: *Americamysis bahia* Chronic Toxicity Test, EPA 1007.0

Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan		09-01-19
Quality Assurance Officer	Jim Sumner		09-01-19

Document Revision History

Effective Date	Revision number	Review Type	Evaluators	Revisions
12-01-00	0	Internal	Jim Sumner (ETS)	Original document
06-01-11	1	Internal	Jim Sumner (ETS)	• Updated references and exhibits. • Statistical analyses and data review moved to QAP-Q12.
07-01-12	2	External (NC DENR) Internal	Lance Ferrell (NC DENR) Jim Sumner (ETS)	• The measurement of pH, DO and conductivity and salinity of each new, full-strength, undiluted sample was added. • The light intensity was amended to reflect that it is a recommended range as specified in the EPA manuals.
11-01-14	3	Internal	Jim Sumner (ETS)	• Updated exhibits during document review. • Increased Mysid chronic feeding rate to 100 µl (from 50 µL) per feeding twice daily. • Changed renewal time recommendation to ± 2-hours from test initiation. • Provided additional clarification to testing procedure. • Added acceptance criteria with Table AT43.1.
09-01-19	5	Internal	Jim Sumner (ETS)	• Updated procedure to include NELAP requirements. • Additional guidance included in SOP.

Scope and Application

To measure the chronic toxicity of water samples to Mysid shrimp, *Americamysis bahia*, during a 7-day, static renewal test.

Summary of Method

The chronic toxicity test generally involves the exposure of test organisms to up to five effluent concentrations and a control water. The test duration is 7-days. Test solutions are renewed daily, and observations of survival are documented. At the end of the 7-day exposure period, organisms are killed, and a dry weight is determined. In addition, observations of fecundity (presence of females with eggs in the oviduct and/or brood pouch) may be documented.

A summary of the *Americamysis bahia* chronic method is provided in Exhibit AT43.1.

Subject: *Americamysis bahia* Chronic Toxicity Test, EPA 1007.0

Quality Control

Acceptance Criteria: The test acceptance criteria are indicated in the table below. If acceptability criteria are not met, the test must be invalidated.

Table AT20.1: *Pimephales promelas* chronic toxicity test acceptability criteria.

Test Acceptability Criteria	USEPA
Control survival	≥ 80%
Mean dry weight of surviving control larvae (mg)	≥ 0.20
Guidance control growth coefficient of variation	< 20%
Guidance percent minimum significant difference (PMSD)	11 – 37%

Equipment and Materials

Mysid shrimp (*Americamysis bahia*)
Temperature-controlled incubator (set to maintain test temperature = 25.0 ± 1.0°C, photoperiod of 16-hour light / 8-hours dark, light intensity = 50 – 100 ft-c)
Control / Dilution water
(salt synthetic water made with Bioassay Grade 40-Fathoms Sea Salt, MarineMix®)
Microbalance accurate to 0.00001 mg (e.g. Cahn)
Class S or Class I certified weights
Microweight aluminum pans (e.g. Cahn)
Drying oven
Desiccator
Scintillation vials
Plastic tray
250-mL Glass beakers
Graduated cylinders
Large glass finger bowls
Serological, fixed, or adjustable volume pipette (e.g., Eppendorf)
Transfer pipettes

Siphoning hose (rubber tubing affixed with a plastic tube fitted with a soft, angled rubber tip)
White plastic photographic tray
Fine mesh sieve
Forceps
Ice water
Aquarium pump, tubing, and air stones
Plexiglas® slides
Thermometer
1-oz medicine cups
Newly hatched brine shrimp
Light box or table
Disposable gloves
Americamysis bahia Chronic Toxicity Test Bench Sheet
Randomization template

Procedure

A. Test Preparation.

1. Prepare the glassware.

   a. Obtain eight replicate 250-mL plastic glass beakers for each site/sample and concentration tested, including the control. Label each replicate cup with the following information.
   - Facility/sample name
   - Concentration
   - Replicate number

   b. Label the appropriate graduated cylinder with the facility/sample name and concentration.

   c. Prepare the *Americamysis bahia* Chronic Toxicity Test Benchsheet (Exhibit T43.2). Record the following information on the Benchsheet:
   - Client/facility name
   - Permit number
   - County
   - Outfall/Pipe number
   - ETS project and sample numbers
   - Control/Dilution water type and batch

- Test concentrations and dilution preparation information (sample, dilution and total volumes)

2. Weigh the microweight pans (This step may be completed at any time before test termination on day 7).

   a. Label 20-mL glass beakers or Coors® spot plates with the facility or sample name, concentration, and replicate number.

   b. Obtain the microweight aluminum pans from the desiccator.

   c. Using forceps, place one microweight pan into each of the 20-mL glass beakers or each of the wells of the spot plates.

   d. Place the 20-mL glass beakers or spot plates in a drying oven and let the contents dry a minimum of 24-hours at 60 ± 2°C or 6-hours at 100 ± 2°C.

   e. Remove the 20-mL glass beakers or spot plates from the oven and place in a desiccator a minimum of 4-hours to cool the pans before they are weighted on a calibrated microbalance.

   f. Verify the accuracy of the microbalance as described in SOP-G10.

   g. Using forceps, remove a microweight pan and place it on the balance pan. Allow the reading to stabilize and record the measurement on the chronic benchsheet. Record the date, beaker/spot plate color identification and analyst initials on the chronic benchsheet. Return the microweight pan to the appropriate 20-mL glass beaker or well on the spot plate.

   h. Repeat Step 2.g to obtain the initial weight of each pan needed for the test. After all the initial weights are obtained, place the 20-mL glass beakers or spot plates in a desiccator until needed on day 7.

B. Test Initiation (Day 0).

1. Prepare the test concentrations according to SOP-G5. It may be necessary to salt-up the sample prior to making the test concentrations. Refer to SOP-G5 for the appropriate procedures for salting-up samples.

a. The control/dilution water is salt synthetic water (SaltSW, SOP-AT1). SaltSW must have an alkalinity of 57 – 64 mg $CaCO_3$/L, hardness of 80 – 100 mg $CaCO_3$/L, and an initial pH of 6.5 – 8.5 S.U. (guidance range = 7.4 – 7.8 S.U.).

b. Measure and record the pH (SOP-C3), dissolved oxygen [SOP-C2, ensure that the dissolved is within the acceptable range (4.0 to 9.0 mg/L), aerate the sample if necessary according to SOP-G5] and conductivity (SOP-C4) of each concentration tested and control. Measure and record the pH (SOP-C3), dissolved oxygen (SOP-C2), conductivity (SOP-C4), total residual chlorine (SOP-C8), total alkalinity (SOP-C6), total hardness (SOP-C7) and sample characteristics of each new, full-strength, undiluted sample. Measure and record the alkalinity (SOP-C6) and hardness (SOP-C7) of the control/dilution water.

c. Pour 250 mL of control water into each of the control cups.

d. Pour 250 mL of each test concentration into each of the labeled test cups.

e. Maintain the test temperature (25.0 ± 1.0°C) of the test concentrations. This may be accomplished by placing the test cups into a temperature-controlled incubator.

2. Isolate the larvae for the test.

a. Obtain a batch of larvae (SOP-AT17), which are < 24 hours old. The test organisms must come from a pool of larvae consisting of at least three separate spawnings. Please refer to Exhibit AT20.2: Weekly *Pimephales promelas* Spawning / Egg Collection Log. Record the spawning date, age and hatch dates and times of the organisms to be used in the test on the chronic bench sheet. Transfer the larvae from the tank to a large finger bowl.

b. After the larvae have acclimated to the test conditions, the larvae may be transferred by transfer pipette to the test solutions. The end of the pipette tip should be cut to a size that will not injure or harm the larvae during transfer. Larvae should be transferred gently in a manner that will not expose the organisms to the air.

c. Two techniques may be used for transferring 10 organisms to each test cup from the finger bowl. The technique used is dependent on the sample being evaluated for toxicity.

- If the transferred water may alter the toxicity of the sample (decrease toxicity), organisms should be transferred directly by pipette. This technique is predominately used by the laboratory. Organisms should be transferred in a manner that allows them to swim from the pipette into the test solutions. This will minimize the volume of transfer water introduced into the sample. Follow procedures outlined in step 3 for transferring the organisms to the randomly placed test cups. The average transfer volume by this technique for each analyst must be determined yearly. For an example transfer volume log sheet refer to Exhibit AT20.4.

- If pathogenic interferences have been identified or there is risk of cross contamination between replicates, organisms should be transferred to medicine cups (10 per cup) and then introduced into the test solutions. The final volume contained in the medicine cups after the organisms have been transferred should be between 10 and 15 ml. With a transfer pipette, remove any food or debris that was placed in the cup during transfer. The average transfer volume by this technique for each analyst must be determined yearly. For an example transfer volume log sheet refer to Exhibit AT20.4. Continue this process until enough medicine cups containing 10 larvae each have been obtained to initiate the test. 1 medicine cup containing 10 larvae will be needed for each sample concentration and replicate. If a test consists of 5 concentrations and a control, 24 medicine cups containing 10 larvae each will be required. If a test consists of 1 concentration and a control, 8 medicine cups containing 10 larvae each will be required.

d. Save approximately 30 mL of transfer water to be measured for pH (SOP-C3). Measure and record the transfer water pH and temperature on the chronic bench sheet.

3. Transfer the larvae to the randomly placed test cups.

a. Obtain a randomization template (Exhibit AT20.5). Order the test cups according to the randomization template and record the template name on the bench sheet.

b. Measure and record the temperature in one of the test cups for each concentration and control. The temperature must be 25.0 ± 1.0^{o}C before the test organisms are placed in the test cups. Warm the test cups in a warm water bath or temperature-controlled incubator, if necessary, until the desired test

temperature is obtained. Measure and record the temperature of an arbitrary transfer cup.

c. Place 10 larvae in the first test cup of the first row (by pipette or medicine cup). Continue in this manner (placing the larvae in the test cups from left to right in the first row and then the second row) until all the test cups contain 10 larvae.

d. Record the initiation date, time and analyst's initials on the chronic bench sheet. Record the average transfer volume by the technique used on the chronic bench sheet. **The test must be initiated within 36-hours of completion of the first sampling period.**

e. Verify that each cup received the required number of larvae (i.e., 10) by conducting a repeat count. Remove excess larvae or add larvae as necessary. Record the initial number of larvae on the bench sheet. Place the lids on each cup.

f. Place the test cups in order according to the randomization template in a temperature-controlled incubator. The organisms must be maintained at 25.0 ± 1.0°C with a photoperiod of 16-hours light and 8-hours dark and a recommended light intensity of 50 to 100 ft-c. Record the incubator number used on the bench sheet.

g. Using a transfer pipette, feed the larvae in each test cup 3 drops (150 µL) newly hatched brine shrimp (1050 to1500 shrimp). To obtain the appropriate suspension of brine shrimp, refer to SOP-AT16. [Note: The test larvae are fed twice daily at a 6 ± 1-hour interval (generally at the beginning and at the end of the workday).] Record the time(s) the larvae were fed on the *Pimephales promelas* Chronic Toxicity Test Bench Sheet.

Note: Since the larvae are fed in holding prior to test initiation, the larvae may be fed only once in the test cups on the first day.

C. Daily Test Renewal (Days 1-6).

Repeat this process each day during the test period. The test must be renewed within ± 2 hours from test initiation. **When new samples are used for test solution renewal, the test must be renewed within 36-hours of completion of the first sampling period for each new sample.**

1. Prior to renewal of the test water in the cups, carefully pour ~30 mL of test water from at least one replicate cup for each test concentration and control into a labeled 1-oz

medicine cup. This water will be used to determine final pH and dissolved oxygen concentrations.

2. Feed the larvae in the test cup 150 µL of newly-hatched brine shrimp a minimum of 2-hours prior to renewal of the test concentrations. Record the feeding time on the *Pimephales promelas* Chronic Toxicity Test Bench Sheet.

3. Measure and record the temperature in an arbitrarily selected test replicate for each concentration and control.

4. Prepare fresh test water in accordance with SOP-G5. Maintain the test temperature (25.0 ± 1.0°C) of the fresh test water until needed by storing in a temperature-controlled incubator.

5. Remove the test cups from the incubator and remove the lids. Place the cups on a light box or table for ease of viewing.

6. Change the test water in all four replicate cups before starting the next four-cup series. To change the test water, test cups may be either siphoned or decanted.

   a. Siphoning method: Siphon off old water, excess shrimp and detritus from the cups using rubber tubing affixed with a plastic tube fitted with a soft, angled rubber tip. Slowly siphon the water from the cup into a white plastic photographic tray until ~ 50 mL of old test water remains. Control the flow through the tubing by holding one gloved finger over the end of the tubing.

      Decanting method: Using a transfer pipette, remove any debris, dead artemia and dead larvae that may have accumulated on the bottom of the test cup. Carefully decant the water from the cup into a white plastic photographic tray until ~ 50 mL of old test water remains. This technique is predominately used by the laboratory.

   b. If any larvae are accidentally siphoned off or decanted with the water, retrieve them from the plastic tray, using a transfer pipette. The end of the transfer pipette tip should be cut to a size that will not injure or harm the larvae during transfer. Larvae should be transferred gently in a manner that will not expose the organisms to the air. Return the larvae to the appropriate replicate cup. Record the number of larvae siphoned out or decanted (per replicate). Discard any dead larvae.

   c. Record the following information on the chronic bench sheet.

- Number of larvae surviving in each replicate cup
- Number of dead larvae in each replicate cup (if applicable)
- Any comments (injured, sick or larvae siphoned out)

d. Fill each replicate cup to 250 mL using fresh test water. Pour the test water down the side of the cup to avoid unnecessarily disturbing the larvae.

h. After all test cups have been renewed, record the renewal time and the analyst's initials on the chronic bench sheet.

i. Place the lids on each cup. Place the test cups in order according to the randomization template in a temperature-controlled incubator.

7. At 6 ± 1-hour after the first feeding, feed the test larvae 3 drops (150 µL) of newly-hatched brine shrimp. Record the feeding time on the chronic bench sheet.

Note: Test solutions may be renewed prior to the first feeding.

D. Test Termination (Day 7, not to exceed 7 days + 2 hours).

Terminate the test after the organisms have been exposed to the test concentrations for 7 consecutive days ± 2-hours.

1. Measure and record the temperature in an arbitrarily selected test cup for each concentration and control.

2. Remove the test cups from the incubator and remove the lids. Place the cups on a light box or table for ease of viewing.

3. Carefully pour ~30 mL of test water from at least one replicate cup for each test concentration and control into a labeled 1-oz medicine cup. This water will be used to determine final pH and dissolved oxygen concentrations.

4. Obtain the appropriately labeled 20-mL glass beakers or spot plates containing pre-weighed microweight pans.

5. Fill a 600-mL beaker or equivalent with ice water and obtain a fine mesh sieve with a handle.

6. Beginning with the first replicate cup of the control.

a. Count and record (in the appropriate section) the number of living and dead larvae in each replicate cup on the chronic bench sheet. Record comments, if applicable. Discard any dead larvae.

b. Holding the mesh sieve over an empty beaker, pour the test water containing the larvae from one replicate cup through the sieve. The larvae will be retained on the mesh.

c. Immediately submerge the sieve containing the larvae into the ice water. Keep the larvae in the ice water for 3 to 4 seconds.

d. Remove the sieve from the ice water. Carefully rinse the larvae with deionized water to remove any excess food or detritus.

e. Using forceps, remove the microweight pan from the appropriate 20-mL glass beaker or well on the spot plate. Using the forceps, transfer the larvae from the mesh to the microweight pan. In the process, to ensure the larvae are dead, sever their spinal cords with forceps. Ensure that all the larvae have been transferred to the microweight pan. Verify against the number recorded in Step 6.a. above.

   A study was performed to determine if solids are lost by this method of killing the larvae before they are placed on the microweight pans. The study determined that the amount of solids lost from larvae killed by severing the spinal cords was not significantly different than the amount of moisture lost during the weighing process (study performed using wet weights, Exhibit AT20.6).

f. Return the pan to the appropriate 20-mL glass beaker or well on the spot plate.

g. Repeat Step 6 for the remaining test cups for each test concentration (from lowest to highest).

7. Place the 20-mL glass beakers or spot plates in a drying oven and let the contents dry a minimum of 24-hours at 60 ± 2°C or 6-hours at 100 ± 2°C. Yearly laboratory studies have confirmed that drying the larvae longer than the recommended time will not alter the final dry weight.

8. Remove the 20-mL glass beakers or spot plates from the oven and place in a desiccator a minimum of 4-hours to cool the larvae before weighing them on a calibrated microbalance.

9. Measure the final pan weights.

   a. Verify the accuracy of the microbalance as described in SOP-G10.

   b. Using forceps, remove the microweight pan from the 20-mL glass beaker or well on the spot plate and place it on the balance pan. Allow the reading to stabilize and record the measurement on the chronic benchsheet. Return the microweight pan to the 20-mL glass beaker or well on the spot plate. Record the date the weights were measured and analyst initials on the chronic benchsheet.

   c. Repeat Step 9.b. to obtain the final weight of each remaining pan. After all the final weights are obtained, return the 20-mL glass beakers or spot plates to a desiccator until the survival and weight data have been verified.

E. Statistical Analyses and Test Data Verification.

Statistical analyses and data review are performed according to QAP-Q12.

Safety and Hazardous Waste Management

Safety glasses, gloves and lab coats should be worn at all times.

Review Policy-P6: General Safety Policy and Policy-P9: Radiation Protection Policy for additional safety requirements.

References

USEPA. October 2002. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, 4th ed. **EPA-821-R-02-013, Method 1000.0**. US Environmental Protection Agency, Cincinnati, OH.

TNI Standard. Management and Technical Requirements for Laboratories Performing Environmental Analysis. EL-V1-ISO-2016-Rev2.0. The NELAC Institute, PO Box 2439, Weatherford, TX 76086.

Exhibits

Exhibit AT20.1: Summary of Test Conditions for the *Pimephales promelas* Chronic Toxicity Test.
Exhibit AT20.2: Weekly *Pimephales promelas* Spawning / Egg Collection Log.
Exhibit AT20.3: *Pimephales promelas* Chronic Toxicity Test Bench Sheet.

Exhibit AT20.4: Average Transfer Volume Log Sheet.
Exhibit AT20.5: Randomization Template.
Exhibit AT20.6: Determination of Solids Loss from Killing of Larvae at Test Termination.

Subject: *Americamysis bahia* Chronic Toxicity Test, EPA 1007.0

Exhibit AT20.1: Summary of Test Conditions for the *Pimephales promelas* Chronic Toxicity Test.

SUMMARY OF TEST CONDITIONS FOR THE *PIMEPHALES PROMELAS* CHRONIC TOXICITY TEST

Test type:	Static renewal
Test duration:	7-days
Temperature:	25.0 ± 1.0°C
Light quality:	Cool-white fluorescent
Light intensity:	50 – 100 ft-candles (recommended)
Photoperiod:	16-hours light, 8-hours dark
Test chamber size:	500 mL Solo® cups
Test solution volume:	250 mL
Renewal of test solutions:	Daily
Age of test organisms:	< 24-hours old.
Number of organisms per test chamber:	10
Number of replicate test chambers per concentration:	4
Number of organisms per concentration:	40
Test concentrations:	Multiple concentration tests: 5 and a control with ≥ 0.5 dilution series (recommended) Single dilution tests: 100% and a control
Test chamber cleaning:	Daily, test chambers are cleaned immediately before test solution renewal.
Aeration:	None, unless the dissolved oxygen concentration falls below 4.0 mg/L.
Feeding regime:	On days 0 through 6, organisms in each test cup are fed 150 µL *Artemia nauplii* twice daily at 6-hour intervals.
Control / Dilution water:	Moderately hard synthetic water
Sampling and sample holding:	3-gallon grab or composite samples collected on days one, three and five. Each sample must first be used within 36-hours of completion of each sampling period.
Endpoint:	Survival and growth (dry weight per initial number of larvae)
Test acceptability criterion:	≥ 80% control survival, control growth ≥ 0.25 mg/surviving larvae

Exhibit AT20.2: Weekly *Pimephales promelas* Spawning / Egg Collection Log.

ETS

Page ______

Weekly *Pimephales promelas* Spawning / Egg Collection Log

Date (Spawn date and Batch ID)	Time	Analyst	Total # Tiles	Start Hatching		End Hatching		Comments
				Date	Time	Date	Time	
07-28-19								Large stock: 1 ____, 2 ____, 3 ____, 4 ____, 5 ____, 6 ____ Removed larvae hatched prior to: ________ Analyst ______
07-29-19								Large stock: 1 ____, 2 ____, 3 ____, 4 ____, 5 ____, 6 ____ Removed larvae hatched prior to: ________ Analyst ______
07-30-19								Large stock: 1 ____, 2 ____, 3 ____, 4 ____, 5 ____, 6 ____ Removed larvae hatched prior to: ________ Analyst ______
07-31-19								Large stock: 1 ____, 2 ____, 3 ____, 4 ____, 5 ____, 6 ____ Removed larvae hatched prior to: ________ Analyst ______
08-01-19								Large stock: 1 ____, 2 ____, 3 ____, 4 ____, 5 ____, 6 ____ Removed larvae hatched prior to: ________ Analyst ______
08-02-19								Large stock: 1 ____, 2 ____, 3 ____, 4 ____, 5 ____, 6 ____ Removed larvae hatched prior to: ________ Analyst ______
08-03-19								Large stock: 1 ____, 2 ____, 3 ____, 4 ____, 5 ____, 6 ____ Removed larvae hatched prior to: ________ Analyst ______

Note: Unhatched eggs on tiles at the end hatch time are moved to the next spawn date to allow the remaining eggs to hatch. These eggs will all hatch in < 24-hours.

Egg Collection Source by Spawn Date:

Date	Tank ID (X locations where tiles with eggs were collected)																																																	
(Spawn date and Batch ID)	A						B						C						D						E						F						G			H			Stock							
	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	1	2	3	1	2	3	4	5	6	7	8
07-28-19																																																		
07-29-19																																																		
07-30-19																																																		
07-31-19																																																		
08-01-19																																																		
08-02-19																																																		
08-03-19																																																		

Comments:

SOP-AT17-Revision 4–Exhibit AT17.4

Subject: *Americamysis bahia* Chronic Toxicity Test, EPA 1007.0

Exhibit AT20.3: *Pimephales promelas* Chronic Toxicity Test Bench Sheet.

Chronic Whole Effluent Toxicity Test (EPA-821-R-02-013 Method 1000.0)
Species: *Pimephales promelas*

Client: City of Chattanooga, Moccasin Bend WWTP
NPDES #: TN 0024210
Project #: _____

County: Hamilton
Outfall #: 001
Permit Limit: 5.5%

Dilution preparation information:						***Comments:***
Dilution prep (%)	1.38	2.8	5.5	11	22	
Effluent volume (mL)	20.7	42	82.5	165	330	
Diluent volume (mL)	1479.3	1458	1417.5	1335	1170	
Total volume (mL)	1500	1500	1500	1500	1500	

Test organism information:		***Test information:***	
Organism source:	In-house culture	Randomizing template:	
Age:	< 24-hours old	Incubator number and shelf location:	
Spawn date:		*Artemia* CHM number:	CHM984
Hatch dates and times:		***Drying information for weight determination:***	
Transfer vessel information:	pH = S.U. Temperature = °C	Date / Time in oven:	
		Initial oven temperature:	
Average transfer volume:	< 0.25 mL	Date / Time out of oven:	
		Final oven temperature:	
		Total drying time:	

Daily feeding and renewal information:

Day	Date	Morning feeding		Afternoon feeding		Test initiation, renewal, or termination		Sample numbers used	MHSW batch used
		Time	Analyst	Time	Analyst	Time	Analyst		
0	05-07-19								
1	05-08-19								
2	05-09-19								
3	05-10-19								
4	05-11-19								
5	05-12-19								
6	05-13-19								
7	05-14-19								

Control information:		Acceptance criteria	***Summary of test endpoints:***	
% Mortality:		≤ 20%	7-day LC_{50} (%)	
Average weight per initial larvae:			NOEC (%)	
Average weight per surviving larvae:		≥ 0.25mg/larvae	LOEC (%)	
			ChV (%)	
			IC_{25} (%)	

SOP AT20-Revision 5–Exhibit AT20.3

Species: *Pimephales promelas*

Client: City of Chattanooga, Moccasin Bend WWTP Date: 05-07-19

Survival and Growth Data

Day	CONTROL				1.38%				2.8%			
	A	B	C	D	E	F	G	H	I	J	K	L
0	10	10	10	10	10	10	10	10	10	10	10	10
1												
2												
3												
4												
5												
6												
7												
A = Pan weight (mg) Tray color code:: _____ Analyst: _____ Date: _____												
B = Pan + Larvae weight (mg) Analyst: _____ Date: _____												
C = Larvae weight (mg) = B – A Analyst: _____												
Weight per initial number of larvae (mg) = C / Initial number of larvae Analyst: _____												
Average weight per initial number of larvae (mg) / Percent reduction from control (%)												

Comment codes: c = clear, d = dead, fg = fungus, k = killed, m = missing, sk = sick, sm = unusually small, lg = unusually large, d&r = decanted and returned, w = wounded.

