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Guidance on Toxicity Identification Evaluations

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SOP-AT80	Toxicity Characterizations	08-01-04		
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Subject: Preparation of Synthetic Water

Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan	\mathcal{N}	09-01-19
Quality Assurance Officer	Jim Sumner	Un funse	09-01-19

Document Revision History

Effective	Revision	Review	Evaluators	Revisions
Date	number	Туре		
12-01-00	0	Internal	Jim Sumner (ETS)	Original document
07-20-09	1	External	William Rogers (TVA)	 Document revision history initiated.
		(TVA,	Cynthia Russell (TVA)	 Procedure and Exhibits AT1.1 and AT1.2 amended to include
		Environmental	Rick Sherrard (TVA)	documentation of synthetic water pH.
		Standard, Inc.)	Rock Vitale	 Table AT1.1 was revised to distinguish between approximate and
			(Environmental	required analytical ranges of synthetic water.
			Standards, Inc.)	
		Internal	Jim Sumner (ETS)	
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	William Rogers (TVA)	 Procedure and Exhibits AT1.1 amended to include the approximate
		(TVA)	Donald Snodgrass (TVA)	resistivity limit. Included course of action if the resistivity limit is exceeded.
			Rick Sherrard (TVA)	
		Internal	Jim Sumner (ETS)	
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.



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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.

Parameter	Moderately hard	Hard	Salt	
	synthetic water	synthetic water	synthetic water	
	(MHSW)	(HSW)	(SaltSW)	
рН (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	Approximate average	Approximate average	Not applicable	
(µmhos/cm)	= 310	= 700		
Alkalinity	Required range	Approximate average	Approximate range	
(mg/L CaCO₃)	= 57 – 64	= 110 – 120	= 80 – 120	
Hardness	Required range	Approximate average	Not applicable	
(mg/L CaCO ₃)	= 80 - 100	= 160 – 180		
Salinity 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.



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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 μ g/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 <u>recommends</u> < 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 (NaHCO₃) Calcium sulfate dihydrate (CaSO₄ • 2H₂O) Magnesium sulfate (MgSO₄) 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



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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 NaHCO₃, CaSO₄ \cdot 2H₂O, MgSO₄ 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.



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Subject: Preparation of Synthetic Water

Chemical	Moderately hard synthetic water (MHSW)	Hard synthetic water (HSW)
	(Total volume = 50 L)	(Total volume = 50 L)
Sodium bicarbonate (NaHCO₃)	5.20 g	10.50 g
Calcium sulfate dihydrate (CaSO ₄ \cdot 2H ₂ O)	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.

Table AT1.2: Guide to preparing synthetic 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.



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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 NaHCO₃ or deionized water may be added to bring the synthetic water into range. Record the amount of NaHCO₃ 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.



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- 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 CaSO₄ 2H₂O and MgSO₄ (equal proportions) or deionized water may be added to bring the synthetic water into range. Record the amount of CaSO₄ 2H₂O and MgSO₄ 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.



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- 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.



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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.



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Subject: Preparation of Synthetic Water

Exhibit AT1.1: Synthetic Water Preparation Log.

	-	S		1			N	NHSW			Salt	sw
Analyst					CHM # Quick Ch	ick Check resu	lts	CHM #	1000			
	yst Water type Preparation date Expiration date (MHSW or SatSW) (Satch) (Satch) (Satchy from preparation)		NaHCO	Ca504. 2H20	MgSO4	ĸci	pH (5.0.) Required Limits: = 6.5 - 8.5 Approximate Range: = 7.4 - 7.8*	Alkalinity (mg CaCO ₉ /L) Required Limits: = 37 - 64	Hardness (mg CaCO ₂ /L) Required Limits: = 80 – 100	Marinemix ^e	Salinity (ppt) Required Limi = 24 - 26	
							-					
) (
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SOP AT1-Revision 6-Exhibit AT1.1

	Aquatic Toxicity P	rocedures
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Exhibit AT1.2: MHSW, Alkalinity and Hardness Quick Check Log.

Analyst	Analyst	Date	MHSW Batch	(samp	inity* (mg C ile volume = ceptance Cri = 57 – 64	100 mL)	(sam)	ness* (mg C ple volume = ceptance Cri = 80 – 100	= 50 mL) iteria
		I	Begin mL	End mL	Total mL X 10	Begin mL	End mL	Total mL X 2	
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			la	·					
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*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



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Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan	\mathcal{N}	09-01-19
Quality Assurance Officer	Jim Sumner	Un fune	09-01-19

Document Revision History

Effective	Revision	Review	Evaluators	Revisions
Date	number	Туре		
12-01-00	0	Internal	Jim Sumner (ETS)	Original document
07-10-10	1	External	Lance Ferrell	Exhibit AT2.3 revised to include calculations for determining the North
		(NC DENR)	(NC DENR)	Carolina cell concentration.
		Internal	Jim Sumner (ETS)	
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	Rick Sherrard,	Included a diagram showing the process of preparing starter and food
		(TVA)	Donald Snodgrass	cultures (Figure AT2.1).
			(TVA)	
		Internal	Jim Sumner (ETS)	
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).



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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



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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 $MgCl_2 \cdot 6H_20$ $CaCl_2 \cdot 2H_20$ NaNO₃ $MgSO_4 \cdot 7H_20$ K₂HPO₄ NaHCO₃ ZnCl₂ $CoCl_2\cdot 6H_20$ $Na_2MoO_4\cdot 2H_20$ $CuCl_2\cdot 2H_20$ Na₂SeO₄ H₃BO₃ $MnCl_2 \cdot 2H_20$ $FeCl_3\cdot 4H_20$ Na₂EDTA · 6H₂0



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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°C to stop the algal growth.



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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 MgCl₂ · 6H₂0 2.20 g of CaCl₂ · 2H₂0 12.75 g NaNO₃
 - 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 MgSO₄ \cdot 7H₂0

- 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.



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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 NaHCO₃

- 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.



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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 H₃BO₃ 0.2080 g of MnCl₂ · 4H₂0 0.0799 g of FeCl₃ · 6H₂0 0.1500 g of Na₂EDTA · 2H₂0
 - 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 Na₂SeO₄ 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.



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7. Preparation of the **<u>ALGAE MEDIA</u>**.

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.



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- 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.

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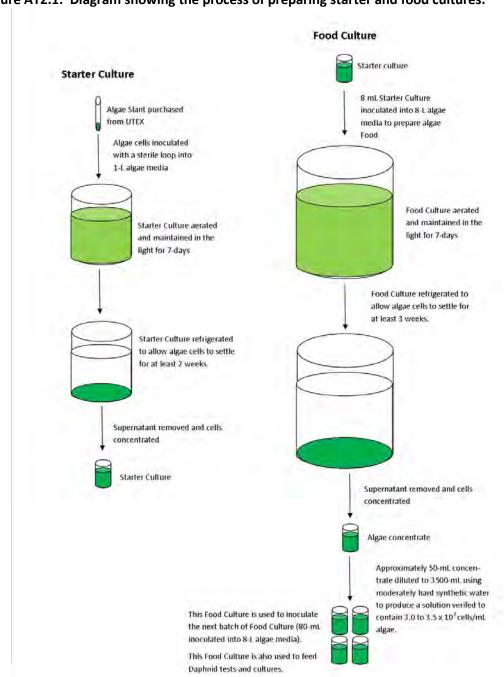


Figure AT2.1: Diagram showing the process of preparing starter and food cultures.



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- 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)

 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.



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- 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 10⁷ 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.
- 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.



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- 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.

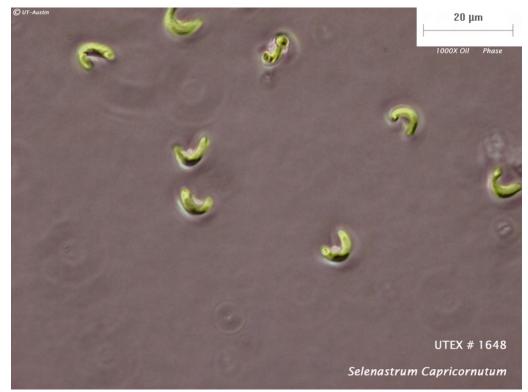


Figure AT2.1: Selenastrum capricornutum cells.

- 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.



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- 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.

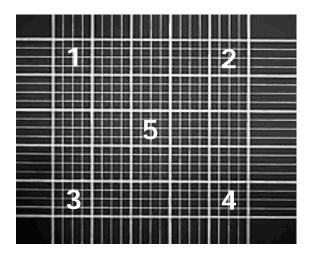


Figure AT2.2: Hemocytometer

- 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 X 10⁻⁶] = 3.07×10^7 cells/mL

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



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- 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 10⁷ 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 10⁷ 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**

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.



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- i. A portion of this *Selenastrum* is diluted further to the North Carolina cell concentration of 1.71 x 10⁷ cells/mL.
 - Determine the necessary volume of moderately hard synthetic water needed to dilute 100 mL full strength algae to 1.71 x 10⁷ 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.



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

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

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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.



Exhibit AT2.1: Raphidocelis subcapitata Shipment Log.





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:	

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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):	0
Volume Prepared (mL):	1
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

Negative Control	Sample	1	Initiation		Te	rmination	1000	Turbid
		Date	Time	Analyst	Date	Time	Analyst	(+ or -)
(10 ml algae media)	Negative Control		1			1.7		
(10 mc aigae media)	(10 mL algae media)			1.000		1	12	
Positive Control	(10 mL algae media + E. coli)							

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Exhibit AT2.3: Raphidocelis subcapitata Culture Log Sheet.



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. Cell count Number of cells on Average number Final cell count one grid of cells (average number of cells / 4.0x10⁻⁶ mL) Correction for dilution factor (cell count X 5) 3 Algae concentrate dilution to 3.5 x 10⁷ cells/mL: Total volume of diluted algae Total volume of **Cell concentration of** Desired cell Dilution algae concentrate algae concentrate concentration factor concentrate (mL) (A, cells/mL) (B, cells/mL) (C = A/B)(mL = C X volume of algae concentrate) 3.5 x 10 Verification of cell count (3.0 to 3.5 x 10⁷ cells/mL): Number of cells Average number Cell count (cells/mL =average number of cells / 4.0x10⁻⁶) On one grid of cells 3 North Carolina algae dilution (1.71 x 10⁷ cells/mL): Total volume of diluted algae Dilution Volume of algae Cell concentration of Desired cell at 3.0 to 3.5 X 107 algae 3.0 to 3.5 X 107 concentration factor (mL = C X volume of cells/mL cells/mL (A, cells/mL) (B, cells/mL) (C = A/B)algae at 3.0 to 3.5 X 107 cells/mL (mL) 1.71 X 107 cells/mL 100

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

Approval

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

Document Revision History

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



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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 <u>recommends</u> that YWT be verified to contain < 50 ng/L organochlorine pesticides plus PCBs, < 1 μ g/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 <u>should</u> 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).



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

<u>Table AT6.1</u>: Concentration of metals (μ g/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 μ L 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
Со	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



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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)



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

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)



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

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 μ m mesh). Discard the particulate fraction that was retained on the screen.
 - c. Remove the wheat grass from the refrigerator.
 - 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.



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

- 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).

(YV	VT Batch: 04-01-09	
Da	ete thawed:	
lni	tials:	,

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.



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

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.



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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



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Subject: Maintenance of Daphnid Cultures

Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan	\mathcal{N}	03-01-20
Quality Assurance Officer	Jim Sumner	Un fune	03-01-20

Document Revision History

Effective	Revision	Review Type	Evaluators	Revisions
Date	number			
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)	• Provided clarification that under normal circumstances, brood boards are maintained for less than 10 days.
01-01-18	4	Internal	Jim Sumner (ETS) 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 <i>Ceriodaphnia</i> cultures are initiated from third broods.



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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 = $25.0 \pm 1.0^{\circ}$ 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



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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 \leq 24 hours old. Neonates used to establish new brood boards must come from third broods.
 - 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 $25.0 \pm 1.0^{\circ}$ 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 3.5×10^7 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.



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- 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.



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- 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 3.5×10^7 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.



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- 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 $25.0 \pm 1.0^{\circ}$ C to each beaker. Add 20 mL YWT and 20 mL *Selenastrum* (cell concentration = 3.0 to 3.5×10^{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.



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- 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.
 - 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.



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- c. Add approximately 2000 mL of MHSW warmed to $25.0 \pm 1.0^{\circ}$ C to each beaker. Add 25 mL YWT and 25 mL *Selenastrum* (cell concentration = 3.0 to 3.5×10^{7} cells/mL) to each beaker. Note: These are designated as "new" cultures.
- 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 $25.0 \pm 1.0^{\circ}$ 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.
 - 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.



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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.

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Exhibit AT7.1: Ceriodaphnia dubia Culture and Neonate Collection Log.



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Ceriodaphnia dubia Culture and Neonate Collection Log

Culture:

Test organism information:		Culture information:			
Organism age:	< 24-hours old	Incubator number:	4		
Date and times		YWT batch:			
organisms were born between:		Selenostrum batch:			
Organism source:		• 14 THE			

	1.20	1.000	1. S. 1. 1		Acti	vity (V)	2
Day	Date	Time	Analyst	MHSW batch	*Fed (100 μL Selenastrum and 100 μL YWT)	Renewed	Cleared
0 (Initiation)							
1							
2							
3							
4							
5						1	
6		ļ					
7					1		
8	0	1			·	·	
9							
10		1			1		1
11	1						
12							
13							
14		1		1			

*Organisms fed using HandyStep repeat pipettor SN 17E59354.

Comments:	

SOP AT7-Revision 6- Exhibit AT7.1



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SOP AT7-Revision 6- Exhibit AT7.1



SECTION	SOP-AT7
REVISION NUMBER	6
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Subject: Maintenance of Daphnid Cultures

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Subject: Maintenance of Daphnid Cultures

ure board: _		eriodaphnia dubia C <u>- C</u>	ulture and N	leonate Colle		izing temp	olate: <u>PINK</u>
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Subject: Maintenance of Daphnid Cultures

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Subject: Maintenance of Daphnid Cultures

Exhibit AT7.2: Daphnia Culture Log.



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Daphnia magna Culture Log

Culture:

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

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SOP AT7-Revision 6- Exhibit AT7.2



SECTION	SOP-AT8
REVISION NUMBER	5
EFFECTIVE DATE	03-01-20
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Subject: Ceriodaphnia dubia Neonate Collection

Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan	\mathcal{N}	03-01-20
Quality Assurance Officer	Jim Sumner	Un funse	03-01-20

Document Revision History

Effective	Revision	Review	Evaluators	Revisions
Date	number	Туре		
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 \leq 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.



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Subject: Ceriodaphnia dubia Neonate Collection

Neonates used to initiate toxicity tests must come from brood boards with \leq 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 = $25.0 \pm 1.0^{\circ}$ C, photoperiod of 16hour 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.



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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.



SECTION	SOP-AT9
REVISION NUMBER	6
EFFECTIVE DATE	03-01-20
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Subject: Daphnid Acute Toxicity Test, EPA 2002.0 and EPA 2021.0

Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan	\mathcal{N}	03-01-20
Quality Assurance Officer	Jim Sumner	Un fune	03-01-20

Document Revision History

Effective	Revision	Review Type	Evaluators	Revisions
Date	number			
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	Lance Ferrell	The measurement of pH, DO and conductivity of each new, full-strength,
		(NC DENR)	(NC DENR)	undiluted sample was added.
				 The light intensity was amended to reflect that it is a <u>recommended</u>
		Internal	Jim Sumner (ETS)	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)	Rick Sherrard (TVA)	 Updated bench sheet (Exhibits AT9.2 and AT9.3) to include reporting
				limits, method numbers, meters and serial numbers used for chemical
		Internal	Jim Sumner (ETS)	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.



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

Quality Control

Acceptance Criteria: The test acceptance criterion is \geq 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 = $25.0 \pm 1.0^{\circ}$ C, photoperiod of 16hour 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



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

- 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 $57 64 \text{ mg CaCO}_3/L$, hardness of $80 100 \text{ mg CaCO}_3/L$, 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 fullstrength, 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 $(25.0 \pm 1.0^{\circ}C)$ 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



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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.
 - 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.



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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 \pm 1 hour from test initiation.



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- 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 $(25.0 \pm 1.0^{\circ}\text{C})$ 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 \pm 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.



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- 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.



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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.



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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 <i>Selenastrum</i> made available while holding prior to test initiation (2 to 5-hours prior to initiation). Organisms in each test cup are fed 100 μ L YWT and 100 μ L <i>Selenastrum</i> 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



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

					en en er							
Client	v	Vater (Quality Lab	& Operat	tions, Inc.	_	- 11	NP	DES #	NCOO	75965	
Facilit	y B	Burnsvi	lle WTP	-				Ou	tfall	001	_	
Projec	t#		_					Co	unty	Yance	ey	
Sample w	vas not aerat	nted or treat	Acute Limit) ed unless otherwise th and then diluted		rm. Sample was w			Dilutio prepar		mL Sample	mL Dilution water	Total volum mL
	betic water		and then debled	to the test could	intraction using mos	on a cory				270	30	300
Hour		Date	Feed	ling	Test Initiation	or Termination	Location (Incubator/She	Randomizing Te	molate	Sample N	lumber M	HSW Batch
0	-	oute	Time	Analyst	Time	Analyst		in the second se	- mpmis	Sample i	tuininer inn	ion butch
initiatic	on			1.1		(1222)	1					
24	- 1											
		10.11	A DESCRIPTION OF A		1.1.1.1.1	1.1.1.1	I second second	10.000			and the second second	
48				-				-	-	-		-
Terminat		re fed in hold	ding 2 to 5 hours pri	or to test initiatio	n. Test organisms	were not fed duri	ng the test.					
Terminat "Test org	anisms were		ding 2 to 5 hours pri	or to test initiatio		were not fed duri	ng the test.				1	
Terminat "Test org			ding 2 to 5 hours pri		Hours		Analyst identified for				nd conductivity measure	
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Survival Data (number of living organisms):

pH (S.U.)

100%

Dissolved oxygen (mg/L)

Conductivity (µmhos/cm) Total residual chlorine (mg/L)

		Con	trol		Te	st Cond	entrat	ion		
Hours		Repl	icate	Replicate						
	A	В	c	D	ε	F	G	н		
0 Initiation	10	10	10	10	10	10	10	10		
24				0						
48 Termination										
	Mean su	rvival (%)			Mean su	rvival (%)	:			

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



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Exhibit AT9.3: Acute Toxicity Test Bench Sheet.

					EPA-8	321-R-02	-012, Method 2002	.0						
lient	Chemtrade	Perfor	nance	Chemica	Is LLC	-		N	PDES #	SC 0	022756			
acility	Leeds Plant							o	utfall	002				
roject #		San	nple #				County				ster			
	reparation:	-		-										
222.1	trations (%)	. 1		10.10	70	100	Sample was not aerated or	treated upless of	henvise note	rd.				
L Sample	trations (%)	4	8	15.7 31.4	58 116	200	on this form. Sample was	warmed to 25.0 ±	1.0°C in a wa	rm				
L Dilution	water	192	184	168.6	84	0	water bath and then dilute moderately hard synthetic		entrations us	ang				
otal volum	e (mL)	200	200	200	200	200								
hamical	Analyses:		-		Hours	_	_							
enneur	Analyses.			0	24	41	3							
Concentration		A	nalyst								oxygen and conductiv			
-	pH (s.u.)					1	the analyst perform	ning the toxicity t	est. Alkalinit	y, hardn	e of test initiation or ess and total residual	chlorine		
	Dissolved oxyge	n (mg/L)				-					bench sheets and tran			
Control,	Conductivity (un	nhos/cm)					Chemical ana	lyses:						
MHSW	Alkalinity (mg/L	CaCO ₁)				1	Parameter	Reporting limit	Method num	nber	Meter	Serial number		
	Hardness (mg/L)	caco,)					pH	0.15.U.	SM 4500-H+	B-2011	Accumet AR20	93312452		
	Temperature (°C	:)					Dissolved oxygen	1.0 mg/L	SM 4500-0 0	5-2011	YSI Model 52CE	18D104324		
	pH (5,U.)		-				Conductivity	14.9 µmhos/cm	SM 2510 B-2	011	Accumet AR20	93312452		
	Dissolved oxygen (mg/L) Conductivity (µmhos/cm) Temperature (°C)			-	1	111	Alkalinity	5.0 mg CaCO ₃ /L	SM 2320 8-2	011	Accumet AR20	93312452		
4.0%				_			Hardness	5.0 mg CaCO ₁ /L	SM 2340 C-2	011	Not applicable	Not applicable		
					-	-	Total residual chlorine	0.1 mg/L	ORION 97-70	-1977	Accumet A8250	92349123		
-	pH (S.U.)				()		Temperature	0.1°C	SM 25508-20	010	Digital Thermometer	1.1		
	Dissolved oxyge	n (mg/L)						1				-		
8.0%	% Conductivity (umhos/cm)			-		-	-							
	Temperature (°C	:)	-			-								
-	pH (5,U.)		-											
	Dissolved oxyge	n (mg/L)												
15.7%	Conductivity (umhos/cm)					1								
	Temperature (°C	3	-	-			_							
-	pH (5.U.)		-				-							
	Dissolved oxyge	n (mg/L)	+				-							
58%	Conductivity (µm	nhos/cm)				-								
	Temperature ("C	:)	-											
-	pH (S.U.)		-	-			-							
	Dissolved oxyge	n (mg/L)	-		-									
	Conductivity (µn			-										
100%	Alkalinity (mg/L)		-			-								
100/0	Hardness (mg/L													
	11		-		-									
	Total residual ch	norine (m												



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Acute LC₅₀ Whole Effluent Toxicity Test, Species: <u>Ceriodaphnia dubia</u> EPA-821-R-02-012, Method 2002.0

Client Chemtrade Performance Chemicals LLC

Project # Sample #

		Fee	ading	Test Initiation	or Termination	Location	Randomizing	MHSW Batch	
tiours	Date	Time	Analyst	t Time Analyst		Incubator/Shelf	Template	MINDAA Berch	
0			11-01		19-11 (d)		17	. 1	
24		1	1		1.000				
48		1	1	-	1			-	

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:	рн (5.0.):	-
	Temperature (°C):	

Survival Data (number of living organisms);

1		Con	trol			4.0	0%			8.	0%	
Hours		Repl	icate	-	1	Repl	icate		-	Repl	icate	_
	A	В	c	D	E	F	G	н	t.	L	к	L
0 Institution	5	5	5	5	5	5	5	5	5	5	5	5
24											(
48 Termination									E		Ū,	ľ.
Mean Survival (%)						-						
		15.	7%		1	58	3%	100%				
Hours		Rep	icate		-	Repl	icate			Rep	icate	_
	м	N	o	P	Q	R	s	Ţ	U	v	w	x
0 Initiation	5	5	5	5	5	5	5	5	5	5	5	5
24												
48 Termination		Ē	Ī									T
					_							

mment codes: d = dead, u = u

Method	
Lower 95% confidence limit (%)	
Upper 95% confidence limit (%)	
48-hour LC ₁₀ (%)	

SOF AT9-birdstein & Fahiluly AT9.3



Exhibit AT9.4: Acute Test Holding Rack.