Comments:

SOP AT20-Revision 5–Exhibit AT20.3

Species: *Pimephales promelas*
Client: City of Chattanooga, Moccasin Bend WWTP Date: 05-07-19

Survival and Growth Data

Day	5.5%				11%				22%			
	M	N	O	P	Q	R	S	T	U	V	W	X
0	10	10	10	10	10	10	10	10	10	10	10	10
1												
2												
3												
4												
5												
6												
7												
A = Pan weight (mg) Tray color code:: _____ Analyst: _____ Date: _____												
B = Pan + Larvae weight (mg) Analyst: _____ Date: _____												
C = Larvae weight (mg) = B – A Analyst: _____												
Weight per initial number of larvae (mg) = C / Initial number of larvae Analyst: _____												
Average weight per initial number of larvae (mg) / Percent reduction from control (%)												

Comment codes: c = clear, d = dead, fg = fungus, k = killed, m = missing, sk = sick, sm = unusually small, lg = unusually large, d&r = decanted and returned, w = wounded.

Comments:

SOP AT20-Revision 5–Exhibit AT20.3

Subject: *Americamysis bahia* Chronic Toxicity Test, EPA 1007.0

Environmental Testing Solutions, Inc.

Species: ***Pimephales promelas*** **Date:** **05-07-19**

Client: **City of Chattanooga, Moccasin Bend WWTP**

Daily Chemistry:

		Day (Analyst identified for each day, performed pH, D.O. and conductivity measurements only.)					
		0		1		2	
	Analyst						
Concentration	Parameter						
CONTROL, MHSW	pH (S.U.)						
	DO (mg/L)						
	Conductivity (µmhos/cm)						
	*Alkalinity (mg $CaCO_3$/L)						
	*Hardness (mg $CaCO_3$/L)						
	*Temperature (°C)						
1.38%	pH (S.U.)						
	DO (mg/L)						
	Conductivity (µmhos/cm)						
	*Temperature (°C)						
2.8%	pH (S.U.)						
	DO (mg/L)						
	Conductivity (µmhos/cm)						
	*Temperature (°C)						
5.5%	pH (S.U.)						
	DO (mg/L)						
	Conductivity (µmhos/cm)						
	*Temperature (°C)						
11%	pH (S.U.)						
	DO (mg/L)						
	Conductivity (µmhos/cm)						
	*Temperature (°C)						
22%	pH (S.U.)						
	DO (mg/L)						
	Conductivity (µmhos/cm)						
	*Temperature (°C)						
100%	pH (S.U.)						
	DO (mg/L)						
	Conductivity (µmhos/cm)						
	*Alkalinity (mg $CaCO_3$/L)						
	*Hardness (mg $CaCO_3$/L)						
	*TR chlorine (mg/L)						
	*Temperature (°C)						
		Initial	Final	Initial	Final	Initial	Final

*Temperatures performed at the time of test initiation, renewal or termination by the analyst identified in the Daily Renewal Information table located on Page 1. Alkalinity, hardness and total residual chlorine performed by the analyst identified on the bench sheet specific for each analysis and transcribed to this bench sheet by: _____.

SOP AT20-Revision 5–Exhibit AT20.3

Subject: *Americamysis bahia* Chronic Toxicity Test, EPA 1007.0

Species: *Pimephales promelas*

Client: City of Chattanooga, Moccasin Bend WWTP Date: 05-07-19

Concentration	Parameter	Day 3 Initial	Day 3 Final	Day 4 Initial	Day 4 Final	Day 5 Initial	Day 5 Final	Day 6 Initial	Day 6 Final
	Analyst								
CONTROL	pH (S.U.)								
	DO (mg/L)								
	Conductivity (µmhos/cm)								
	*Alkalinity (mg $CaCO_3$/L)								
	*Hardness (mg $CaCO_3$/L)								
	*Temperature (°C)								
1.38%	pH (S.U.)								
	DO (mg/L)								
	Conductivity (µmhos/cm)								
	*Temperature (°C)								
2.8%	pH (S.U.)								
	DO (mg/L)								
	Conductivity (µmhos/cm)								
	*Temperature (°C)								
5.5%	pH (S.U.)								
	DO (mg/L)								
	Conductivity (µmhos/cm)								
	*Temperature (°C)								
11%	pH (S.U.)								
	DO (mg/L)								
	Conductivity (µmhos/cm)								
	*Temperature (°C)								
22%	pH (S.U.)								
	DO (mg/L)								
	Conductivity (µmhos/cm)								
	*Temperature (°C)								
100%	pH (S.U.)								
	DO (mg/L)								
	Conductivity (µmhos/cm)								
	*Alkalinity (mg $CaCO_3$/L)								
	*Hardness (mg $CaCO_3$/L)								
	*TR chlorine (mg/L)								
	*Temperature (°C)								

*Temperatures performed at the time of test initiation, renewal or termination by the analyst identified in the Daily Renewal Information table located on Page 1. Alkalinity, hardness and total residual chlorine performed by the analyst identified on the bench sheet specific for each analysis and transcribed to this bench sheet by: _____.

SOP AT20-Revision 5–Exhibit AT20.3

Subject: *Americamysis bahia* Chronic Toxicity Test, EPA 1007.0

Exhibit AT20.4: Average Transfer Volume Log Sheet.

ETS

Page 1 of 1

Larval Fish Transfer Volume

Analyst: J. Sumner Species: *P. promelas*

Date: 12-05-17 Source / Batch: Spawn date: 11-29-17

Ambient temperature: 24.3°C Wet Weight of 10 Larvae (g): 0.0063 g

Estimate transfer volume, where minnows are allowed to swim from the pipette into the test vessel.

Numerically label 10 medicine cups.
Add 10 mL MHSW to each of the 10 cups.
Measure and record the weight of each cup containing MHSW.
Transfer 10 larvae to each cup, following procedures identified in SOP-AT18, AT47, or AT53 for vertebrate acute toxicity tests.
Transfer the larvae in a manner that allows them to swim from the pipette into the MHSW contained in each cup.
Measure and record the weight of each cup containing MHSW with 10 larvae.
Determine each transfer volume and average transfer volume.

Replicate Number	Initial Weight Medicine cup + 10 mL MHSW (g)	Final Weight Medicine cup + 10 mL MHSW + 10 Larval Fish transferred (g)	Transfer Volume Final - Initial Weight (g = mL)
1	11.3649	11.4693	0.1044
2	11.3957	11.4430	0.0473
3	11.4323	11.6293	0.1970
4	11.3821	11.4334	0.0513
5	11.3271	11.4008	0.0737
6	11.3224	11.4435	0.1211
7	11.4096	11.8059	0.3963
8	11.1915	11.2037	0.0122
9	11.2186	11.3718	0.1532
10	11.3001	11.3103	0.0102
		Average volume to transfer 10 organisms (mL):	0.1167

Estimate transfer volume, where the minnows are transferred with MHSW into the test vessels.

Numerically label 10 medicine cups.
Measure and record the weight of each cup.
Add approximately 10 mL MHSW to each of the 10 cups.
Transfer 10 larvae to each cup, following procedures identified in SOP-AT18, AT47, or AT53 for vertebrate acute toxicity tests.
Transfer the larvae in a manner that allows them to swim from the pipette into the MHSW contained in each cup.
Measure and record the weight of each cup containing MHSW with 10 larvae.
Determine each transfer volume and average transfer volume.

Replicate Number	Initial Weight Medicine cup (g)	Final Weight Medicine cup + 10 mL MHSW + 10 Larval Fish transferred (g)	Transfer Volume Final - Initial Weight (g = mL)
1	1.6179	11.4693	9.8514
2	1.6074	11.4430	9.8356
3	0.6279	11.6293	11.0014
4	1.5349	11.4334	9.8985
5	1.6472	11.4008	9.7536
6	1.5997	11.4435	9.8438
7	1.5972	11.8059	10.2087
8	1.5358	11.2037	9.6679
9	1.5956	11.3718	9.7762
10	1.6018	11.3103	9.7085
		Average volume to transfer 10 organisms (mL):	9.9546

SOP AT18-Revision 5-Exhibit AT18.4

Exhibit AT20.5: Randomization Template.

Randomizing template: **BLUE**

Replicate #	1	2	3	4
Concentrations	**1**	**7**	**3**	**5**
	7	**3**	**4**	**6**
1 = Control	**4**	**2**	**6**	**1**
2 = Lowest concentration	**3**	**5**	**5**	**2**
3 - 5 = Intermediate concentrations	**6**	**4**	**2**	**4**
6 = Highest concentration	**2**	**1**	**1**	**7**
7 = Intake/Upstream	**5**	**6**	**7**	**3**

Random number seeds: 10 through 13

SOP AT18-Revision 5-Exhibit AT18.5

Exhibit AT20.6: Determination of Solids Loss from Killing of Larvae at Test Termination.

Study to determine the amount of solids lost by killing the minnows (severing the spinal cords) at test termination.

Study performed using 1 minnow per replicate.

Analyst: J. Sumner

Date: 08-23-08

Replicate	Initial Pan Weight (mg)	Pan + Larvae weight (mg)	Larvae removed, killed, and returned to pan. Pan + Larvae weight (mg)	Weight loss (mg)
1	14.53	16.14	16.06	**0.08**
2	14.98	16.87	16.80	**0.07**
3	14.60	16.13	16.05	**0.08**
4	14.73	16.53	16.46	**0.07**
5	12.55	13.79	13.70	**0.09**
6	13.73	16.15	16.05	**0.10**
7	13.89	15.30	15.21	**0.09**
8	15.65	17.40	17.30	**0.10**
9	13.19	14.35	14.27	**0.08**
10	14.14	15.52	15.45	**0.07**
11	13.34	14.11	14.04	**0.07**
12	14.95	16.96	16.86	**0.10**
13	14.09	14.92	14.84	**0.08**
14	13.02	15.06	14.96	**0.10**
15	14.15	15.79	15.70	**0.09**
16	13.01	14.36	14.28	**0.08**
17	13.55	14.57	14.51	**0.06**
18	14.20	15.68	15.60	**0.08**
19	14.21	15.57	15.49	**0.08**
20	12.85	13.90	13.82	**0.08**
			Average:	**0.08**

Method:

Pan + Larvae weight =

a. Holding the mesh sieve over an empty beaker, pour the test water containing the larvae from one replicate cup through the sieve. The larvae will be retained on the mesh.

b. Immediately submerge the sieve containing the larvae into the ice water. Keep the larvae in the ice water for 3 to 4 seconds.

c. Remove the sieve from the ice water. Carefully rinse the larvae with deionized water to remove any excess food or detritus.

d. Using forceps, carefully remove the larvae by the tail and place on the pan.

Larvae killed and re-weighed =

a. Using forceps, sever the spinal cord of the larvae on the pan.

Larvae never removed from pan.

SOP AT-20, page: 9 of 13

Subject: *Americamysis bahia* Chronic Toxicity Test, EPA 1007.0

Study to determine the amount of moisture lost during weighing.

Study performed using 1 minnow per replicate.

Analyst: J. Sumner

Date: 08-23-08

Replicate	Initial Pan Weight (mg)	Pan + Larvae weight (mg)	Pan + Larvae reweighed after 3-5 seconds. Pan + Larvae weight (mg)	Weight loss (mg)
1	13.17	14.69	14.62	**0.07**
2	14.02	15.66	15.58	**0.08**
3	14.93	17.14	17.06	**0.08**
4	14.48	16.00	15.93	**0.07**
5	14.53	15.82	15.77	**0.05**
6	14.42	17.15	17.05	**0.10**
7	14.71	17.27	17.18	**0.09**
8	14.88	16.75	16.70	**0.05**
9	13.50	16.17	16.09	**0.08**
10	14.32	16.87	16.79	**0.08**
11	14.29	16.83	16.75	**0.08**
12	14.80	16.78	16.71	**0.07**
13	15.69	18.72	18.63	**0.09**
14	14.16	16.59	16.50	**0.09**
15	14.65	16.91	16.83	**0.08**
16	13.47	15.71	15.62	**0.09**
17	13.81	16.24	16.15	**0.09**
18	15.10	17.08	16.98	**0.10**
19	14.12	16.60	16.49	**0.11**
20	13.42	17.31	17.22	**0.09**
			Average:	**0.08**

Method:

Pan + Larvae weight =

a. Holding the mesh sieve over an empty beaker, pour the test water containing the larvae from one replicate cup through the sieve. The larvae will be retained on the mesh.

b. Immediately submerge the sieve containing the larvae into the ice water. Keep the larvae in the ice water for 3 to 4 seconds.

c. Remove the sieve from the ice water. Carefully rinse the larvae with deionized water to remove any excess food or detritus.

d. Using forceps, carefully remove the larvae by the tail and place on the pan.

Larvae re-weighed = a. Pan + larvae reweighed after 3 to 5 seconds.
(length of time to kill minnow by severing spinal cord)
Larvae never removed from pan.

Subject: *Americamysis bahia* Chronic Reference Toxicity Test, EPA 1007.0

Document Revision History

Revision Date	Surveillance number	Surveillance Type	Evaluators	Revisions
12-01-00				Original document
06-01-11	Not applicable.	Internal	Jim Sumner (ETS)	• Updated references and exhibits.
11-01-14	Not applicable.	Internal	Jim Sumner (ETS)	• Updated exhibits during document review.

Subject: *Americamysis bahia* Chronic Reference Toxicity Test, EPA 1007.0

Purpose

To assess the sensitivity of Mysid shrimp (*Americamysis bahia*) and the overall credibility of the mysid chronic toxicity test. Reference toxicant data are part of the laboratory's QA/QC program to evaluate the performance of laboratory personnel and the robustness and sensitivity of the test organisms.

References

USEPA. October 2002. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms, 3rd ed. **EPA-821-R-02-014, Method 1007.0**. US Environmental Protection Agency, Cincinnati, OH.

USEPA. 2000. Understanding and Accounting for Method Variability in Whole Effluent Toxicity Applications Under the National Pollutant Discharge Elimination Program. EPA-833-R-00-003. US Environmental Protection Agency, Cincinnati, OH.

USEPA. 2001. Final Report: Inter-laboratory Variability Study of EPA Short-term Chronic and Acute Whole Effluent Toxicity Test Methods, Volumes 1 and 2 - Appendix. EPA-821-B-01-004 and EPA-821-B-01-005. US Environmental Protection Agency, Cincinnati, OH.

North Carolina Department of Environment and Natural Resources, Division of Water Quality. Biological Laboratory Certification / Criteria Procedures, Version 3.0. December 2010.

Definitions

Acceptance Criteria: Specified limits placed on characteristics of an item, process, or service defined in codes, standards, or other required documents.

Precision: The extent to which measurement results repeat themselves when repeat measurements are made on the same unit of product.

Equipment and Materials

Mysid shrimp (*Americamysis bahia*)
Temperature-controlled incubator (set to maintain test temperature = 26.0 ± 1.0ºC, photoperiod of 16-hour light / 8-hours dark, light intensity = 50 – 100 ft-c)
Control water (salt synthetic water made with Bioassay Grade 40-Fathoms Sea Salt, MarineMix®)
Microbalance accurate to 0.00001 mg (e.g. Cahn)
Class S or Class I certified weights
Microweight aluminum pans (e.g. Cahn)
Drying oven
Desiccator
Scintillation vials

250-ml glass beakers
Graduated cylinders
Large glass finger bowls
Serological, fixed, or adjustable volume pipette (e.g., Eppendorf)
Transfer pipettes
Siphoning hose (rubber tubing affixed with a plastic tube fitted with a soft, angled rubber tip)
White plastic photographic tray
Fine mesh sieve
Forceps
Ice water
Aquarium pump, tubing, and air stones
Thermometer
1-oz medicine cups
Newly hatched brine shrimp
Light box or table
Disposable gloves
Americamysis bahia Shipment Log and Organism History Information Sheet
Potassium chloride (KCl, reagent grade)
500-ml volumetric flask
2000-ml graduated cylinder
2000-ml Erlenmeyer flask
1 and 10-ml serological pipettes
Americamysis bahia Chronic Reference Toxicity Test Benchsheet
Randomization template

Procedure

A. Frequency of Testing and Requirements.

1. An *Americamysis bahia* chronic reference toxicant test must be performed on each batch of organisms obtained from a supplier and used for chronic whole effluent toxicity tests. At a minimum, the *Americamysis bahia* chronic reference toxicant tests must be performed quarterly to meet certification requirements.

B. Test Preparation.

1. Prepare the glassware.

   a. Obtain eight replicate 250-ml glass beakers (or equivalent) for each concentration tested including the control. Label each replicate cup with the following information.
      - Concentration
      - Replicate number

b. Obtain enough 2000 ml Erlenmeyer flasks for each test concentration and the control. These flasks will be used in the preparation of the test concentrations. Label each flask with the test concentration.

c. Label the appropriate graduated cylinder.

d. Prepare the *Americamysis bahia* Chronic Reference Toxicity Test Benchsheet (see Exhibit AT44.1). Record the *Americamysis bahia* KCl Chronic (AbKClCR) test number on the benchsheet.

C. Preparation of the Stock Solution.

1. Using a calibrated top-loading balance, carefully weigh out 50 g of KCl (SOP-G10). Place approximately 900 ml of Milli-Q water in a 1000-ml volumetric flask. Add the KCl to the flask. Dissolve the KCl by swirling the flask and bring to volume with Milli-Q water. Label the volumetric flask with the concentration (50 g/L), analyst's initials, preparation date and stock standard number (INSS, SOP-G15). Record the INSS number of the KCl stock solution on the benchsheet.

D. Preparation of the Test Concentrations.

1. Prepare all test concentrations using graduated cylinders and serological pipettes. The precision of test concentrations is increased when smaller volume graduated cylinders and/or serological pipettes are used to prepare the dilutions. For this reference toxicant test, stock solution volumes should be measured using 10-ml serological pipettes and the total volumes should be measured using a 2000-ml graduated cylinder.

2. Beginning with the lowest concentration, add approximately 500 ml of salt synthetic water to a 2000-ml graduated cylinder, add the required volume of stock solution using a 10-ml serological pipette (refer to Table AT44.1), bring to volume (1500 ml) with salt synthetic water. Mix the solution well by pouring the solution into a 2000-ml Erlenmeyer flask.

3. Pour 150 ml of test solution into each of the replicate test beakers for that concentration. Pour 30 ml of test solution into a 1-oz medicine cup for chemical analyses. For each concentration, measure and record the salinity (SOP-C5), pH (SOP-C3), and dissolved oxygen (SOP-C2).

4. Rinse the graduated cylinder well with deionized water and repeat steps C.2 through C.4 for preparing the next test concentration. Record the batch date of salt synthetic water used to prepare the dilutions on the benchsheet.

Table AT44.1: Test concentration, stock volumes, salt synthetic water volumes and final volumes for the *Americamysis bahia* KCl chronic reference toxicant tests.

Test Concentration (mg KCl/L)	Volume of Stock Required (ml)	Volume of Salt Synthetic Water (ml)	Final Volume (ml)
250	7.50	1492.50	1500
375	11.25	1488.75	1500
500	15.00	1485.00	1500
750	22.50	1477.50	1500
1000	30.00	1470.00	1500

5. Once all test concentrations have been prepared, follow the procedure described in SOP-AT43 for conducting *Americamysis bahia* Chronic Toxicity Tests.

E. Preparation of Control Charts.

1. Refer to QAP-Q5 for how control charts are prepared and procedures for interpreting outlier tests. Refer to Exhibit AT44.2 for an example control chart.

F. Exhibits.

Exhibit AT44.1: *Americamysis bahia* Chronic Reference Toxicant Test Benchsheet.
Exhibit AT44.2: Example *Americamysis bahia* Chronic Reference Toxicant Control Chart.

Subject: *Americamysis bahia* Chronic Reference Toxicity Test, EPA 1007.0

Exhibit AT44.1: *Americamysis bahia* Chronic Reference Toxicant Test Benchsheet.

Potassium Chloride Chronic Reference Toxicant Test
(EPA-821-R-02-014, Method 1007.0)
Species: *Americamysis (Mysidopsis) bahia*

AbKClCR Test Number: 126

Dilution preparation information:						*Comments:*
KCl Stock INSS number:		INSS				
Stock preparation:		50 g KCl/L: Dissolve 50 g KCl in 1-L Deionized water				
Dilution prep (mg/L)	250	375	500	750	1000	
Stock volume (mL)	5	7.5	10	15	20	
Diluent volume (mL)	995	992.5	990	985	980	
Total volume (mL)	1000	1000	1000	1000	1000	

Test organism information:		*Test information:*	
Organism age:		Randomizing template:	
Date and times organisms were born between:		Incubator number and shelf location:	
Organism source:	AI Batch Ab:	*Artemia* CHM number:	CHM780
		Drying information for weight determination:	
Transfer bowl information:	pH = S.U. Temperature = °C	Date / Time in oven:	
		Initial oven temperature:	
Average transfer volume:	0.1271 mL	Date / Time out of oven:	
		Final oven temperature:	
		Total drying time:	

Daily feeding and renewal information:

Day	Date	Morning feeding		Afternoon feeding		Test initiation, renewal, or termination		Salt SW batch used
		Time	Analyst	Time	Analyst	Time	Analyst	
0	10-07-14							
1	10-08-14							
2	10-09-14							
3	10-10-14							
4	10-11-14							
5	10-12-14							
6	10-13-14							
7	10-14-14							

Control information:		Acceptance criteria	*Summary of test endpoints:*	
% Mortality:		≤ 20%	7-day LC_{50}	
Average weight per initial shrimp:			NOEC	
Average weight per surviving shrimp:		≥ 0.20 mg/shrimp	LOEC	
			ChV	
			IC_{25}	

Subject: *Americamysis bahia* Chronic Reference Toxicity Test, EPA 1007.0

AbKClCR Test Number: 126

Survival and Growth Data

Day	CONTROL								250 mg KCl/L							
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
1																
2																
3																
4																
5																
6																
7																
# females with eggs in brood sac																
# females with developing ova in oviducts																
# immature females																
# males																
A = Pan weight (mg) Tray color code:: _____ Analyst:_____ Date: _____																
B = Pan + Shrimp weight (mg) Analyst:_____ Date: _____																
C = Shrimp weight (mg) = B – A Hand calculated. Analyst:_____																
Weight per initial number of shrimp (mg) = C / Initial number of shrimp Hand calculated. Analyst:_____																
	Average weight per initial number of shrimp (mg)								Average weight per initial number of shrimp (mg)				Percent reduction from control (%)			

Comment codes: c = clear, d = dead, fg = fungus, k = killed, m = missing, sk = sick, sm = unusually small, lg = unusually large, d&r = decanted and returned, w = wounded.