Randomizing template: <u>RED</u>				
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				



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Exhibit AT9.5: Average Transfer Volume Log Sheet.

		Page 1 of
Ceriodaphnia di	ubia Transfer Volume	
icine cups.		
n of the 10 cups.		
veight of each cup containin	ng MHSW.	
		phnid acute toxicity tests.
veight of each cup containin	ng MHSW with 5 Ceriodaphnia.	
volume, average transfer vo	lume, and estimated volume to tra	ansfer 1 Ceriodaphnia.
Initial Woight	Final Weight	Transfer
		Volume
•		Final - Initial Weight
IS HIL WITSW		(g = mL)
(g)	· · ·	(g – IIIL)
\6/	(6/	
	+ +	
	+	
	+	
Average volur	me to transfer 5 organisms (mL):	
	icine cups. n of the 10 cups. veight of each cup containir co each cup, following proce veight of each cup containir volume, average transfer vo Initial Weight Medicine cup + 15 mL MHSW (g) (g) Average volur	In of the 10 cups. veight of each cup containing MHSW. to each cup, following procedures identified in SOP-AT9 for Da veight of each cup containing MHSW with 5 Ceriodaphnia. volume, average transfer volume, and estimated volume to transfer volume, and estimated volume to transfer cup + Initial Weight Medicine cup + Medicine cup + 15 mL MHSW + 5 Ceriodaphnia transferred



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Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan	N	03-01-20
Quality Assurance Officer	Jim Sumner	Un / unse	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	Rick Sherrard,	 Updated Table AT10.1 for test concentrations and conductivity
		(TVA)	Donald Snodgrass	measurement guidance values.
			(TVA)	Deleted statement: "Verify that the conductivity measured for each
				test concentration is within the acceptance criteria (refer to table Table
		Internal	Jim Sumner (ETS)	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)	Rick Sherrard (TVA)	Updated bench sheet to include reporting limits, method numbers,
				meters and serial numbers used for chemical analyses.
		Internal	Jim Sumner (ETS)	



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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 \geq 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.



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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 = $25.0 \pm 1.0^{\circ}$ C, photoperiod of 16hour 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.

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



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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 watervolumes, final volumes, and conductivity measurements guidance values for the*Ceriodaphnia dubia* and *Daphnia* NaCl acute reference toxicant tests.

Test Concentration	Volume of Stock	Volume of Moderately hard	Final Volume	Conductivity Guidance Values
(mg NaCl/L)	Required (mL)	synthetic water (mL)	(mL)	(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
aphnia:				
Daphnia: Test	Volume of	Volume of	Final	Conductivity
•	Volume of Stock Required	Volume of Moderately hard synthetic water	Final Volume (mL)	Conductivity Guidance Values (μmhos/cm)
Test Concentration	Stock	Moderately hard	Volume	Guidance Values
Concentration	Stock Required	Moderately hard synthetic water	Volume	Guidance Values (μmhos/cm)
Test Concentration (mg NaCl/L)	Stock Required (mL)	Moderately hard synthetic water (mL)	Volume (mL)	Guidance Values (µmhos/cm) Not determined
Test Concentration (mg NaCl/L) 2000	Stock Required (mL) 4.0	Moderately hard synthetic water (mL) 196.0	Volume (mL)	Guidance Values (µmhos/cm) Not determined Not determined
Test Concentration (mg NaCl/L) 2000 3000	Stock Required (mL) 4.0 6.0	Moderately hard synthetic water (mL) 196.0 194.0	Volume (mL) 200 200	Guidance Values

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.



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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.



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

Hours

24

48

.*	-		-
-		4	
3	c		-7)
4		-	

Acute LC₅₀ Whole Effluent Toxicity Test, Species: <u>Ceriodaphnia dubia</u> EPA-821-R-02-012, Method 2002.0

Ceriodaphnia dubia Sodium Chloride Acute Reference Toxicant Test

0

CdNaCIAC #

Chemical Analyses:

pH (S.U.)

Dissolved oxygen (mg/L) Conductivity (µmhos/cm)

Alkalinity (mg/L CaCO₃) Hardness (mg/L CaCO₃) Temperature (°C) pH (S.U.)

Dissolved oxygen (mg/L)

Conductivity (µmhos/cm) Temperature (°C) pH (S.U.)

Dissolved oxygen (mg/L)

Conductivity (µmhos/cm) Temperature (°C) pH (S.U.)

Dissolved oxygen (mg/L)

Conductivity (µmhos/cm) Temperature (°C) pH (S.U.)

Dissolved oxygen (mg/L)

Conductivity (µmhos/cm) Temperature (°C) pH (S.U.)

Dissolved oxygen (mg/L)

Conductivity (µmhos/cm) Temperature (°C)

Concentration

Control, MHSW

1750 mg/L

2000 mg/L

2250 mg/L

2500 mg/L

2750 mg/L

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	195.0	195.5	195.0	194.5
Total volume (ml.)	200	200	200	200	200

Analys

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.

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:

Stock solution INSS #:

Parameter	Reporting limit	Method number	Meter	Serial number
рН	0.1 S.U.	SM 4500-H+ B-2011	Accumet AR20	93312452
Dissolved oxygen	1.0 mg/L	SM 4500-0 G-2011	VSI Model 52CE	18D104324
Conductivity	14.9 µmhos/cm	SM 2510 8-2011	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 25508-2010	Digital Thermometer	

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ETS

Acute LC₅₀ Whole Effluent Toxicity Test, Species: <u>Ceriodaphnia dubia</u> EPA-821-R-02-012, Method 2002.0

Ceriodaphnia dubia Sodium Chloride Acute Reference Toxicant Test

CdNaCIAC#

- C		Feeding		Test Initiation	or Termination	Location	Randomizing	MHSW Batch	
Hours	Date	Time	Analyst	Time	Analyst	Incubator/Shelf	Template	THESE W BATCH	
0	1.1	•	151.0.5		1		E . 19		
24		1							
48				-					

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 (5.U.):	
	Temperature (² C):	

Survival Data (number of living organisms):

		Con	trol			1750	mg/L) II	2000	mg/L	
Hours		Rep	icate		_	Repl	icate	Replicate				
	A	В	c	D	E	F	G	н	1	1	ĸ	L
0 Indiation	5	5	5	5	5	5	5	5	5	5	5	5
24												
48 Termination												
Mean Survival	2			· · · · ·		0-0-0			1	-		

		2250	mg/L	-		2500	mg/L	-	<u>,</u>	2750	mg/L	
Hours	_	Repl	icate	_		Repl	icate			Rep	icate	_
	м	N	0	р	Q	R	s	7	U	v	w	×
0 lectulation	5	5	5	5	5	5	5	5	5	5	5	5
24			111	1.1					ų ir st		111	
48 Terminailan												
Mean Survival					-			1.1				

Comment codes: d = dead, u = unhealthy

Statistics:

Method	Comments:	
Lower 95% confidence limit (mg NaCl/L)		
Upper 95% confidence limit (mg NaCl/L)		
48-hour LC ₁₀ (mg NaCl/L)		

Confidential

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

			Log	g ₁₀ Conversion	1	-		Anti-loga	arithmic Values	(g/L NaCl)		
Fest number	Test date	48-hour LC ₅₀ ToxCal Determination	48-hour LC ₅₀	ст	s	ст		l Limits	Warnin	Calculated CV og Limits	Warnin	centile CV ng Limits
		(g/L NaCl)					CT - 25	CT + 2S	CT - 2CV	CT + 2CV	CT - 54.10	CT + SA.
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	2.460
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	2.468
6	10-09-18	2.3918	0.3787	0.3682	0.0086	2.3346	2.2438	2.4290	2.2957	2.3750	2.1945	2.474
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	2.490
9	11-14-18	2.3569	0.3723	0.3715	0.0071	2.3521	2.2764	2.4303	2.3199	2.3854	2.2110	2.493
10	12-04-18	2.3918	0.3787	0.3724	0.0067	2.3574	2.2862	2.4308	2.3272	2.3885	2.2159	2.498
11	12-12-18	2.3442	0.3700	0.3726	0.0065	2.3586	2.2890	2.4302	2.3291	2.3889	2.2170	2.500
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	2.503
16	04-03-19	2.3212	0.3657	0.3729	0.0065	2.3600	2.2906	2.4316	2.3306	2,3903	2.2184	2.501
17	04-09-19	2.3436	0.3699	0.3723	0.0062	2.3568	2.2907	2.4247	2.3288	2.3856	2.2154	2.498
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

48-hour LC₅₀ = 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 LC₅₀ values. Note:

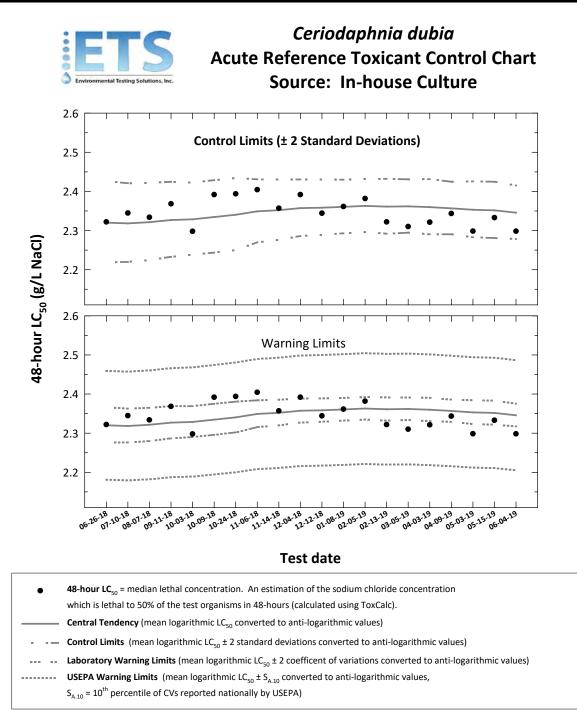
S = Standard deviation of the LC₅₀ values.

 $S_{A,10} =$ Standard deviation corresponding to the 10th percentile of CVs reported nationally by USEPA. ($S_{A,10} = 0.06$). CV = Coefficient of variation.



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

Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan	\mathcal{N}	03-01-20
Quality Assurance Officer	Jim Sumner	Un funse	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	Lance Ferrell	• The measurement of pH, DO and conductivity of each new, full-strength,
		(NC DENR)	(NC DENR)	undiluted sample was added.
				 The light intensity was amended to reflect that it is a <u>recommended</u>
		Internal	Jim Sumner (ETS)	range as specified in the EPA manuals.
07-01-13	3	External	Lance Ferrell	Added North Carolina acceptance and termination criteria: Testing in
		(NC DENR)	(NC DENR)	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.
		Internal	Jim Sumner (ETS)	
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)	Rick Sherrard (TVA)	Updated bench sheet to include reporting limits, method numbers,
				meters and serial numbers used for chemical analyses.
		Internal	Jim Sumner (ETS)	



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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.

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 3 rd 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%

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



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Equipment and Materials

Ceriodaphnia dubia Temperature-controlled incubator (set to maintain test temperature = 25.0 ± 1.0°C, photoperiod of 16hour 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 WWT mixture

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)



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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 $57 64 \text{ mg CaCO}_3/L$, hardness of $80 100 \text{ mg CaCO}_3/L$, 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 10⁷ 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 ($25.0 \pm 1.0^{\circ}$ C) of the test concentrations. This may be accomplished by placing the holding rack into a temperature-controlled incubator.



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- 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.
 - 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.



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- g. Verify that each test cup received one neonate by conducting a repeat count. Remove excess neonates or add neonates as necessary.
- 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 μ L *Selenastrum capricornutum* and 100 μ L YWT mixture to each cup. The cell density of the *Selenastrum* mixture must be between 3.0 to 3.5 x 10⁷ 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 $(25.0 \pm 1.0^{\circ}C)$ 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.



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- 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.



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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.



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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.



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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 <i>Selenastrum</i> 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



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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 NPDES #: TN 0020168 Project #:

County: Rhea Outfall #: 101

Dilution preparation:

Dilution prep (%)	0.7	.1.4	2,8	5.6	11.2	Sample was not aerated or treated unless otherwise
Effluent volume (mL)	14	28	56	112	224	noted on this form. Sample was warmed to 25.0 ± 1.0 °C
Diluent volume (mL)	1986	1972	1944	1888	1776	in a warm water bath and then diluted to the test
Total volume (mL)	2000	2000	2000	2000	2000	concentrations with moderately hard synthetic water (MHSW).

-.

Test organism source:											Test randomization and location:
Organism age:	< 24	4-hou	rs old								Randomizing template color:
Date and times organisms were born between:										1	Incubator number
Culture board:										7	and shelf location:
Replicate number:	1	2	3	4	5	6	7	8	9	10	
Culture board cup number:				1					1		
Transfer vessel information:	pH	(S.U.)	:		Tem	perat	ure (C):		-	
Average transfer volume (mL):	< 0.	25 m	L				-			_	

Daily renewal:

Day	Date	Test initiation and feeding,	*Feeding B	atches	MHSW	Sample num	bers used	Analyst
		renewal and feeding, or termination time	Selenastrum	YWT	batch used	Outfall 101	Intake	1
0	1.1			1.1	1			
1								
2								
3	1.1.1							
4						E		1
5			l l		1	(m	1	1
6							-	1
7	-		and the second se	in the second	1	and the second se		

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 4500-0 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:	and the second sec	100.00		Summary of test endpoints:
CELOS DO LOS COMOS DE LA COMOS DE LA COMOS DE LA COMOS DE LA COMOS DE LA COMOS DE LA COMOS DE LA COMOS DE LA C	Control-1	Control-2	Acceptance criteria	
% of Male Adults:		y	≤ 20%	7-day LCso (%)
% Adults having 3rd Broods:			≥ 60% surviving adults	NOEC (%)
% Mortality:			≤ 20%	LOEC (%)
Mean Offspring/Female:		(= = 1	≥ 15.0 offspring/female	ChV (%)
% CV:		h	< 42.0 %	IC25 (%)

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lient:	: <u>Ceriodaphnia dul</u> TVA / Watts Bar Ni		lant, O							e:	
CONTR	ROL-1	_		Si	urvival	and Re	product	tion Da	ta		
		21			F	Replicat	e numbe	r			
Day		1	2	3	4	5	6	7	8	9	10
1	Young produced								1		
-	Adult mortality	_				-			-		
2	Young produced Adult mortality									-	-
3	1			-					-	<u> </u>	<u> </u>
3	Young produced Adult mortality			-	-			-			-
4	Young produced					-	1				-
7	Adult mortality										
5	Young produced	-				-				-	1
	Adult mortality		-			-					
6	Young produced		-				1		-		-
	Adult mortality			1000	1.2						
	Young produced			12-1	0		1		100		
7					_	_		-	-		-
	ng produced			1	1.000						
otal you	ng produced									$\sim =$	-
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ONC: 1	.4%			S	urvival	and Re	product	ion Dat	a		
						Replicat	e number				
Day		1	2	3	4	5	6	7	8	9	10
1	Young produced	1000	1		· · · ·		2	1		1 T	_
1.11	Adult mortality				-	1.0	1. 22.	1			-
2	Young produced		· · · · ·	Sec. 1.	1	-				1.000	
	Adult mortality	1			-	1				1.000	1.00
3	Young produced	30.5	1000		h	1.0					
_	Adult mortality	1			1				_	2.00	_
4	Young produced					1	1.1.1	1.000		1	-
1. st (Adult mortality				Ú	· · · · · · · ·		-			
5	Young produced							1			
	Adult mortality	1.00	1.00	A	1.1		1.1	1.1			1.000
6	Young produced					100		1			-
141	Adult mortality	-	1		In succession		1.00				
7	Young produced	2	-		1		11	-			
otal you	ing produced	1.1	10.21			1					
							_				-
	i lt Mortality mortality (L = live, D = dead), Si	B = split brood	(single brood	d split betwee	n two days),		er (offspring ca Concentr % Mortali Mean Off % Reduct	ation: ity: spring/Fe	emale:		
ote' Adult	mortality (L = live, D = dead), Sł	B = split brood	(single brood				<i>Concentr</i> % Mortal Mean Off	ation: ity: spring/Fe ion from	emale: Control-J		
onc: 2	mortality (L = live, D = dead), Sł			S	urvival	and Re Replicat	Concentro % Mortal Mean Off % Reduct product e number	ation: ity: ispring/Fe ion from tion Dat	emale: Control-1		
onc: 2	mortality (L = live, D = dead), Si	B = split brood	(single brood			and Re	Concentr % Mortal Mean Off % Reduct product	ation: ity: spring/Fe ion from	emale: Control-J		10
onc: 2	mortality (L = live, D = dead), Si 2.8% Young produced			S	urvival	and Re Replicat	Concentro % Mortal Mean Off % Reduct product e number	ation: ity: ispring/Fe ion from tion Dat	emale: Control-1		10
ONC: 2 Day 1	mortality (L = live, D = dead), Si 2.8% Young produced Adult mortality			S	urvival	and Re Replicat	Concentro % Mortal Mean Off % Reduct product e number	ation: ity: ispring/Fe ion from tion Dat	emale: Control-1		10
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onc: 2 Day 1 2 3	Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality			S	urvival	and Re Replicat	Concentro % Mortal Mean Off % Reduct product e number	ation: ity: ispring/Fe ion from tion Dat	emale: Control-1		10
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% Reduction from Control-1:



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ONC: 5	.6%	_	-	S	urvival	and Rep	product	ion Dat	a		
		Replicate number									
Day		1	2	3	4	5	6	7	8	9	10
1	Young produced	_				-	-	2			-
-	Adult mortality		-		1	-					-
2	Young produced Adult mortality	-				-	-				
2		-	-	-	-	-		-		-	-
3	Young produced Adult mortality		-	(-	-	-			-	-
4	and a set of the set of the set of the set		-	-		-		-	L	-	
4	Young produced Adult mortality					-				-	-
5							-	-		_	-
,	Young produced Adult mortality		-	-	-	-	-	-		-	-
6	Young produced						-				-
0	Adult mortality	-		-			-		_		
7	Young produced		-	a	-	-	-	-	_	-	-
	122	-	-	-	-	-	-	-	_	_	_
otal you	ing produced			1 - 1	· · · · · ·	1.5	1.1	12.1		()	
						-				1	
ore: Adult	It Mortality mortality (L = live, D = dead). S	B = spiit brond	i (single broo				Concentro % Mortali Mean Off % Reduct	ation: ity: spring/Fe ion from	emale: Control-1		_
iore; Aduit	mortality (L = iive, D = dead). S	8 = split brood	i (single broo			and Rep	Concentro % Mortali Mean Off % Reduct product	ation: ity: spring/Fe ion from	emale: Control-1		
iore: Adult	mortality (L = iive, D = dead). S			S	urvival	and Rej Replicate	Concentro % Mortali Mean Off % Reduct product e number	ation: ity: spring/Fe ion from tion Dat	emale: Control-1	L;	1
iote: Adult	mortality (L=live, D=dead). 5 1.2%	B = aplit brood	i (single broo			and Rep	Concentro % Mortali Mean Off % Reduct product	ation: ity: spring/Fe ion from	emale: Control-1		10
CONC: 1 Day	mortality (L = iive, D = dead). S			S	urvival	and Rej Replicate	Concentro % Mortali Mean Off % Reduct product e number	ation: ity: spring/Fe ion from tion Dat	emale: Control-1	L;	10
conc: 1 Day	mortality (L = live, D = dead). 5 1.2% Young produced Adult mortality			S	urvival	and Rej Replicate	Concentro % Mortali Mean Off % Reduct product e number	ation: ity: spring/Fe ion from tion Dat	emale: Control-1	L;	10
iore: Adult CONC: 1 Day 1	mortality (L = live, D = dead). 5 1.2% Young produced			S	urvival	and Rej Replicate	Concentro % Mortali Mean Off % Reduct product e number	ation: ity: spring/Fe ion from tion Dat	emale: Control-1	L;	10
iore: Adult CONC: 1 Day 1	nortality (L = live, D = dead). 5 1.2% Young produced Adult mortality Young produced			S	urvival	and Rej Replicate	Concentro % Mortali Mean Off % Reduct product e number	ation: ity: spring/Fe ion from tion Dat	emale: Control-1	L;	10
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conc: 1 Day 1 2 3	Nortality (L = live, D = dead). S 1.2% Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality			S	urvival	and Rej Replicate	Concentro % Mortali Mean Off % Reduct product e number	ation: ity: spring/Fe ion from tion Dat	emale: Control-1	L;	10
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conc: 1 Day 1 2 3 4	Nortality (L = live, D = dead). S 1.2% Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality Young produced			S	urvival	and Rej Replicate	Concentro % Mortali Mean Off % Reduct product e number	ation: ity: spring/Fe ion from tion Dat	emale: Control-1	L;	
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conc: 1 Day 1 2 3 4 5 6 7	Nortality (L = live, D = dead). S Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality			S	urvival	and Rej Replicate	Concentro % Mortali Mean Off % Reduct product e number	ation: ity: spring/Fe ion from tion Dat	emale: Control-1	L;	
conc: 1 Day 1 2 3 4 5 6 7 7 otal you	1.2% Young produced Adult mortality Young produced			S	urvival	and Rej Replicate	Concentro % Mortali Mean Off % Reduct product e number	ation: ity: spring/Fe ion from tion Dat	emale: Control-1	L;	