Comments:

SOP AT44 – Exhibit AT44.1, revision 11-01-14

Subject: *Americamysis bahia* Chronic Reference Toxicity Test, EPA 1007.0

AbKClCR Test Number: 126

Survival and Growth Data

Day	375 mg KCl/L								500 mg KCl/L							
	Q	R	S	T	U	V	W	X	Y	Z	AA	BB	CC	DD	EE	FF
0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
1																
2																
3																
4																
5																
6																
7																
# females with eggs in brood sac																
# females with developing ova in oviducts																
# immature females																
# males																
A = Pan weight (mg) Tray color code:: _____ Analyst:_____ Date: _____																
B = Pan + Shrimp weight (mg) Analyst:_____ Date: _____																
C = Shrimp weight (mg) = B – A Hand calculated. Analyst:_____																
Weight per initial number of shrimp (mg) = C / Initial number of shrimp Hand calculated. Analyst:_____																
	Average weight per initial number of shrimp (mg)				Percent reduction from control (%)				Average weight per initial number of shrimp (mg)				Percent reduction from control (%)			

Comment codes: c = clear, d = dead, fg = fungus, k = killed, m = missing, sk = sick, sm = unusually small, lg = unusually large, d&r = decanted and returned, w = wounded.

Comments:

SOP AT44 – Exhibit AT44.1, revision 11-01-14

Subject: *Americamysis bahia* Chronic Reference Toxicity Test, EPA 1007.0

AbKClCR Test Number: 126

Survival and Growth Data

Day	750 mg KCl/L								1000 mg KCl/L							
	GG	HH	II	JJ	KK	LL	MM	NN	OO	PP	QQ	RR	SS	TT	UU	VV
0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
1																
2																
3																
4																
5																
6																
7																
# females with eggs in brood sac																
# females with developing ova in oviducts																
# immature females																
# males																
A = Pan weight (mg) Tray color code: _____ Analyst:_____ Date: _____																
B = Pan + Shrimp weight (mg) Analyst:_____ Date: _____																
C = Shrimp weight (mg) = B – A Hand calculated. Analyst:_____																
Weight per initial number of shrimp (mg) = C / Initial number of shrimp Hand calculated. Analyst:_____																
	Average weight per initial number of shrimp (mg)				Percent reduction from control (%)				Average weight per initial number of shrimp (mg)				Percent reduction from control (%)			

Comment codes: c = clear, d = dead, fg = fungus, k = killed, m = missing, sk = sick, sm = unusually small, lg = unusually large, d&r = decanted and returned, w = wounded.

Comments:

SOP AT44 – Exhibit AT44.1, revision 11-01-14

Subject: *Americamysis bahia* Chronic Reference Toxicity Test, EPA 1007.0

AbKClCR Test Number: 126

Daily Chemistry:

Conc.	Parameter	Day (Analyst identified for each day, performed pH, D.O. and salinity measurements only.) 0		1		2	
	Analyst						
CONTROL	pH (S.U.)						
	DO (mg/L)						
	Salinity (ppt)						
	*Alkalinity (mg $CaCO_3$/L)						
	*Temperature (°C)						
250 mg KCl/L	pH (S.U.)						
	DO (mg/L)						
	Salinity (ppt)						
	*Temperature (°C)						
375 mg KCl/L	pH (S.U.)						
	DO (mg/L)						
	Salinity (ppt)						
	*Temperature (°C)						
500 mg KCl/L	pH (S.U.)						
	DO (mg/L)						
	Salinity (ppt)						
	*Temperature (°C)						
750 mg KCl/L	pH (S.U.)						
	DO (mg/L)						
	Salinity (ppt)						
	*Temperature (°C)						
1000 mg KCl/L	pH (S.U.)						
	DO (mg/L)						
	Salinity (ppt)						
	*Temperature (°C)						
		Initial	Final	Initial	Final	Initial	Final

*Temperatures performed at the time of test initiation, renewal or termination by the analyst identified in the Daily Renewal Information table located on Page 1.
Alkalinity performed by the analyst identified on the bench sheet specific for this analysis and transcribed to this bench sheet by: _____

Subject: *Americamysis bahia* Chronic Reference Toxicity Test, EPA 1007.0

AbKClCR Test Number: 126

Conc.	Parameter	Day (Analyst identified for each day, performed pH, D.O. and salinity measurements only.) 3		4		5		6	
	Analyst								
CONTROL	pH (S.U.)								
	DO (mg/L)								
	Salinity (ppt)								
	*Alkalinity (mg $CaCO_3$/L)								
	*Temperature (°C)								
250 mg KCl/L	pH (S.U.)								
	DO (mg/L)								
	Salinity (ppt)								
	*Temperature (°C)								
375 mg KCl/L	pH (S.U.)								
	DO (mg/L)								
	Salinity (ppt)								
	*Temperature (°C)								
500 mg KCl/L	pH (S.U.)								
	DO (mg/L)								
	Salinity (ppt)								
	*Temperature (°C)								
750 mg KCl/L	pH (S.U.)								
	DO (mg/L)								
	Salinity (ppt)								
	*Temperature (°C)								
1000 mg KCl/L	pH (S.U.)								
	DO (mg/L)								
	Salinity (ppt)								
	*Temperature (°C)								
		Initial	Final	Initial	Final	Initial	Final	Initial	Final

*Temperatures performed at the time of test initiation, renewal or termination by the analyst identified in the Daily Renewal Information table located on Page 1.
Alkalinity performed by the analyst identified on the bench sheet specific for this analysis and transcribed to this bench sheet by: _____.

Subject: *Americamysis bahia* Chronic Reference Toxicity Test, EPA 1007.0

Exhibit AT44.2: Example *Americamysis bahia* Chronic Reference Toxicant Control Chart.

Environmental Testing Solutions, Inc.

Americamysis bahia
Chronic Reference Toxicant Control Chart

Test number	Test date	7-day IC_{25} (g KCl/L)	CT (g KCl/L)	S	Control Limit CT - 2S	Control Limit CT + 2S	$S_{A.75}$	Warning Limit CT - $S_{A.75}$	Warning Limit CT + $S_{A.75}$	$S_{A.90}$	Control Limit CT - $S_{A.90}$	Control Limit CT + $S_{A.90}$	CV
1	06-04-13	0.444											
2	07-09-13	0.450	0.45	0.00	0.44	0.46	0.14	0.30	0.59	0.18	0.27	0.63	0.01
3	07-11-13	0.436	0.44	0.01	0.43	0.46	0.14	0.30	0.59	0.18	0.27	0.62	0.02
4	08-06-13	0.431	0.44	0.01	0.42	0.46	0.14	0.30	0.58	0.18	0.26	0.62	0.02
5	09-10-13	0.441	0.44	0.01	0.43	0.46	0.14	0.30	0.58	0.18	0.26	0.62	0.02
6	10-08-13	0.436	0.44	0.01	0.43	0.45	0.14	0.30	0.58	0.18	0.26	0.62	0.02
7	11-05-13	0.461	0.44	0.01	0.42	0.46	0.14	0.30	0.58	0.18	0.27	0.62	0.02
8	12-03-13	0.464	0.45	0.01	0.42	0.47	0.14	0.30	0.59	0.18	0.27	0.62	0.03
9	01-07-14	0.475	0.45	0.02	0.42	0.48	0.14	0.31	0.59	0.18	0.27	0.63	0.03
10	01-09-14	0.483	0.45	0.02	0.42	0.49	0.14	0.31	0.60	0.18	0.27	0.63	0.04
11	01-14-14	0.448	0.45	0.02	0.42	0.49	0.14	0.31	0.60	0.18	0.27	0.63	0.04
12	02-04-14	0.457	0.45	0.02	0.42	0.48	0.14	0.31	0.60	0.18	0.27	0.63	0.04
13	02-06-14	0.470	0.45	0.02	0.42	0.49	0.15	0.31	0.60	0.18	0.27	0.64	0.04
14	03-04-14	0.459	0.45	0.02	0.42	0.49	0.15	0.31	0.60	0.18	0.27	0.64	0.03
15	04-08-14	0.438	0.45	0.02	0.42	0.48	0.14	0.31	0.60	0.18	0.27	0.63	0.03
16	05-06-14	0.418	0.45	0.02	0.42	0.49	0.14	0.31	0.59	0.18	0.27	0.63	0.04
17	06-03-14	0.446	0.45	0.02	0.42	0.48	0.14	0.31	0.59	0.18	0.27	0.63	0.04
18	07-08-14	0.414	0.45	0.02	0.41	0.49	0.14	0.30	0.59	0.18	0.27	0.63	0.04
19	08-05-14	0.420	0.45	0.02	0.41	0.49	0.14	0.30	0.59	0.18	0.27	0.63	0.04
20	09-09-14	0.424	0.45	0.02	0.41	0.48	0.14	0.30	0.59	0.18	0.27	0.62	0.04

Note: **7-d IC_{25}** = 7-day 25% inhibition concentration. An estimation of the concentration of potassium chloride that would cause a 25% reduction in *Americamysis* growth for the test population.

CT = Central tendency (mean IC_{25}).

S = Standard deviation of the IC_{25} values.

$S_{A.75}$ = Standard deviation corresponding to the the 75^{th} percentile CV. ($S_{A.75}$ = 0.32)

$S_{A.90}$ = Standard deviation corresponding to the the 90^{th} percentile CV. ($S_{A.90}$ = 0.40)

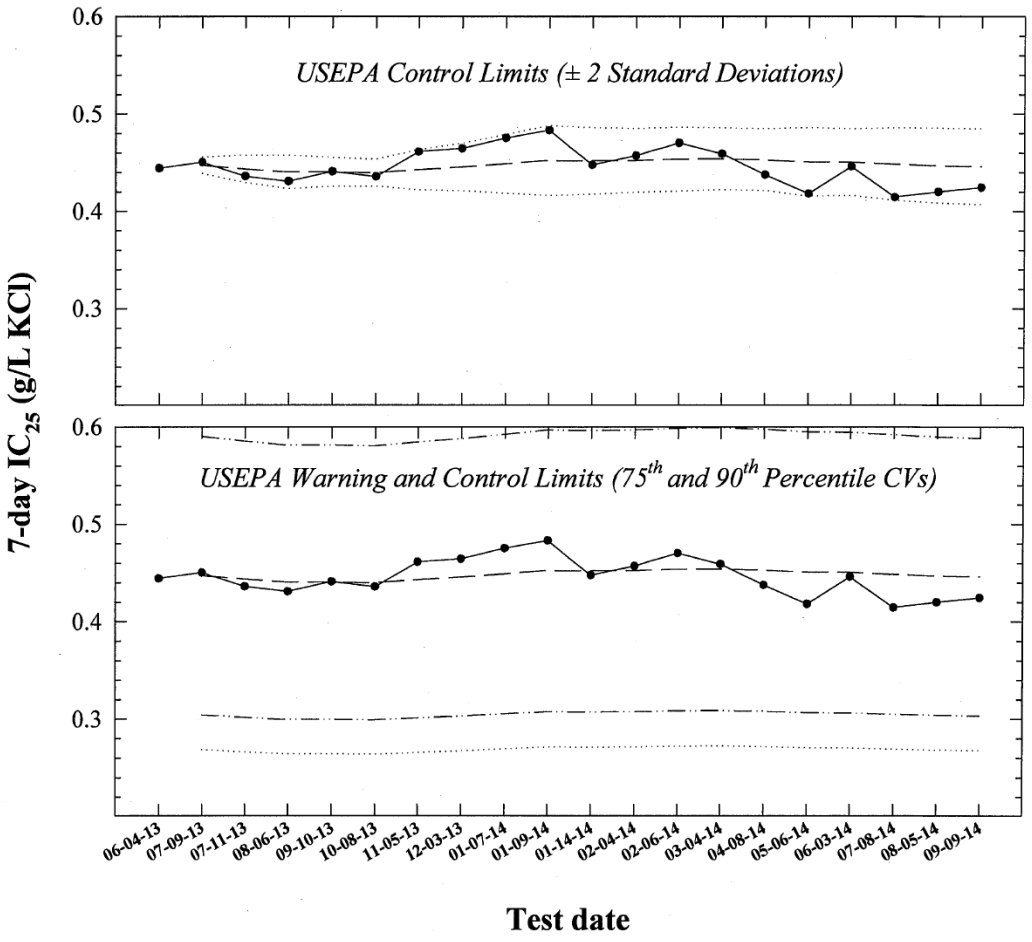
CV = Coefficient of variation of the IC_{25} values.

USEPA. 2000. Understanding and Accounting for Method Variability in Whole Effluent Toxicity Applications Under the National Pollutant Discharge Elimination Program. EPA-833-R-00-003. US Environmental Protection Agency, Cincinnati, OH.

Subject: *Americamysis bahia* Chronic Reference Toxicity Test, EPA 1007.0

Environmental Testing Solutions, Inc.

Americamysis bahia
Chronic Reference Toxicant Control Chart



—●— **7-day IC_{25}** = 25% inhibition concentration. An estimation of the concentration of potassium chloride that would cause a 25% reduction in *Americamysis* growth for the test population.

— — **Central Tendency** (mean IC_{25})

—·—·— **Warning Limits** (mean $IC_{25} \pm S_{A.75}$)

····· **Control Limits** (mean $IC_{25} \pm S_{A.90}$ or 2 Standard Deviations)

Subject: *Americamysis bahia* Chronic Reference Toxicity Test, EPA 1007.0

Environmental Testing Solutions, Inc.

Precision of Endpoint Measurements

Americamysis bahia

Chronic Reference Toxicant Data

Test number	Test date	Control Survival (%)	Control Mean Growth (mg/shrimp)	CT for Control Growth (mg/shrimp)	CV (%)	CT for Control Growth CV (%)	MSD	PMSD (%)	CT for PMSD (%)
1	06-04-13	100	0.333		10.2		0.03	7.6	
2	07-09-13	100	0.368	0.351	8.3	9.3	0.03	8.9	8.2
3	07-11-13	100	0.265	0.322	8.8	9.1	0.03	9.9	8.8
4	08-06-13	100	0.307	0.318	10.1	9.4	0.03	9.0	8.9
5	09-10-13	100	0.293	0.313	12.4	10.0	0.04	15.3	10.1
6	10-08-13	100	0.207	0.295	8.8	9.8	0.02	11.9	10.4
7	11-05-13	100	0.326	0.300	7.1	9.4	0.04	13.6	10.9
8	12-03-13	100	0.298	0.300	10.2	9.5	0.04	13.9	11.3
9	01-07-14	100	0.270	0.296	9.0	9.4	0.03	11.6	11.3
10	01-09-14	100	0.263	0.293	8.3	9.3	0.03	9.7	11.1
11	01-14-14	100	0.202	0.285	15.7	9.9	0.03	13.4	11.4
12	02-04-14	100	0.268	0.283	11.8	10.1	0.03	12.1	11.4
13	02-06-14	100	0.249	0.281	8.3	9.9	0.03	10.4	11.3
14	03-04-14	100	0.253	0.279	7.7	9.8	0.03	13.6	11.5
15	04-08-14	100	0.212	0.274	14.1	10.1	0.04	16.6	11.8
16	05-06-14	100	0.289	0.275	18.9	10.6	0.04	14.5	12.0
17	06-03-14	100	0.286	0.276	8.7	10.5	0.03	9.8	11.9
18	07-08-14	100	0.232	0.273	15.2	10.8	0.03	13.5	12.0
19	08-05-14	100	0.245	0.272	7.9	10.6	0.03	11.4	11.9
20	09-09-14	100	0.315	0.274	9.7	10.6	0.03	8.9	11.8

Note: **CV** = Coefficient of variation for control growth.

Lower CV bound determined by USEPA (10^{th} percentile) = 8.8%.

Upper CV bound determined by USEPA (90^{th} percentile) = 28%

MSD = Minimum Significant Difference

PMSD = Percent Minimum Significant Difference

PMSD is a measure of test precision. The PMSD is the minimum percent difference between the control and treatment that can be declared statistically significant in a whole effluent toxicity test.

Lower PMSD bound determined by USEPA (10^{th} percentile) = 11%.

Upper PMSD bound determined by USEPA (90^{th} percentile) = 37%.

CT = Central Tendancy (mean Control Growth, CV, or PMSD)

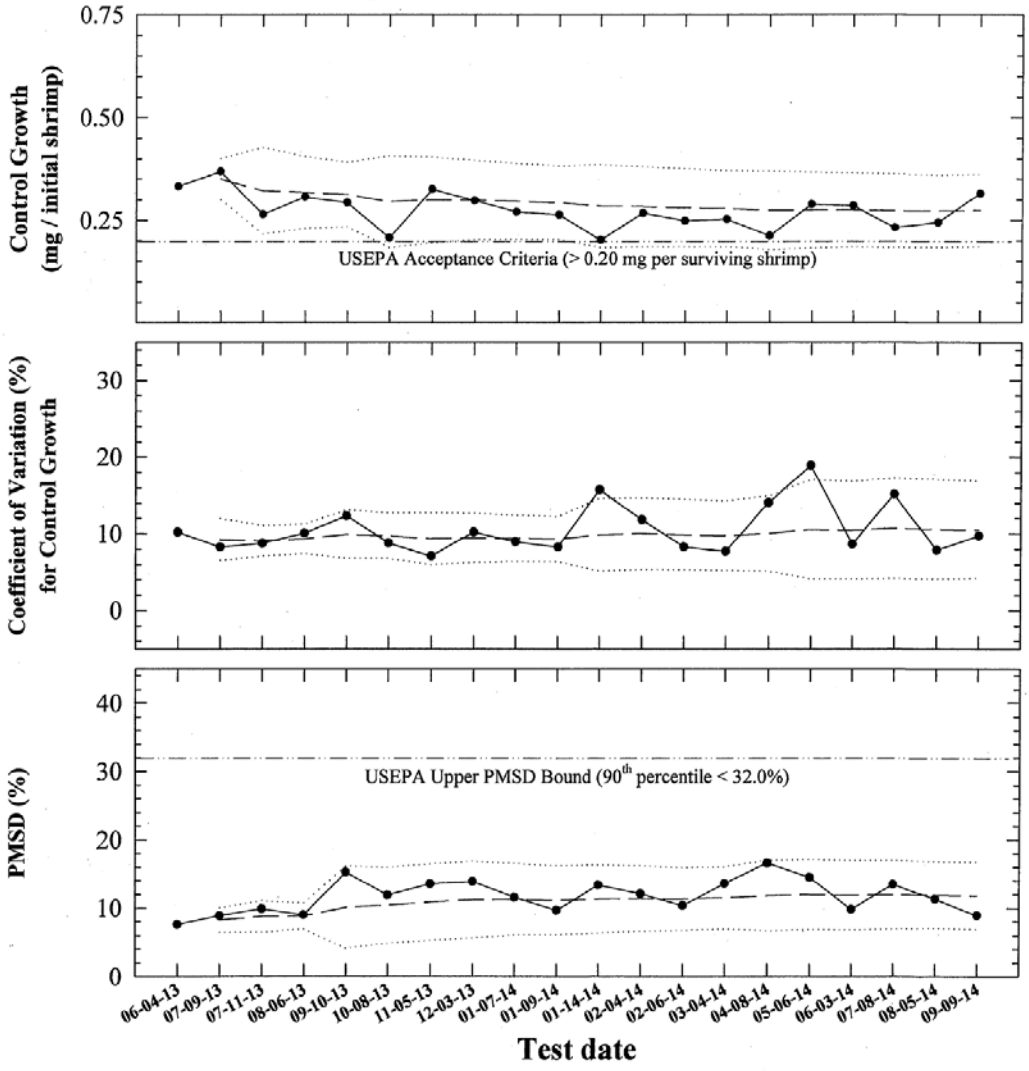
USEPA. 2000. Understanding and Accounting for Method Variability in Whole Effluent Toxicity Applications Under the National Pollutant Discharge Elimination Program. EPA-833-R-00-003. US Environmental Protection Agency, Cincinnati, OH.

USEPA. 2001a, 2001b. Final Report: Interlaboratory Variability Study of EPA Short-term Chronic and Acute Whole Effluent Toxicity Test Methods, Volumes 1 and 2 Appendix. EPA-821-B-01-004 and EPA-821-B-01-005. US Environmental Protection Agency, Cincinnati, OH.

Subject: *Americamysis bahia* Chronic Reference Toxicity Test, EPA 1007.0

Environmental Testing Solutions, Inc.

Americamysis bahia
Chronic Reference Toxicant Control Chart
Precision of Endpoint Measurements



Control Reproduction, Coefficient of Variation (CV), or Percent Minimum Significant Difference (PMSD) PMSD is the minimum significant difference between the control and treatment that can be declared statistically significant.

Central Tendency (mean Control Growth, CV, or PMSD)

Control Limits (mean Control Growth, CV, or PMSD ± 2 Standard Deviations)

Subject: Taxonomic Identification of *Americamysis bahia*

Document Revision History

Revision Date	Surveillance number	Surveillance Type	Evaluators	Revisions
12-01-00				Original document
06-01-11	Not applicable.	Internal	Jim Sumner (ETS)	• Exhibit AT45.2 revised for the key taxonomic characteristics and to provide a more efficient logsheet.
11-01-14	Not applicable.	Internal	Jim Sumner (ETS)	• Updated exhibits during document review.

Subject: Taxonomic Identification of *Americamysis bahia*

Purpose

To verify the genus and species of *Americamysis bahia* breeder cultures used by the laboratory for a source of shrimp in toxicity tests.

References

USEPA. October 2002. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, 5th ed. EPA-821-R-02-012. US Environmental Protection Agency, Cincinnati, OH.

Stuck, Kenneth C., Harriet M. Perry, and Richard W. Heard. 1979. An Annotated Key to the Mysidacea of the North Central Gulf of Mexico. Gulf Research Reports, Vol. 6, No. 3.

Price, W. Wayne. 1982. Key to the Shallow Water Mysidacea of the Texas Coast with Notes on their Ecology. Hydrobiologia 93, 9-21.

Douglas H. Farrell. April 1979. Guide to Shallow-Water Mysids from Florida. State of Florida Department of Environmental Regulation. Technical Series Vol. 4, No. 1.

Equipment and Materials

Adult, *Americamysis bahia*
1-oz medicine cups
Reagent alcohol
CMC-9AF Mounting Media®, manufactured by Masters Chemical Company
Clear fingernail polish
Glass slides and cover slips
Compound microscope equipped with an oil emersion lense
Pasteur® pipettes
Bulbs
Forceps
Americamysis bahia Taxonomic Log and Logsheet

Procedure

A. Frequency of Taxonomic Identification.

1. Certification of the identification of *Americamysis bahia* must be obtained from each approved supplier of test organisms. Refer to Exhibit AT45.1 for supplier certifications.

2. Taxonomic identification of *Americamysis bahia* breeder cultures used by the laboratory for a source of shrimp in toxicity tests must be performed yearly.

B. Preparation.

1. Order adult *Americamysis bahia* from an approved supplier (e.g., Aquatic Indicators, Inc. – St. Augustine, FL), which is used to obtain shrimp by the laboratory to perform toxicity tests. The representative shrimp must be from the supplier's breeding cultures.

2. Prepare the *Americamysis bahia* Taxonomic Identification Logsheet (Exhibit AT45.2).

C. Receipt of Adult Shrimp.

1. Remove the shrimp from the shipping container and transfer the water containing the shrimp to plastic beaker (or equivalent).

2. Record the following information on the Organism History information sheet provided by the supplier (Exhibit AT45.3).
   - Date received at the laboratory
   - Initials of the analyst that received the shipment

3. Place the Organism History information sheet in the *Americamysis bahia* Taxonomic Identification Log.

4. Using a transfer pipette, transfer 10 adult shrimp to a 1-oz medicine cup containing approximately 20 ml of reagent alcohol.

5. After 15 minutes, transfer the euthanized organisms to a medicine cup containing 2 to 3 ml CMC-9AF Mounting Media® using a transfer pipette.

6. Keep the organisms in the mounting media for a minimum of 2 hours to allow the organisms to be fully stained.

7. Using a transfer pipette, transfer 1 stained organism to a glass slide.

8. Cover the shrimp with a few additional drops of mounting media.

9. Using a pair of fine tipped forceps, gently position the shrimp on its back.

10. Allow the mounting media to thicken by air-drying the slide for approximately 10 minutes. Allowing the media to become thick will prevent the specimens from becoming damaged when the cover slip is placed over the organisms.

11. Pick up a cover slip by its edges and place one edge on the slide. Slowly lower the slip to cover the stained specimen. The media will spread out under the slip.

12. Repeat steps 4 through 8 until all of the stained organisms have been mounted on slides.

13. Allow the slides to air dry overnight.

14. Seal the mounts by covering the edges of the cover slips with clear fingernail polish (overlapping the edge by approximately 1 cm). Label the mounted specimens with the species, source of organisms, date and analyst's initials.

15. Once preserved and mounted, taxonomic identification of the specimens can be performed at a later date.

D. Taxonomic Identification.

1. Record the date the taxonomic identification was performed, analyst's initials and source of the mounted specimens on the *Americamysis bahia* Taxonomic Identification Logsheet.

2. Place a slide under the compound microscope. Identify each of the distinguishing characteristics of *Americamysis bahia* in the mounted specimens as indicated on the logsheet. Any deviations from these characteristics should be noted. For additional information on the taxonomic identification of *Americamysis bahia*, refer to the references sited at the beginning of this SOP.

3. If several of the distinguishing characteristics are not represented in the preserved specimens as determined by internal verification, and outside consultant should be contacted to provide guidance and confirm the taxonomy of the specimens.

4. These taxonomic specimens must be maintained in the laboratory for a minimum of 1 year.

E. Exhibits.

Exhibit AT45.1: Supplier Certification of *Americamysis bahia*.
Exhibit AT45.2: *Americamysis bahia* Taxonomic Identification Logsheet.
Exhibit AT45.3: Organism History Information Sheet.

Subject: Taxonomic Identification of *Americamysis bahia*

Exhibit AT45.1: Supplier Certification of *Americamysis bahia*

AQUATIC INDICATORS

P.O. BOX 632 ST. AUGUSTINE, FL 32085 (904) 829-2780

ORGANISM DOCUMENTATION
January 1, 2013

Aquatic Indicators currently produces two marine organisms:
Mysidopsis bahia (aka Americamysis bahia) and Menidia beryllina.

Our organisms are raised in natural seawater obtained from offshore (Atlantic) sources and filtered to five (5) microns or below. Culture methods are based on Appendix A of the EPA Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms 4th Edition edited by Cornelius I. Weber dated August 1993.

ENVIRONMENTAL REGIME:
Salinity - 20 ppt
Ph - 8.0 to 8.2
Temperature - 25 C ± 1 C
Photo period - 16 hours light, 8 hours dark

SPECIES HISTORY AND IDENTIFICATION:
Mysidopsis bahia - Our cultures were initially obtained from the Environmental Protection Agency, Gulf Breeze, Florida in 1985. Original taxonomic identification was personally established by Dr. Wayne Price, University of Tampa. Supplemental broodstock periodically purchased from other suppliers to ensure genetic diversity. Current taxonomic identification performed yearly by Raymond H. Lewis on January 1 by utilizing the following reference:

> Price, W. W. 1982. Key to the shallow water Mysidacea of the Texas coast with notes on their ecology. Hydrobiologia. 93(1-2):9-21.

Menidia beryllina - Our cultures were initially captured by seine nets in North Florida estuaries in 1985. Original taxonomic identification was personally established by Mr. Robert Thompson (Ichthyologist), Florida Atlantic University. Supplemental broodstock annually collected to ensure genetic diversity. Current taxonomic identification performed yearly by Raymond H. Lewis on January 1 by utilizing the following reference:

> Middaugh, D. P., M. J. Hemmer and L. R. Goodman. 1987. EPA. Methods for Spawning, Culturing and Conducting Toxicity-Tests with Early Life Stages of Four Atherinid Fishes: The Inland Silverside, *Menidia beryllina*, Atlantic silverside, *M. menidia*, Tidewater Silverside, *M. peninsulae* and California grunion, *Leuresthes tenuis.* p4.

Preserved samples of our culture organisms (5% formalin) are available upon request, as are monthly Standard Reference Toxicant tests.

Raymond H. Lewis

Subject: Taxonomic Identification of *Americamysis bahia*

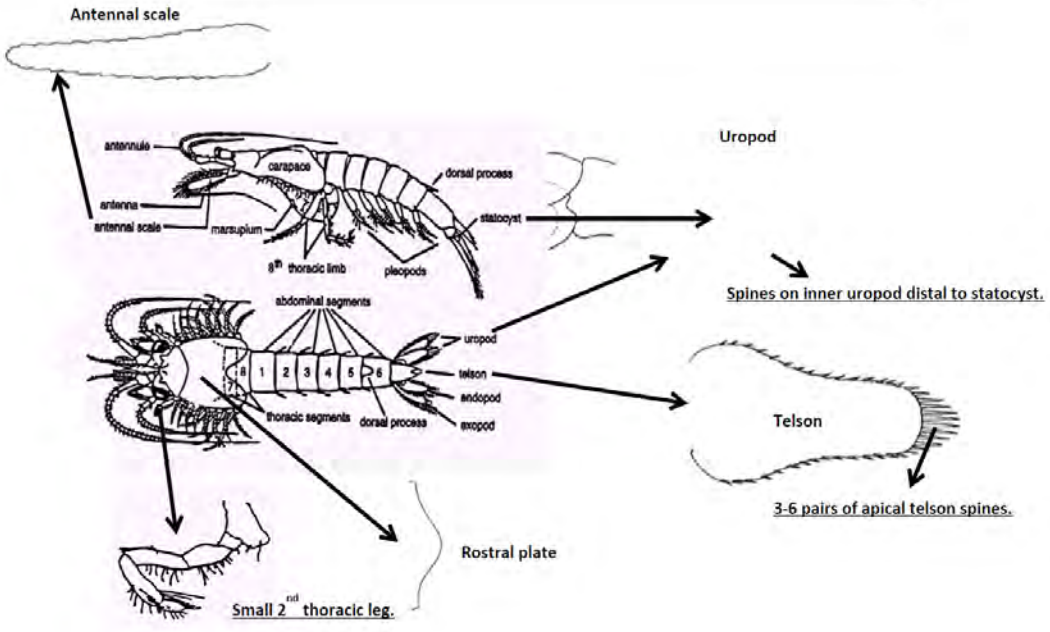
Exhibit AT45.2: *Americamysis bahia* Taxonomic Identification Logsheet.

Page 1 of 1

***Americamysis bahia* Taxonomic Identification Logsheet**

Date identification performed: _____ **Analyst:** _____
Date specimens received: _____ **Supplier:** _____

Comments:



SOP AT45 – Exhibit AT45.2, revision 11-01-14

Subject: Taxonomic Identification of *Americamysis bahia*

Exhibit AT45.3: Organism History Information Sheet.

Aquatic Indicators, Inc.
P.O. Box 632 • St. Augustine, FL 32085-0632 • (904) 829-2780

Date 9-8-14

Species:
1. M. bahia
2. M. beryllina
3.

Total Supplied: HATCH 09.07.14 HATCH 09.02.14
1. 700 @ 1 day + 500 @ 6 days
2. 120
3.

09.09.14 1005 TEMP = 24.8°C

Brood Description:
1. EPA
2. EPA
3.

	Ab 09.07.14	Ab 09.02.14	Mb 08.30.14
pH	7.74	7.77	7.79
DO(mg/L)	9.2	10.2	10.4
SALINITY (ppt)	21.6	22.0	21.9
# DEAD / TOTAL	0/700†	0/500†	1/120†

Age:
1. see above
2. 9 days - HATCH 08.30.14
3.

All organisms appear healthy
Fed at 1010. JH

Environmental Regime

Feeding: Zooplankton, Artemia NH

Photo: L 16 D 8

P.H.: 8.1

Temp: 25°C

Salinity: 20‰

Comments: Thanks.

Subject: Acclimation and Maintenance of *Menidia beryllina* Cultures

Document Revision History

Revision Date	Surveillance number	Surveillance Type	Evaluators	Revisions
04-01-09				Original document
06-01-11	Not applicable.	Internal	Jim Sumner (ETS)	• Updated exhibits.
11-01-14	Not applicable.	Internal	Jim Sumner (ETS)	• Updated exhibits during document review.

Subject: Acclimation and Maintenance of *Menidia beryllina* Cultures

Purpose

To provide procedures for the acclimation and maintenance of healthy *Menidia beryllina* cultures.

References

USEPA. October 2002. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, 5th ed. EPA-821-R-02-012. US Environmental Protection Agency, Cincinnati, OH.

USEPA. October 2002. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms, 3rd ed. EPA-821-R-02-014. US Environmental Protection Agency, Cincinnati, OH.

Equipment and Materials

Inland silversides (*Menidia beryllina*)
Temperature-controlled incubator (set to maintain test temperature = 25.0 ± 1.0°C, photoperiod of 16-hour light / 8-hours dark, light intensity = 50 – 100 ft-c)
Salt synthetic water
Large glass jars
Transfer pipettes
Aquarium pump and tubing
Thermometer
1-oz medicine cups
Newly hatched brine shrimp
Light box or table
Disposable gloves
Test Organism Shipment Log and Test Organism History Information Sheet
P. promelas, *C. variegates*, *M. beryllina*, and *A. bahia* Culture Log

Procedure

A. Receipt of Test Silversides, Acclimation, and Holding.

1. Order Inland silverside larvae (*Menidia beryllina*) from an approved supplier (e.g., Aquatic Indicators, Inc. – St. Augustine, FL).

2. Obtain the Test Organism Shipment Log and Culture Log.

3. Organisms are shipped next day air in insulated boxes and are contained in clear plastic bags. Remove the plastic bags containing the larvae from the shipping container

(insulated box). Carefully transfer the water containing the larvae from each plastic bag to large glass jars (or equivalent). Measure the temperature (SOP-C1), pH (SOP-C3), salinity (SOP-C5), and dissolved oxygen (SOP-C2) of the transferred water in the jar. Record the following information on the Test Organism History Information Sheet provided by the supplier (Exhibit AT46.1).

- Date and time received at the laboratory
- Initials of the analyst that received the shipment
- Water temperature
- Dissolved oxygen (DO) concentration, pH, and salinit
- Appearance and health of the organisms. Unhealthy or diseased larvae (fungus present) must be discarded and may not be used for testing. Document the number of unhealthy or diseased larvae which are discarded.
- Number of dead larvae and the total number of larvae received
- Date and time the organisms were fed

4. Place the Test Organism History Information Sheet in the Test Organism Shipment Log.

5. Record the following information on the Culture Log (Exhibit AT46.2).
   - Organism source (Aquatic Indicators, Inc.)
   - Organism type (*Menidia beryllina*)
   - Organism batch (hatch date)
   - Organism age upon receipt
   - Dates and times organisms were born between
   - Incubator number (cultures are stored in Toxicity Incubator # 4)
   - Synthetic water type (Salt synthetic water is used to culture *Menidia beryllina*)

6. Remove any debris or dead larvae from the jar with a transfer pipette and replace approximately ¾ of the water with salt synthetic water. This activity should be performed daily, until the organisms are used in a toxicity test. Document in the Culture Log the date and time water is renewed. If at any time before a test is initiated the larvae appear unhealthy, diseased (fungus present), or > 10% mortality is identified; the larvae must be discarded and may not be used for testing. Document in the Culture Log the number of dead, diseased, and discarded larvae, total number of larvae and the date the entire culture is discarded.

7. Feed the larvae in the jar twice daily newly hatched brine shrimp (*Artemia nauplii*) which are < 24-hours old (SOP-AT16), until the organisms are used in a toxicity test. Test organisms are typically fed in the morning and afternoon (6 hours between feedings). Organisms must be fed a minimum of 2 hours to a maximum of 5 hours prior to initiating acute tests. Sufficient numbers of nauplii should be provided to assure that some remain alive in the jar at the next feeding, but not in excessive amounts which will result in the depletion of DO below acceptable levels (< 4.0 mg/L). Document in the Culture Log the

times that the organisms are fed daily. If the organisms are used for initiating tests, record in the Culture Log the tests that were initiated on that day.

8. Place the jar in a temperature-controlled incubator. Gently aerate the water using an aquarium pump and tubing. The organisms are initially acclimated to 25.0 ± 1.0ºC such that no more than a 3 ºC change in temperature occurs over a 12 hour period. It may be necessary to place the organisms in an incubator set at a lower temperature to acclimate the organisms gradually. Once acclimated, the organisms are maintained at 25.0 ± 1.0ºC with a photoperiod of 16-hours light and 8-hours dark and a recommended light intensity of 50 to 100 ft-c.

B. Exhibits.

Exhibit AT46.1: Test Organism History Information Sheet.
Exhibit AT46.2: *P. promelas, C. variegates, M. beryllina*, and *A. bahia* Culture Log.

Subject: Acclimation and Maintenance of *Menidia beryllina* Cultures

Exhibit AT46.1: Test Organism History Information Sheet.

Aquatic Indicators, Inc.
P.O. Box 632 • St. Augustine, FL 32085-0632 • (904) 829-2780

Date 9-8-14

Species:
1. M. bahia
2. M. beryllina
3.

Total Supplied: HATCH 09.07.14 HATCH 09.02.14
1. 700 @ 1 day + 500 @ 6 days
2. 120
3.

09.09.14 1005 JH
TEMP = 24.8°C

	Ab 09.07.14	Ab 09.02.14	Mb 08.30.14
pH	7.74	7.77	7.79
DO(g/L)	9.2	10.2	10.4
SALINITY (ppt)	21.6	22.0	21.9
# DEAD / TOTAL	0/700+	0/500+	1/120+

Brood Description:
1. EPA
2. EPA
3.

Age:
1. see above
2. 9 days - HATCH 08.30.14
3.

All organisms appear healthy
Fed at 1010. JH

Environmental Regime — Feeding: Zooplankton, Artemia NH ✓ — Photo: L 16 D 8

P.H.: 8.1 — Temp: 25°C — Salinity: 20‰

Comments: Thanks.

Subject: Acclimation and Maintenance of *Menidia beryllina* Cultures

Exhibit AT46.2: *P. promelas, C. variegates, M. beryllina,* and *A. bahia* Culture Log.

P. promelas, C. variegates, M. beryllina,* and *A. bahia
Culture Log

Test organism information:		*Culture information:*	
Organism source:		Incubator number:	4
Organism type:		Synthetic water type:	
Organism batch:			
Organism age upon receipt:			
Date and times organisms were born between:			

Note: Suppliers of *C. variegatus, M. beryllina* and *A. bahia* do not provide the time that organisms were born between.

Day	Date	Analyst	Synthetic water batch	Activity					
				Feeding Time		Renewal Time	Number of living organisms received from vendor	# Dead, Diseased, Fungused and Discarded	Tests initiated from organism batch
				AM	PM				
0 (day received)									
1									
2									
3									
4									
5									
6									
7									
8									
9									
10									
11									
12									
13									
14									

Comments:

SOP-AT17, Exhibit AT17.2 – revision 11-01-14, SOP-AT40, Exhibit AT40.2 – revision 11-01-14,
SOP-AT46, Exhibit AT46.2 – revision 11-01-14, SOP-AT52, Exhibit AT52.2 – revision 11-01-14

Subject: *Menidia beryllina* Acute Toxicity Test, EPA 2006.0

Document Revision History

Revision Date	Surveillance number	Surveillance Type	Evaluators	Revisions
12-01-00				Original document
06-01-11	Not applicable.	Internal	Jim Sumner (ETS)	• Updated exhibits and references. • Statistical analyses and data review moved to QAP-Q12.
07-01-12	Not applicable.	External (NC DENR) Internal	Lance Ferrell (NC DENR) Jim Sumner (ETS)	• The measurement of pH, DO, conductivity and salinity of each new, full-strength, undiluted sample was added. • The light intensity was amended to reflect that it is a recommended range as specified in the EPA manuals.
11-01-14	Not applicable.	Internal	Jim Sumner (ETS)	• Updated exhibits during document review. • Removed loading weight determination.

Subject: *Menidia beryllina* Acute Toxicity Test, EPA 2006.0

Purpose

To measure the acute toxicity of water samples to inland silverside larvae (*Menidia beryllina*) during 24 or 48-hour exposure.

A summary of the *Menidia beryllina* acute method is provided in Exhibit AT47.1.

References

USEPA. October 2002. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, 5th ed. **EPA-821-R-02-012, Method 2006.0**. US Environmental Protection Agency, Cincinnati, OH.

North Carolina Department of Environment and Natural Resources, Division of Water Quality. Pass/Fail Methodology for Determining Acute Toxicity in a Single Effluent, Version 3.0. December 2010.

Equipment and Materials

Inland silverside larvae (*Menidia beryllina*)
Temperature-controlled incubator (set to maintain test temperature = 25.0 ± 1.0ºC, photoperiod of 16-hour light / 8-hours dark, light intensity = 50 – 100 ft-c)
Control water (salt synthetic water made with Bioassay Grade 40-Fathoms Sea Salt, MarineMix®)
500-ml plastic Solo® Cups
Solo® Cup Lids
Graduated cylinders
Large glass finger bowls
Serological, fixed, or adjustable volume pipette (e.g., Eppendorf)
Transfer pipettes
Aquarium pump, tubing, and air stones
Thermometer
1-oz medicine cups
Newly hatched brine shrimp
Light box or table
Disposable gloves
Menidia beryllina Shipment Log and Organism History Information Sheet
Acute Toxicity Test or Pass/Fail Acute Toxicity Test Benchsheet
Randomization template

Subject: *Menidia beryllina* Acute Toxicity Test, EPA 2006.0

Procedure

A. Test Preparation.

1. Prepare the plasticware.

   a. Obtain enough 500-ml plastic Solo® cups with lids for each site/sample and concentration tested, including the control. For Pass/Fail acute tests, four replicates are used for the test concentration and control. For multiple concentration acute tests, two replicates are used for each concentration and control. Label each replicate cup with the following information.
   - Facility/sample name
   - Concentration
   - Replicate number

   b. Label the appropriate graduated cylinder with the facility/sample name and concentration.

   c. Prepare the Acute Toxicity Test Benchsheet (for multiple concentration tests, Exhibit AT47.3) or Pass/Fail Acute Toxicity Test Benchsheet (for Pass/Fail acute tests, Exhibits AT47.2). Record the following information on the Benchsheet:
   - Client/facility name
   - Permit number
   - County
   - Outfall/Pipe number
   - ETS project and sample number
   - Control/Dilution water type and batch
   - Test concentrations and dilution preparation information (sample, dilution and total volumes)

B. Test Initiation.

1. Prepare the test concentrations according to SOP-G5. It may be necessary to salt-up the sample prior to making the test concentrations. Refer to SOP-G5 for the appropriate procedures for salting-up samples.

   a. Measure and record the pH (SOP-C3), dissolved oxygen (SOP-C2), and salinity (SOP-C5) of each concentration tested and control. Ensure that the dissolved oxygen is within the acceptable range (4.0 to 9.0 mg/L), aerate the sample if necessary according to SOP-C2. Measure and record the pH (SOP-C3), dissolved oxygen (SOP-C2), conductivity (SOP-C4), salinity (SOP-C5) total residual chlorine (SOP-C8), total alkalinity (SOP-C6) and sample characteristics of each new, full-strength, undiluted sample. Measure and record the alkalinity

(SOP-C6) control/dilution water. The alkalinity of full-strength, undiluted samples for North Carolina tests is not required.

b. Pour 250 ml of control water into each of the replicate control cups.

c. Pour 250 ml of each test concentration into each of the labeled replicate test cups.

d. Maintain the test temperature (25.0 ± 1.0ºC) of the test concentrations. This may be accomplished by placing the test cups into a temperature-controlled incubator.

2. Isolate the larvae for the test.

a. Obtain a batch of larvae (SOP-AT46), which are 9 to 14-days old (with a maximum of 24-hour range in age). Record the source, age, and hatch date of the organisms to be used in the test on the acute benchsheet. Feed the larvae a minimum of 2 hours prior to test initiation to a maximum of 5 hours prior to test initiation. Record the date and time the organisms were fed on the benchsheet. Transfer the larvae to a large glass finger bowl.

b. After the larvae have fed for a minimum of 2-hours to a maximum of 5 hours, transfer 10 larvae from the finger bowl to a 1-oz medicine cup using a transfer pipette.

c. Two techniques may be used for transferring 10 organisms to each test cup from the finger bowl. The technique used is dependent on the sample being evaluated for toxicity. In both methods, larvae are transferred by plastic pipette. The end of the pipette tip should be cut to a size that will not injure or harm the larvae during transfer. Larvae should be transferred gently in a manner that will not expose the organisms to the air.

- If the transferred water may alter the toxicity of the sample (decrease toxicity), organisms should be transferred directly by pipette. This technique is predominately used by the laboratory. Organisms should be transferred in a manner that allows them to swim from the pipette into the test solutions. This will minimize the volume of transfer water introduced into the sample. Follow procedures outlined in step 3 for transferring the organisms to the randomly placed test cups. The average transfer volume by this technique for each analyst must be determined yearly. For an example transfer volume logsheet refer to Exhibit AT47.4.

- If pathogenic interferences have been identified or there is risk of cross contamination between replicates, organisms should be transferred to medicine cups (10 per cup) and then introduced into the test solutions. The

final volume contained in the medicine cups after the organisms have been transferred should be between 10 and 15 ml. With a pipette, remove any food or debris that was placed in the cup during transfer. The average transfer volume by this technique for each analyst must be determined yearly. For an example transfer volume logsheet refer to Exhibit AT47.4. Continue this process until enough medicine cups containing 10 larvae each have been obtained to initiate the test. 1 medicine cup containing 10 larvae will be needed for each sample concentration and replicate. If a test consists of 5 concentrations and a control, 12 medicine cups containing 10 larvae each will be required. If a test consists of 1 concentration and a control, 8 medicine cups containing 10 larvae each will be required.

- A limit is placed on the loading (weight) of organisms per liter of test solution to minimize the depletion of dissolved oxygen, the accumulation of injurious concentrations of metabolic waste products and/or stress induced by crowding, any of which could significantly affect the test results. The loading in the test solutions must not exceed or 0.40 g live weight/L at 25°C. Through testing, ETS has determined that this loading requirement is not exceeded using *M. beryllina* larvae which are 9 to 14 days old.

d. Save approximately 30 ml of transfer water to be measured for pH (SOP-C3). Measure and record the transfer water pH and temperature on the acute benchsheet.

3. Transfer the larvae to the randomly placed test cups.

a. Obtain a randomization template (Exhibit AT47.5). Order the test cups according to the randomization template and record the template name (color) on the benchsheet.

b. Measure and record the temperature in one of the test cups for each concentration and control. The temperature must be 25.0 ± 1.0°C before the test organisms are placed in the test cups. Warm the test cups in a warm water bath or temperature-controlled incubator, if necessary, until the desired test temperature is obtained. Measure and record the temperature of an arbitrary transfer cup.

c. Place 10 larvae in the first test cup of the first row (by pipette or medicine cup). Continue in this manner (placing the larvae in the test cups from left to right in the first row and then the second row) until all the test cups contain 10 larvae.

d. Record the initiation date, time and analyst's initials on the acute benchsheet. **The acute test must be initiated within 36-hours of completion of the sampling period.**

e. Verify that each cup received the required number of larvae (i.e., 10) by conducting a repeat count. Remove excess larvae or add larvae as necessary. Record the initial number of larvae on the benchsheet. Place lids on each cup.

f. Place the test cups in order, according to the randomization template, in a temperature-controlled incubator. The organisms must be maintained at 25.0 ± 1.0°C with a photoperiod of 16-hours light and 8-hours dark and a recommended light intensity of 50 to 100 ft-c. Record the incubator number and shelf used on the benchsheet.

g. Place the test cups in order according to the randomization template in a temperature-controlled incubator. The organisms must be maintained at 25.0 ± 1.0°C with a photoperiod of 16-hours light and 8-hours dark and a recommended light intensity of 50 to 100 ft-c. Record the incubator number and shelf used on the benchsheet.

C. Record Daily Survival.

Repeat this process daily, starting at 24-hours ± 1-hour after test initiation and continuing until test termination.