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% Reduction from Control-1:



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Total young produced Final Adult Mortality

± live, D

ad), 58 = split brood (single b

ood sp

CONTR	C C & C	uclear P	lant, Ou	tfall 101						e:	
CONTR	ROL-2	-		S	urvival	and Re	product	ion Da	ta		_
100	100 million (1997)	1			F	Replicate	numbe	r			_
Day		1	2	3	4	5	6	7	8	9	10
1	Young produced	-		$\sim - 4$					-		
	Adult mortality	1		1							
2	Young produced			2117		u i i i					
	Adult mortality							_	1		
3	Young produced			1	· · · · · · · · ·	i	1		2 4	اختكر	
	Adult mortality			2.011.2	1	i = 19		-			
4	Young produced	·		5		5					
	Adult mortality	· · · · · · · · ·		1	· · · · · · · · · · · · · · · · · · ·		·				
5	Young produced			1255	12-51	1-10	12-12	_	2224		
	Adult mortality	(a)				1					1
6	Young produced		-	1				-	1.00		
	Adult mortality					·					
7	Young produced	-									
otal your	ng produced	-		1.000	1.1	0.00			1.0.0		
				-	-					-	
for 3 rd B	It Mortality	-		-	-	-				-	-
and the second second	mortality (L=live, D = dead), SI	3 = split brood	[single broo	d split betwee	n two days), (0 = carry ove	r (offspring ca	rried over wi	th adult durin	g transfer).	
								-		2	
							Concentr			1	_
							% Mortal	ity:	emale:		_
ONC: 11	00% Intaka				unvival	E	% Mortal Mean Off	ity: fspring/F			_
CONC: 10	00% Intake			s	urvival	and Rej	% Mortal Mean Off product	ity: fspring/F			_
	00% Intake	1	2	5	urvival	and Rej	% Mortal Mean Off	ity: fspring/F		9	10
Day	00% Intake	1	2		_	and Rej	% Mortal Mean Off product number	ity: spring/F ion Da	ta	9	10
Day		1	2		_	and Rej	% Mortal Mean Off product number	ity: spring/F ion Da	ta	9	10
Day	Young produced Adult mortality	1	2		_	and Rej	% Mortal Mean Off product number	ity: spring/F ion Da	ta	9	10
Day 1	Young produced	1	2		_	and Rej	% Mortal Mean Off product number	ity: spring/F ion Da	ta	9	10
Day 1	Young produced Adult mortality Young produced Adult mortality	1	2		_	and Rej	% Mortal Mean Off product number	ity: spring/F ion Da	ta	9	10
Day 1 2	Young produced Adult mortality Young produced	1	2		_	and Rej	% Mortal Mean Off product number	ity: spring/F ion Da	ta	9	10
Day 1 2	Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality	1	2		_	and Rej	% Mortal Mean Off product number	ity: spring/F ion Da	ta	9	10
Day 1 2 3	Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality Young produced	1	2		_	and Rej	% Mortal Mean Off product number	ity: spring/F ion Da	ta	9	10
Day 1 2 3 4	Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality	1	2		_	and Rej	% Mortal Mean Off product number	ity: spring/F ion Da	ta	9	10
Daγ 1 2 3	Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality Young produced	1	2		_	and Rej	% Mortal Mean Off product number	ity: spring/F ion Da	ta	9	10
Day 1 2 3 4 5	Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality	1	2		_	and Rej	% Mortal Mean Off product number	ity: spring/F ion Da	ta	9	
Day 1 2 3 4	Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality Young produced		2		_	and Rej	% Mortal Mean Off product number	ity: spring/F ion Da	ta	9	

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

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Date:

Species: <u>Ceriodaphnia dubia</u> Client: <u>TVA / Watts Bar Nuclear Plant, Outfall 101</u>

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.

		(Analyst iden	luctivity measure	ements only			
			0	-	1		2
	Analyst						-
Concentration	Parameter		C. Constanting of the	1		C	
100 C	pH (S.U.)		· · · · · · · · · · · · · · · · · · ·	1	-	-	-
	Dissolved oxygen (mg/L)			1. I	;		
CONTROL,	Conductivity (umhos/cm)		a second second	C	100 mar 100 k		
MHSW	Alkalinity (mg CaCO ₁ /L)	i: :		· · · · · · · · · · · · · · · · · · ·			
	Hardness (mg CaCO ₃ /L)	P. 1		/ /			
	Temperature ("C)						
	pH (S.U.)						
0.70/	Dissolved oxygen (mg/L)		1				
0.7%	Conductivity (µmhos/cm)		1.0	1			
	Temperature (°C)		2	· · · · · · · · · · · · · · · · · · ·			
	pH (S.U.)		1		1.		
	Dissolved oxygen (mg/L)						
1.4%	Conductivity (umhos/cm)				1		-
	Temperature (°C)						
	pH (5.U.)						
	Dissolved oxygen (mg/L)		1				
2.8%	Conductivity (umhos/cm)		Concession of the		the second second second second second second second second second second second second second second second se		
	Temperature (°C)				_		
	pH (S.U.)						
	Dissolved oxygen (mg/L)		-				-
5.6%	Conductivity (µmhos/cm)		1		1		
	Temperature (°C)						-
	pH (S.U.)						-
	Dissolved oxygen (mg/L)						
11.2%	Conductivity (umhos/cm)		-		1	-	
	Temperature (°C)						
				-			
	pH (S.U.) Dissolved oxygen (mg/l.)		-				_
	Conductivity (µmhos/cm)	_		-			_
100%	Alkalinity (mg CaCO ₁ /L)			_			
100%				-			_
	Hardness (mg CaCO ₃ /L)				-		-
	Chlorine (mg/L)						
	*Temperature (°C)	-	1.00	-			A Designation
	pH (S.U.)		-				
	Dissolved oxygen (mg/L)	11	-		-	-	-
100%	Conductivity (umhos/cm)		-	-	-		_
Intake	Alkalinity (mg CaCO ₁ /C)		-		-		
	Hardness (mg CaCO ₃ /L)		-				_
	Chlorine (mg/L)		-		-		
	Temperature (°C)						

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		(Analyst ident	ified for each day, perforn	Day and pH_D_O_and conducti	uity measurements or	(uda
		3	4	5	vity measurements of	5
	Analyst				100	
Concentration	Parameter	-				
	pH (S.U.)					
	Dissolved oxygen (mg/L)		·			
CONTROL,	Conductivity (jumhos/cm)	The second second second second second second second second second second second second second second second se				
MHSW	Alkalinity (mg CaCO JL)	1		-	and the second se	
	Hardness (mg CaCO ₄ /L)	Sec.			and the second s	
	Temperature ("C)				1.1	
	pH (S.U.)					
2.00	Dissolved oxygen (mg/L)		· · · · · · · · · · · · · · · · · · ·			
0.7%	Conductivity (µmhos/cm)	A DESCRIPTION OF			CONTRACTOR OF THE OWNER.	1
	Temperature ("C)					
	pH (S.U.)					
	Dissolved oxygen (mg/L)		· · · · · · · · · · · · · · · · · · ·			
1.4%	Conductivity (µmhos/cm)	a summer sum	and the second second	-	and the second se	
	Temperature (*C)					
	pH (S.U.)					
	Dissolved oxygen (mg/L)					
2.8%	Conductivity (µmhos/cm)	Concession and			and the second se	1
	Temperature (*C)					
	pH (S.U.)	_			1.1	
	Dissolved oxygen (mg/L)			1		
5.6%	Conductivity (jumhos/cm)	The second second	· · · · ·			1
	Temperature (*C)					
-	pH (S.U.)					
	Dissolved oxygen (mg/L)				5.	-
11.2%	Conductivity (umhos/cm)	1 million (199				
	Temperature (*C)					_
	pH (S.U.)				100	
	Dissolved oxygen (mg/L)			-		
	Conductivity (µmhos/cm)					_
100%	Alkalinity (mg CaCO ₂ /L)	-				
10070	Hardness (mg CaCO,/L)	-				-
	Chlorine (mg/L)		-			-
	*Temperature (*C)	_				-
	pH (S.U.)				-	
	Dissolved oxygen (mg/l)					-
	Conductivity (µmhos/cm)	1			100	1
100%	Alkalinity (mg CaCO _V /L)			-	201	100
Intake	Hardness (mg CaCD_s/L)			-	1	
	Childrine (mg/L)					-
	Temperature ("C)				22.42	-

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Final

Initial

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Initial

Final

Initial

Final



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Randomizing template: <u>RED</u>										
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										

Exhibit AT11.3: Example Chronic Test Holding Rack.



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Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan	2	09-01-19
Quality Assurance Officer	Jim Sumner	Un / un re-	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)	Lance Ferrell (NC DENR)	 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.
		Internal	Jim Sumner (ETS)	 The light intensity was amended to reflect that it is a <u>recommended</u> 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.



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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 3 rd 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 16hour 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



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

Transfer pipettes Eppendorf Repeater Pipetter Chronic test holding rack Plexiglas® slides Thermometer YWT mixture *Selenastrum capricornutum* (cell concentration = 1.71 x 10⁷ 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 $57 64 \text{ mg CaCO}_3/L$, hardness of $80 100 \text{ mg CaCO}_3/L$, 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



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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 10⁷ 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 ($25.0 \pm 1.0^{\circ}$ 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.



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- 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 $25.0 \pm 1.0^{\circ}$ 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.
 - 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.



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C. Feed Test Organisms on Day 1 (Thursday)

- 1. Remove the holding rack containing the test organisms from the incubator.
- 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 10⁷ 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.
- 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).
- 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 10⁷ 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 $(25.0 \pm 1.0^{\circ}C)$ 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.



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- 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 $25.0 \pm 1.0^{\circ}$ 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.
- 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.



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

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.



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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.



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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.

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 <i>Selenastrum</i> (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%

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



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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.

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			Initial		Final		Initial		Final		Initial		Final	
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		pH (s.u.)			_				-					
Test		Dissolved oxygen (mg/L)	-								1		-	
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100%		Conductivity (µmhos/cm)												
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Test was initiated using Sample 1. Sample 2 was used for Renewals One (day 2) and Two (day 5). synthetic water and warmed to 25.0 ± 1.0°C in a warm water bath.

Comments:

Sealer Dane Tonice

Test Results and Statistical Analyses: Test results

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SOP AT12-Revision 4-Exhibit AT12.2

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Exhibit AT12.3: Control Bench Sheet.

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

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

Randomizing template: **<u>RED</u>**

Random number seeds: 4 through 13

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Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan	2	09-01-19
Quality Assurance Officer	Jim Sumner	Un / un re-	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 <u>recommended</u> 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.



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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 3 rd broods in the control	≥ 80%
Percentage of male adults in the control	≤ 20%
Guidance percent minimum significant difference (PMSD)	No criteria



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Equipment and Materials

Ceriodaphnia dubia

Temperature-controlled incubator (set to maintain test temperature = 25.0 ± 1.0°C, photoperiod of 16hour 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 10⁷ 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



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• 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 $57 64 \text{ mg CaCO}_3/L$, hardness of $80 100 \text{ mg CaCO}_3/L$, and an initial pH of 6.5 8.5 S.U. (guidance range = 7.4 7.8 S.U.).
 - 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 10⁷ 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.



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- g. Maintain the test temperature $(25.0 \pm 1.0^{\circ}C)$ 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 $25.0 \pm 1.0^{\circ}$ 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.



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- 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.
- 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.
- 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 10⁷ 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.



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- 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).
- 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 10⁷ 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 $(25.0 \pm 1.0^{\circ}C)$ 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 $25.0 \pm 1.0^{\circ}$ 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.



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- 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)



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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.



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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.



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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.

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 <i>Selenastrum</i> (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%

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.

Client	Duke En	ergy Progres	s				NPDES #	NCOOD	0396	
Facility	Asheville	Ash Pond					Outfall	001		
Project #							County	Bunco	mbe	
Chronic	Limit 1	1.9%								
Dilution	Preparation:									
fest conce	entrations (%)	0.45	0.90	1.8	3.6	7.2			treated unless other med to 25.0 ± 1.0°0	
mL Sample	e	0,9 1,8 3		3.6	7.2	14.4	water bath an moderately h		d to the test concer water.	ntrations with
nL Dilution water		199.1	199.1 198.2 1		192.8	185.6				
Total volu	me (mL)	200	200	200	200	200				
Daily Re	newal Inform	ation Test Initiation, res	neural feading	~	_			- 1. (A)		-
Day	Date	termin	nation		Number	MHSW Batch	Selenastrum	YWT	Location	Randomizing
0	04-10-19	Time Initiation/Feeding	Analyst	Sample 1			Batch	Batch	Incubator/Shelf	Template
1	04-11-19	Feeding								
2	04-12-19	Renewal 1/Feeding	-	Sample 2						1
3	04-13-19	Feeding	-			-				
4	04-14-19	Feeding.								
5	04-15-19	Renewal 2/Feeding		Sample 2				-	-	1
6	04-16-19	Feeding	1			-				1
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):		1				11				
Replicate #	1	2	3	4	5	6	7	8	9	##
Culture board cup #										17
Date and time organisms were born between:		į.				ł				ļ
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



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	lle Ash Pond										
35.1											
nd Reproc	luction Data :										
ontrol											
Day	Observations			-	Re	-	numbe	er	_	-	
2	Adult mortality	1	2	3	4	5	6	1	8	9	10
Renewal One	(L = Live, D = dead)		10.1	$1 \le 1$	1.22	1.1		LUU:	100	1.1	P
	Number of broods present										
5	Number of young produced	4.1	1	1.11		1		$1 \leq 1$		1.1	
enewal Two	Adult mortality (L = Live, D = dead)		1.1.1					1.11			
-	Number of broods present								- 123		11 100
l	Number of young produced	1.1.2.5	1.5	16.1		12-11					143
7	Total young produced			1	1			1.1			
Final	Final adult mortality	-			-				-		
	(L = Live, D = dead)			1.41		h 11	, E.,	$[\ldots]_{i}$	- 14 J	0.11,1	
	X for 3rd Broods	100	14.5	1	1771	1-6		1-11	100	145	
_		1.1.		-	-	-	-				_
Survival		an offspri			1						
- Leafe Co		er female			-						
est conc	entration: 0.45%										
Day	Observations		-		Re	plicate	numbe	er		-	
2	Adult mortality	1	2	3	4	5	6	7	8	9	10
2 tenewal One	(L = Live, D = dead)		1.1.4			L. 11	-	$1 \le 1$			
	Number of broods present							1.25		11	
5 Renewal Two					-				-		
	(L = Live, D = dead) Number of broods present	-	-			_		-	-	_	
	Number of young produced					-	4	-	-	<u> </u>	4
7	Total young produced	-			10.000				-		
Final							1	1.1		1.1.1	
	Final adult mortality (L = Live, D = dead)	1.0	111	1.1				1.4.1	· · · · ·	11.1	
		-	_	-	-	_	-	-		-	_
		an offspri er female						duction			
Survival		si remale			-	1.2	non	contro			
Survival											
	entration: 0.90%									_	
est conc	- 2	F					numbe	er		-	10
est conc _{Day}	entration: 0.90%	1	2	3	R 4	eplicate 5	numbe 6	ar 7	8	9	10
est conc	entration: 0.90%	1	2	3				ar 7	8	9	10
est conc Day 2 Renewal One	entration: 0.90% Observations Adult mortality	1	2	3				ar 7	8	9	10
est conc Day 2 Renewal One 5	entration: 0.90% Observations Adult mortality (L=Live, D=dead) Number of broods present Number of young produced Adult mortality	1	2	3				ər 7	8	9	
est conc Day 2 Renewal One 5	entration: 0.90% Observations Adult mortality (L = Live, D = dead) Number of broods present Number of young produced Adult mortality (L = Live, D = dead)		2	3				ar 7	8	9	
est conc Day 2 Renewal One 5	entration: 0.90% Observations Adult mortality (t = live, D = dead) Number of broads present Number of young produced Adult mortality (t = live, D = dead) Number of broads present		2	3				ar 7	8	9	
est conc Day 2 Renewal One 5	entration: 0.90% Observations Adult mortality (L = Live, D = dead) Number of broods present Number of young produced Adult mortality (L = Live, D = dead)		2	3				ər 7	8	9	
est conc Day 2 Renewal One 5 Renewal Two	entration: 0.90% Observations Adult mortality (L = live, D = dead) Number of broods present Number of young produced Adult mortality (L = Live, D = dead) Number of broods present Number of broods present		2	3					8	9	



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Ashowi	lle Ash Pond											
Ashevi	ile Asir Fond											
nd Reprod	luction Data :											
Tost cons	entration: 1.8%											
	Observations	1	-	_	R	eplicate	oumbe	er	-			
Day		1	2	3	4	5	6	7	8	9	10	
2 Renewal One	Adult mortality (L+Live, D = dead)	1.5		1	11	17.2	1.20	1.11	121	1.0		
	Number of broods present		1		1.1							
5	Number of young produced		1.1	1.2.2		1			1	151	A. 1	
Renewal Two	Adult mortality (L = Live, D = dead)	111	i = j	Left 1	11	$1 \le 1$	1.000		1.11	1.00	21	
-	Number of broods present	L										
	Number of young produced		1	1.00	ere in		1		1.4.4	1.0		
7 Final	Total young produced		÷Ξr			-						
- inter	Final adult mortality	-				1	1			-		
1 m	(L = Live, D = dead)		1.5		211	110			11.0		1.1	
22.45		an offspri	ng	-	-	n d	% Re	duction	T	_	-	
Survival		er female			-			Contro				
Test cond	entration: 3.6%											
Day	Observations				_	-	numbe	_			10	
2	Adult mortality	1	2	3	4	5	6	7	8	9	10	
Renewal One	(L + Live, D = dead)				911	i E.	1.21		5 I U		11	
	Number of broods present			10								
5 Renewal Two	Number of young produced Adult mortality			-		104			100			
inchewat i Wo	(L = Live, D = dead)											
-	Number of broods present				100							
	Number of young produced	-	1.14	1.00		15	$r \simeq 0$		1	1.0	1.1	
				1		1				1		
7 Final	Total young produced								-			
7 Final	Total young produced Final adult mortality		-				1 1					
			i.						11	1		
Final	Final adult mortality (L = Live, D = dead)						94 P-	duction			_	
	Final adult mortality (L = Live, D = dead) Me	an offspri						duction				
Final Survival	Final adult mortality (L = Live, D = dead) Me											
Final Survival	Final adult mortality (L = Live, D = dead) Me P entration: 7.2%						from	Contro			Ξ	
Final Survival	Final adult mortality (L = Live, D = dead) Me	er female		3	_	eplicate	from e numbe	Contro		9	10	
Final Survival	Final adult mortality (L = Live, D = dead) Me P entration: 7.2%			3	R 4	-	from	Contro		9	10	
Final Survival Test conc Day	Final adult mortality (L = Live, D = dead) entration: 7.2% Observations Adult mortality (L = Live, D = dead)	er female		3	_	-	from e numbe	Contro		9	10	
Final Survival Fest conc Day 2 Renewal One	Final adult mortality (L = Live, D = dead) entration: 7.2% Observations Adult mortality (L = Live, D = dead) Number of broods present	er female		3	_	-	from e numbe	Contro		9	10	
Final Survival Fest conc Day 2	Final adult mortality (L = Live, D = dead) Me pentration: 7.2% Observations Adult mortality (L = Live, D = dead) Number of broods present Number of young produced	er female		3	_	-	from e numbe	Contro		9	10	
Final Survival Fest conc Day 2 Renewal One 5	Final adult mortality (L = Live, D = dead) entration: 7.2% Observations Adult mortality (L = Live, D = dead) Number of broods present	er female		3	_	-	from e numbe	Contro		9	10	
Final Survival Fest conc Day 2 Renewal One 5	Final adult mortality (L = Live, D = dead) entration: 7.2% Observations Adult mortality (L = Live, D = dead) Number of broods present Number of young produced Adult mortality (L = Live, D = dead) Number of proods present Number of broods present	er female		3	_	-	from e numbe	Contro		9	10	
Final Survival Fest conc Day 2 Renewal One 5 Renewal Two	Final adult mortality (L = Live, D = dead) Me pentration: 7.2% Observations Adult mortality (L = Live, D = dead) Number of broods present Number of broods present	er female		3	_	-	from e numbe	Contro		9	10	
Final Survival Fest conce Day 2 Renewal One 5 Renewal Two 7	Final adult mortality (L = Live, D = dead) entration: 7.2% Observations Adult mortality (L = Live, D = dead) Number of broods present Number of young produced Adult mortality (L = Live, D = dead) Number of proods present Number of proods present	er female		3	_	-	from e numbe	Contro		9		
Final Survival Fest conc Day 2 Renewal One 5 Renewal Two	Final adult mortality (L = Live, D = dead) Me pentration: 7.2% Observations Adult mortality (L = Live, D = dead) Number of broods present Number of broods present	er female		3	_	-	from e numbe	Contro		9		



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ity Asl	neville Ash Pond						
ect #							
mical Analy	ises:	Init	iation Final	Renev	val One Final	Renev	val Two Final
oncentration	Analyst	Initiat	Final	Initial	Final	Initial	Pinal
	pH (S.U.)	14	1000		TI II	12 31	11
	Dissolved oxygen (mg/L)	1		1.1.1.1.		1	
1.55	Conductivity (sumhos/cm)				1		
Control MH5W	*Alkalinity (mg/L CaCO ₁)		-				
	*Hardness (mg/L CaCO ₁)	1					1
	*Temperature (°C)				-	-	
_	pH (S.U.)	-		-		-	
	Dissolved oxygen (mg/1)	-	-			1	-
0.45%	Conductivity (µmhos/em)		1	-	1.0		1
	*Temperature (°C)	-		-			
	pH (5.0.)		-	-			
	Dissolved oxygen (mg/L)					-	
0.90%	Conductivity (µmhos/cm)		-				
		-		_		-	
	*Temperature (°C)	1.1				1.1	
	pH (5.U.)		15.41		1	1	100
1.8%	Dissolved oxygen (mg/L)						
	Conductivity (umhoi/cm)	Plane I		i 1		$l = \mathbf{g} $	
0.90%	*Temperature (°C)	100	1000		11.19		
	pH (s.u.)	1				1	
	Dissolved oxygen (mg/L)			1.1.1.1	11011	12	
3.6%	Conductivity (umhos/cm)			1	1		
	"Temperature (°C)				1		
	pH (S.U.)	1	1	1		1 A.	
	Dissolved oxygen (mg/L)		1.1	1.1			
7.2%	Conductivity (µmhos/cm)	-	-	1			-
	*Temperature ("C)			-	-	-	10000
-	pH (S.U.)				1	-	1
	Dissolved oxygen (mg/L)			-	-	-	
100%	Conductivity (umhos/cm)					-	
100/6	*Total residual chlorine (mg/L)	-	-		-		
	*Temperature (°C)					-	

enewal or termination by the analysi identified in the Daily Renewal Information table. Alkalimity, hardness and total residual chlorine performed by the analysis identified on the test specific bench sheets and transcribed to this bench sheet.