1. Measure and record the temperature in an arbitrarily selected test cup for each test concentration and control.

2. Remove the test cups from the incubator and remove the lids. Place the cups on a light box or table for ease of viewing.

3. Count and record (in the appropriate section) the number of larvae surviving in each replicate cup on the acute benchsheet. The criteria that establish death are: (1) no movement and (2) no reaction to gentle prodding. Record comments, if applicable.

4. Remove any dead larvae and discard with a transfer pipette.

5. Record the date, time and the analyst's initials on the benchsheet.

6. Carefully pour ~30 ml of test water from at least one replicate cup for each test concentration and control into a labeled 1-oz medicine cup. Measure and record the pH (SOP-C3), dissolved oxygen (SOP-C2), and salinity (SOP-C5) of this water.

7. Place the lids on the test cups and place the test cups back in order, according to the randomization template, in a temperature-controlled incubator.

Subject: *Menidia beryllina* Acute Toxicity Test, EPA 2006.0

D. Test Termination.

Terminate the test after the organisms have been exposed to the test concentrations for the required time (i.e. 24 or 48-hours). The test may be terminated ± 1-hour from the time it was initiated.

1. Measure and record the temperature in an arbitrarily selected test cup for each concentration and control.

2. Remove the test cups from the incubator and remove the lids. Place the cups on a light box or table for ease of viewing.

3. Count and record (in the appropriate section) the number of larvae surviving in each replicate cup on the acute benchsheet. Record comments, if applicable.

4. Record the termination date, time, and the analyst's initials on the benchsheet.

5. Measure and record the pH (SOP-C3), dissolved oxygen (SOP-C2), and salinity (SOP-C5) in one of the test cups for each concentration and control.

6. Once all analyses have been completed and documented, discard the test water and larvae according to established laboratory protocol.

E. Statistical Analyses and Data Verification.

Statistical analyses and data review is performed according to QAP-Q12.

F. Acceptance Criteria.

The test acceptance criterion is ≥ 90% survival in the control. If the control survival is < 90%, notify the Laboratory Supervisor.

G. Exhibits.

Exhibit AT47.1: Summary of Test Conditions for the *Menidia beryllina* Acute Toxicity Test.
Exhibit AT47.2: Pass/Fail Acute Toxicity Test Benchsheet.
Exhibit AT47.3: Acute Toxicity Test Benchsheet.
Exhibit AT47.4: Average Transfer Volume Logsheet.
Exhibit AT47.5: Randomization Templates.

Subject: *Menidia beryllina* Acute Toxicity Test, EPA 2006.0

Exhibit AT47.1: Summary of Test Conditions for the *Menidia beryllina* Acute Toxicity Test.

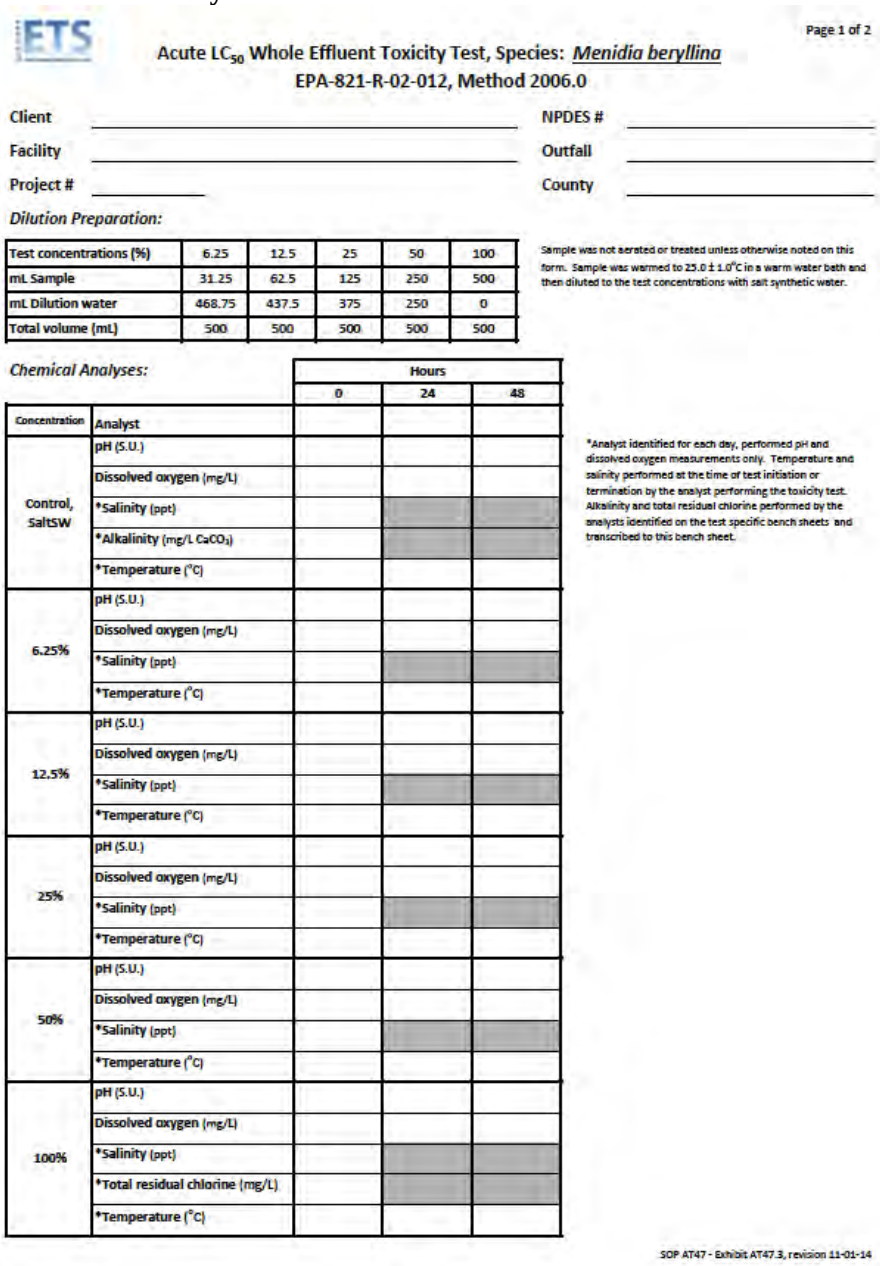
SUMMARY OF TEST CONDITIONS FOR THE *MENIDIA BERYLLINA* ACUTE TOXICITY TEST

Test type:	Static non-renewal or static renewal
Test duration:	24 or 48 hours
Temperature:	25.0 ± 1.0ºC
Light quality:	Cool-white fluorescent
Light intensity:	50 – 100 ft-candles (recommended)
Photoperiod:	16-hours light, 8-hours dark
Test chamber size:	500 mL Solo® cups
Test solution volume:	250 mL
Renewal of test solutions:	None
Age of test organisms (days old):	9 to 14 days old, ≤ 24 hour range in age
Number of organisms per test chamber:	10
Number of replicate test chambers per concentration:	Multiple concentration tests: 2 Single dilution tests: 4
Number of organisms per concentration:	Multiple concentration tests: 20 Single dilution tests: 40
Test concentrations:	Multiple concentration tests: 5 and a control with ≥ 0.5 dilution series (recommended) Single dilution tests: 90% or 100% and a control
Test chamber cleaning:	Dead larvae removed daily.
Aeration:	None, unless the dissolved oxygen concentration falls below 4.0 mg/L.
Feeding regime:	*Artemia nauplii* made available while holding prior to test initiation (2 to 5-hours prior to initiation).
Control / Dilution water:	Salt synthetic water (25.0 ± 2.0 ppt)
Sampling and sample holding:	1-gallon grab or composite sample first used within 36-hours of completion of the sampling period.
Endpoint:	Mortality
Test acceptability criterion:	≥ 90% control survival

Subject: *Menidia beryllina* Acute Toxicity Test, EPA 2006.0

Exhibit AT47.2: Pass/Fail Acute Toxicity Test Benchsheet.
(Pass/Fail acute tests are currently not performed at ETS. An exhibit will be provided when pass/fail acute tests are performed.)

Exhibit AT47.3: Acute Toxicity Test Benchsheet.



ETS

Page 1 of 2

Acute LC_{50} Whole Effluent Toxicity Test, Species: *Menidia beryllina*
EPA-821-R-02-012, Method 2006.0

Client _____ NPDES # _____

Facility _____ Outfall _____

Project # _____ County _____

Dilution Preparation:

Test concentrations (%)	6.25	12.5	25	50	100
mL Sample	31.25	62.5	125	250	500
mL Dilution water	468.75	437.5	375	250	0
Total volume (mL)	500	500	500	500	500

Sample was not aerated or treated unless otherwise noted on this form. Sample was warmed to 25.0 ± 1.0°C in a warm water bath and then diluted to the test concentrations with salt synthetic water.

Chemical Analyses:

Concentration	Analyst	Hours 0	24	48
Control, SaltSW	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	*Salinity (ppt)			
	*Alkalinity (mg/L $CaCO_3$)			
	*Temperature (°C)			
6.25%	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	*Salinity (ppt)			
	*Temperature (°C)			
12.5%	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	*Salinity (ppt)			
	*Temperature (°C)			
25%	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	*Salinity (ppt)			
	*Temperature (°C)			
50%	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	*Salinity (ppt)			
	*Temperature (°C)			
100%	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	*Salinity (ppt)			
	*Total residual chlorine (mg/L)			
	*Temperature (°C)			

*Analyst identified for each day, performed pH and dissolved oxygen measurements only. Temperature and salinity performed at the time of test initiation or termination by the analyst performing the toxicity test. Alkalinity and total residual chlorine performed by the analysts identified on the test specific bench sheets and transcribed to this bench sheet.

SOP AT47 - Exhibit AT47.3, revision 11-01-14

Subject: *Menidia beryllina* Acute Toxicity Test, EPA 2006.0

ETS

Acute LC_{50} Whole Effluent Toxicity Test, Species: *Menidia beryllina*
EPA-821-R-02-012, Method 2006.0

Client _____

Project # _____

Hours	Date	Feeding		Test Initiation or Termination		Location	Randomizing	Sample Number	Salt SW Batch
		Time	Analyst	Time	Analyst	Incubator/Shelf	Template		
0 initiation		*							
24									
48 Termination									

*Test organisms were fed in holding 2 to 5 hours prior to test initiation. Test organisms were not fed during the test.

Test Organism Information:

Organism Source:	Aquatic Indicators, Inc.
Batch (AI Batch Mb):	
Age (9 to 14 days old):	
Date organisms were born: (time organisms were born between is not provided by supplier)	
Average transfer volume:	0.2542 mL
Transfer bowl information:	pH (S.U.):
	Temperature (°C):

EPA loading requirement for freshwater species of < 0.40 g/L at 25.0°C has been documented by ETS to never be exceeded using 9 to 14 day old *M. beryllina*.

Survival Data **(number of living organisms):**

Hours	Control		6.25%		12.5%		25%		50%		100%	
	Replicate		Replicate		Replicate		Replicate		Replicate		Replicate	
	A	B	C	D	E	F	G	H	I	J	K	L
0 initiation	10	10	10	10	10	10	10	10	10	10	10	10
24												
48 Termination												
Mean Survival												

Comment codes: d = dead, u = unhealthy, s = stressed

Statistics:

Method	
Upper 95% confidence limit	
Lower 95% confidence limit	
48-hour LC_{50}	

Comments:

Subject: *Menidia beryllina* Acute Toxicity Test, EPA 2006.0

Exhibit AT47.4: Average Transfer Volume Logsheet.

***Pimephales promelas* are used as surrogate organisms to determine the average transfer volume.**

ETS Environmental Testing Solutions, Inc. Page 1 of 1

Larval Fish Transfer Volume

Analyst: J. Sumner | Species: *P. promelas*

Date: 02-12-14 | Source / Batch: ATOX Batch Pp 02-03-14

Ambient temperature: 24.1°C | Wet Weight of 10 Larvae (g): 0.0209

Estimate transfer volume, where minnows are allowed to swim from the pipette into the test vessel.

Numerically label 10 medicine cups.
Add 10 mL MHSW to each of the 10 cups.
Measure and record the weight of each cup containing MHSW.
Transfer 10 larvae to each cup, following procedures identified in SOP-AT18, AT47, or AT53 for vertebrate acute toxicity tests.
Transfer the larvae in a manner that allows them to swim from the pipette into the MHSW contained in each cup.
Measure and record the weight of each cup containing MHSW with 10 larvae.
Determine each transfer volume and average transfer volume.

Replicate Number	Initial Weight Medicine cup + 10 mL MHSW (g)	Final Weight Medicine cup + 10 mL MHSW + 10 Larval Fish transferred (g)	Transfer Volume Final - Initial Weight (g = mL)
1	11.3533	11.6989	**0.3456**
2	11.3337	11.4850	**0.1513**
3	11.3607	11.6976	**0.3369**
4	11.2950	11.5662	**0.2712**
5	11.2945	11.5634	**0.2689**
6	11.3349	11.3360	**0.0011**
7	11.3539	11.7630	**0.4091**
8	11.3883	11.7335	**0.3452**
9	11.2812	11.4930	**0.2118**
10	11.3321	11.5329	**0.2008**
		Average volume to transfer 10 organisms (mL):	**0.2542**

Estimate transfer volume, where the minnows are transferred with MHSW into the test vessels.

Numerically label 10 medicine cups.
Measure and record the weight of each cup.
Add approximately 10 mL MHSW to each of the 10 cups.
Transfer 10 larvae to each cup, following procedures identified in SOP-AT18, AT47, or AT53 for vertebrate acute toxicity tests.
Transfer the larvae in a manner that allows them to swim from the pipette into the MHSW contained in each cup.
Measure and record the weight of each cup containing MHSW with 10 larvae.
Determine each transfer volume and average transfer volume.

Replicate Number	Initial Weight Medicine cup (g)	Final Weight Medicine cup + 10 mL MHSW + 10 Larval Fish transferred (g)	Transfer Volume Final - Initial Weight (g = mL)
1	1.7092	11.6989	**9.9897**
2	1.6929	11.4850	**9.7921**
3	1.7092	11.6976	**9.9884**
4	1.6728	11.5662	**9.8934**
5	1.6967	11.5634	**9.8667**
6	1.6740	11.3360	**9.6620**
7	1.7015	11.7630	**10.0615**
8	1.6870	11.7335	**10.0465**
9	1.7009	11.4930	**9.7921**
10	1.7152	11.5329	**9.8177**
		Average volume to transfer 10 organisms (mL):	**9.8910**

SOP AT18 - Exhibit AT18.5, SOP AT47 - Exhibit AT47.5, SOP AT53 - Exhibit AT53.5, revision 11-01-14

Subject: *Menidia beryllina* Acute Toxicity Test, EPA 2006.0

Exhibit AT47.5: Randomization Templates.

BLUE

Replicate number	Concentrations
1	1 7 4 3 6 2 5
2	7 3 2 5 4 1 6
3	3 4 6 5 2 1 7
4	5 6 1 2 4 7 3

Random number seeds 10 through 13.

Subject: *Menidia beryllina* Acute Reference Toxicity Test, EPA 2006.0

Document Revision History

Revision Date	Surveillance number	Surveillance Type	Evaluators	Revisions
12-01-00				Original document
06-01-11	Not applicable.	Internal	Jim Sumner (ETS)	• Updated references and exhibits.
11-01-14	Not applicable.	Internal	Jim Sumner (ETS)	• Updated exhibits during document review.

Subject: *Menidia beryllina* Acute Reference Toxicity Test, EPA 2006.0

Purpose

To assess the sensitivity of inland silverside larvae (*Menidia beryllina*) and the overall credibility of the inland silverside acute toxicity test. Reference toxicant data are part of the laboratory's QA/QC program to evaluate the performance of laboratory personnel and the robustness and sensitivity of the test organisms.

References

USEPA. October 2002. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, 5th ed. **EPA-821-R-02-012, Method 2006.0**. US Environmental Protection Agency, Cincinnati, OH.

USEPA. 2000. Understanding and Accounting for Method Variability in Whole Effluent Toxicity Applications Under the National Pollutant Discharge Elimination Program. EPA-833-R-00-003. US Environmental Protection Agency, Cincinnati, OH.

USEPA. 2001. Final Report: Inter-laboratory Variability Study of EPA Short-term Chronic and Acute Whole Effluent Toxicity Test Methods, Volumes 1 and 2 - Appendix. EPA-821-B-01-004 and EPA-821-B-01-005. US Environmental Protection Agency, Cincinnati, OH.

North Carolina Department of Environment and Natural Resources, Division of Water Quality. Biological Laboratory Certification / Criteria Procedures, Version 3.0. December 2010.

Definitions

Acceptance Criteria: Specified limits placed on characteristics of an item, process, or service defined in codes, standards, or other required documents.

Precision: The extent to which measurement results repeat themselves when repeat measurements are made on the same unit of product.

Equipment and Materials

Inland silverside larvae (*Menidia beryllina*)
Temperature-controlled incubator (set to maintain test temperature = 25.0 ± 1.0ºC, photoperiod of 16-hour light / 8-hours dark, light intensity = 50 – 100 ft-c)
Control water (salt synthetic water made with Bioassay Grade 40-Fathoms Sea Salt, MarineMix®)
Potassium chloride (KCl, reagent grade)
1000-ml volumetric flask
Deionized water
500-ml plastic Solo® cups

Solo® cup lids
500-ml graduated cylinder
1000-ml Erlenmeyer flask
Large glass finger bowls
10-ml serological pipettes
Transfer pipettes
Calibrated top-loading balance (e.g. Sartorius)
Thermometer
1-oz disposable medicine cups
Newly hatched brine shrimp
Light box or table
Disposable gloves
Menidia beryllina Acute Reference Toxicity Test Benchsheet
Randomization template

Procedure

A. Frequency of Testing and Requirements.

1. An *Menidia beryllina* acute reference toxicant test must be performed such that all acute whole effluent toxicity tests are conducted within 1 week of a reference toxicant test. At a minimum, the *Menidia beryllina* acute reference toxicant tests must be performed on a quarterly basis in order to maintain certification requirements. Since *Menidia beryllina* are obtained from an outside supplier, an acute reference toxicant test must be performed on each batch of organisms used for acute whole effluent toxicity tests.

B. Test Preparation.

1. Prepare the glassware.

   a. Obtain two replicate 500-ml plastic Solo® cups and lids for each of the five KCl concentrations tested and the control. Label each replicate cup with the following information.
      - Concentration
      - Replicate number

   b. Label the appropriate graduated cylinders.

   c. Prepare the *Menidia beryllina* Acute Reference Toxicity Test Benchsheet (see Exhibit AT48.1). Record the *Menidia beryllina* Potassium Chloride Acute (MbKClAC) test number on the benchsheet.

C. Preparation of the Stock Solution.

1. Using a calibrated top-loading balance, carefully weigh out 50 g of KCl (SOP-G10). Place approximately 900 ml of Milli-Q water in a 1000-ml volumetric flask. Add the KCl to the flask, dissolve the KCl by swirling the flask; bring to volume with Milli-Q water. Label the volumetric flask with the concentration (50 g/L), analyst's initials, preparation date and stock standard number (INSS, SOP-G15). Record the INSS number of the KCl stock solution on the benchsheet.

D. Preparation of the Test Concentrations.

1. Prepare all test concentrations using graduated cylinders and serological pipettes. The precision of preparing the test concentrations is increased when smaller volume graduated cylinders and/or serological pipettes are used. For this reference toxicant test, stock solution volumes should be measured using a 10-ml serological pipette and the total volumes should be measured using a 500-ml graduated cylinder.

2. Beginning with the lowest concentration, add approximately 100 ml of salt synthetic water to a 500-ml graduated cylinder, add the required volume of stock solution using a 10-ml serological pipette (refer to Table AT42.1), bring to volume (500 ml) with salt synthetic water. Mix the solution well by pouring the solution into a 1000-ml Erlenmeyer flask.

3. Pour 250 ml of test solution into each of the replicate test cups for that concentration. 30 ml should be saved for chemical analyses. Measure and record the pH (SOP-C3), dissolved oxygen (SOP-C2) and salinity (SOP-C5) of the test solution.

4. Rinse the graduated cylinder well with deionized water and repeat steps C.2 through C.3 for preparing the next test concentration. Record the batch date of salt synthetic water used to prepare the dilutions.

Table AT48.1: Test concentration, stock volumes, salt synthetic water volumes, and final volumes for the *Menidia beryllina* KCl acute reference toxicant tests.

Test Concentration (mg KCl/L)	Volume of Stock Required (ml)	Volume of Moderately Hard Synthetic Water (ml)	Final Volume (ml)
1000	10.0	490.0	500
1250	12.5	487.5	500
1500	15.0	485.0	500
1750	17.5	482.5	500
2000	20.0	480.0	500

5. Once all test concentrations have been prepared, follow the procedure described in SOP-AT47 for conducting *Menidia beryllina* Acute Toxicity Tests.

E. Preparation of Control Charts.

1. Refer to QAP-Q5 for how control charts are prepared and procedures for interpreting outlier tests. Refer to Exhibit AT48.2 for an example control chart.

F. Exhibits.

Exhibit AT48.1: *Menidia beryllina* Acute Reference Toxicity Test Benchsheet.
Exhibit AT48.2: Example of a *Menidia beryllinas* Acute Reference Toxicant Control Chart.

Subject: *Menidia beryllina* Acute Reference Toxicity Test, EPA 2006.0

Exhibit AT48.1: *Menidia beryllina* Acute Reference Toxicity Test Benchsheet.

Page 1 of 2

Acute LC_{50} Whole Effluent Toxicity Test, Species: *Menidia beryllina*
EPA-821-R-02-012, Method 2006.0

Menidia beryllina Potassium Chloride Acute Reference Toxicant Test

MbKCIAC # _____

Dilution Preparation:

Test concentrations (mg/L KCl)	1000	1250	1500	1750	2000
mL Stock solution	10.0	12.5	15.0	17.5	20.0
mL Dilution water	490.0	487.5	485.0	482.5	480.0
Total volume (mL)	500	500	500	500	500

A stock solution was prepared by diluting 100 g KCl into 2000 mL Milli-Q water. This 50,000 mg/L KCl stock solution was used to prepare the concentrations evaluated for toxicity.

Stock solution INSS #: _____

Chemical Analyses:

Concentration	Analyst	Hours 0	24	48
Control, SaltSW	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	*Salinity (ppt)			
	*Alkalinity (mg/L $CaCO_3$)			
	*Temperature (°C)			
1000 mg/L	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	*Salinity (ppt)			
	*Temperature (°C)			
1250 mg/L	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	*Salinity (ppt)			
	*Temperature (°C)			
1500 mg/L	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	*Salinity (ppt)			
	*Temperature (°C)			
1750 mg/L	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	*Salinity (ppt)			
	*Temperature (°C)			
2000 mg/L	pH (S.U.)			
	Dissolved oxygen (mg/L)			
	*Salinity (ppt)			
	*Temperature (°C)			

*Analyst identified for each day, performed pH and dissolved oxygen measurements only. Temperature and salinity performed at the time of test initiation or termination by the analyst performing the toxicity test. Alkalinity performed by the analyst identified on the test specific bench sheet and transcribed to this bench sheet.

SOP AT48 - Exhibit AT48.1, revision 11-01-14

Subject: *Menidia beryllina* Acute Reference Toxicity Test, EPA 2006.0

ETS

Acute LC_{50} Whole Effluent Toxicity Test, Species: *Menidia beryllina*
EPA-821-R-02-012, Method 2006.0

Menidia beryllina Potassium Chloride Acute Reference Toxicant Test

MbKClAC # _____

Hours	Date	Feeding		Test Initiation or Termination		Location	Randomizing	SaltSW Batch
		Time	Analyst	Time	Analyst	Incubator/Shelf	Template	
0 Initiation		*						
24								
48 Termination								

*Test organisms were fed in holding 2 to 5 hours prior to test initiation. Test organisms were not fed during the test.

Test Organism Information:

Organism Source:	Aquatic Indicators, Inc.
Batch (AI Batch Mb):	
Age (9 to 14 days old):	
Date organisms were born: (time organisms were born between is not provided by supplier)	
Average transfer volume:	0.2542 mL
Transfer bowl information:	pH (S.U.):
	Temperature (°C):

EPA loading requirement for freshwater species of < 0.40 g/L at 25.0°C has been documented by ETS to never be exceeded using 9 to 14 day old *M. beryllina*.

Survival Data **(number of living organisms):**

Hours	Control		1000 mg/L		1250 mg/L		1500 mg/L		1750 mg/L		2000 mg/L	
	Replicate		Replicate		Replicate		Replicate		Replicate		Replicate	
	A	B	C	D	E	F	G	H	I	J	K	L
0 Initiation	10	10	10	10	10	10	10	10	10	10	10	10
24												
48 Termination												
Mean Survival												

Comment codes: d = dead, u = unhealthy, bs = bent spines, s = stressed

Statistics:

Method	
Upper 95% confidence limit	
Lower 95% confidence limit	
48-hour LC_{50}	

Comments:

Test Reviewed by: _____

SOP AT48 - Exhibit AT48.1, revision 11-01-14

Subject: *Menidia beryllina* Acute Reference Toxicity Test, EPA 2006.0

Exhibit AT48.2: Example of a *Menidia beryllina* Acute Reference Toxicant Control Chart.

Environmental Testing Solutions, Inc.

Menidia beryllina
Acute Reference Toxicant Control Chart

Test number	Test date	48-h LC_{50}	CT	S	Control Limits		$S_{A.75}$	Warning Limits		$S_{A.90}$	Control Limits		CV
		(g/L KCl)	(g/L KCl)		CT - 2S	CT + 2S		CT - $S_{A.75}$	CT + $S_{A.75}$		CT - $S_{A.90}$	CT + $S_{A.90}$	
1	12-06-11	1.50											
2	01-10-12	1.38	1.44	0.08	1.27	1.60	0.58	0.86	2.01	0.79	0.65	2.23	0.06
3	03-07-12	1.40	1.42	0.06	1.30	1.55	0.57	0.85	1.99	0.78	0.64	2.21	0.04
4	04-12-12	1.37	1.41	0.06	1.30	1.53	0.56	0.85	1.98	0.78	0.64	2.19	0.04
5	05-12-12	1.42	1.41	0.05	1.31	1.51	0.57	0.85	1.98	0.78	0.64	2.19	0.04
6	06-07-12	1.42	1.41	0.05	1.32	1.51	0.57	0.85	1.98	0.78	0.64	2.19	0.03
7	09-19-12	1.50	1.43	0.05	1.32	1.53	0.57	0.86	2.00	0.79	0.64	2.21	0.04
8	12-04-12	1.53	1.44	0.06	1.32	1.56	0.58	0.86	2.02	0.79	0.65	2.23	0.04
9	03-05-13	1.49	1.45	0.06	1.33	1.56	0.58	0.87	2.02	0.80	0.65	2.24	0.04
10	04-05-13	1.51	1.45	0.06	1.33	1.57	0.58	0.87	2.03	0.80	0.65	2.25	0.04
11	06-04-13	1.51	1.46	0.06	1.34	1.58	0.58	0.87	2.04	0.80	0.66	2.26	0.04
12	07-26-13	1.45	1.46	0.06	1.34	1.57	0.58	0.87	2.04	0.80	0.66	2.26	0.04
13	09-13-13	1.39	1.45	0.06	1.34	1.57	0.58	0.87	2.03	0.80	0.65	2.25	0.04
14	10-14-13	1.38	1.45	0.06	1.33	1.56	0.58	0.87	2.03	0.80	0.65	2.24	0.04
15	12-04-13	1.54	1.45	0.06	1.33	1.58	0.58	0.87	2.03	0.80	0.65	2.25	0.04
16	01-14-14	1.50	1.46	0.06	1.33	1.58	0.58	0.87	2.04	0.80	0.66	2.26	0.04
17	04-08-14	1.51	1.46	0.06	1.34	1.58	0.58	0.88	2.04	0.80	0.66	2.26	0.04
18	06-03-14	1.37	1.45	0.06	1.33	1.58	0.58	0.87	2.04	0.80	0.65	2.25	0.04
19	07-08-14	1.54	1.46	0.06	1.33	1.59	0.58	0.88	2.04	0.80	0.66	2.26	0.04
20	09-10-14	1.41	1.46	0.06	1.33	1.58	0.58	0.87	2.04	0.80	0.66	2.26	0.04

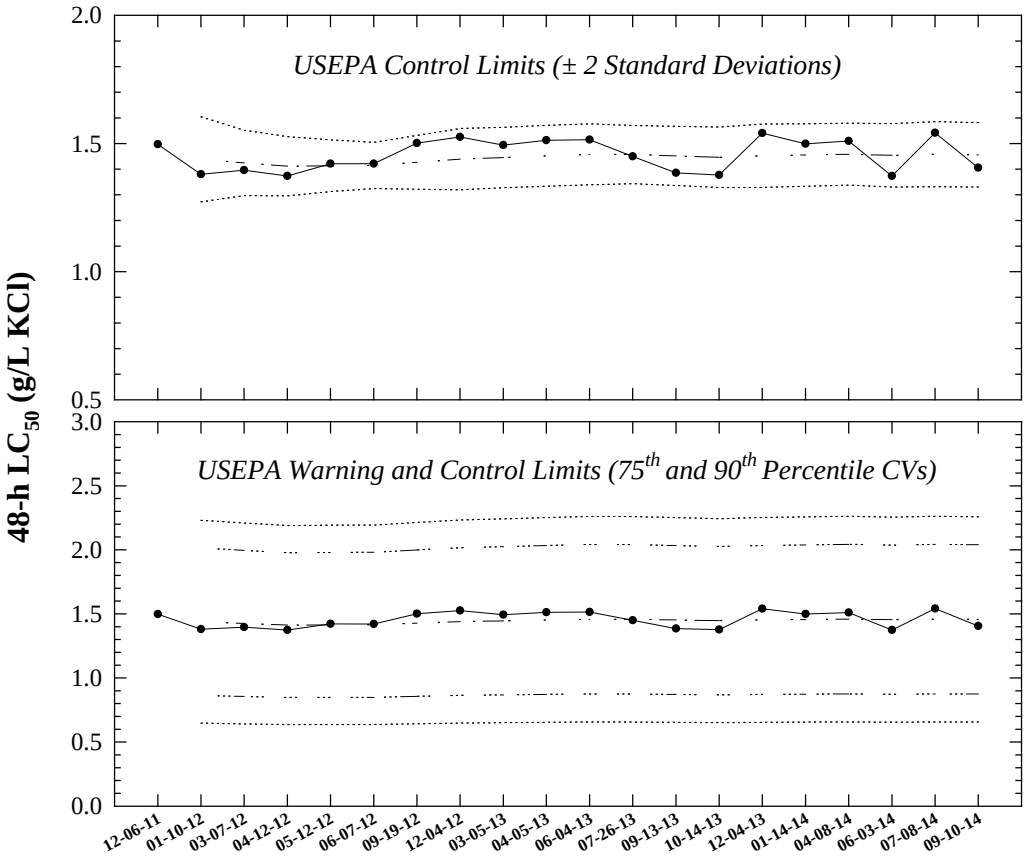
Note:
48-h LC_{50} = 48-hour median lethal concentration. An estimate of the concentration of potassium chloride which is lethal to 50% of the test organisms in 48-hours.
CT = Central tendency (mean LC_{50}).
S = Standard deviation of the LC_{50} values.
$S_{A.75}$ = Standard deviation corresponding to the the 75th percentile CV. $S_{A.75}$ = 0.40, as determined by USEPA for the method and endpoint.
$S_{A.90}$ = Standard deviation corresponding to the the 90th percentile CV. $S_{A.90}$ = 0.55, as determined by the USEPA for the method and endpoint.
CV = Coefficient of variation of the LC_{50} values.

USEPA. 2000. Understanding and Accounting for Method Variability in Whole Effluent Toxicity Applications Under the National Pollutant Discharge Elimination Program. EPA-833-R-00-003. US Environmental Protection Agency, Cincinnati, OH.

Subject: *Menidia beryllina* Acute Reference Toxicity Test, EPA 2006.0

Environmental Testing Solutions, Inc.

Menidia beryllina
Acute Reference Toxicant Control Chart



Test date

——●—— **48-hour LC_{50}** = median lethal concentration. An estimation of the concentration of potassium chloride which is lethal to 50% of the test organisms in 48-hours.

. . . . **Central Tendency** (mean LC_{50})

... . **Warning Limits** (mean $LC_{50} \pm S_{A.75}$)

..... **Control Limits** (mean $LC_{50} \pm S_{A.90}$ or 2 Standard Deviations)

Graphs generated from associated excel spreadsheet.
Excel spreadsheet entered by: J. Sumner
Reviewed by: _____

Subject: *Menidia beryllina* Chronic Toxicity Test, EPA 1006.0

Document Revision History

Revision Date	Surveillance number	Surveillance Type	Evaluators	Revisions
12-01-00				Original document
07-10-10	Not applicable.	External (NC DENR) Internal	Lance Ferrell (NC DENR) Jim Sumner (ETS)	• Section B.2.a and Exhibit AT49.1 amended to indicate that 9 to 11 day old larvae are used to initiate chronic *Menidia* tests.
06-01-11	Not applicable.	Internal	Jim Sumner (ETS)	• Updated exhibits. • Statistical analyses and data review moved to QAP-Q12.
07-01-12	Not applicable.	External (NC DENR) Internal	Lance Ferrell (NC DENR) Jim Sumner (ETS)	• The measurement of pH, DO, conductivity and salinity of each new, full-strength, undiluted sample was added. • The light intensity was amended to reflect that it is a recommended range as specified in the EPA manuals.
11-01-14	Not applicable.	Internal	Jim Sumner (ETS)	• Updated exhibits during document review. • Changed renewal time recommendation to ± 2-hours from test initiation. • Provided additional clarification to testing procedure. • Added acceptance criteria with Table AT49.1.

Subject: *Menidia beryllina* Chronic Toxicity Test, EPA 1006.0

Purpose

To measure the chronic toxicity of water samples to *Menidia beryllina* in 7 day static renewal exposures.

A summary of the *Menidia beryllina* chronic method is provided in Exhibit AT49.1.

References

USEPA. October 2002. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms, 3rd ed. **EPA-821-R-02-014, Method 1006.0**. US Environmental Protection Agency, Cincinnati, OH.

Equipment and Materials

Inland silverside larvae (*Menidia beryllina*)
Temperature-controlled incubator (set to maintain test temperature = 25.0 ± 1.0ºC, photoperiod of 16-hour light / 8-hours dark, light intensity = 50 – 100 ft-c)
Control water (salt synthetic water made with Bioassay Grade 40-Fathoms Sea Salt, MarineMix®)
Microbalance accurate to 0.00001 mg (e.g. Cahn)
Class S or Class I certified weights
Microweight aluminum pans (e.g. Cahn)
Drying oven
Desiccator
20-ml glass beakers
Coors® spot plates
600-ml glass beakers
Graduated cylinders
Large glass finger bowls
Serological, fixed, or adjustable volume pipette (e.g., Eppendorf)
Transfer pipettes
Siphoning hose (rubber tubing affixed with a plastic tube fitted with a soft, angled rubber tip)
White plastic photographic tray
Fine mesh sieve
Forceps
Ice water
Aquarium pump, tubing, and air stones
Plexiglas® slides
Thermometer
1-oz medicine cups
Newly hatched brine shrimp
Light box or table
Disposable gloves
Menidia beryllina Shipment Log and Organism History Information Sheet

Subject: *Menidia beryllina* Chronic Toxicity Test, EPA 1006.0

Menidia beryllina Chronic Toxicity Test Benchsheet
Randomization template

Procedure

A. Test Preparation.

1. Prepare the glassware.

   a. Obtain four replicate 600-ml glass beakers for each site/sample and concentration tested, including the control. Label each replicate beaker with the following information.
   - Facility/sample name
   - Concentration
   - Replicate number

   b. Label the appropriate graduated cylinder with the facility/sample name and concentration.

   c. Prepare the *Menidia beryllina* Chronic Toxicity Test Benchsheet (Exhibit T49.2). Record the following information on the Benchsheet:
   - Client/facility name
   - Permit number
   - County
   - Outfall/Pipe number
   - ETS project and sample numbers
   - Control/Dilution water type and batch
   - Test concentrations and dilution preparation information (sample, dilution and total volumes)

2. Weigh the microweight pans (This step may be completed at any time before test termination on day 7).

   a. Label the 20-ml glass beakers or Coors® spot plates with the facility or sample name, concentration, and replicate number.

   b. Obtain the microweight aluminum pans.

   c. Using forceps, place one microweight pan into each of the 20-ml glass beakers or each of the wells of the spot plates.

   d. Place the 20-ml glass beakers or spot plates in a drying oven and let the contents dry a minimum of 24-hours at 60 ± 2ºC or 6-hours at 100 ± 2ºC.

e. Remove the 20-ml glass beakers or spot plates from the oven and place in a desiccator a minimum of 4-hours to cool the pans before they are weighted on a calibrated microbalance.

f. Verify the accuracy of the microbalance as described in SOP-G10 Step B.

g. Using forceps, remove a microweight pan and place it on the balance pan. Allow the reading to stabilize and record the measurement on the chronic benchsheet. Record the date, beaker/spot plate color identification and analyst initials on the chronic benchsheet. Return the microweight pan to the appropriate 20-ml glass beaker or well on the spot plate.

h. Repeat Step 2.g. to obtain the initial weight of each pan needed for the test. After all the initial weights are obtained, place the 20-ml glass beakers or spot plates in a desiccator until needed on day 7.

B. Test Initiation (Day 0).

1. Prepare the test concentrations according to SOP-G5. It may be necessary to salt-up the sample prior to making the test concentrations. Refer to SOP-G5 for the appropriate procedures for salting-up samples.

   a. Measure and record the pH (SOP-C3), dissolved oxygen [SOP-C2, ensure that the dissolved is within the acceptable range (4.0 to 9.0 mg/L), aerate the sample if necessary according to SOP-G5] and salinity (SOP-C5) of each concentration tested and control. Measure and record the pH (SOP-C3), dissolved oxygen (SOP-C2), conductivity (SOP-C4), salinity (SOP-C5), total residual chlorine (SOP-C8), total alkalinity (SOP-C6) and sample characteristics of each new, full-strength, undiluted sample. Measure and record the alkalinity (SOP-C6) of the control/dilution water.

   b. Pour 500 ml of each test concentration into each of the labeled replicate beakers.

   c. Pour 500 ml of control water into each of the replicate control beakers.

   d. Maintain the test temperature (25.0 ± 1.0°C) of the test concentrations. This may be accomplished by placing the test beakers into a temperature-controlled incubator.

2. Isolate the larvae for the test.

   a. Obtain a batch of larvae (SOP-AT46), which are 9 to 11 days old at test initiation, with a maximum of 24-hour range in age. Record the source, age and hatch date and time of the organisms to be used in the test on the chronic benchsheet. Transfer the larvae from the jar to a large finger bowl.

   b. After the larvae have acclimated to the test conditions, the larvae may be transferred by transfer pipette to the test solutions. The end of the pipette tip should be cut to a size that will not injure or harm the larvae during transfer. Larvae should be transferred gently in a manner that will not expose the organisms to the air.

   c. Two techniques may be used for transferring 10 organisms to each test beaker from the finger bowl. The technique used is dependent on the sample being evaluated for toxicity.

      - If the transferred water may alter the toxicity of the sample (decrease toxicity), organisms should be transferred directly by pipette. This technique is predominately used by the laboratory. Organisms should be transferred in a manner that allows them to swim from the pipette into the test solutions. This will minimize the volume of transfer water introduced into the sample. Follow procedures outlined in step 3 for transferring the organisms to the randomly placed test cups. The average transfer volume by this technique for each analyst must be determined yearly. For an example transfer volume logsheet refer to Exhibit AT49.3.

      - If pathogenic interferences have been identified or there is risk of cross contamination between replicates, organisms should be transferred to medicine cups (10 per cup) and then introduced into the test solutions. The final volume contained in the medicine cups after the organisms have been transferred should be between 10 and 15 ml. With a transfer pipette, remove any food or debris that was placed in the cup during transfer. The average transfer volume by this technique for each analyst must be determined yearly. For an example transfer volume logsheet refer to Exhibit AT49.3. Continue this process until enough medicine cups containing 10 larvae each have been obtained to initiate the test. 1 medicine cup containing 10 larvae will be needed for each sample concentration and replicate. If a test consists of 5 concentrations and a control, 24 medicine cups containing 10 larvae each will be required. If a test consists of 1 concentration and a control, 8 medicine cups containing 10 larvae each will be required.

d. Save approximately 30 ml of transfer water to be measured for pH (SOP-C3). Measure and record the transfer water pH and temperature on the chronic benchsheet.

3. Transfer the larvae to the randomly placed test cups.

a. Obtain a randomization template (Exhibit AT49.4). Order the test beakers according to the randomization template and record the template name on the benchsheet.

b. Measure and record the temperature in one of the test beaker for each concentration and control. The temperature must be 25.0 ± 1.0°C before the test organisms are placed in the test beakers. Warm the test beakers in a warm water bath or temperature-controlled incubator, if necessary, until the desired test temperature is obtained. Measure and record the temperature of an arbitrary transfer cup.

c. Place 10 larvae in the first test beaker of the first row (by pipette or medicine cup). Continue in this manner (placing the larvae in the test beakers from left to right in the first row and then the second row) until all the test beakers contain 10 larvae.

d. Record the initiation date, time and analyst's initials on the chronic benchsheet. Record the average transfer volume by the technique used on the chronic benchsheet. **The test must be initiated within 36-hours of completion of the first sampling period.**

e. Verify that each beaker received the required number of larvae (i.e., 10) by conducting a repeat count. Remove excess larvae or add larvae as necessary. Record the initial number of larvae on the benchsheet.

f. Place the test beakers in order according to the randomization template in a temperature-controlled incubator and cover with a Plexiglas® slide. The organisms must be maintained at 25.0 ± 1.0°C with a photoperiod of 16-hours light and 8-hours dark and a recommended light intensity of 50 to 100 ft-c. Record the incubator number used on the benchsheet.

g. Using a transfer pipette, feed the larvae in each test cup 3 drops (150 µl) newly hatched brine shrimp (1050 to1500 shrimp). To obtain the appropriate suspension of brine shrimp, refer to SOP-AT16. [Note: The test larvae are fed twice daily at a 6 ± 1-hour interval (generally at the beginning and at the end of the workday).] Record the time(s) the larvae were fed on the *Menidia beryllina* Chronic Toxicity Test Benchsheet.

Note: Since the larvae are fed in holding prior to test initiation, the larvae may be fed only once in the test cups on the first day.

C. Daily Test Renewal (Days 1-6).

Repeat this process each day during the test period. The test must be renewed within ± 2 hours from test initiation. **When new samples are used for test solution renewal, the test must be renewed within 36-hours of completion of the first sampling period for each new sample.**

1. Prior to renewal of the test water in the beakers, carefully pour ~30 ml of test water from at least one replicate beaker for each test concentration and control into a labeled 1-oz medicine cup. This water will be used to determine final pH, dissolved oxygen and salinity.

2. Feed the larvae in the test beakers 150 µl of newly-hatched brine shrimp a minimum of 2-hours prior to renewal of the test concentrations. Record the feeding time on the *Menidia beryllina* Chronic Toxicity Test Benchsheet.

3. Measure and record the temperature in an arbitrarily selected test replicate for each concentration and control.

4. Prepare fresh test water in accordance with SOP-G5. Maintain the test temperature (25.0 ± 1.0°C) of the fresh test water until needed by storing in a temperature-controlled incubator.

5. Remove the test beakers from the incubator. Place the beakers on a light box or table for ease of viewing.

6. Change the test water in all four replicate beakers before starting the next four-beaker series. To change the test water, test beakers may be either siphoned or decanted.

   a. Siphoning method: Siphon off old water, excess shrimp and detritus from the cups using rubber tubing affixed with a plastic tube fitted with a soft, angled rubber tip. Slowly siphon the water from the cup into a white plastic photographic tray until ~ 50 ml of old test water remains. Control the flow through the tubing by holding one gloved finger over the end of the tubing.

   Decanting method: Using a transfer pipette, remove any debris, dead artemia and dead larvae that may have accumulated on the bottom of the test beaker. Carefully decant the water from the cup into a white plastic photographic tray until ~ 50 ml of old test water remains. This technique is predominately used by the laboratory.

b. If any larvae are accidentally siphoned off or decanted with the water, retrieve them from the plastic tray, using a transfer pipette. The end of the transfer pipette tip should be cut to a size that will not injure or harm the larvae during transfer. Larvae should be transferred gently in a manner that will not expose the organisms to the air. Return the larvae to the appropriate replicate beaker. Record the number of larvae siphoned out or decanted (per replicate). Discard any dead larvae.

c. Record the following information on the chronic benchsheet.
- Number of larvae surviving in each replicate beaker
- Number of dead larvae in each replicate beaker (if applicable)
- Any comments (injured, sick or larvae siphoned out)

d. Fill each replicate beaker to 500 ml using fresh test water. Pour the test water down the side of the beaker to avoid unnecessarily disturbing the larvae.

h. After all of the test beakers have been renewed, record the renewal time and the analyst's initials on the chronic benchsheet.

i. Place the test cups in order according to the randomization template in a temperature-controlled incubator and cover with a Plexiglas® slide.

7. At 6 ± 1-hour after the first feeding, feed the test larvae 3 drops (150 µl) of newly-hatched brine shrimp. Record the feeding time on the chronic benchsheet.

Note: Test solutions may be renewed prior to the first feeding.

D. Test Termination (Day 7, not to exceed 7 days + 2 hours).

Terminate the test after the organisms have been exposed to the test concentrations for 7 consecutive days ± 2-hours.

1. Measure and record the temperature in an arbitrarily selected test beaker for each concentration and control.

2. Remove the test beakers from the incubator. Place the beakers on a light box or table for ease of viewing.

3. Carefully pour ~30 ml of test water from at least one replicate beaker for each test concentration and control into a labeled 1-oz medicine cup. This water will be used to determine final pH, dissolved oxygen and salinity.

4. Obtain the appropriately labeled 20-ml glass beakers or spot plates containing pre-weighed microweight pans.

5. Fill a 600-ml beaker or equivalent with ice water and obtain a fine mesh sieve with a handle.

6. Beginning with the first replicate beaker of the control.

    a. Count and record (in the appropriate section) the number of living and dead larvae in each replicate beaker on the chronic benchsheet. Record comments, if applicable. Discard any dead larvae.

    b. Holding the mesh sieve over an empty beaker, pour the test water containing the larvae from one replicate beaker through the sieve. The larvae will be retained on the mesh.

    c. Immediately submerge the sieve containing the larvae into the ice water. Keep the larvae in the ice water for 3 to 4 seconds.

    d. Remove the sieve from the ice water. Carefully rinse the larvae with deionized water to remove any excess food or detritus.

    e. Using forceps, remove the microweight pan from the appropriate 20-ml glass beaker or well on the spot plate. Using the forceps, transfer the larvae from the mesh to the microweight pan. In the process, to ensure the larvae are dead, sever their spinal cords with forceps. Ensure that all the larvae have been transferred to the microweight pan. Verify against the number recorded in Step 6.a. above.

    f. Return the pan to the appropriate 20-ml glass beaker or well on the spot plate.

    g. Repeat Step 6 for the remaining test beakers for each test concentration (from lowest to highest).

7. Place the 20-ml glass beakers or spot plates in a drying oven and let the contents dry a minimum of 24-hours at 60 ± 2°C or 6-hours at 100 ± 2°C. Yearly laboratory studies have confirmed that drying the larvae longer than the recommended time will not alter the final dry weight.

8. Remove the 20-ml glass beakers or spot plates from the oven and place in a desiccator a minimum of 4-hours to cool the larvae before weighing them on a calibrated microbalance.