Confidential

SOP AT13-Revision 4-Eshibit AT13.2



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

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

Randomizing template: **<u>RED</u>**

Random number seeds: 4 through 13

SOP AT11-Revision 5-Exhibit AT11.3



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

Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan	N	03-01-20
Quality Assurance Officer	Jim Sumner	Un / unse	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)	Rick Sherrard (TVA)	Updated bench sheet to include reporting limits, method numbers, meters and serial numbers used for chemical analyses.
		Internal	Jim Sumner (ETS)	



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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 (IC₂₅) 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.

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 3 rd 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%

 Table AT14.1: Ceriodaphnia dubia chronic toxicity test acceptability criteria.



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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 = $25.0 \pm 1.0^{\circ}$ C, photoperiod of 16hour 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.



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- 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.

 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.
- 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 watervolumes, 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.



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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.



Exhibit AT14.1: Example Ceriodaphnia dubia Chronic Reference Toxicity Test Bench Sheet.



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Sodium Chloride Chronic Reference Toxicant Test (EPA-821-R-02-013 Method 1002.0) Species: <u>Ceriodaphnia dubia</u>

CdNaCICR	#:	241
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Dilution prepara	tion info	Comments:				
NaCl Stock INSS number	r:	INSS				
Stock preparation:	100 g NaC Dissolve 5		00 mL deioni			
Dilution prep (mg/L)	600	800	×		1400	11 T
Stock volume (mL)	9	12	15	18	5	
Diluent volume (mL)	1491	1488	1485	1482		
Total volume (mL)	1500	1500	1500	1500	1500	A REAL PROPERTY OF THE PROPERT

Test organism source:

Test randomization and location:

Organism age:		< 24-hours old								Randomizing template color:	
Date and times organisms were born between:										Incubator number and shelf	
Culture board:											location:
Replicate number:	1	2	3	4	5	6	7	8	9	10	
Culture board cup number:			1	1.1			1.1				
Transfer vessel information:	pH (S.U.): Temperature (°C):						ure (°	C):	1 m		
Average transfer volume (mL):	< 0.25 mL										

Daily renewal:

Day	Date	Test initiation and feeding, renewal and feeding, or termination time	*Feeding B Selenastrum	Batches YWT	MHSW batch used	Analyst
0		termination unic				
1					1	
2					-	
3						-
4				-	1	
5						
6					· · · · · · · · · · · · · · · · · · ·	
7			19.00000001	-		1

*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
pН	0.1 S.U.	SM 4500-H+ B-2011	Accumet AR20	93312452
Dissolved Oxygen (D.O.)	1.0 mg/L	SM 4500-0 G-2011	YSI Model 52CE	18D104324
Conductivity	14.9 µmhos/cm	SM 2510 B-2011	Accumet AR20	93312452
Alkalinity	5.0 mg CaCO ₁ /L	SM 2320 B-2011	Accumet AR20	93312452
Hardness	5.0 mg CaCO ₁ /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 LCso (mg/L NaCl)
% Adults having 3 rd Broods:	> 80%	NOEC (mg/L NaCl)
% Mortality:	≤ 20%	LOEC (mg/L NaCl)
Mean Offspring/Female:	\geq 15.0 offspring/female	ChV (mg/L NaCl)
% CV:	< 40.0 %	IC25 (mg/L NaCl)

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Day				urvival	-			u		_
Dav				-	-	e numbe	-			
	1	2	3	4	5	6	7	8	9	10
1 Young produced		-	11	1	1					-
Adult mortality				1 1	11 11	11 11	1.1	11		1.1.1
2 Young produced					·	1				1
Adult mortality	t	1				1	-	2	2	1
3 Young produced				11		1		1		-
Adult mortality	-							2.111	1	1
4 Young produced		1	1					1 11		-
Adult mortality						-				
5 Young produced			1	-		12.23	-			
Adult mortality	-		100			-	-	1		24-1
6 Young produced		-0-04	+ $ -$	1.5	5.5	5-5-		1.51		1
Adult mortality		1.00	1.000			1.00		2.2	1.200	1.27%
7 Young produced	-									1.1
otal young produced		1.000	1			10000		11	0.1011	1.000
and the second second			the second second					1 I	1	
				1 = 1		-	-	-		2
inal Adult Mortality for 3 rd Broods ote: Adult mortality (L=live, D=deed), t	8 = split brood	d (single brod	d i gilt betwee	n two days),	CO ≈ carry ov	Concent % Morta	tration: ality:			
inal Adult Mortality for 3 st Broods ote: Adult mortality (L = irve, D = dead), S	B = 1pht brood	d (single brod				Concent % Morta Mean O	tration: ality: ffspring,	/Female:		
inal Adult Mortality for 3 rd Broods ate: Adult mortality (t = Inve. D = dead), 5 ate: Adult mortality (t = Inve. D = dead), 5 ate: Adult mortality (t = Inve. D = dead), 5 ate: Adult mortality (t = Inve. D = dead), 5 ate: Adult Mortality (t = In			S	urvival	and Re Replicat	Concent % Morta Mean O product	tration: ality: ffspring, tion Da	/Female:		
inal Adult Mortality for 3 rd Broods oter Adult mortality (L=line, D=dead), 5 500 mg NaCl/L Day	B = split brood	d (single brod			and Re	Concent % Morta Mean O product	tration: ality: ffspring,	/Female:		10
inal Adult Mortality for 3 rd Broods for Adult mortality (L=live, D=deed), 5 600 mg NaCl/L Day 1 Young produced			S	urvival	and Re Replicat	Concent % Morta Mean O product	tration: ality: ffspring, tion Da	/Female:		10
inal Adult Mortality for 3 rd Broods for Adult mortality (L=live, D=deed), 5 600 mg NaCl/L Day 1 Young produced Adult mortality			S	urvival	and Re Replicat	Concent % Morta Mean O product	tration: ality: ffspring, tion Da	/Female:		10
inal Adult Mortality for 3 rd Broods for Adult mortality (L=live, D=deed), 5 600 mg NaCl/L Day 1 Young produced Adult mortality 2 Young produced			S	urvival	and Re Replicat	Concent % Morta Mean O product	tration: ality: ffspring, tion Da	/Female:		10
inal Adult Mortality for 3 rd Broods for Adult mortality (L=live, D=deed), 5 600 mg NaCl/L Day 1 Young produced Adult mortality 2 Young produced Adult mortality			S	urvival	and Re Replicat	Concent % Morta Mean O product	tration: ality: ffspring, tion Da	/Female:		10
inal Adult Mortality for 3 rd Broods for Adult mortality (L=live, D=deed), 5 600 mg NaCl/L Day 1 Young produced Adult mortality 2 Young produced Adult mortality 3 Young produced			S	urvival	and Re Replicat	Concent % Morta Mean O product	tration: ality: ffspring, tion Da	/Female:		10
inal Adult Mortality for 3 rd Broods ate: Adult mortality (L=live, D=dead), 1 500 mg NaCl/L Day 1 Young produced Adult mortality 2 Young produced Adult mortality 3 Young produced Adult mortality			S	urvival	and Re Replicat	Concent % Morta Mean O product	tration: ality: ffspring, tion Da	/Female:		10
inal Adult Mortality for 3 rd Broods are Adult mortality (t = Iwe, D = dead), 1 are Adult mortality (t = Iwe, D = dead), 1 are Adult mortality 1 Young produced Adult mortality 3 Young produced Adult mortality 4 Young produced			S	urvival	and Re Replicat	Concent % Morta Mean O product	tration: ality: ffspring, tion Da	/Female:		10
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inal Adult Mortality for 3 rd Broods ote: Adult mortality (t = Ine, D = dead), the Adult mortality (t = Ine, D = dead), the Adult mortality 2 Young produced Adult mortality 3 Young produced Adult mortality 4 Young produced Adult mortality 5 Young produced			S	urvival	and Re Replicat	Concent % Morta Mean O product	tration: ality: ffspring, tion Da	/Female:		10
inal Adult Mortality for 3 rd Broods ote: Adult mortality (t = Ine, D = dead), the Adult mortality (t = Ine, D = dead), the Adult mortality 2 Young produced Adult mortality 3 Young produced Adult mortality 4 Young produced Adult mortality 5 Young produced Adult mortality			S	urvival	and Re Replicat	Concent % Morta Mean O product	tration: ality: ffspring, tion Da	/Female:		10
inal Adult Mortality for 3 rd Broods ote: Adult mortality (L = Inve, D = deed), adult mortality (L = Inve, D = deed), adult mortality 2 2 3 4 4 4 4 4 5 5 5 5 4 5 5 5 5 4 5 5 5 5			S	urvival	and Re Replicat	Concent % Morta Mean O product	tration: ality: ffspring, tion Da	/Female:		10
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Inal Adult Mortality for 3 rd Broods ate: Adult mortality (L = Inve, D = deed), S 200 mg NaCl/L Day 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			S	urvival	and Re Replicat	Concent % Morta Mean O product	tration: ality: ffspring, tion Da	/Female:		30
Inal Adult Mortality for 3 rd Broods ate: Adult mortality (L = Inve, D = deed), S 200 mg NaCl/L Day 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			S	urvival	and Re Replicat	Concent % Morta Mean O product	tration: ality: ffspring, tion Da	/Female:		10

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			5	urvival	and Re	product	tion Da	ta		
					Replicat	e numbe	er			
Day	1	2	3	4	5	6	7	8	9	10
1 Young produced					13	1.57			111	
Adult mortality			1	L	2				·	
2 Young produced		100		5 · · · · ·				1		1
Adult mortality		1.20	11.21	1000	2-1-1				1	1.000
3 Young produced			1.2.2		200		-	1		
Adult mortality		1000		1	1				1	-
4 Young produced										
Adult mortality										
5 Young produced				1	1					
Adult mortality		1		1		1				
6 Young produced					1			-		
Adult mortality			1.1			-	-		-	1
7 Young produced							-			-
otal young produced									_	1177
	_	1		1						
nal Adult Mortality te. Adult mortality (L= live, D = dead).	5B = split broos	d (single broo	d split betwee	en two days),	2 %	oncent Mortali Mean Off	r ation: ty: spring/F	emale:		
nal Adult Mortality te, Adult mortality (L= iive, D = dead).	68 = split broos	d (single broo			2% N %	Mortali Mean Off	ration: ty: spring/F ion from	emale: Control		
nal Adult Mortality	5B = split broos	d (single broo			and Re	oncent Mortali Mean Off Reduct product	ration: ty: spring/F ion from	emale: Control		
nal Adult Mortality te, Adult mortality (L= iive, D = dead).	SB = split broos	d (single broo			and Re	Mortali Mean Off	ration: ty: spring/F ion from	emale: Control		10
nal Adult Mortality te. Adult mortality (L= ive, D = dead). 000 mg NaCl/L			s	urvival	and Replicat	Mortali Mean Off Reduct product	ration: ty: spring/F ion from tion Dat	emale: Control ta	E I	10
nal Adult Mortality te: Adult mortality (L= live, D= dead). 000 mg NaCl/L Day			s	urvival	and Replicat	Mortali Mean Off Reduct product	ration: ty: spring/F ion from tion Dat	emale: Control ta	E I	10
nal Adult Mortality te: Adult mortality (L= live, D = dead). 000 mg NaCl/L Day 1 Young produced			s	urvival	and Replicat	Mortali Mean Off Reduct product	ration: ty: spring/F ion from tion Dat	emale: Control ta	E I	10
Adult Mortality te: Adult mortality (L= live, D = dead). 000 mg NaCl/L Day 1 Young produced Adult mortality			s	urvival	and Replicat	Mortali Mean Off Reduct product	ration: ty: spring/F ion from tion Dat	emale: Control ta	E I	10
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Adult Mortality Adult mortality (L= live, D = dead). 0000 mg NaCl/L Day 1 Young produced Adult mortality 2 Young produced Adult mortality 3 Young produced Adult mortality			s	urvival	and Replicat	Mortali Mean Off Reduct product	ration: ty: spring/F ion from tion Dat	emale: Control ta	E I	10
Adult Mortality Adult mortality (L=live, D=dead). Adult mortality 2 Young produced Adult mortality 3 Young produced Adult mortality 4 Young produced			s	urvival	and Replicat	Mortali Mean Off Reduct product	ration: ty: spring/F ion from tion Dat	emale: Control ta	E I	10
Adult Mortality Adult mortality (L=live, D=dead). Adult mortality 2 Young produced Adult mortality 3 Young produced Adult mortality 4 Young produced Adult mortality			s	urvival	and Replicat	Mortali Mean Off Reduct product	ration: ty: spring/F ion from tion Dat	emale: Control ta	E I	10
Adult Mortality Adult mortality (L= ive, D = dead). Adult mortality (L= ive, D = dead). Day 1 Young produced Adult mortality 2 Young produced Adult mortality 3 Young produced Adult mortality 4 Young produced Adult mortality 5 Young produced			s	urvival	and Replicat	Mortali Mean Off Reduct product	ration: ty: spring/F ion from tion Dat	emale: Control ta	E I	10
Adult Mortality Adult mortality (L= ive, D = dead). Adult mortality (L= ive, D = dead). Day 1 Young produced Adult mortality 2 Young produced Adult mortality 4 Young produced Adult mortality 5 Young produced Adult mortality			s	urvival	and Replicat	Mortali Mean Off Reduct product	ration: ty: spring/F ion from tion Dat	emale: Control ta	E I	10
Adult Mortality te: Adult mortality (L= live, D = dead). 0000 mg NaCl/L Day 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 5 Young produced			s	urvival	and Replicat	Mortali Mean Off Reduct product	ration: ty: spring/F ion from tion Dat	emale: Control ta	E I	10
Adult Mortality te: Adult mortality (L= ive, D = dead). Adult mortality (L= ive, D = dead). Day 1 Young produced Adult mortality 2 Young produced Adult mortality 3 Young produced Adult mortality 5 Young produced Adult mortality 5 Young produced Adult mortality 6 Young produced Adult mortality			s	urvival	and Replicat	Mortali Mean Off Reduct product	ration: ty: spring/F ion from tion Dat	emale: Control ta	E I	10
Adult Mortality te: Adult mortality (L= ive, D = dead). Adult mortality (L= ive, D = dead). Day 1 Young produced Adult mortality 2 Young produced Adult mortality 3 Young produced Adult mortality 5 Young produced Adult mortality 5 Young produced Adult mortality 5 Young produced Adult mortality 5 Young produced Adult mortality 7 Young produced			s	urvival	and Replicat	Mortali Mean Off Reduct product	ration: ty: spring/F ion from tion Dat	emale: Control ta	E I	10
Adult Mortality te: Adult mortality (L= ive, D = dead). Adult mortality (L= ive, D = dead). Day 1 Young produced Adult mortality 2 Young produced Adult mortality 3 Young produced Adult mortality 5 Young produced Adult mortality 5 Young produced Adult mortality 6 Young produced Adult mortality			s	urvival	and Replicat	Mortali Mean Off Reduct product	ration: ty: spring/F ion from tion Dat	emale: Control ta	E I	10

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1200 r	ng NaCl/L			S	urvivai	and Re	product	ion Dat	ta		
					1	Replicate	e numbe	r			
Day		1	2	3	4	5	6	7	8	9	10
1	Young produced	-	1.00	1.1			1.	1.00			
	Adult mortality	_			_		_	_		-	
2	Young produced	_	1000	1000	1						
	Adult mortality			1.1.1			12.23				
3	Young produced		1								
-	Adult mortality										
4	Young produced		1.000	16-24				1.00		· · · · ·	1
	Adult mortality		1000	12				1		1	
5	Young produced		1.000							1 mar 1	
	Adult mortality	_	-						-		1.0
6	Young produced			1						-	-
	Adult mortality			17. 24						1.000	-
7	Young produced			1.1.1.1							1
fotal you	ng produced		1.7.7.1	1.0.00						1 1 1	
Final Adu	It Mortality							1		-	-
100	na Na Cl (I					N %	Mortali Iean Off Reducti	spring/F on from	Control	:	
1400 r	ng NaCl/L			s	urvival	and Re	lean Off Reducti product	spring/F on from	Control	:	
1	ng NaCl/L	1				and Replicate	lean Off Reducti product	spring/F on from tion Dat	Control ta		1 10
Day		1	2	3	urvival	and Re	lean Off Reducti product	spring/F on from	Control	9	10
1	ng NaCI/L Young produced Adult mortality	1	2			and Replicate	lean Off Reducti product	spring/F on from tion Dat	Control ta		10
Day	Young produced Adult mortality	1	2			and Replicate	lean Off Reducti product	spring/F on from tion Dat	Control ta		10
Day 1	Young produced	1	2			and Replicate	lean Off Reducti product	spring/F on from tion Dat	Control ta		10
Day 1	Young produced Adult mortality Young produced	1	2			and Replicate	lean Off Reducti product	spring/F on from tion Dat	Control ta		10
Day 1 2	Young produced Adult mortality Young produced Adult mortality	1	2			and Replicate	lean Off Reducti product	spring/F on from tion Dat	Control ta		10
Day 1 2	Young produced Adult mortality Young produced Adult mortality Young produced	1	2			and Replicate	lean Off Reducti product	spring/F on from tion Dat	Control ta		10
Day 1 2 3	Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality	1	2			and Replicate	lean Off Reducti product	spring/F on from tion Dat	Control ta		10
Day 1 2 3	Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality Young produced	1	2			and Replicate	lean Off Reducti product	spring/F on from tion Dat	Control ta		
Day 1 2 3 4	Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality	1	2			and Replicate	lean Off Reducti product	spring/F on from tion Dat	Control ta		10
Day 1 2 3 4	Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality Young produced	1	2			and Replicate	lean Off Reducti product	spring/F on from tion Dat	Control ta		
Day 1 2 3 4 5	Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality	1	2			and Replicate	lean Off Reducti product	spring/F on from tion Dat	Control ta		
Day 1 2 3 4 5	Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality Young produced		2			and Replicate	lean Off Reducti product	spring/F on from tion Dat	Control ta		
Day 1 2 3 4 5 6 7	Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality		2			and Replicate	lean Off Reducti product	spring/F on from tion Dat	Control ta		
Day 1 2 3 4 5 6 7 fotal you	Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality Young produced ng produced		2			and Replicate	lean Off Reducti product	spring/F on from tion Dat	Control ta		
Day 1 2 3 4 5 6 7 rotal you	Young produced Adult mortality Young produced It Mortality			3	4	Replicate s	1ean Off: Reducti product e number 6	spring/F on from tion Dat	Control ta 8	9	
Day 1 2 3 4 5 6 7 rotal you	Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality Young produced Adult mortality Young produced ng produced			3	4	Replicate 5	1ean Off: Reducti product e number 6	spring/F on from tion Dat 7	Control a 8	9	
Day 1 2 3 4 5 6 7 Total you	Young produced Adult mortality Young produced It Mortality			3	4	Replicato	Tean Off: Reduction Product e number 6	spring/F on from tion Dat 7	Control a 8	9	
Day 1 2 3 4 5 6 7 Total you Final Adu	Young produced Adult mortality Young produced It Mortality			3	4	Replicate 5	1ean Off: Reducti product e number 6	spring/F on from tion Dat 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7	Control a 8	g transfer):	

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Species: Ceriodaphnia dubia

CdNaCICR #: 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.

		Analust Ideas	ifted for each de	Da ay, performed pH			incase only
		(Analyst iden		ay, performed pr	1, 0.0, and cond 1		2
	Analyst				-		
Concentration	Parameter		-	The summer of			-
	pH (S.U.)						
	Dissolved oxygen (mg/L)				1		
CONTROL, MHSW	Conductivity (µmhos/cm)					1	
CONTROL, MILISW	Alkalinity (mg CaCO3/L)		1	1			
	Hardness (mg CaCO ₃ /L)			100		1	_
	Temperature (°C)					1	
	pH (S.U.)			100 C		1	11
600 mg NaCl/L	Dissolved oxygen (mg/L) Conductivity	-		-			
	(µmhos/cm)			1.1		1 ·····	
	Temperature ("C)	1		17 *		n	
	pH (S.U.)						-
800 mg NaCl/L	Dissolved oxygen (mg/L)						
BOU HIS NACI/L	Conductivity (µmhos/cm)					· · · · · · · · · · ·	
	Temperature (°C)			1			
	pH (S.U.)						
1000 mg NaCl/L	Dissolved oxygen (mg/L)						
2000 mg much 2	Conductivity (µmhos/cm)		-				
	Temperature (°C)						
	pH (S.U.)						-
1200 mg NaCl/L	Dissolved oxygen (mg/L)		1000			1 = =0	
	Conductivity (µmhos/cm)						
	Temperature (°C)					· · · · · · · · ·	
	pH (S.U.)						
1400 mg NaCl/L	Dissolved oxygen (mg/L)						
/-	Conductivity (µmhos/cm)		-	·			-
	Temperature (°C)	Initial	Final	Initial	Final	Initial	Final

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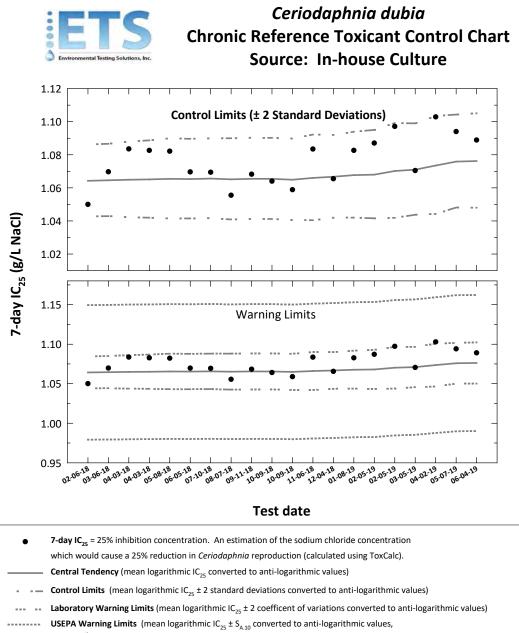
CdNaCICR #: 241

		Day							
		 (Analyst identified for each day, performed pH, D.O. and conductivity measu 3 4 5						irements only.) 6	
	Analyst	 -	-			-	~	Î	
Concentration	Parameter		1	1		1	1		
CONTROL, MHSW	pH (S.U.)		-						
	Dissolved oxygen (mg/L)						1		
	Conductivity (µmhos/cm)						3		
	Alkalinity (mg CaCO ₃ /L)	 1					1		
	Hardness (mg CaCO ₃ /L)				1		1		
	Temperature (°C)	1	1	1					
600 mg NaCl/L	pH (S.U.)	 0			24	1.000	·		
	Dissolved oxygen (mg/L)		1, 11, 1	-		1.1	<u> </u>		
	Conductivity (µmhos/cm)		1				·		
	Temperature (°C)	 1	C	3 — T		1			
800 mg NaCl/L	pH (S.U.)	 - I	1			and the	J. 6.		
	Dissolved oxygen (mg/L)		1 1			·			
	Conductivity (µmhos/cm)	 	1				1		
	Temperature (°C)	c c	1		-	*/	1.000	1	
1000 mg NaCl/L	pH (S.U.)	š	1			1	1		
	Dissolved oxygen (mg/L)	1				, L			
	Conductivity (µmhos/cm)		+		1		i		
	Temperature (°C)	 1	A			i			
1200 mg NaCl/L	pH (S.U.)			-			2.2		
	Dissolved oxygen (mg/L)	1	0710			1	(?~~~~		
	Conductivity (µmhos/cm)			-	1000	1	2		
	Temperature ("C)	1					12		
1400 mg NaCl/L	pH (S.U.)	 1	-	1	-				
	Dissolved oxygen (mg/L)	1	1	-					
	Conductivity (µmhos/cm)		1						
	Temperature (°C)		Contraction in the	1			. C	_	

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	Aquatic Toxicity P	rocedures
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 $S_{A,10} = 10^{th}$ percentile of CVs reported nationally by USEPA)





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Subject: Ceriodaphnia dubia Chronic Reference Toxicity Test, EPA 1002.0

E					Ceriodo Referenco Source: In		Control C	hart				
-			Le	og ₁₀ Conversio	n	5		Anti-loga	rithmic Values	(g/L NaCl)		
Test number	Test date	ToxCal Determination 7	7-day IC ₂₅ foxCal Determination 7-day IC ₂₅ CT S		-	ст		l Limits	Laboratory (Warnin	Calculated CV ng Limits	Warnin	centile CV Ig Limits
	02.06.18	(g/L NaCl)	0.0212	0.0270	0.0044	1.0642	CT-25 1.0427	CT + 2S 1.0862	CT - 2CV 1.0440	CT + 2CV 1.0844	CT - S _{A.10} 0.9791	CT + SA.10 1.1494
2	02-06-18	1.0500 1.0697	0.0212	0.0270	0.0044	1.0642	1.042/	1.0862	1.0440	1.0844	0.9791	1.1494
3	04-03-18	1.0835	0.0295	0.0272	0.0043	1.0649	1.0430	1.0880	1.0443	1.0861	0.9794	1.1498
4	04-03-18	1.0835	0.0348	0.0275	0.0047	1.0651	1.0424	1.0888	1.0437	1.0869	0.9797	1.1501
5	05-08-18	1.0822	0.0343	0.0274	0.0048	1.0655	1.0420	1.0899	1.0434	1.0809	0.9799	1.1503
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	1.0413	1.0900	1.0430	1.0880	0.9804	1.1508
8	08-07-18	1.0555	0.0235	0.0274	0.0050	1.0652	1.0409	1.0900	1.0432	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

7-day IC25 = 25% inhibition concentration. An estimation of the sodium chloride concentration that would cause a 25% reduction in Ceriodophnia reproduction (calculated using ToxCalc). Note:

CT = Central tendency of the IC25 values.

 $\label{eq:standard deviation of the (<math>\frac{1}{25}$ values. **Control Limits =** Mean logarithmic ($\frac{1}{25}$ ± 2 standard deviations converted to anti-logarithmic values. **Warning Limits =** Mean logarithmic ($\frac{1}{25}$ ± 2CV or $\frac{1}{8,10}$ converted to anti-logarithmic values.

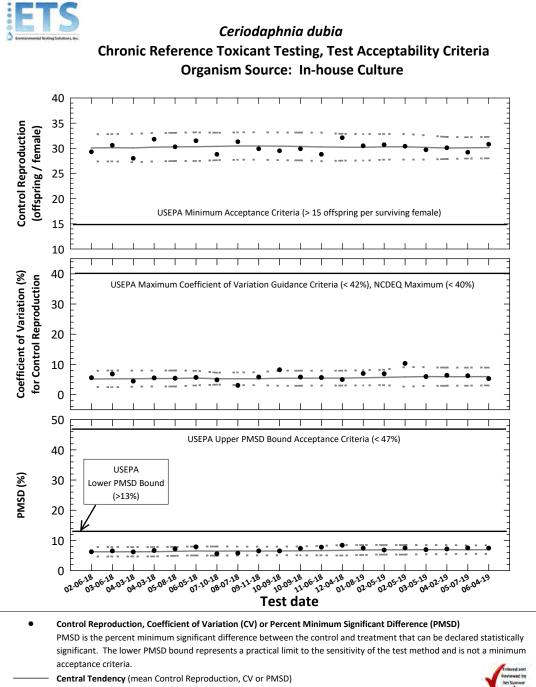
 $S_{A,30} = Standard deviation corresponding to the 10th percentile of CVs reported nationally by USEPA (S_{A,30} = 0.08).$ CV = Coefficient of variation.





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Subject: Ceriodaphnia dubia Chronic Reference Toxicity Test, EPA 1002.0



= = 95% Confidence Interval (mean Control Reproduction, CV or PMSD ± 2 Standard Deviations)



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Subject: Ceriodaphnia dubia Chronic Reference Toxicity Test, EPA 1002.0

	-	· · · · ·	ToxCall	Determinati	on		Co	ontrol Reprodu	ction	Con	trol Reproduct	ion CV		Test PMSD	-
1		Control	Control Repro	duction	T	est	1.000	(offspring/fema	le)		(%)			(96)	
Test number	Test date	Survival	Mean	CV	MSD	PMSD	CT	95% Confid	ence Interval	CT	95% Confid	ence Interval	CT	95% Confid	ence Interva
		(%)	(offspring/female)	(%)		(16)		CT - 25	CT + 25		CT - 25	CT + 25		CT - 25	CT + 25
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 2 80% survival.

 Control Mean Reproduction =
 USEPA minimum test acceptability criteria 2 15 offsping/surviving female.

 CV =
 Coefficient of variation for control reproduction, USEPA maximum CV guidance criteria (90° percentile) < 42%. NCDEQ maximum CV acceptance criteria < 40%.</td>

 MSD =
 Notimum 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° percentile) > 13%. The lower PMSD bound acceptance criteria determined by USEPA (10° percentile) < 47%.</td>

 CT =
 Central tendency of the reproduction, CV or PMSD values.

 S =
 Standard deviation of the reproduction, CV or PMSD values.





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Subject: Taxonomic Identification of *Ceriodaphnia dubia*

Approval

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

Document Revision History

Effective	Revision	Review	Evaluators	Revisions
Date	number	Туре		
12-01-00	0	Internal	Jim Sumner (ETS)	Original document
07-10-10	1	Internal	Lance Ferrell	Exhibit AT15.1 revised for the key taxonomic characteristics of
			(NC DENR)	Ceriodaphnia dubia and to provide a more efficient logsheet.
			Jim Sumner (ETS)	
06-01-11	2	Internal	Jim Sumner (ETS)	Updated exhibits.
11-01-14	3	External	Jim Sumner (ETS)	 Updated exhibits during document review.
		(TVA)		 Updated procedure for current slide preparation techniques.
		Internal		
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.



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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



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Subject: Taxonomic Identification of Ceriodaphnia dubia

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.



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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.

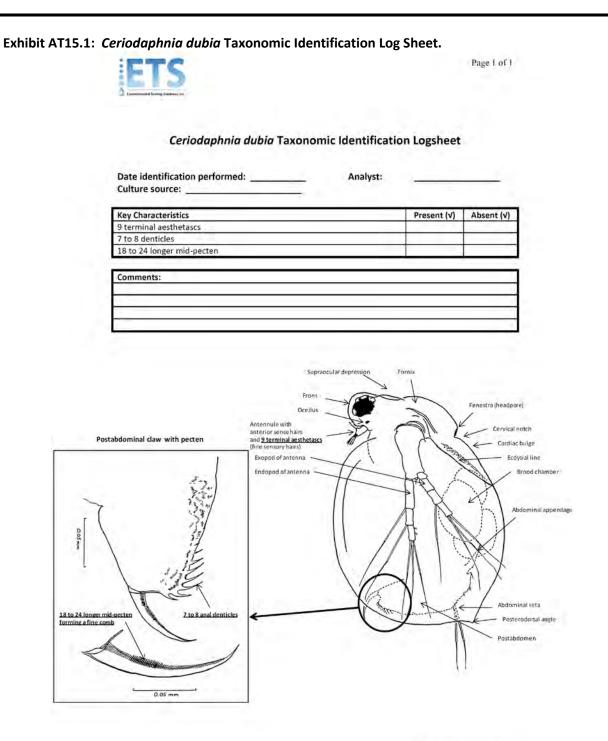
H. B. Ward and G. C. Wipple, Fresh-Water Biology, Second Edition, John Wiley & Sons, Inc., 1959.

Exhibits

Exhibit AT15.1: Ceriodaphnia dubia Taxonomic Identification Log Sheet.



Subject: Taxonomic Identification of Ceriodaphnia dubia



SOP AT15-Revision 4-Exhibit AT15.1



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Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan	2	04-25-22
Quality Assurance Officer	Jim Sumner	Un / un re-	04-25-22

Document Revision History

Effective	Revision	Review	Evaluators	Revisions
Date	number	Туре		
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)	Rick Sherrard (TVA)	Updated table AT16.1 to include the date that each Artemia CHM number was submitted for analytical analyses.
		Internal	Jim Sumner (ETS)	
04-25-22	5	Internal	Jim Sumner (ETS)	 Additional feeding guidance added to SOP.



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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 <u>recommends</u> that brine shrimp be verified to contain < 50 ng/L organochlorine pesticides plus PCBs, < 1 μ g/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 <u>should</u> 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).



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Table AT16.1: Concentration of metals (μ g/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 μ L 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
Со	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.



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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 μ m 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.



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- 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 $50 \ \mu$ L) 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 μ L) and the percent hatch-out and record on the log sheet.
 - i. The number of nauplii contained in one drop (equal to $50 \ \mu$ L) 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.



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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.
- 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 <u>2 times</u> 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.



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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.



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Exhibits

Exhibit AT16.1: Artemia Shipment Log Sheet.



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Exhibit AT16.2: Artemia Shipment Log Sheet.



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Artemia Shipment Log

Shipment receipt:

Source:	Brine Shrimp Direct
Lot number:	the second second second second second second second second second second second second second second second se
Date received:	
Expiration date:	
Received by (initials):	
Analytical submitted:	
Reference testing performed:	

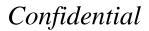
Artemia count verification:

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:			

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Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan	\mathcal{N}	08-28-23
Quality Assurance Officer	Jim Sumner	Upp / upp e	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)	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.



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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.



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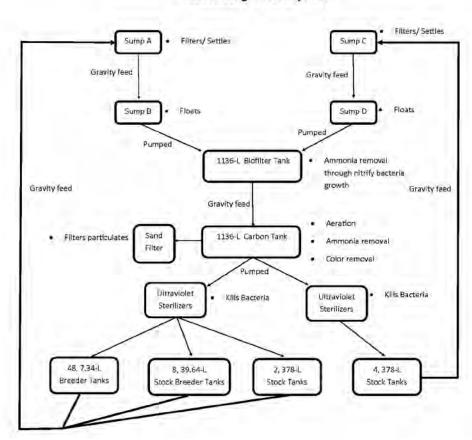
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- 4. Low pH (< 7.0 S.U.) will result in reduced spawns and low-quality embryos. NaHCO₃ may be added to increase the pH back to > 7.5 S.U. when low pH occurs.
- 5. Fungal growth on eggs must be completely removed. Thoroughly rinsing of eggs after their 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.



Fathead minnow (*Pimephales promelas*) Recirculating Culture System



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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:

<u>Water Supply</u>: 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 NaHCO₃, increase aeration, exchange culture water.)

<u>Sumps</u>: 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.

<u>Hayward® High Rate Sand Filter</u>: 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.

<u>Biofilter tank</u>: 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.

<u>Carbon Tank</u>: 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



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carbon. This provides additional ammonia removal and removes color. The carbon tank is washed, and activated carbon replaced as needed.

<u>Ultraviolet Sterilizers</u>: 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.

<u>External Pumps</u>: Flow and pressure are provided by two external pumps and flow rate is regulated by ball valves at each culture tank.

<u>Temperature Control</u>: Temperature control ($25.0 \pm 2.0^{\circ}$ 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.

<u>Standpipes</u>: Each tank is fitted with an overflow standpipe which causes outflow water to be drawn from the bottom of the tanks.

<u>Cleaning Culture Tanks</u>: Accumulated wastes (excess food and solid metabolic material) are siphoned as needed. Tanks are removed from the system and cleaned as needed.

<u>Spawning Substrates</u>: 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.

<u>Aeration</u>: 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.

<u>Lighting</u>: 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



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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

<u>Adults Sources</u>: 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).

<u>Acclimation</u>: 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.

<u>Treatment</u>: 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.

<u>Isolation</u>: 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.

<u>Identification</u>: 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



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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.

<u>Spawning</u>: 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.

<u>Adominant Males</u>: 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.

<u>Daily Observation</u>: 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.

<u>Diagnostics</u>: 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.

<u>Treatment</u>: 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:



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- 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

<u>Collection</u>: 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.

<u>Incubation</u>: 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.

<u>Maintenance</u>: 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.

<u>Hatching</u>: 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).



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<u>Larvae Collection</u>: 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.

<u>Holding</u>: Larvae are fed twice daily and may be maintained in the culture system for up to 14days.

<u>Health</u>: Fungus is typically not a problem after embryos hatch. If larvae do become fungusinfested 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-I0 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.



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• Periphyton that grows naturally in the tanks also provides a good dietary supplement for the fish.

<u>Frequency of Feedings</u>: 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

<u>Brushes, and Miscellaneous Cleaning Equipment</u>: 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.

<u>Tiles, Beakers, Siphon Tubes, Air stones, etc.</u>: Scrub items using a brush and hot, soapy water, and rinse well with tap water. Disinfect by soaking, as described above.



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.



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)

	Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
System Maintenance		_					
Temperature (°C), Acceptable range = 25.0 ± 2.0°C	1			F	1		
Check and repair: leaks, water flow / aeration in all tanks, amber alarms?, all pumps flowing					J T		
Backwash sand filter, add DI water to system, add chemicals		T				-	
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.			1				
Transfer hatched larvae to a new tank and place into culture system.	1						1.0
Remove any dead larvae or food in all larval tanks.			1				
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)			-	11	1.00		101.0
Record the spawn date/time and tank ID's where eggs were collected, replace missing tiles in tanks							
Make 1 tray for next day			1	-	<u>}</u>		
Adult Minnow Maintenance		*	4		£		-
Feed adults (AM feeding): flake food, <u>if collecting tiles</u> : feed frozen food to breeder tanks, record time in feeding log				- I			
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			4				
Feed adults (MID-DAY feeding): flake food, record feeding time in feeding log		Í		Í			T T
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				1			
Feed larvae artemia and remake artemia, record feeding time in feeding log		1 *****	1	1			
Feed adults (PM feeding): flake food, record feeding time in feeding log		1	12 1	1	18 3		
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				1			1
Temperature (°C), Acceptable range = 25.0 ± 2.0°C				1			-
Front and metal doors locked			1				
Check and repair: leaks, water flow / aeration in all tanks, amber alarms?, all pumps flowing						-	
Comments		•		•			

SOP-AT17-Revision 5-Exhibit AT17.1



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Subject: Maintenance of Fathead minnow (Pimephales promelas) Cultures

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Pimephales promelas Culture Maintenance

	Oct	Nov	Dec
	2023	2023	2023
Breeder Tanks (replace tank, air stone/weight, clean air line/w	hite shelving a	nd PVC drain pi	pes)
Breeder tanks: A 1-6, verify 1 male to 5 females per tank	1 m m m m		1
Breeder tanks: B 1-6, verify 1 male to 5 females per tank	1		
Breeder tanks: C1-6, verify 1 male to 5 females per tank	·		
Breeder tanks: D 1-6, verify 1 male to 5 females per tank	1 +	11	
Breeder tanks: E1-6, verify 1 male to 5 females per tank	1		
Breeder tanks: F1-6, verify 1 male to 5 females per tank	1		
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			
	d waights don	n aidina tubina	
Black Stock Tanks (clean sides, replace standpipes/air bars and Black stock tank: 1	a weights, clea	annue cubing,	
Black stock tank: 1 Black stock tank: 2			-
	1		
Black stock tank: 3			
Black stock tank: 4			_
Black stock tank: 5	24		
Black stock tank: 6	1 ++ (1	
Sumps			
Clean filters in sumps (remove and spray with hose to remove solids)		17	
Replace filters in sumps	·	l	ī
Clean sides of sumps and sump covers	11 16	1	1
Vacuum solids out of sumps			
Clean screens to pump intakes	-		
Clean floats		1	
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 Tank (clean sides/ainine tubing, replace air stones/weights) Carbon tube: replace carbon, clean PVC pipe (inside and outside),			-
clean tubing and resecure coupling to PVC pipe		10	
Clean water delivery gutters in bio-tank			
Flush drainpipes			
Pumps		1	-
Rotate pump order			-
Oil pumps (4-5 drops vacuum pump oil to each side)			
General Housekeeping		-	1
Stock bathroom (toilet paper, paper towels)	-	-	
Make bleach	24		-
Clean bathroom			
Refill soap dispensers	tt		
Clean counters, artemia table and light table		1	
Clean top and bottom shelves of culture system			-
cital top and social sheres of cutal constant		1	



Exhibit AT17.2: Pimephales promelas Culture Water Chemistry Log.

FTS

Date	Analyst	Tank JD			Ical Addition base (Hardness Values) hity and Hardness > 100 Hardn	Obtained mg/L CaCO ₁)				
			рн (S.U.)	Dissolved Oxygen (mg/L)	Conductivity (µmhos/cm)	Alkalinity (mg/L CaCO ₂)	Hardness (mg/L CaCOs)	Sodium bicarbonate (NaHCO3) (R)	Calcium sulfate defiydrate (CaSO4 · 2H2O) (g)	Magneslun sulfate (MgSO4) (g)
			1							
		E E								
			1					_		
			1							
				-				-		

Chemical analyses:

Parameter	Reporting Limit	Method number	Meter	Serial number
pli	aisu,	SM 4500 H4 8-2011	Accumet AR20	93312452
Dissolved Oxygen (D.O.)	1.0 mg/l	SM 4500-0 G-2016	YSI Model SZCE	130104324
LandorWelly	14.9 µmhos/cm	SM 2510 8-2011	Accumet AR20	93312452
Alkalimity	5.0 mg CaCOi/L	SM 2320 B-2011	Accumet AR20	93312452
Hardness.	5.0 mg CaCO ₀ /L	SM 2340 C-2011	Not applicable	Not applicable

SCP ATT7-Revision 5 Exhibit ATT7.1

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Exhibit AT17.3: *Pimephales promelas* Culture Feeding Log.