9. Measure the final pan weights.

a. Verify the accuracy of the microbalance as described in SOP-G10 Step B.

b. Using forceps, remove the microweight pan from the 20-ml glass beaker or well on the spot plate and place it on the balance pan. Allow the reading to stabilize and record the measurement on the chronic benchsheet. Return the microweight pan to the 20-ml glass beaker or well on the spot plate. Record the date the weights were measured and analyst initials on the chronic benchsheet.

c. Repeat Step 9.b. to obtain the final weight of each remaining pan. After all the final weights are obtained, return the 20-ml glass beakers or spot plates to a desiccator until the survival and weight data have been verified.

E. Acceptance Criteria.

The test acceptance criteria are indicated in the table below. In general, the most stringent acceptability criteria are used by the laboratory.

Table AT49.1: *Menidia beryllina* chronic toxicity test acceptability criteria.

Test Acceptability Criteria	USEPA
Control survival	≥ 80%
Mean dry weight of surviving control larvae (mg)	≥ 0.50
Guidance control growth coefficient of variation	< 18%
Guidance percent minimum significant difference (PMSD)	11 – 28%

F. Statistical Analyses and Test Data Verification.

Statistical analyses and data review is performed according to QAP-Q12.

G. Exhibits.

Exhibit AT49.1: Summary of Test Conditions for the *Menidia beryllina* Chronic Toxicity Test.
Exhibit AT49.2: *Menidia beryllina* Chronic Toxicity Test Benchsheet.
Exhibit AT49.3: Average Transfer Volume Logsheet.
Exhibit AT49.4: Randomization Templates.

Subject: *Menidia beryllina* Chronic Toxicity Test, EPA 1006.0

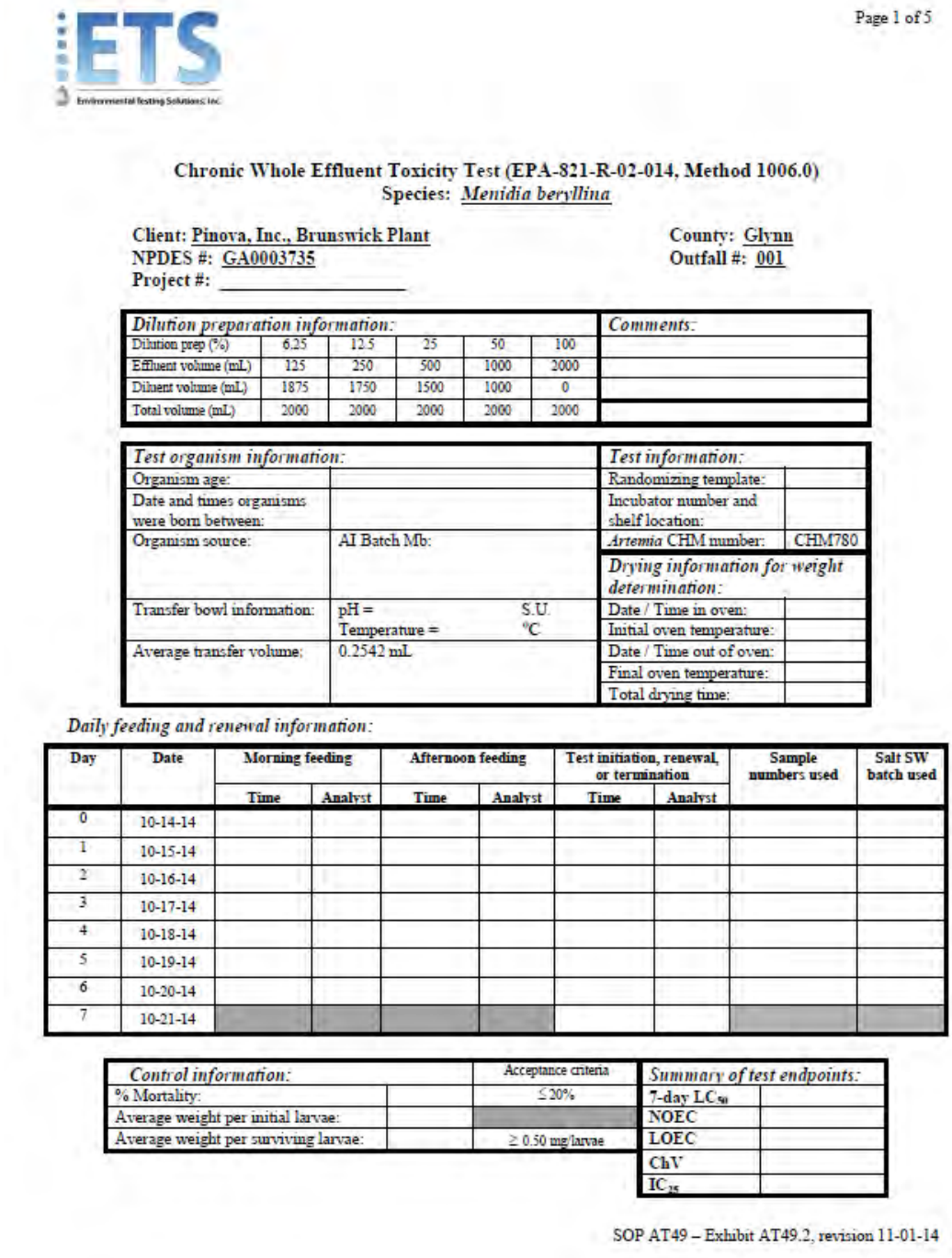
Exhibit AT49.1: Summary of Test Conditions for the *Menidia beryllina* Chronic Toxicity Test.

SUMMARY OF TEST CONDITIONS FOR THE *MENIDIA BERYLLINA* CHRONIC TOXICITY TEST

Test type:	Static renewal
Test duration:	7-days
Temperature:	25.0 ± 1.0°C
Light quality:	Cool-white fluorescent
Light intensity:	50 – 100 ft-candles (recommended)
Photoperiod:	16-hours light, 8-hours dark
Test chamber size:	600 mL glass beakers
Test solution volume:	500 mL
Renewal of test solutions:	Daily
Age of test organisms:	9 to 11-days with ≤ 24 hour range in age.
Number of organisms per test chamber:	10
Number of replicate test chambers per concentration:	4
Number of organisms per concentration:	40
Test concentrations:	Multiple concentration tests: 5 and a control with ≥ 0.5 dilution series (recommended) Single dilution tests: At chronic permit limit and a control.
Test chamber cleaning:	Daily, test chambers are cleaned immediately before test solution renewal.
Aeration:	None, unless the dissolved oxygen concentration falls below 4.0 mg/L.
Feeding regime:	On days 0 through 6, organisms in each test cup are fed 150 µL *Artemia nauplii* twice daily at 6-hour intervals.
Control / Dilution water:	Salt synthetic water (25.0 ± 2.0 ppt)
Sampling and sample holding:	3-gallon grab or composite samples collected on days one, three and five. Each sample must first be used within 36-hours of completion of each sampling period.
Endpoint:	Survival and growth (dry weight per initial number of larvae)
Test acceptability criterion:	≥ 80% control survival, control growth ≥ 0.50 mg/surviving larvae

Subject: *Menidia beryllina* Chronic Toxicity Test, EPA 1006.0

Exhibit AT49.2: *Menidia beryllina* Chronic Toxicity Test Benchsheet.



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Chronic Whole Effluent Toxicity Test (EPA-821-R-02-014, Method 1006.0)
Species: *Menidia beryllina*

Client: Pinova, Inc., Brunswick Plant **County: Glynn**
NPDES #: GA0003735 **Outfall #: 001**
Project #: _____

Dilution preparation information:						*Comments:*
Dilution prep (%)	6.25	12.5	25	50	100	
Effluent volume (mL)	125	250	500	1000	2000	
Dilnent volume (mL)	1875	1750	1500	1000	0	
Total volume (mL)	2000	2000	2000	2000	2000	

Test organism information:			*Test information:*	
Organism age:			Randomizing template:	
Date and times organisms were born between:			Incubator number and shelf location:	
Organism source:	AI Batch Mb:		*Artemia* CHM number:	CHM780
			Drying information for weight determination:	
Transfer bowl information:	pH =	S.U.	Date / Time in oven:	
	Temperature =	°C	Initial oven temperature:	
Average transfer volume:	0.2542 mL		Date / Time out of oven:	
			Final oven temperature:	
			Total drying time:	

Daily feeding and renewal information:

Day	Date	Morning feeding		Afternoon feeding		Test initiation, renewal, or termination		Sample numbers used	Salt SW batch used
		Time	Analyst	Time	Analyst	Time	Analyst		
0	10-14-14								
1	10-15-14								
2	10-16-14								
3	10-17-14								
4	10-18-14								
5	10-19-14								
6	10-20-14								
7	10-21-14								

Control information:		Acceptance criteria	*Summary of test endpoints:*	
% Mortality:		≤ 20%	7-day LC_{50}	
Average weight per initial larvae:			NOEC	
Average weight per surviving larvae:		≥ 0.50 mg/larvae	LOEC	
			ChV	
			IC_{25}	

SOP AT49 – Exhibit AT49.2, revision 11-01-14

Subject: *Menidia beryllina* Chronic Toxicity Test, EPA 1006.0

Species: *Menidia beryllina*
Client: Pinova, Inc.

Date: 10-14-14

Survival and Growth Data

Day	CONTROL				6.25%				12.5%			
	A	B	C	D	E	F	G	H	I	J	K	L
0	10	10	10	10	10	10	10	10	10	10	10	10
1												
2												
3												
4												
5												
6												
7												
A = Pan weight (mg) Tray color code:: _____ Analyst: _____ Date: _____												
B = Pan + Larvae weight (mg) Analyst: _____ Date: _____												
C = Larvae weight (mg) = B – A Hand calculated. Analyst: _____												
Weight per initial number of larvae (mg) = C / Initial number of larvae Hand calculated. Analyst: _____												
Average weight per initial number of larvae (mg) / Percent reduction from control (%)												

Comment codes: c = clear, d = dead, fg = fungus, k = killed, m = missing, sk = sick, sm = unusually small, lg = unusually large, d&r = decanted and returned, w = wounded.

Comments:

SOP AT49 – Exhibit AT49.2, revision 11-01-14

Subject: *Menidia beryllina* Chronic Toxicity Test, EPA 1006.0

Species: *Menidia beryllina*
Client: Pinova, Inc.

Date: 10-14-14

Survival and Growth Data

Day	25%				50%				100%			
	M	N	O	P	Q	R	S	T	U	V	W	X
0	10	10	10	10	10	10	10	10	10	10	10	10
1												
2												
3												
4												
5												
6												
7												
A = Pan weight (mg) Tray color code:: _____ Analyst: _____ Date: _____												
B = Pan + Larvae weight (mg) Analyst: _____ Date: _____												
C = Larvae weight (mg) = B – A Hand calculated. Analyst: _____												
Weight per initial number of larvae (mg) = C / Initial number of larvae Hand calculated. Analyst: _____												
Average weight per initial number of larvae (mg) / Percent reduction from control (%)												

Comment codes: c = clear, d = dead, fg = fungus, k = killed, m = missing, sk = sick, sm = unusually small, lg = unusually large, d&r = decanted and returned, w = wounded.

Comments:

Subject: *Menidia beryllina* Chronic Toxicity Test, EPA 1006.0

Species: *Menidia beryllina*
Client: Pinova, Inc.

Date: 10-14-14

Daily Chemistry:

		Day (Analyst identified for each day, performed pH, D.O. and salinity measurements only.)					
		0		1		2	
	Analyst						
Concentration	**Parameter**						
CONTROL	**pH** (S.U.)						
	DO (mg/L)						
	Salinity (ppt)						
	***Alkalinity** (mg $CaCO_3$/L)						
	***Temperature** (°C)						
6.25%	**pH** (S.U.)						
	DO (mg/L)						
	Salinity (ppt)						
	***Temperature** (°C)						
12.5%	**pH** (S.U.)						
	DO (mg/L)						
	Salinity (ppt)						
	***Temperature** (°C)						
25%	**pH** (S.U.)						
	DO (mg/L)						
	Salinity (ppt)						
	***Temperature** (°C)						
50%	**pH** (S.U.)						
	DO (mg/L)						
	Salinity (ppt)						
	***Temperature** (°C)						
100%	**pH** (S.U.)						
	DO (mg/L)						
	Salinity (ppt)						
	***Temperature** (°C)						
100% (Not salted-up)	**pH** (S.U.)						
	DO (mg/L)						
	Conductivity (µmhos/cm)						
	Salinity (ppt)						
	***Alkalinity** (mg $CaCO_3$/L)						
	***TR chlorine** (mg/L)						
	***Temperature** (°C)						
		Initial	**Final**	**Initial**	**Final**	**Initial**	**Final**

*Temperatures performed at the time of test initiation, renewal or termination by the analyst identified in the Daily Renewal Information table located on Page 1. Alkalinity and total residual chlorine performed by the analyst identified on the bench sheet specific for each analysis and transcribed to this bench sheet by: _____.

Subject: *Menidia beryllina* Chronic Toxicity Test, EPA 1006.0

Species: *Menidia beryllina*
Client: Pinova, Inc.

Date: 10-14-14

Conc.	Parameter	Day 3 Initial	Day 3 Final	Day 4 Initial	Day 4 Final	Day 5 Initial	Day 5 Final	Day 6 Initial	Day 6 Final
	Analyst								
CONTROL	pH (S.U.)								
	DO (mg/L)								
	Salinity (ppt)								
	*Alkalinity (mg $CaCO_3$/L)								
	*Temperature (°C)								
6.25%	pH (S.U.)								
	DO (mg/L)								
	Salinity (ppt)								
	*Temperature (°C)								
12.5%	pH (S.U.)								
	DO (mg/L)								
	Salinity (ppt)								
	*Temperature (°C)								
25%	pH (S.U.)								
	DO (mg/L)								
	Salinity (ppt)								
	*Temperature (°C)								
50%	pH (S.U.)								
	DO (mg/L)								
	Salinity (ppt)								
	*Temperature (°C)								
100%	pH (S.U.)								
	DO (mg/L)								
	Salinity (ppt)								
	*Temperature (°C)								
100% (Not salted-up)	pH (S.U.)								
	DO (mg/L)								
	Conductivity (µmhos/cm)								
	Salinity (ppt)								
	*Alkalinity (mg $CaCO_3$/L)								
	*TR chlorine (mg/L)								
	*Temperature (°C)								

*Temperatures performed at the time of test initiation, renewal or termination by the analyst identified in the Daily Renewal Information table located on Page 1. Alkalinity and total residual chlorine performed by the analyst identified on the bench sheet specific for each analysis and transcribed to this bench sheet by: _____.

SOP AT49 – Exhibit AT49.2, revision 11-01-14

Subject: *Menidia beryllina* Chronic Toxicity Test, EPA 1006.0

Exhibit AT49.3: Average Transfer Volume by Medicine Cup for each Analyst.

***Pimephales promelas* are used as surrogate organisms to determine the average transfer volume.**

ETS Environmental Testing Solutions, Inc. Page 1 of 1

Larval Fish Transfer Volume

Analyst: J. Sumner | Species: *P. promelas*

Date: 02-12-14 | Source / Batch: ATOX Batch Pp 02-03-14

Ambient temperature: 24.1°C | Wet Weight of 10 Larvae (g): 0.0209

Estimate transfer volume, where minnows are allowed to swim from the pipette into the test vessel.

Numerically label 10 medicine cups.
Add 10 mL MHSW to each of the 10 cups.
Measure and record the weight of each cup containing MHSW.
Transfer 10 larvae to each cup, following procedures identified in SOP-AT18, AT47, or AT53 for vertebrate acute toxicity tests.
Transfer the larvae in a manner that allows them to swim from the pipette into the MHSW contained in each cup.
Measure and record the weight of each cup containing MHSW with 10 larvae.
Determine each transfer volume and average transfer volume.

Replicate Number	Initial Weight Medicine cup + 10 mL MHSW (g)	Final Weight Medicine cup + 10 mL MHSW + 10 Larval Fish transferred (g)	Transfer Volume Final - Initial Weight (g = mL)
1	11.3533	11.6989	**0.3456**
2	11.3337	11.4850	**0.1513**
3	11.3607	11.6976	**0.3369**
4	11.2950	11.5662	**0.2712**
5	11.2945	11.5634	**0.2689**
6	11.3349	11.3360	**0.0011**
7	11.3539	11.7630	**0.4091**
8	11.3883	11.7335	**0.3452**
9	11.2812	11.4930	**0.2118**
10	11.3321	11.5329	**0.2008**
	Average volume to transfer 10 organisms (mL):		**0.2542**

Estimate transfer volume, where the minnows are transferred with MHSW into the test vessels.

Numerically label 10 medicine cups.
Measure and record the weight of each cup.
Add approximately 10 mL MHSW to each of the 10 cups.
Transfer 10 larvae to each cup, following procedures identified in SOP-AT18, AT47, or AT53 for vertebrate acute toxicity tests.
Transfer the larvae in a manner that allows them to swim from the pipette into the MHSW contained in each cup.
Measure and record the weight of each cup containing MHSW with 10 larvae.
Determine each transfer volume and average transfer volume.

Replicate Number	Initial Weight Medicine cup (g)	Final Weight Medicine cup + 10 mL MHSW + 10 Larval Fish transferred (g)	Transfer Volume Final - Initial Weight (g = mL)
1	1.7092	11.6989	**9.9897**
2	1.6929	11.4850	**9.7921**
3	1.7092	11.6976	**9.9884**
4	1.6728	11.5662	**9.8934**
5	1.6967	11.5634	**9.8667**
6	1.6740	11.3360	**9.6620**
7	1.7015	11.7630	**10.0615**
8	1.6870	11.7335	**10.0465**
9	1.7009	11.4930	**9.7921**
10	1.7152	11.5329	**9.8177**
	Average volume to transfer 10 organisms (mL):		**9.8910**

SOP AT18 - Exhibit AT18.5, SOP AT47 - Exhibit AT47.5, SOP AT53 - Exhibit AT53.5, revision 11-01-14

Exhibit AT49.4: Randomization Templates.

BLUE

Replicate number	Concentrations
1	1 7 4 3 6 2 5
2	7 3 2 5 4 1 6
3	3 4 6 5 2 1 7
4	5 6 1 2 4 7 3

Random number seeds 10 through 13.

Subject: *Menidia beryllina* Chronic Reference Toxicity Test, EPA 1006.0

Document Revision History

Revision Date	Surveillance number	Surveillance Type	Evaluators	Revisions
12-01-00				Original document
06-01-11	Not applicable.	Internal	Jim Sumner (ETS)	• Updated references and exhibits.
11-01-14	Not applicable.	Internal	Jim Sumner (ETS)	• Updated references and exhibits during document review.

Subject: *Menidia beryllina* Chronic Reference Toxicity Test, EPA 1006.0

Purpose

To assess the sensitivity of inland silverside larvae (*Menidia beryllina*) and the overall credibility of the inland silverside chronic toxicity test. Reference toxicant data are part of the laboratory's QA/QC program to evaluate the performance of laboratory personnel and the robustness and sensitivity of the test organisms.

References

USEPA. October 2002. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms, 3rd ed. **EPA-821-R-02-014, Method 1006.0**. US Environmental Protection Agency, Cincinnati, OH.

USEPA. 2000. Understanding and Accounting for Method Variability in Whole Effluent Toxicity Applications Under the National Pollutant Discharge Elimination Program. EPA-833-R-00-003. US Environmental Protection Agency, Cincinnati, OH.

USEPA. 2001. Final Report: Inter-laboratory Variability Study of EPA Short-term Chronic and Acute Whole Effluent Toxicity Test Methods, Volumes 1 and 2 - Appendix. EPA-821-B-01-004 and EPA-821-B-01-005. US Environmental Protection Agency, Cincinnati, OH.

North Carolina Department of Environment and Natural Resources, Division of Water Quality. Biological Laboratory Certification / Criteria Procedures, Version 3.0. December 2010.

Definitions

Acceptance Criteria: Specified limits placed on characteristics of an item, process, or service defined in codes, standards, or other required documents.

Precision: The extent to which measurement results repeat themselves when repeat measurements are made on the same unit of product.

Equipment and Materials

Inland silverside larvae (*Menidia beryllina*)
Temperature-controlled incubator (set to maintain test temperature = 25.0 ± 1.0ºC, photoperiod of 16-hour light / 8-hours dark, light intensity = 50 – 100 ft-c)
Control water (salt synthetic water made with Bioassay Grade 40-Fathoms Sea Salt, MarineMix®)
Microbalance accurate to 0.00001 mg (e.g. Cahn)
Class S or Class I certified weights
Microweight aluminum pans (e.g. Cahn)
Drying oven
Desiccator

Scintillation vials
Plastic tray
600-ml glass or plastic beakers
Graduated cylinders
Large glass finger bowls
Serological, fixed, or adjustable volume pipette (e.g., Eppendorf)
Transfer pipettes
Siphoning hose (rubber tubing affixed with a plastic tube fitted with a soft, angled rubber tip)
White plastic photographic tray
Fine mesh sieve
Forceps
Ice water
Aquarium pump, tubing, and air stones
Plexiglas® slides
Thermometer
1-oz medicine cups
Newly hatched brine shrimp
Light box or table
Disposable gloves
Menidia beryllina Shipment Log and Organism History Information Sheet
Copper sulfate ($CuSO_4$, reagent grade)
500-ml volumetric flask
2000-ml graduated cylinder
1 and 10-ml serological pipettes
Menidia beryllina Chronic Reference Toxicity Test Benchsheet
Randomization template

Procedure

A. Frequency of Testing and Requirements.

1. A *Menidia beryllina* chronic reference toxicant test must be performed on each batch of organisms obtained from a supplier and used for chronic whole effluent toxicity tests. At a minimum, *Menidia beryllina* chronic reference toxicant tests must be performed quarterly to meet certification requirements.

B. Test Preparation.

1. Prepare the glassware.

   a. Obtain four replicate 600-ml glass beakers for each of the five Cu concentrations tested and the control. Label each replicate beaker with the following information.

- Concentration
- Replicate number

b. Label the appropriate graduated cylinder.

c. Prepare the *Menidia beryllina* Chronic Reference Toxicity Test Benchsheet (see Exhibit AT50.1). Record the *Menidia beryllina* Cu Chronic (MbCuCR) test number on the benchsheet.

C. Preparation of the Stock Solution.

1. Using a calibrated top-loading balance, carefully weigh out 0.1965 g of $CuSO_4$ (SOP-G10). Place approximately 400 ml of Milli-Q water in a 500-ml volumetric flask. Add the $CuSO_4$ to the flask, dissolve the $CuSO_4$ by swirling the flask; bring to volume with Milli-Q water. Label the volumetric flask with the concentration (100 mg Cu/L), analyst's initials, preparation date and stock standard number (INSS, SOP-G15). Record the INSS number of the Cu stock solution on the benchsheet.

D. Preparation of the Test Concentrations.

1. Prepare all test concentrations using graduated cylinders and serological pipettes. The precision of conductivity measurements is increased when smaller volume graduated cylinders and/or serological pipettes are used to prepare the test concentrations. For this reference toxicant test, stock solution volumes should be measured using 1 and 10-ml serological pipettes and the total volumes should be measured using a 2000-ml graduated cylinder.

2. Beginning with the lowest concentration, add approximately 500 ml of salt synthetic water to a 2000-ml graduated cylinder, add the required volume of stock solution using a 1 and/or 10-ml serological pipettes (refer to Table AT50.1), bring to volume (2000 ml) with salt synthetic water. Mix the solution well by pouring the solution into the respective 2000 ml Erlenmeyer flask and swirling the solution in the flask.

3. Pour 500 ml of test solution into each of the replicate test beakers for that concentration. Pour 30 ml of test solution into a 1-oz medicine cup for chemical analyses. For each concentration, measure and record salinity (SOP-C5), pH (SOP-C3), and dissolved oxygen (SOP-C2).