FTS

Day	AM	Fo	od Type (check)	1	Analyst.	Mid-	Fo	od Type (check)		Analyst	PM	Fo	ood Type (check	1	Analyst
	Time	Artemia nauplii	Frozen or Freeze Dried Brine Shrimp	Flake Food		dəy Time	Artemia nauplii	Frozen or Freeze Dried Brine Shrimp	Flake Food		Time	Artemia nauplii	Frozen or Freeze Dried Brine Shrimp	Flake Food	
1	_	1					1								
2							1	1							
3		1						0							
4									1						
5	_								1 1	i					
6		1							1 1			1			
7	-								1 1					1	
8				1 1				1	11	()	-			-	
9									11	j				-	
10	÷			i				i	11	i	· · · ·		2		
n	_		1					1	1						
12		·	-				·		1			· · · · · · · · · · · · · · · · · · ·			
13	-	1			· · · · · · · · · · · · · · · · · · ·			J	1						
14									1						+
15		1					-	1	1. 1. 11	1 m					
15	_							1	1			-			
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18	-					-	-		-		-	-			-
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20	-			-		-	-				-				
21	_				_	-				_	-	-			-
22	_					-					_				
23 24	-					-	-				-	-			
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25	-	-				-	-				-	-			
20	-	-	-	-		-						-		-	
28	-	-				-	-	-	-						
28	-		-			-									
30	-	-				-	-			-		1		-	
31		-	-			-		/	-	-	-	-			-

Pimephales promelas Culture Feeding Log, Month: December 2023

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Artenia naugli: Bithe Shring Eggs (Bithe Sining Direct), Frozen Bring Stating: Flat Packs (Brine Shring Direct), Freeze Dried Bring Stating: Cubes (Brine Shring Direct), Flate Food Aquator Flah Direct), Elster Food Stop-Att3 Prevalues 5-(Autor Mitta Att3.)



Exhibit AT17.4: Weekly Pimephales promelas Spawning / Egg Collection Log.

ETS

Date	Time	Analyst	Total #	Start Ha	tching	End Ha	tching	Comments
(Spawn date and Barch (D)	(-1)	· · · · · · · · · · · · · · · · · · ·	Tiles	Date	Time	Date	Time	
09-24-23	10.1		1000		12		1	Large stock: 1 2 3 4 5 6 Removed larvae hatched prior to: Analyst
09-25-23			LTE I			1		Large stock: 1, 2, 3, 4, 5 6 Removed larvae batched prior to: Analyst
09-26-23		1	1			;;		Large stock: 1 2, 3 4, 5 6 Removed larves hetched prior to: Anelyst
09-27-23		1		1	11220	1001	1	Large stock: 3, 2, 3, 4, 5, 6 Removed larvae hatched prior to;Analysi
09-28-23		· · · · · ·						Large stock: 1, 2, 3, 4, 5, 6 Removed larvae hatched prior to: Analysi
09-29-23		1	li					Large stock: 1 2 3, 4, 5 6 Removed larvae hatched prior to: Analyst
09-30-23	÷	1.2	15.11	1.00	1.24	122.5	1	Large stock: 1 2, 3, 4 5 6 Removed larvae hatched prior to: Analyst

Weekly Pimephales promelas Spawning / Egg Collection Log

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

preven stat: ped 1 2 with (D)	2 3	A	_		_		6			Tank ID (X locations where tiles with eggs were collected)																																											
	2 3				B			8						THE THEFT						C.			Γ			D	1		Т		-	E	1					F	-	_			G	Γ	H					Stor	ck		
	1.1	4	5	6	1	2	3	4	5	6	ì	2	ŝ	4	5	ô	1	2	3	1	4 3	5	<u>6</u> .	1	2	3	4	5	6	x	2	3	4	5	ġ.	1	2	3 1	2	3	1	2	3 4	1	5 6	1	7						
P-24-23								Ī		L			1			T	Γ	Γ	Τ	T			T		Т				T															Τ	T								
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627-Ú	T	1.1												Γ			Γ	T	T	T			T			í.										1	T		Π				T	T			T						
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1.10-25																	Г	T		T			T		T															Π				T	T		T						

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Subject: *Pimephales promelas* Acute Toxicity Test, EPA 2000.0

Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan	N	03-01-20
Quality Assurance Officer	Jim Sumner	Upa/usase-	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 <u>recommended</u> 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)	Rick Sherrard (TVA)	• Updated bench sheet (Exhibits AT18.2 and AT18.3) to include reporting limits, method numbers, meters and serial numbers used for chemical
		Internal	Jim Sumner (ETS)	analyses.



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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 \geq 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 = $25.0 \pm 1.0^{\circ}$ 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



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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.



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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 CaCO₃/L, hardness of 80 100 mg CaCO₃/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 fullstrength, 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 $(25.0 \pm 1.0^{\circ}C)$ 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.



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- 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.



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- 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.
 - 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.



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- 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.

- 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.
- 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 $(25.0 \pm 1.0^{\circ}C)$ 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.



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- 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 \pm 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.



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- 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.



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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 \geq 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:	<i>Artemia nauplii</i> made available while holding prior to test initiation (2 to 5-hours prior to initiation). Organisms in each test cup are fed 200 μL <i>Artemia nauplii</i> 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

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Exhibit AT18.2: Pass/Fail Acute Toxicity Test Bench Sheet.

Test Concentration 100% Survival Do Hours 0 Initiation 24 Termination	pH (S.U.) Dissolved o Conductivit Temperatur pH (S.U.) Dissolved o Conductivit Total residu ato (numbri A 10	xygen (mg/L) y (µmhos/cm) re (°C) xygen (mg/L) y (µmhos/cm) ial chlorine (n er of living Cor	rg/L) organisms) htrol licate c 10	D E		duras metra	5.0 mg CaCO ₄ /L 0.1 mg/L 0.1 °C Test Org Organism Spawn da Age (1 to Date and born betw Average t Transfer B EFA loading been docum pomeios. Statistic Method:	SM 2340 C-2021 ORION 97-70-19 SM 23508-2010 Carnism Infor Source: te: 14 days old): time organism reen: ransfer volums requirement for fn ented by ETS to m	Net app 77. Accume Digital 1 mation: 15 were e: ion: reshwater spec	olicable et A8250 Thermometer In-house C C D D H (5.U.); Temperatur	92349123 ulture e (°C): Lat 25.0°C has
100% Survival Da Hours 0 Initiation	pH (S.U.) Dissolved o Conductivit Temperatur pH (S.U.) Dissolved o Conductivit Total residu	xygen (mg/L) y (jumhos/cm) re (°C) xygen (mg/L) y (jumhos/cm) rail chlorine (m er of living Cor Rep B	organisms). htrol licate C	D E	Repli	Temperature Cate G H	5.0 mg CaCO ₂ /L 0.1 mg/L 0.1 °C Test Org Organism Spawn da Age (1 to) Date and born betw EPA loading been docum promeibs. Statistic	SM 2340 C-2021 ORION 97-70-19 SM 23508-2010 Carnism Infor Source: te: 14 days old): time organism reen: ransfer volums requirement for fn ented by ETS to m	Net app 77. Accume Digital 1 mation: 15 were e: ion: reshwater spec	olicable et A8250 Thermometer In-house C C D D H (5.U.); Temperatur	Not applicat 92349125 ulture e (°C): Lat 25.0°C has
Concentration 100% Survival Da Hours 0	pH (S.U.) Dissolved o Conductivit Temperatur pH (S.U.) Dissolved o Conductivit Total residu	xygen (mg/L) y (jumhos/cm) re (°C) xygen (mg/L) y (jumhos/cm) rail chlorine (m er of living Cor Rep B	organisms). htrol licate C	D E	Repli	Temperature Cate G H	5.0 mg CaCO ₀ /L 0.1 mg/L 0.1 °C Test Org Organism Spawn da Age (1 to) Date and born betw EPA loading Been docum promebs.	SM 2340 C-2021 ORION 97-70-19 SM 23508-2010 Carnism Infor Source: te: 14 days old): time organism reen: ransfer volums requirement for fn ented by ETS to m	Net app 77. Accume Digital 1 mation: 15 were e: ion: reshwater spec	olicable et A8250 Thermometer In-house C C D D H (5.U.); Temperatur	Not applicat 92349125 ulture e (°C): Lat 25.0°C has
100% Survival Da Hours	pH (S.U.) Dissolved o Conductivit Temperatur pH (S.U.) Dissolved o Conductivit Total residu	xygen (mg/L) y (jumhos/cm) re (°C) xygen (mg/L) y (jumhos/cm) rail chlorine (m er of living Cor Rep B	organisms). htrol licate C	D E	Repli	Temperature Cate G H	5.0 mg CaCO ₄ /L 0.1 mg/L 0.1 °C Test Org Organism Spawn da Age (1 to Date and born betw Average t Transfer b EPA loading	SM 2340 C-2021 ORION 97-70-19 SM 25508-2020 Canism Infor Source: te: 14 days old): time organism recen: ransfer volum ransfer volum noswi informati	Net app 77. Accume Digital 1 mation: 15 were e: ion: reshwater spec	olicable et A8250 Thermometer In-house C C D D H (5.U.); Temperatur	Not applicab 92349128 ulture e (°C): Lat 25.0°C has
100%.	pH (S.U.) Dissolved o Conductivit Temperatur pH (S.U.) Dissolved o Conductivit Total residu	xygen (mg/L) y (µmhos/cm) re (°C) xygen (mg/L) y (µmhos/cm) ial chlorine (n cor Rep	organisms). htrol licate		Repli	Hardness Total residual dhiorine Temperature Temperature	5.0 mg CaCO ₂ /L 0.1 mg/L 0.1 ℃ Test Org Organism Spawn da Age (1 to Date and born betw Average t	SM 2340 C-2021 ORION 97-70-19 SM 25508-2010 Itanism Infor Source: te: 14 days old): time organism recen: ransfer volum	Noc app 77 Accume Digital 1 mation: 15 were e:	alisable at A8250 Thermometer In-house C C C 0.25 mL PH (5.U.);	Not applicat 92349123 ulture
Concentration 100% Survival Do	pH (S.U.) Dissolved o Conductivit Temperatur pH (S.U.) Dissolved o Conductivit Total residu	xygen (mg/L) y (µmhos/cm) re (°C) xygen (mg/L) y (µmhos/cm) ial chlorine (n er of living Cor	organisms). htrol			Hardness Total residual dhorine Temperature	5.0 mg CaCO ₂ /L 0.1 mg/L 0.1 ℃ Test Org Organism Spawn da Age (1 to Date and born betw Average t	SM 2340 C-2021 ORION 97-70-19 SM 25508-2010 Itanism Infor Source: te: 14 days old): time organism recen: ransfer volum	Noc app 77 Accume Digital 1 mation: 15 were e:	alisable at A8250 Thermometer In-house C C C 0.25 mL PH (5.U.);	Not applicat 92349123 ulture
Concentration	pH (S.U.) Dissolved o Conductivit Temperatur pH (S.U.) Dissolved o Conductivit Total residu	xygen (mg/L) y (µmhos/cm) re (°C) xygen (mg/L) y (µmhos/cm) al chlorine (n er of living	organisms)		Tast Core	Hardness Total residual chlorine Temperature	5.0 mg CaCO ₀ /L 0.1 mg/L 0.1 % <i>Test Org</i> Organism Spawn da Age (1 to Date and born betw	SM 2340 C-2011 ORION 97-70-19 SM 25508-2010 Cource: te: 14 days old): time organism reen:	Not app 77 Accume Digital 1 mation:	plicable at A8250 Thermometer	Not applicab 92349123
Concentration	pH (S.U.) Dissolved o Conductivit Temperatur pH (S.U.) Dissolved o Conductivit Total residu	xygen (mg/L) y (µmhos/cm) re (°C) xygen (mg/L) y (µmhos/cm) rai chlorine (m				Hardness Total residual chlorine	5.0 mg CaCO ₃ /L 0.1 mg/L 0.1 %	SM 2340 C-2011 ORION 97-70-19 SM 25508-2010 anism Infor Source: te: 14 days old): time organism	Not app 77 Accume Digital 1 rmation:	olicable et A8250 Thermometer	Not applicab 92349123
Concentration	pH (S.U.) Dissolved o Conductivit Temperatur pH (S.U.) Dissolved o Conductivit	xygen (mg/L) y (µmhos/cm) re (°C) xygen (mg/L) y (µmhos/cm)	ng/L)			Hardness Total residual chlorine	5.0 mg CaCO ₃ /L 0.1 mg/L 0.1 °C Test Org Organism Spawn da Age (1 to	SM 2340 C-2011 ORION 97-70-19 SM 25508-2010 Manism Infor Source: te: 14 days old):	Not app 77 Accume Digital 1 rmation:	olicable et A8250 Thermometer	Not applicab 92349123
Concentration	pH (S.U.) Dissolved o Conductivit Temperatur pH (S.U.) Dissolved o	xygen (mg/L) γ (μmhos/cm) re (°¢) xygen (mg/L)				Hardness Total residual chlorine	5.0 mg CaCO ₄ /L 0.1 mg/L 0.1 °C Test Org Organism Spawn da	SM 2340 C-2011 ORION 97-70-19 SM 25508-2010 manism Infor Source: te:	Not app 77 Accume Digital 1	olicable et A8250 Thermometer	Not applicat 92349123
Concentration	pH (S.U.) Dissolved o Conductivit Temperatur pH (S.U.)	xygen (mg/L) y (μmhos/cm) re (°C)				Hardness Total residual chlorine	5.0 mg CaCO _a /L 0.1 mg/L 0.1 °C Test Org Organism	SM 2340 C-2011 ORION 97-70-19 SM 25508-2010 Canism Infor Source:	Not app 77 Accume Digital 1	olicable et A8250 Thermometer	Not applicat
	pH (S.U.) Dissolved o Conductivit Temperatur	xygen (mg/L) γ (μmhos/cm)				Hardness Total residual chlorine	5.0 mg CaCO ₀ /L 0.1 mg/L 0.1 °C Test Org	SM 2340 C-2011 ORION 97-70-19 SM 2550B-2010 anism Infor	Not app 77 Accume Digital 1	olicable et A8250 Thermometer	Not applicat
	pH (S.U.) Dissolved o Conductivit	xygen (mg/L) γ (μmhos/cm)				Hardness Total residual chlorine	5.0 mg CaCO ₀ /L 0.1 mg/L 0.1 °C	SM 2340 C-2011 ORION 97-70-19 SM 25508-2010	Not app 77 Accume Digital 1	alicable at AB250	Not applicat
	pH (S.U.) Dissolved o	xygen (mg/L)				Hardness Total residual chlorine	5.0 mg CaCO _b /L 0.1 mg/L	SM 2340 C-2011 ORION 97-70-19	Not app	alicable at AB250	Not applicat
Test	рН (s.u.)				=	Hardness Total residual	5.0 mg CaCO _b /L	SM 2340 C-2011	Not app	plicable	Not applicat
-		re ("¢)							_		
	Temperatur	re (°¢)				Alkalinity	5.0 mg CaCO ₃ /L	SM 2320 B-2011	Accume	et AR20	93512452
	Temperature (°¢)		-	-	1	5		5.0 mg CaCO ₃ /L SM 2320 B-2011		Accumet AR20	
MHSW	Hardness (n	ng/L CaCO ₃)				Conductivity	14.9 µmhos/cm	SM 2510 8-2011 Accume		et AR20 93312452	
-	Alkalinity (n	ng/L CaCO ₁)	-03-			Dissolved oxygen	1.0 mg/L	SM 4500-0 G-2011 YSI Mod		odel 52CE 18D1043	
	Conductivit	y (µmhos/cm)	2-03			рН	0.1 S.U.	SM 4500-H+ B-2	011 Accume	et AR20	93312452
	Dissolved o	xygen (mg/L)				Parameter	Reporting limit	Method number	Meter		Serial numb
	pH (S.U.)					Chemical and					
Concentration	-		Analyst			test. Alkalinity, hard specific bench sheet	iness and total residu	al chlorine perfor			
Chemical A	Analyses:			nitial F	inal	Analyst identified fo Temperatures perfo					
1111111111	were fed in hold	ling 2 to 5 hours	prior to test initia	tion. Test organism	ts were not fed durin	g the test.					
24		1	1			-	-				
0 mitiation								1.1			
Hours	Date	Fee	Analyst	Test Initiation	n or Termination Analyst	Location incubator/Shelf	Randomizi Template C		ample Numbe		MHSW Batch
		-					- C	_	-		-
25.0 ± 1.0°C in a synthetic water.		h and then dilute	nd to the test cond	centration with mo	derately hard		prepa		990	110	1100
	entration (A			90% form. Sample was	warmed to		Dilutio	and the second se	mL Imple	mL Dilution water	Total volum
Project #				C	ounty R	utherfo	rd				
Facility	2	Riverstone Industrial Park WWTP				001					
					_					04	
Client	Forest C	itu					N	PDES # N	00870	84	
		Acut	C 1 333/1 4			12, Method 20		ares prom	icius		
	-	Acut	e Pass/Fa	il Whole Ff	fluent Toxic	ity Test, Speci	es: Pimenh	ales prom	elas		Page 1

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SOF AT18-Revision 6-Exhibit AT18.2

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EIS	2	۵	cute I	C. Who	le Efflue	nt Toxi	rity Te	st, Species: <u>P</u>	menhales	promela	•		Page 1
8	-		cute c	C50 00110				Method 2000		Ji Oniciu.	2		
Client	Nutrien A	urora Phe	osphat	e						PDES #	NCO	003255	
		1.1.1.1.1.1.1.1.1.1.1.1.1.1.1.1.1.1.1.									0.3		
Facility	PCS Phose	Phosphate, Inc.							0	utfall	007	akv	
Project #		Sam	ple #		_				Co	ounty	Beau	ufort	
Dilution P	reparation:												
Test concent	trations (%)	6.25	12.5	25	50	100		ole was not aerated or is form. Sample was t					
mL Sample 31.25 62.5				125	250	500	wate	r bath and then dilute	d to the test conce				
mL Dilution		468.75 500	437.5 500	375 500	250 500	0 500	mode	erately hard synthetic	water (WHSW).				
							1						
Chemical A	Analyses:		-	0	Hours 24	1	48						
Concentration	Concentration			U	24	-	40	Analyst identified	or each day, perfe	ormed oH, dis	ssolved	oxygen and conductiv	vity
1	pH (5.U.)	Ar	alyst	-			-	measurements on	y. Temperatures	performed at	t the tim	e of test initiation or ess and total residual	termination
	Dissolved oxy	gen (mg/L)		-		-	-					ench sheets and tran	
Control	Conductivity (-	-				Chemical ana	lyses:				
Control, MHSW	Alkalinity (mg)		-			-	-	Parameter Reporting limit Method nu				Meter	Serial numb
	Hardness (mg/		-		1	-		pH	0.1 S.U.	SM 4500-H+	B-2011	Accumet AR20	93312452
	Temperature ("C)			-		1	_	Dissolved oxygen	1.0 mg/L	SM 4500-0 G	-2011	YSI Model SZCE	180104824
	pH (s.u.)			-	-	1		Conductivity	14.9 µmhos/cm	SM 2510 8-2	011	Accumet AR20	93312452
	Dissolved oxygen (mg/L)			-	-	1		Alkalinity	5.0 mg CaCO ₃ /L	SM 2320 B-2	011	Accumet AR20	93312452
6.25%	Conductivity (µmhos/cm)		-	-		-		Hardness	5.0 mg CaCO ₁ /L	SM 2840 C-2	011	Not applicable	Not applicat
	Temperature (°C)					-		Total residual chlorine	0.1 mg/L	ORION 97-70	-1977	Accumet AB250	92349123
	pH (S.U.)		- 1.	1.1.1	-	-10	-	Temperature	0.1 °C	SM 25508-20	010	Digital Thermometer	
	Dissolved oxy	gen (mg/L)		-					-		-		
12.5%	Conductivity (µmhos/cm)		1	1.								
	Temperature	(°C)		_									
	pH (S.U.)												
	Dissolved oxy	gen (mg/L)	- 1		£	-							
25%	Conductivity (µmhas/cm)			1								
	Temperature	(°C)			-	1							
	pH (S.U.)					11							
	Dissolved oxy	gen (mg/L)				1							
50%	Conductivity (µmhos/cm)											
	Temperature	(°C)											
	pH (S.U.)					1)							
	Dissolved oxy	gen (mg/L)				(1)							
	Conductivity (µmhos/cm)											
100%	Alkalinity (mg/	/L CaCO ₃)											
	Hardness (mg/	L CaCO ₃)			1								
	Total residual	chlorine (ms	/1)		1								



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			EP	A-82	1-R-02-0	12, Metho	od 2000.0				
ent	PCS Phosp	hate, Inc									
oject #		Sam	nple #								
Hours	Date		eeding	_		or Termination	Location	Randomizing	MHSW Batch	1	
0		Time •	Analys	-	Time	Analyst	Incubator/Shelf	Template		1	
24											
48				1	-						
Organism s		nation:	in-house (Culture	6						
Spawn date	e:										
Age (1 to 1	4 days old):		< 24-hour	s old							
Date and ti were born	ime organisms between:	1	1								
Average tra	ansfer volume	c	< 0.25 mL							1	
Transfer bo	owl informatio	n:	pH (S.U.):							1	
			Temperatu	re ("C):						1	
rvival Dat	a (number	of living or	rganisms):						-		
	Con	trol	6.259	%	12.	.5%	25%	50	0%	10	0%
Hours	Repli	licate	Replicat	te	Repl	licate	Replicate	Rep	licate	Repl	licate
	A	в	c	D	E	F	G H	9	j.	к	1
0	10	10	10	10	10	10	10 10	10	10	10	1
Initiation	-		_								
24								1111			

Mean Survival
Comment codes: d = dead, u = unhealthy, bs = bent spines, s = stressed

Statistics:

48

Method	1.1.1.	
Lower sonfidence limit (%)	95%	
Upper 95% confidence limit (%)	1 E	
48-hour LC ₅₀ (%)		

Comments:

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Exhibit AT18.4: Average Transfer Volume Log Sheet.

			Page 1 of
	Larval Fi	sh Transfer Volume	
Analyst:		Species:	
Date:		Source / Batch:	
Ambient temperature:		Wet Weight of 10 Larvae (g):	
Estimate transfer vol	ume, where minnows are	allowed to swim from the	pipette into the test vessel.
Numerically label 10 med	licine cups.		
Add 10 mL MHSW to eac			
Transfer 10 larvae to eac			T53 for vertebrate acute toxicity test ISW 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	Initial Weight	Final Weight	Transfer
Number	Medicine cup + 10 mL MHSW	Medicine cup + 10 mL MHSW	Volume Final - Initial Weight
	10 HIL WHISW	+ 10 Larval Fish transferred	(g = mL)
	(g)	(g)	
1 2			
3			
4			
5			
6 7			
8			
9			
10			
	Average volume	to transfer 10 organisms (mL):	
Estimate transfer vol	ume, where the minnows	are transferred with MHS	V into the test vessels.
lumerically label 10 mer	liaina auna		
Numerically label 10 med Measure and record the			
	MHSW to each of the 10 cup	s.	
			T53 for vertebrate acute toxicity tes
	anner that allows them to sw weight of each cup containing	im from the pipette into the MH MHSW with 10 larvae.	ISW contained in each cup.
	volume and average transfer		
Replicate	Initial Weight	Final Weight	Transfer
Number	Medicine cup	Medicine cup +	Volume
		10 mL MHSW	Final - Initial Weight
	(g)	+ 10 Larval Fish transferred (g)	(g = mL)
1	10/	10/	
2			
3			
4			
5			
6			
6 7			
6 7 8			
6 7			



Exhibit AT18.5: Randomization Template.

Randomizing template: <u>RED</u>						
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		·	·	·		



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Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan	\mathcal{N}	03-01-20
Quality Assurance Officer	Jim Sumner	Un fune	03-01-20

Document Revision History

Effective	Revision	Review	Evaluators	Revisions
Date	number	Туре		
12-01-00	0	Internal	Jim Sumner (ETS)	Original document
07-10-10	1	External	Lance Ferrell	• Section D.5 updated for the current <i>Pimephales</i> Acute SOP, AT18.
		(NC DENR)	(NC DENR)	 Exhibits updated and included 96-hour acute reference toxicant
				benchsheets and control charts.
		Internal	Jim Sumner (ETS)	
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	Rick Sherrard,	 Updated Table AT19.1 for conductivity measurement guidance values.
		(TVA)	Donald Snodgrass	Deleted statement: "Verify that the conductivity measured for each
			(TVA)	test concentration is within the acceptance criteria (refer to table Table
				AT19.1) before proceeding with the preparation of next concentration.
		Internal	Jim Sumner (ETS)	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)	Rick Sherrard (TVA)	Updated bench sheet to include reporting limits, method numbers,
				meters and serial numbers used for chemical analyses.
		Internal	Jim Sumner (ETS)	



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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 \geq 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



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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 (PpKCIAC) 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 KCI (SOP-G10). Place approximately 900 mL of deionized water in a 1000-mL volumetric flask. Add the KCI to the flask, dissolve the KCI 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.



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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.
- 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.



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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.

	Aquatic Toxicity F	Procedures
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Exhibit AT19.1: Pimephales promelas Acute Reference Toxicity Test Bench Sheet.

Hours

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	2.000			· · ·
	_			_
				_
- 2	-	-	-	

Acute LC₅₀ Whole Effluent Toxicity Test, Species: <u>Pimephales promelas</u> EPA-821-R-02-012, Method 2000.0

Pimephales promelas	Potassium	Chloride Acute	Reference	Toxicant '	Test

PpKCIAC #

Dilution Preparation:

Test concentrations (mg/L KCI)	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

ľ

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.

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Chemical Analyses:

hemical A	nalyses:		Hours	
		0	24	48
Concentration	Analyst		1	
	pH (5.U.)		·	
	Dissolved oxygen (mg/L)			
Control,	Conductivity (µmhos/cm)			(
MHSW	Alkalinity (mg/L CaCO _s)			1
	Hardness (mg/L CaCO ₃)			1
	Temperature (°C)			
	pH (S.∪.)			
	Dissolved oxygen (mg/L)			
500 mg/L	Conductivity (µmhos/cm)		1	
	Temperature ("C)	·		
	pH (S.U.)			
750 mg/L	Dissolved oxygen (mg/L)			
	Conductivity (µmhos/cm)		1	
	Temperature (°C)			
	рН (S.U.)	() () () () () () () () () ()	1	
	Dissolved oxygen (mg/L)	-	1	
1000 mg/L	Conductivity (µmhos/cm)			
	Temperature (°C)	1		
1	pH (S.U.)		1	
	Dissolved oxygen (mg/L)			
1250 mg/L	Conductivity (µmhos/cm)	-	1	
	Temperature (°C)		1	
	pH (S.U.)	1	$r = \pm 1$	1
	Dissolved oxygen (mg/L)	· · · · ·	1	
1500 mg/L	Conductivity (umhos/cm)			-
	Temperature (°C)	1		

*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.

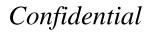
Chemical analyses:

Stock solution INSS #:

1

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 4500-0 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	

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ETS

Acute LC₅₀ Whole Effluent Toxicity Test, Species: <u>Pimephales promelas</u> EPA-821-R-02-012, Method 2000.0

Pimephales promelas Potassium Chloride Acute Reference Toxicant Test

PpKCIAC #

Hours	Date	Fee	eding	Test Initiation	or Termination	Location	Randomizing	MRSW Batch	
Hours	Date	Time	Analyst	Time	Analyst	Incubator/Shelf	Template		
0 ministrom			· · · · ·						
24		-		0.00					
48 trimbellion	1	-				1	The second second	1	

Test Organism Information:

Organism Source:	In-house culture	EPA loading requirement for freshwater species of < 0.40 g/L at 25.0°C has been documented b
Spawning date:		ETS to never be exceeded using 1 to 14 day old
Age (1 to 14 days old):		P. prometos
Hatch date and times:		
Average transfer volume:	< 0.25 mL	
Transfer bowl information:	рН (S.U.):	
	Temperature (°C):	

Survival Data (number of living organisms):

	Con	trol	500	mg/L	750	mg/L	1000	mg/L	1250	mg/L	1500	mg/L
Hours	Repl	icate	Repl	icate	Repl	icate	Repl	icate	Rep	icate	Repl	icate
	A	В	c	D	E	F	G	н	1	J.	к	L
0 Initiation	10	10	10	10	10	10	10	10	10	10	10	10
24	1.1				$1 \sim 1$. 0.		14		
48 Termination				IJ								
lean Survival				-				-	-			

Comment codes: d = dead, u = unhealthy, bs = bent spines, s = stressed

Statistics:

Method	Comments:	
Lower 95% confidence limit (mg KCl/L)		
Upper 95% confidence limit (mg KCl/L)		
48-hour LC ₅₀ (mg KCI/L)		

Test Reviewed by:

SOP AT19 Revision & Exhibit AT19.1



Exhibit AT19.2: Example of a Pimephales promelas Acute Reference Toxicant Control Chart.

	1.000	1	Log	g10 Conversion	n		Anti-logarithmic Values (g/L KCI)						
Test number	Test date	48-hour LC ₅₀ ToxCal Determination		48-hour LC _{so}	ст	s	ст	Contro	ol Limits	and a second second	Calculated CV ng Limits		centile CV og Limits
		(g/L KCI)					CT - 25	CT + 25	CT - 2CV	CT + 2CV	CT - SA.75	CT + 54.7	
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 LC₅₀ = 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 LC₅₀ values.

S = Standard deviation of the LC₅₀ values.

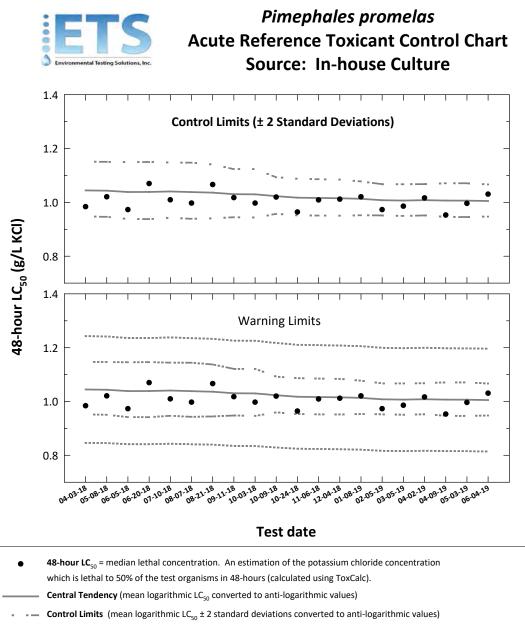
 $\label{eq:control Limits = Mean logarithmic LC_{S0} \pm 2 \mbox{ standard deviations converted to anti-logarithmic values.}$

Warning Limits = Mean logarithmic LC₅₀ \pm 2CV or S_{A.75} converted to anti-logarithmic values.

SA239 = Standard deviation corresponding to the 75th percentile of CVs reported nationally by USEPA. (S_{A25} = 0.19). CV = Coefficient of variation.







- --- Laboratory Warning Limits (mean logarithmic LC₅₀ ± 2 coefficent of variations converted to anti-logarithmic values)
- ------ USEPA Warning Limits (mean logarithmic $LC_{50} \pm S_{A,75}$ converted to anti-logarithmic values,
 - S_{A.75} = 75th percentile of CVs reported nationally by USEPA)





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Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan	N	03-01-20
Quality Assurance Officer	Jim Sumner	Un fune	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	Lance Ferrell	• The measurement of pH, DO and conductivity of each new, full-strength,
		(NC DENR)	(NC DENR)	undiluted sample was added.
				 The light intensity was amended to reflect that it is a <u>recommended</u>
		Internal	Jim Sumner (ETS)	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	Rick Sherrard,	 Updated exhibits during document review.
		(TVA)	Donald Snodgrass	 Updated the isolation of test larvae for using the in-house culture.
			(TVA)	
		Internal	Jim Sumner (ETS)	
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)	Rick Sherrard (TVA)	Updated bench sheet to include reporting limits, method numbers,
				meters and serial numbers used for chemical analyses.
		Internal	Jim Sumner (ETS)	



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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.

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%

Table AT20.1: Pimephales promelas chronic toxicity test acceptability criteria.



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Equipment and Materials

Fathead minnow larvae (Pimephales promelas) Temperature-controlled incubator (set to maintain test temperature = $25.0 \pm 1.0^{\circ}$ C, photoperiod of 16hour 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



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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 $60 \pm 2^{\circ}$ C or 6-hours at $100 \pm 2^{\circ}$ C.



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- 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 $57 64 \text{ mg CaCO}_3/L$, hardness of $80 100 \text{ mg CaCO}_3/L$, and an initial pH of 6.5 8.5 S.U. (guidance range = 7.4 7.8 S.U.).
 - 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.



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- e. Maintain the test temperature $(25.0 \pm 1.0^{\circ}C)$ 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.
 - 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



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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.

- 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 ±





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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 2hours 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 $(25.0 \pm 1.0^{\circ}C)$ 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.



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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 newlyhatched brine shrimp. Record the feeding time on the chronic bench sheet.
- Note: Test solutions may be renewed prior to the first feeding.



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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 \pm 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 preweighed 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



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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 $60 \pm 2^{\circ}$ C or 6-hours at $100 \pm 2^{\circ}$ 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.



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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.



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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 <i>Artemia nauplii</i> 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:	\geq 80% control survival, control growth \geq 0.25 mg/surviving larvae



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Exhibit AT20.2: Weekly Pimephales promelas Spawning / Egg Collection Log.

ETS

Page _____

Date	Time	Analyst	Total	Start Ha	tching	End Ha	tching	Comments
(Spawn date and Batch ID)		1.000	# Tiles	Date	Time	Date	Time	
07-28-19		()	1	1.000		_		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		1						Large stock: 1, 2, 3, 4, 5, 6 Removed larvae hatched prior to: Analyst
08-01-19)					1.1.1	Large stock: 1, 2, 3, 4, 5, 6 Removed larvae hatched prior to: Analyst
08-02-19	11 11	0			11.11	-	1	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

Weekly Pimephales promelas Spawning / Egg Collection Log

Egg Collection Source by Spawn Date:

Date				-	-				_				_		_		Tar	nk I	D (X lo	cati	ons	wh	ere	tiles	wit	he	ggs	wer	e co	olled	ted	0																
(Spawn date			1	4			1		1	в					C)					E	T.					F					G	1	H	H	T				Stoc	k	1	
and Batch ID)	1	2	3	4	5	6	1	2	3	4	5	6	1	z	3	4	5	б	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	1
07-28-19						1													1.1																														
07-29-19						1									1.1																										T	T				1			T
07-30-19			1							11		1									LE I	1É		11																	T	T							T
07-31-19	T İ		1	11			Ĩ.														Ì.				ĩ.																T	T			T	T			T
08-01-19						i.																			-																T	T							T
08-02-19			1				-		Ť															11									-						T		T	T							T
08-03-19	i î		1						1			1										Ĩ																			Т	Т	T						T

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	Aquatic Toxicity P	rocedures
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Exhibit AT20.3: Example *Pimephales promelas* Chronic Toxicity Test Bench Sheet.



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Chronic Whole Effluent Toxicity Test (EPA-821-R-02-013, Method 1000.0) Species: <u>Pimephales promelas</u>

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

Dilution preparation:

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

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
and the second second		Drying information for weight	determination:
Hatch dates and times:		Date / Time in oven:	-
and a state of the second		Initial oven temperature:	· · · · · · · · · · · · · · · · · · ·
Transfer vessel information:	pH (S.U.) =	Date / Time out of oven:	
transier vesser mormation;	Temperature ("C) =	Final oven temperature:	
Average transfer volume (mL):	< 0.25 mL	Total drying time:	

Daily feeding and renewal information:

Day	Date	Morning	g feeding	Afterno	on feeding		on, renewal, ination	Sam number		MHSW batch used
		Time	Analyst	Time	Analyst	Time	Analyst	Outfall 101	Intake	
0					1					
1									-	-
2										
3										
4	1	1								
5							1			
6									1.1	
7		10000	(International Property of the	1	1					

Chemical analyses:

Parameter	Reporting Limit	Method number	Meter		Serial number
pН	0.1 S.U.	SM 4500-H+ B-2011	Accumet AR20		93312452
Dissolved Oxygen (D.O.)	1.0 mg/L	SM 4500-0 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 Not applicable Accumet AB250 Digital Thermometer		93312452 Not applicable
Hardness	5.0 mg CaCO ₃ /L	SM 2340 C-2011			
Chlorine, Total Residual	0.1 mg/L	ORION 97-70-1977			92349123
Temperature	0.1 °C	SM 2550B-2010			
ontrol information:		Acceptance criteria		Summary o	f test endpoints
Mortality:		≤ 20%		7-day LC50 (%)
verage weight per initial larvae:				NOEC (%)	
erage weight per surviving larvae:		≥ 0.25mg/lar	≥0.25mg/larvae		
				ChV (%)	1
				1Car (%)	

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Client: TVA / V	Turto Dur Nu	ereur r	ianty t	audi				· · · · ·					
Day CONTROL				Survival and Growth Data 0.7% 1.4%						_			
Day		A	B	C	D	E	0.,	G	н	in a ca	1.	4% K	L
0		10	10	10	10	10	10	10	10	10	10	10	10
1					121								
2						111				17		11	
3											1		
4				7-1					1-1	1	-		-
5									1			-	
6										The second	-	-	
7						1.0							
A = Pan weight (mg) Tray color code: Analyst: Date: B = Pan + Larvae weight (mg)													
Analyst: Date:								11				_	
C = Larvae weight (mg) = B - A Analyst:					1.1	1.1							
Weight per initial number of = C / Initial number of larvae Analyst:													
Average weight per initial number of larvae (mg)	Percent reduction from control (%)						5	1		1			

Ig = unusually large, d&r = decanted and returned, w = wounded.

Comments:

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client. <u>IV</u>	A / Watts Bar	Nuclea	Fiant	, Outra	11 101					ate:		<u> </u>	
1. C. C. C. C. C. C. C. C. C. C. C. C. C.		_	_			Surviv	al and	Growt	h Data	1.5			
Day		2.8%				5.6	5%			11.	.2%		
		М	N	0	Р	Q	R	S	Т	U	V	w	X
0		10	10	10	10	10	10	10	10	10	10	10	10
1											14		
2							-	-			1.1	-	
3		1											
4			1	1			11			5	111	1	
5									1=1		15.5	1.11	
6			1	1	1 101 1								
7	-	1	1	1	1	1					1.1		
A = Pan weight (mg) Tray color code: Analyst: Date:													
B = Pan + Larvae weight (mg Analyst: Date:	· · · · · · · · · · · · · · · · · · ·												
C = Larvae weight (mg) = B - Analyst:	-A												
Weight per initial number o = C / Initial number of larvad Analyst:													
Average weight per initial number of larvae (mg)	Percent reduction from control (%)	Ĩ.		1		T							

<u>comment codes</u>: c = clear, d = dead, tg = fungus, k = killed, m = missing, sk = sick, sm = unusi lg = unusually large, d&r = decanted and returned, w = wounded.

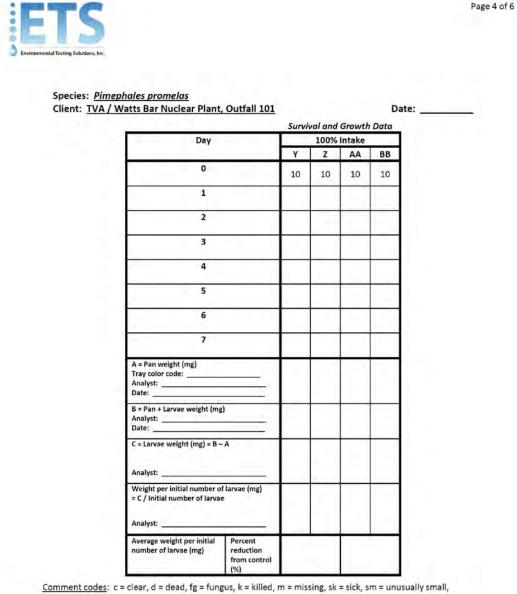
Comments:

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<u>Comment codes</u>: 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:	

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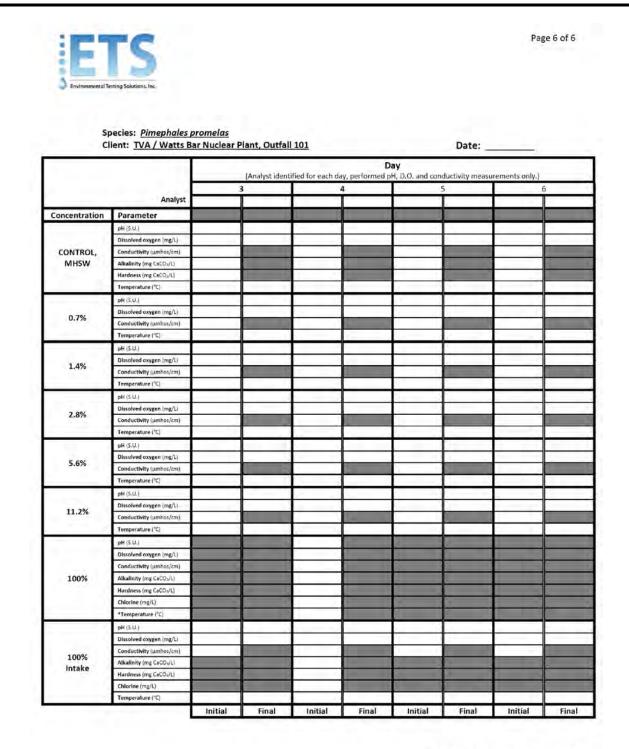
	Inc.						
	phales prometas				Date:		
Client: IVA/	Watts Bar Nuclear Pla	nt, Outfall 1	.01				
ocated on Page 1.	formed at the time of test in Alkalinity, hardness and chl						
analysis and transc	ribed to this bench sheet.				Day		
	1.	(Analyst ide		day, performed	pH, D.O. and con	luctivity measu	
	Analyst		0	-	1		2
Concentration	Parameter		1	1			1
	pH (S.U.)						1
CONTROL, MHSW	Dissolved oxygen (mg/L)			1.1.1.1.1			
	Conductivity (µmhos/cm)			-			
	Alkalinity (mg CaCO ₃ /L)		1				1
	Hardness (mg CaCO ₃ /L)		1	-	-		
	Temperature (°C)	1	-	1000			<u> </u>
0.7%	pH (S.U.)			121.			
	Dissolved oxygen (mg/L) Conductivity (µmhos/cm)				-	-	-
	Temperature (°C)	-	-	-		-	_
		-	-	-		-	<u> </u>
1.4%	pH (S.U.) Dissolved oxygen (mg/L)	-		1	-	-	
	Conductivity (µmhos/cm)		-		-		-
	Temperature (°C)		1			-	
	pH (5.U.)	S		- 1		1	
2.09/	Dissolved oxygen (mg/L)			1			
2.8%	Conductivity (umhos/cm)		Terretori				
2.8%	Temperature (°C)						
	pH (S.U.)		1	1076	1		
5.6%	Dissolved oxygen (mg/L)		-				
	Conductivity (µmhos/cm)		1				
	Temperature (°C)			-		-	
	pH (S.U.) Dissolved oxygen (mg/L)	-	-	1	-	-	
11.2%	Conductivity (µmhos/cm)		1.0				1
	Temperature (°C)		3	-			1
	pH (S.U.)		a second second				
	Dissolved oxygen (mg/L)			1			
	Conductivity (µmhos/cm)			0			
100%	Alkalinity (mg CaCO ₃ /L)		1	1			
	Hardness (mg CaCO ₃ /L)		-	1		-	
	Chlorine (mg/L)		-	-	-	-	-
	*Temperature (°C)		1.	1		_	
	pH (S.U.)	-	-	-			-
	Dissolved oxygen (mg/L) Conductivity (µmhos/cm)		-		-	-	
100%	Alkalinity (mg CaCO_/L)		-	1		-	
Intake	Hardness (mg CaCO ₃ /L)			-		-	
	Chlorine (mg/L)			1			1
	Temperature (°C)						

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Exhibit AT20.4: Average Transfer Volume Log Sheet.