4. Rinse the graduated cylinder well with deionized water and repeat steps C.2 through C.4 for preparing the next test concentration. Record the batch date of salt synthetic water used to prepare the dilutions on the benchsheet.

Table AT50.1: Test concentration, stock volumes, salt synthetic water volumes, and final volumes for the *Menidia beryllina* Cu chronic reference toxicant tests.

Test Concentration (mg Cu/L)	Volume of Stock Required (ml)	Volume of Salt Synthetic Water (ml)	Final Volume (ml)
0.025	0.5	1999.5	2000
0.050	1.0	1999.0	2000
0.100	2.0	1998.0	2000
0.200	4.0	1996.0	2000
0.500	10.0	1990.0	2000
100 (Stock)	NA	NA	NA

5. Once all test concentrations have been prepared, follow the procedure described in SOP-AT49 for conducting *Menidia beryllina* Chronic Toxicity Tests.

E. Preparation of Control Charts.

1. Refer to QAP-Q5 for how control charts are prepared and procedures for interpreting outlier tests. Refer to Exhibit AT50.2 for an example control chart.

F. Exhibits.

Exhibit AT50.1: *Menidia beryllina* Chronic Reference Toxicity Test Benchsheet.
Exhibit AT50.2: Example of a *Menidia beryllina* Chronic Reference Toxicant Control Chart.

Subject: *Menidia beryllina* Chronic Reference Toxicity Test, EPA 1006.0

Exhibit AT50.1: *Menidia beryllina* Chronic Reference Toxicity Test Benchsheet.

Copper Sulfate Chronic Reference Toxicant Test
(EPA-821-R-02-014, Method 1006.0)
Species: *Menidia beryllina*

MbCuCR Test Number: **102**

Dilution preparation information:						*Comments:*
Cu Stock INSS number:		INSS				
Stock preparation:		100 mg Cu/L: Dissolve 0.1965 g $CuSO_4$ in 500-mL Milli-Q water				
Dilution prep (mg/L)	0.025	0.050	0.100	0.200	0.500	
Stock volume (mL)	0.5	1.0	2.0	4.0	10.0	
Dilluent volume (mL)	1999.5	1999.0	1998.0	1996.0	1990.0	
Total volume (mL)	2000	2000	2000	2000	2000	

Test organism information:		*Test information:*	
Organism age:		Randomizing template:	
Date and times organisms were born between:		Incubator number and shelf location:	
Organism source:	AI Batch Mb:	*Artemia* CHM number:	CHM780
		Drying information for weight determination:	
Transfer bowl information:	pH = S.U. Temperature = °C	Date / Time in oven:	
		Initial oven temperature:	
Average transfer volume:	0.2542 mL	Date / Time out of oven:	
		Final oven temperature:	
		Total drying time:	

Daily feeding and renewal information:

Day	Date	Morning feeding		Afternoon feeding		Test initiation, renewal, or termination		Salt SW batch used
		Time	Analyst	Time	Analyst	Time	Analyst	
0	10-14-14							
1	10-15-14							
2	10-16-14							
3	10-17-14							
4	10-18-14							
5	10-19-14							
6	10-20-14							
7	10-21-14							

Control information:		Acceptance criteria	*Summary of test endpoints:*	
% Mortality:		≤ 20%	7-day LC_{50}	
Average weight per initial larvae:			NOEC	
Average weight per surviving larvae:		≥ 0.50 mg/larvae	LOEC	
			ChV	
			IC_{25}	

Subject: *Menidia beryllina* Chronic Reference Toxicity Test, EPA 1006.0

Species: *Menidia beryllina* MbCuCR Test Number: 102

Survival and Growth Data

Day	CONTROL				0.025 mg Cu/L				0.050 mg Cu/L			
	A	B	C	D	E	F	G	H	I	J	K	L
0	10	10	10	10	10	10	10	10	10	10	10	10
1												
2												
3												
4												
5												
6												
7												
A = Pan weight (mg) Tray color code:: _____ Analyst: _____ Date: _____												
B = Pan + Larvae weight (mg) Analyst: _____ Date: _____												
C = Larvae weight (mg) = B – A Hand calculated. Analyst: _____												
Weight per initial number of larvae (mg) = C / Initial number of larvae Hand calculated. Analyst: _____												
Average weight per initial number of larvae (mg) / Percent reduction from control (%)												

Comment codes: c = clear, d = dead, fg = fungus, k = killed, m = missing, sk = sick, sm = unusually small, lg = unusually large, d&r = decanted and returned, w = wounded.

Comments:

SOP AT50 – Exhibit AT50.1, revision 11-01-14

Subject: *Menidia beryllina* Chronic Reference Toxicity Test, EPA 1006.0

Species: *Menidia beryllina* MbCuCR Test Number: 102

Survival and Growth Data

Day	0.100 mg Cu/L				0.200 mg Cu/L				0.500 mg Cu/L			
	M	N	O	P	Q	R	S	T	U	V	W	X
0	10	10	10	10	10	10	10	10	10	10	10	10
1												
2												
3												
4												
5												
6												
7												
A = Pan weight (mg) Tray color code:: _____ Analyst: _____ Date: _____												
B = Pan + Larvae weight (mg) Analyst: _____ Date: _____												
C = Larvae weight (mg) = B – A Hand calculated. Analyst: _____												
Weight per initial number of larvae (mg) = C / Initial number of larvae Hand calculated. Analyst: _____												
Average weight per initial number of larvae (mg) / Percent reduction from control (%)												

Comment codes: c = clear, d = dead, fg = fungus, k = killed, m = missing, sk = sick, sm = unusually small, lg = unusually large, d&r = decanted and returned, w = wounded.

Comments:

Species: *Menidia beryllina* MbCuCR Test Number: 102

Daily Chemistry:

		Day (Analyst identified for each day, performed pH, D.O. and salinity measurements only.)					
		0		1		2	
	Analyst						
Conc.	Parameter						
CONTROL	pH (S.U.)						
	DO (mg/L)						
	Salinity (ppt)						
	*Alkalinity (mg $CaCO_3$/L)						
	*Temperature (°C)						
0.025 mg Cu/L	pH (S.U.)						
	DO (mg/L)						
	Salinity (ppt)						
	*Temperature (°C)						
0.050 mg Cu/L	pH (S.U.)						
	DO (mg/L)						
	Salinity (ppt)						
	*Temperature (°C)						
0.100 mg Cu/L	pH (S.U.)						
	DO (mg/L)						
	Salinity (ppt)						
	*Temperature (°C)						
0.200 mg Cu/L	pH (S.U.)						
	DO (mg/L)						
	Salinity (ppt)						
	*Temperature (°C)						
0.500 mg Cu/L	pH (S.U.)						
	DO (mg/L)						
	Salinity (ppt)						
	*Temperature (°C)						
		Initial	Final	Initial	Final	Initial	Final

*Temperatures performed at the time of test initiation, renewal or termination by the analyst identified in the Daily Renewal Information table located on Page 1.
Alkalinity performed by the analyst identified on the bench sheet specific for this analysis and transcribed to this bench sheet by: _____.

Species: *Menidia beryllina* MbCuCR Test Number: 102

Conc.	Parameter	Day 3 Analyst		Day 4 Analyst		Day 5 Analyst		Day 6 Analyst	
		Initial	Final	Initial	Final	Initial	Final	Initial	Final
CONTROL	pH (S.U.)								
	DO (mg/L)								
	Salinity (ppt)								
	*Alkalinity (mg $CaCO_3$/L)								
	*Temperature (°C)								
0.025 mg Cu/L	pH (S.U.)								
	DO (mg/L)								
	Salinity (ppt)								
	*Temperature (°C)								
0.050 mg Cu/L	pH (S.U.)								
	DO (mg/L)								
	Salinity (ppt)								
	*Temperature (°C)								
0.100 mg Cu/L	pH (S.U.)								
	DO (mg/L)								
	Salinity (ppt)								
	*Temperature (°C)								
0.200 mg Cu/L	pH (S.U.)								
	DO (mg/L)								
	Salinity (ppt)								
	*Temperature (°C)								
0.500 mg Cu/L	pH (S.U.)								
	DO (mg/L)								
	Salinity (ppt)								
	*Temperature (°C)								

*Temperatures performed at the time of test initiation, renewal or termination by the analyst identified in the Daily Renewal Information table located on Page 1.
Alkalinity performed by the analyst identified on the bench sheet specific for this analysis and transcribed to this bench sheet by: _____.

Subject: *Menidia beryllina* Chronic Reference Toxicity Test, EPA 1006.0

Exhibit AT50.2: Example of a *Menidia beryllina* Chronic Reference Toxicant Control Chart.

Environmental Testing Solutions, Inc.

Menidia beryllina
Chronic Reference Toxicant Control Chart

Test number	Test date	7-day IC_{25} (mg/L Cu)	CT (mg/L Cu)	S	State and USEPA Control Limits CT - 2S	State and USEPA Control Limits CT + 2S	$S_{A.75}$	USEPA Warning Limits CT - $S_{A.75}$	USEPA Warning Limits CT + $S_{A.75}$	$S_{A.90}$	USEPA Control Limits CT - $S_{A.90}$	USEPA Control Limits CT + $S_{A.90}$	CV
1	10-08-11	0.180											
2	12-06-11	0.230	0.21	0.04	0.13	0.28	0.09	0.12	0.29	0.11	0.09	0.32	0.17
3	01-14-12	0.228	0.21	0.03	0.16	0.27	0.09	0.12	0.30	0.12	0.10	0.33	0.13
4	03-06-12	0.233	0.22	0.03	0.17	0.27	0.09	0.12	0.31	0.12	0.10	0.34	0.12
5	04-14-12	0.241	0.22	0.02	0.17	0.27	0.10	0.13	0.32	0.12	0.10	0.34	0.11
6	05-12-12	0.210	0.22	0.02	0.18	0.26	0.09	0.13	0.32	0.12	0.10	0.34	0.10
7	06-05-12	0.212	0.22	0.02	0.18	0.26	0.09	0.13	0.31	0.12	0.10	0.34	0.09
8	07-17-12	0.247	0.22	0.02	0.18	0.27	0.10	0.13	0.32	0.12	0.10	0.35	0.10
9	10-06-12	0.228	0.22	0.02	0.18	0.26	0.10	0.13	0.32	0.12	0.10	0.35	0.09
10	11-10-12	0.240	0.23	0.02	0.19	0.26	0.10	0.13	0.32	0.12	0.10	0.35	0.09
11	01-12-13	0.173	0.22	0.02	0.17	0.27	0.09	0.13	0.31	0.12	0.10	0.34	0.11
12	04-05-13	0.251	0.22	0.02	0.17	0.27	0.10	0.13	0.32	0.12	0.10	0.35	0.11
13	05-11-13	0.227	0.22	0.02	0.18	0.27	0.10	0.13	0.32	0.12	0.10	0.35	0.11
14	06-04-13	0.270	0.23	0.03	0.17	0.28	0.10	0.13	0.32	0.12	0.10	0.35	0.12
15	07-13-13	0.252	0.23	0.03	0.18	0.28	0.10	0.13	0.33	0.13	0.10	0.35	0.11
16	10-12-13	0.201	0.23	0.03	0.17	0.28	0.10	0.13	0.32	0.12	0.10	0.35	0.11
17	01-11-14	0.267	0.23	0.03	0.17	0.28	0.10	0.13	0.33	0.13	0.10	0.35	0.12
18	05-09-14	0.263	0.23	0.03	0.18	0.29	0.10	0.13	0.33	0.13	0.10	0.36	0.12
19	06-03-14	0.242	0.23	0.03	0.18	0.28	0.10	0.13	0.33	0.13	0.10	0.36	0.12
20	07-08-14	0.235	0.23	0.03	0.18	0.28	0.10	0.13	0.33	0.13	0.10	0.36	0.11

Note: **7-d IC_{25}** = 7-day 25% inhibition concentration. An estimation of the concentration of copper that would cause a 25% reduction in *Menidia* growth for the test population.

CT = Central tendency (mean IC_{25}).

S = Standard deviation of the IC_{25} values.

USEPA Control and Warning Limits

$S_{A.75}$ = Standard deviation corresponding to the the 75th percentile CV. ($S_{A.75}$ = 0.43)

$S_{A.90}$ = Standard deviation corresponding to the the 90th percentile CV. ($S_{A.90}$ = 0.55)

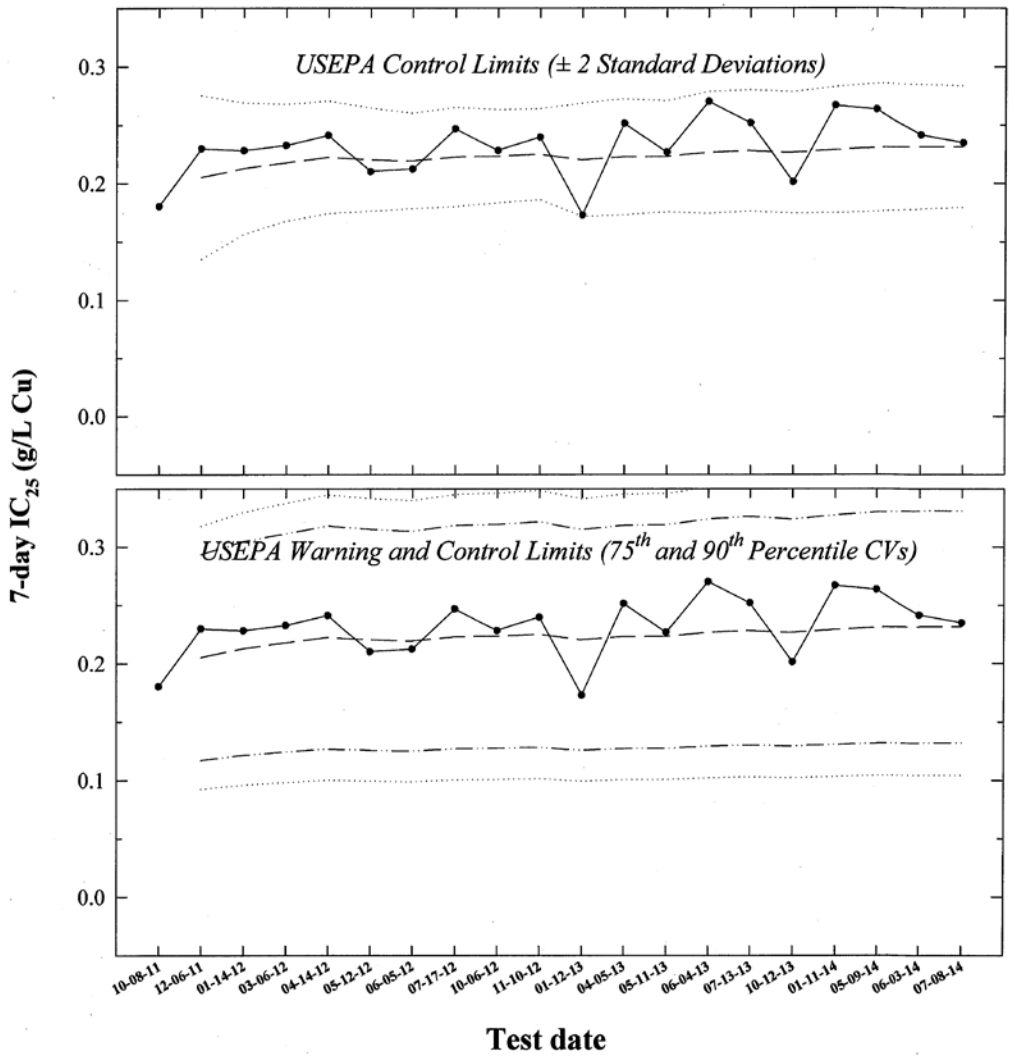
CV = Coefficient of variation of the IC_{25} values.

USEPA. 2000. Understanding and Accounting for Method Variability in Whole Effluent Toxicity Applications Under the National Pollutant Discharge Elimination Program. EPA-833-R-00-003. US Environmental Protection Agency, Cincinnati, OH.

Subject: *Menidia beryllina* Chronic Reference Toxicity Test, EPA 1006.0

Environmental Testing Solutions, Inc.

Menidia beryllina
Chronic Reference Toxicant Control Chart



7-day IC_{25} = 25% inhibition concentration. An estimation of the concentration of copper that would cause a 25% reduction in *Menidia* growth for the test population.

Central Tendency (mean IC_{25})

Warning Limits (mean $IC_{25} \pm S_{A.75}$)

Control Limits (mean $IC_{25} \pm S_{A.90}$ or 2 Standard Deviations)

Subject: *Menidia beryllina* Chronic Reference Toxicity Test, EPA 1006.0

Environmental Testing Solutions, Inc.

Precision of Endpoint Measurements

Menidia beryllina

Chronic Reference Toxicant Data

Test number	Test date	Control Survival (%)	Control Mean Growth (mg/larvae)	CT for Control Growth (mg/larvae)	CV (%)	CT for Control Growth CV (%)	MSD	PMSD (%)	CT for PMSD (%)
1	10-08-11	100.0	0.76		9.8		0.23	30.1	
2	12-06-11	100.0	1.03	0.90	15.6	12.7	0.17	16.4	23.2
3	01-14-12	100.0	1.79	1.20	8.9	11.4	0.16	8.9	18.5
4	03-06-12	100.0	2.46	1.51	4.3	9.6	0.30	12.3	16.9
5	04-14-12	100.0	2.11	1.63	10.2	9.7	0.40	18.9	17.3
6	05-12-12	100.0	1.58	1.62	13.6	10.4	0.32	20.1	17.8
7	06-05-12	100.0	1.76	1.64	1.4	9.1	0.33	18.9	17.9
8	07-17-12	100.0	1.62	1.64	20.2	10.5	0.35	21.4	18.4
9	10-06-12	100.0	1.67	1.64	4.8	9.8	0.29	17.6	18.3
10	11-10-12	100.0	1.53	1.63	9.0	9.8	0.27	17.7	18.2
11	01-12-13	100.0	1.22	1.59	13.2	10.1	0.25	20.4	18.4
12	04-05-13	100.0	1.27	1.57	8.9	10.0	0.17	13.0	18.0
13	05-11-13	100.0	1.11	1.53	6.3	9.7	0.18	16.2	17.9
14	06-04-13	100.0	1.31	1.52	11.9	9.8	0.22	16.5	17.8
15	07-13-13	100.0	1.60	1.52	8.3	9.7	0.25	15.3	17.6
16	10-12-13	100.0	1.55	1.52	3.4	9.3	0.14	9.2	17.1
17	01-11-14	100.0	1.30	1.51	13.3	9.6	0.19	14.7	16.9
18	05-09-14	100.0	1.99	1.54	8.2	9.5	0.36	18.3	17.0
19	06-03-14	100.0	1.80	1.55	3.8	9.2	0.19	10.4	16.7
20	07-08-14	100.0	2.31	1.59	4.8	9.0	0.31	13.4	16.5

Note: **CV** = Coefficient of variation for control growth.

Lower CV bound determined by USEPA (10^{th} percentile) = 4.4%.

Upper CV bound determined by USEPA (90^{th} percentile) = 18%

MSD = Minimum Significant Difference

PMSD = Percent Minimum Significant Difference

PMSD is a measure of test precision. The PMSD is the minimum percent difference between the control and treatment that can be declared statistically significant in a whole effluent toxicity test.

Lower PMSD bound determined by USEPA (10^{th} percentile) = 11%.

Upper PMSD bound determined by USEPA (90^{th} percentile) = 28%.

CT = Central Tendancy (mean Control Growth, CV, or PMSD)

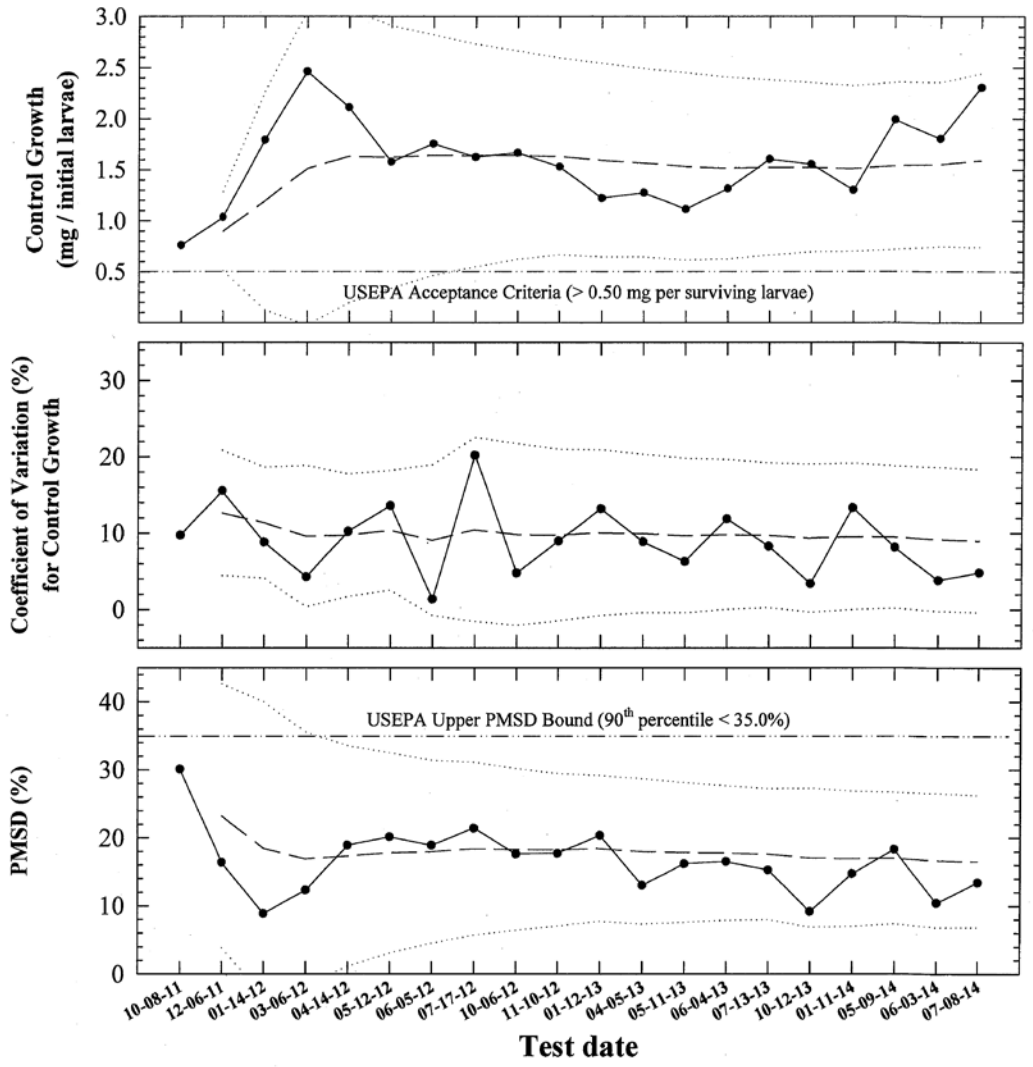
USEPA. 2000. Understanding and Accounting for Method Variability in Whole Effluent Toxicity Applications Under the National Pollutant Discharge Elimination Program. EPA-833-R-00-003. US Environmental Protection Agency, Cincinnati, OH.

USEPA. 2001a, 2001b. Final Report: Interlaboratory Variability Study of EPA Short-term Chronic and Acute Whole Effluent Toxicity Test Methods, Volumes 1 and 2 Appendix. EPA-821-B-01-004 and EPA-821-B-01-005. US Environmental Protection Agency, Cincinnati, OH.

Subject: *Menidia beryllina* Chronic Reference Toxicity Test, EPA 1006.0

Environmental Testing Solutions, Inc.

Menidia beryllina
Chronic Reference Toxicant Control Chart
Precision of Endpoint Measurements



Control Reproduction, Coefficient of Variation (CV), or Percent Minimum Significant Difference (PMSD) PMSD is the minimum significant difference between the control and treatment that can be declared statistically significant.

Central Tendency (mean Control Growth, CV, or PMSD)

Control Limits (mean Control Growth, CV, or PMSD ± 2 Standard Deviations)