Environmental Testing Solutions, Inc.			Page 1 of
	Larval Fi	sh Transfer Volume	1050 2 01
Analyst:		Species:	
Date:		Source / Batch:	
Ambient temperature:		Wet Weight of 10 Larvae (g):	
undent temperature.		wet weight of 10 Laivae (g).	
stimate transfer vol	ume, where minnows are	allowed to swim from the	pipette into the test vessel.
umerically label 10 me	dicine cups.		
dd 10 mL MHSW to ead			
	weight of each cup containing th cup, following procedures ide		T53 for vertebrate acute toxicity tes
		im from the pipette into the MH	
Aeasure and record the	weight of each cup containing	MHSW with 10 larvae.	
Determine each transfer	volume and average transfer	volume.	
Replicate	Initial Weight	Final Weight	Transfer
Number	Medicine cup +	Medicine cup +	Volume
	10 mL MHSW	10 mL MHSW	Final - Initial Weight
	(g)	+ 10 Larval Fish transferred (g)	(g = mL)
1	10/	(8)	
2			
3			
4			
6			
7			
8			
9			
10	A	to transfer 10 organisms (mL):	
	And the second sec		
stimate transfer vol	ume, where the minnows	are transferred with MHSV	V into the test vessels.
umerically label 10 me	dicine cuns		
Aeasure and record the			
	L MHSW to each of the 10 cup	s.	
			T53 for vertebrate acute toxicity tes
	nanner that allows them to sw weight of each cup containing	im from the pipette into the MH	SW contained in each cup.
	volume and average transfer		
	Initial Weight	Final Weight	Transfer
Deallert.			Transfer Volume
Replicate	Medicine cun		
Replicate Number	Medicine cup	Medicine cup + 10 mL MHSW	Final - Initial Weight
		10 mL MHSW + 10 Larval Fish transferred	Final - Initial Weight (g = mL)
Number	Medicine cup (g)	10 mL MHSW	
Number		10 mL MHSW + 10 Larval Fish transferred	
Number		10 mL MHSW + 10 Larval Fish transferred	
Number		10 mL MHSW + 10 Larval Fish transferred	
Number 1 2 3 4 5		10 mL MHSW + 10 Larval Fish transferred	
Number 1 2 3 4 5 6		10 mL MHSW + 10 Larval Fish transferred	
Number 1 2 3 4 5 6 7		10 mL MHSW + 10 Larval Fish transferred	
Number 1 2 3 4 5 6		10 mL MHSW + 10 Larval Fish transferred	
Number 1 2 3 4 5 6 7 8		10 mL MHSW + 10 Larval Fish transferred	
Number 1 2 3 4 5 6 7 8 9	(g)	10 mL MHSW + 10 Larval Fish transferred	



Exhibit AT20.5: Example Randomization Template.

Randomizing template: <u>BLUE</u>						
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						



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Subject: Pimephales promelas Chronic Toxicity Test, EPA 1000.0

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 parter replicate.

study perform	ned using 1 minnow	/ per rep
Analyst:	J. Sumner	

naiyst:	J. Sumner
Date:	08-23-08

			Larvae removed,	Weight loss
			killed, and returned	
			to pan.	
Replicate	Initial Pan Weight	Pan + Larvae weight	Pan + Larvae weight	
	(mg)	(mg)	(mg)	(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

^{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.

- Immediately submerge the sieve containing the larvae into the ice water. Keep b. the larvae in the ice water for 3 to 4 seconds.
- Remove the sieve from the ice water. Carefully rinse the larvae with deionized c. 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 =

Using forceps, sever the spinal cord of the larvae on the pan. Larvae never removed from pan.

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a.



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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

	Non-second second s
Date:	08-23-08

			Pan + Larvae	Weight loss
			reweighed	
	10 C		after 3-5 seconds.	
Replicate	Initial Pan Weight	Pan + Larvae weight	Pan + Larvae weight	
	(mg)	(mg)	(mg)	(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.



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Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan	2	03-01-20
Quality Assurance Officer	Jim Sumner	Un / un re-	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	Rick Sherrard,	 Updated Table AT21.1 for conductivity measurement guidance values.
		(TVA)	Donald Snodgrass	Deleted statement: "Verify that the conductivity measured for each
			(TVA)	test concentration is within the acceptance criteria (refer to table Table
				AT21.1) before proceeding with the preparation of next concentration.
		Internal	Jim Sumner (ETS)	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)	Rick Sherrard (TVA)	Updated bench sheet to include reporting limits, method numbers,
				meters and serial numbers used for chemical analyses.
		Internal	Jim Sumner (ETS)	



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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 (IC₂₅) 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.

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%

Table AT21.1: *Pimephales promelas* chronic toxicity test acceptability criteria.

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.



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Equipment and Materials

Fathead minnow larvae (Pimephales promelas) Temperature-controlled incubator (set to maintain test temperature = $25.0 \pm 1.0^{\circ}$ C, photoperiod of 16hour 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



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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.



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- 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.



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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.

	Aquatic Toxicity F	Procedures
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Exhibit AT21.1: Pimephales promelas Chronic Reference Toxicity Test Bench Sheet.



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Potassium Chloride Chronic Reference Toxicant Test (EPA-822-R-02-013 Method 1000.0) Species: <u>Pimephales promelas</u>

PpKCICR Test Number: 62

Dilution prepara	tion info	rmatio	Comments:					
KCI Stock INSS number:			INSS					
Stock preparation:			50 g KCI/L: Dissolve 50		deionized w	ater.		
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 inform	est organism information:						Test information:	
Organism source:			In-house	culture			Randomizing template:	
Age:			< 24-hours old				Incubator number and shelf location:	
Spawn date:			1.1				Artemia CHM number:	CHM1048
Hatch dates and time	25:						Drying information for weight	determination:
Transfer vessel inform	nation:		pH =		S.U.		Date / Time in oven:	
1	the second second			Temperature = °C			Initial oven temperature:	
Average transfer volume: < 0.		< 0.25 ml	< 0.25 mL			Date / Time out of oven:		
						Final oven temperature:	11	
							Total drying time:	

Daily feeding and renewal information:

Day	Date	Morning	feeding	Afternoo	Afternoon feeding		on, renewal, ination	MHSW batch used
		Time	Analyst	Time	Analyst	Time	Analyst	
0	1				1.00			
1						1		
2								
3				1 1 1 1				
4								
5								
6	1							
7			10.000	1	1		1	

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 4500-0 G-2011	YSI Model 52CE	18D104324
Conductivity	14.9 µmhos/cm	SM 2510 8-2011	Accumet AR20	93312452
Alkalinity	5.0 mg CaCO)/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	1

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 KCI)	
		ChV (mg/L KCl)	
		IC25 (mg/L KCI)	

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PpKCICR Test Number: 62

Subject: Pimephales promelas Chronic Reference Toxicity Test, EPA 1000.0



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Species: Pimephales promelas

				_		Surviv	al and	Growt	h Data	_		_	
Day		Control			-	300 m	g KCI/L		-	450 m	g KCl/L	-	
		Α	В	С	D	E	F	G	Н	11-1 1-1	1	К	- L
.0		10	10	10	10	10	10	10	10	10	10	10	10
1			1.0										
2				-							1-1		11
3													
4		-								1			11
5													
6											Î LL	1.1.1.	1.0
7													
A = Pan weight (mg) Tray color code:: Analyst: Date:		-							1			1	
B = Pan + Larvae weight (mg Analyst: Date:													
C = Larvae weight (mg) = B –	A												11
Analyst:												100 Å	
Weight per initial number of = C / Initial number of larvae												T	
Analyst:									1.1	_	21	tet i	
Average weight per initial number of larvae (mg)	Percent reduction from control								14				

<u>Comment codes</u>: 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:

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Species: Pimephales promelas

PpKClCR Test Number: 62

Day			600 m	g KCI/L	-	750 mg KCI/L					900 m	g KCI/L	
24)		м	N	0	P	Q	R	S	т	U	V	W	x
0		10	10	10	10	10	10	10	10	10	10	10	10
1													
2					TT								
3						1	1	10.00					
4													
5								53					
6			-						-				
7		-		·						1			
A = Pan weight (mg) Tray color code:: Analyst: Date:		Ĩ.											
B = Pan + Larvae weight (mg Analyst: Date:	-												
C = Larvae weight (mg) = B – A Analyst:													
Weight per initial number o = C / Initial number of larva Analyst:													
Analyst: Average weight per initial number of larvae (mg) Percent reduction from control (%)				-									

<u>Comment codes</u>: 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:	

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Species: Pimephales promelas

PpKClCR Test Number: 62

Day		1.2	1050 m	g KCI/L	
	6 - C - M	Y	Z	AA	BB
0		10	10	10	10
1	1.0				1
2					
3					
4					
5					
6					
7	- 1				
A = Pan weight (mg) Tray color code:: Analyst: Date:	_				
B = Pan + Larvae weight (mg Analyst: Date:					
C = Larvae weight (mg) = B -	A				
Analyst:				1	1
Weight per initial number of = C / Initial number of larvae Analyst:		Ţ			
Average weight per initial number of larvae (mg)	Percent reduction from control (%)				

<u>Comment codes</u>: 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.

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PpKCICR Test Number: 62

Subject: Pimephales promelas Chronic Reference Toxicity Test, EPA 1000.0



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Species: Pimephales promelas

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.

		(Analyst ident	ified for each d	Da ay, performed pl-		ductivity measur	ements only
		(Analyst Rent		1	1	and driving measure	2
	Analyst						
Concentration	Parameter		-		-	1	
	pH (S.U.)						
	Dissolved oxygen						-
	(mg/L)						
CONTROL,	Conductivity						
MHSW	(µmhos/cm) Alkalinity (mg CaCO ₁ /L)		_			-	-
	Hardness (mg CaCO ₁ /L)					-	-
	Temperature (°C)				-	-	
			-	-			
	pH (S.U.)					-	
Sec. Sec.	Dissolved oxygen (mg/L)				1. I. I. I.		
300 mg KCI/L	Conductivity		-			1	
	(µmhos/cm)		1		And the second s		
	Temperature (°C)						
	pH (S.U.)		-			-	
450 mg KCI/L	Dissolved oxygen				1	d	
	(mg/L) Conductivity		-				-
	(µmhos/cm)						
	Temperature (°C)						1.00
	pH (S.U.)		-		1	1	
600 mg KCI/L	Dissolved oxygen	1	1		1.00	1	
	(mg/L)						
	Conductivity (µmhos/cm)						
	Temperature (°C)	-		-	-	-	-
	pH (S.U.)	-				-	-
	Dissolved oxygen	-				-	-
750	(mg/L)	1 million (1996)				1	
750 mg KCI/L	Conductivity		-		-		
	(µmhos/cm)	-	-				1
	Temperature (°C)		-	-	_		
	pH (S.U.)						
	Dissolved oxygen (mg/L)			1	1.1.1	1.27	1.1.1
900 mg KCI/L	Conductivity						1
	(µmhos/cm)			· · · · · · ·			
	Temperature (°C)	-			1	1	
	pH (S.U.)				C	·	
1050 mg KCl/L	Dissolved oxygen (mg/L)					Lanna de	
1050 mg KCI/L	Conductivity			1	1	1	1
	(µmhos/cm) Temperature ("C)		-		-	-	
-	hererere (e)	Initial	Final	Initial	Final	Initial	Final

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					D	Day			
			tified for each day, performed pH, D.O. and conductivity measurements only						
	1.000		3	1	4		5		5
	Analyst	_							
Concentration	Parameter		-	1	-	-	-		-
	pH (S.U.)		i			-			
	Dissolved oxygen (mg/L)			1					-
CONTROL,	Conductivity (µmhos/cm)	_	1	1		1			The second
MHSW	Alkalinity (mg CaCO ₃ /L)		· · · · · ·			12			
	Hardness (mg CaCO ₁ /L)			i	Comment.				
	Temperature (°C)		1	· · · · · · · · · · ·					_
	pH (S.U.)			2	2	5		1	
300 mg KCI/L	Dissolved oxygen (mg/L)		1	12				1	
SOO ING KCI/L	Conductivity (µmhos/cm)			i					1
	Temperature (°C)				*				-
450 mg KCI/L	pH (S.U.)								
	Dissolved oxygen (mg/L)			1				1	
	Conductivity (µmhos/cm)	_			[]				1
	Temperature (°C)	-							-
	pH (S.U.)		1	1.02	2.1			1	
600 mg KCl/L	Dissolved oxygen (mg/L)			1					
000 mg Kci/L	Conductivity (µmhos/cm)		1	1					
	Temperature (°C)								_
	pH (S.U.)	-		3	1			1	
750 mg KCl/L	Dissolved oxygen (mg/L)			1	1				
750 mg KCi/L	Conductivity (µmhos/cm)		-			1	1		
	Temperature (°C)			<					
	pH (S.U.)				į.				
900 mg Kcl /	Dissolved oxygen (mg/l)			1	1000				
900 mg KCI/L	Conductivity (µmhos/cm)							1	
E	Temperature ("C)			1				1 · · · · · · · · · · · · · · · · · · ·	
	pH (S.U.)								_
1050 mg KCI/L	Dissolved oxygen (mg/L)			1				=	
1050 mg KCI/L	Conductivity (µmhos/cm)		1						
	Temperature ("C)								

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Exhibit AT21.2: Example of a Pimephales promelas Chronic Reference Toxicant Control Chart.

Pimephales promelas Chronic Reference Toxicant Control Chart Source: In-house Culture												
			Le	og ₁₀ Conversio	n			Anti-log	arithmic Value	s (g/L KCI)		1.1
Test number	Test date	7-day IC ₂₅ ToxCal Determination (g/L KCI)	7-day IC ₂₅	ст	s	ст	Contro CT - 25	ol Limits CT + 2S		Calculated CV ng Limits CT + 2CV		centile CV ng 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	-0.1432	-0.1424	0.0251	0.7205	0.6418	0.8089	0.6112	0.8452	0,4467	1.0004
3	02-06-18	0.7951	-0.0996	-0.1357	0.0244	0.7245	0.6461	0.8211	0.6154	0.8557	0.4516	1.0051
4	03-06-18	0.7002	-0.1548	-0.1387	0.0263	0.7265	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

7-day IC₂₅ = 25% inhibition concentration. An estimation of the potassium chloride concentration that would cause a 25% reduction in Pimepholes growth (calculated using ToxCalc). CT = Central tendency of the IC₂₅ values. Note:

S = Standard deviation of the IC25 values.

 $\label{eq:control Limits = Mean logarithmic IC_{25} \pm 2 \mbox{ standard deviations converted to anti-logarithmic values.}$

Warning Limits = Mean logarithmic IC₂₅ ± 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).

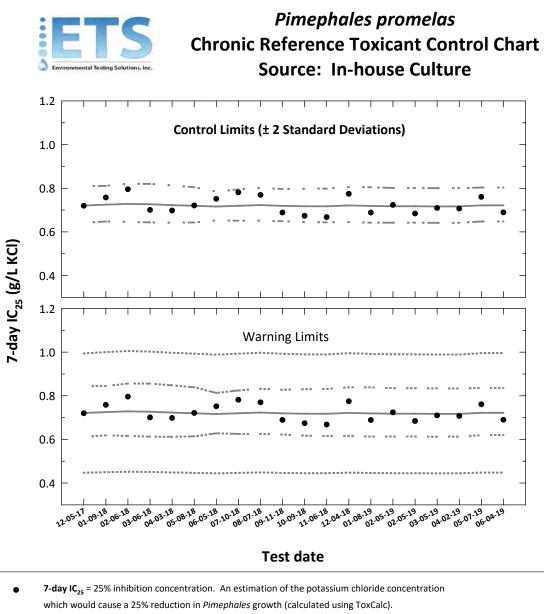
CV = Coefficient of variation.





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- Central Tendency (mean logarithmic IC₂₅ converted to anti-logarithmic values)
- - Control Limits (mean logarithmic IC₂₅ ± 2 standard deviations converted to anti-logarithmic values)
- Laboratory Warning Limits (mean logarithmic IC₂₅ ± 2 coefficent of variations converted to anti-logarithmic values)
 - **USEPA Warning Limits** (mean logarithmic IC₂₅ ± S_{A.75} converted to anti-logarithmic values,
 - $S_{A.75} = 75^{th}$ percentile of CVs reported nationally by USEPA)





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		ToxCal Determination					Control Growth		Control Growth CV		Test PMSD				
		Control	rol Control Growth			Test		(mg/initial larvae)		-	(%)		(%)		
Test number	Test date	Survival	Mean	CV	MSD	PMSD	CT	95% Confidence Interval		CT	95% Confidence Interval		ст	95% Confidence Interval	
		(%)	(mg/initial larvae)	(%)		(%)		CT - 25	CT + 25	-	CT - 25	CT+2S		CT - 25	CT + 25
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:

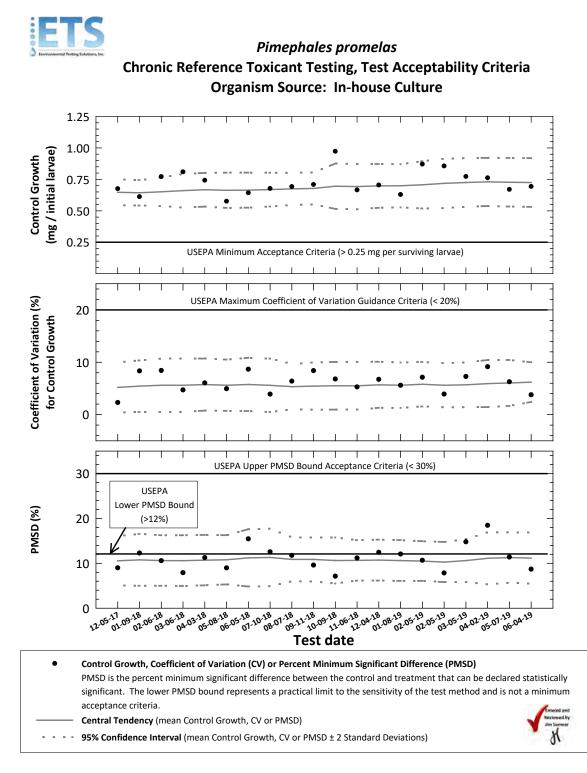
Control Survival = USEPA minimum test acceptability criteria > 0.25 mg/survival
 Control Mean Growth = USEPA minimum test acceptability criteria > 0.25 mg/survival ginvae.
 Control Mean Growth = USEPA minimum Cly positive criteria (00⁻ pricettifiel) < 20%.
 USEPA maximum Cly positive criteria (00⁻ pricettifiel) < 20%.
 MSD = Minimum significant difference.
 PMSD = Anonizom criteria difference.
 PMSD = Anonizom criteria difference.
 PMSD = Anonizom criteria difference.
 PMSD = Anonizom criteria difference.
 Dound for criteria by USEPA (10⁻ percentifiel) = 12%.
 The lower PMSD bound strating criteria a pesticularity of the test method and is not a minimum screptance criteria.
 Upper PMSD bound strating criteria detamined by USEPA (10⁻ percentifiel) = 20%.
 C = Central chancer of the growth, CV or PMSD values.





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Subject: Taxonomic Identification of *Pimephales promelas*

Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan	\mathcal{N}	09-01-19
Quality Assurance Officer	Jim Sumner	Un funse	09-01-19

Document Revision History

Effective	Revision	Review	Evaluators	Revisions
Date	number	Туре		
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)	Rick Sherrard, Donald Snodgrass (TVA)	 Updated exhibits during document review. Updated SOP for using the in-house culture.
		Internal	Jim Sumner (ETS)	
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.



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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



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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.

- 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.

ETS	Page 1 of 1
Pimephales promelas Taxo	nomic Identification Log Sheet
Date identification performed:	Analyst:
Source: <u>In-house Culture</u> Specimens preserved from stock organisms in	n culture system.
Comments:	
ILLUSTRATION OF FATHEAD MIN	NOW WITH ANATOMICAL IDENTIFICATIONS
Fat Pad	
Predorsal scales smaller Doi anteriority than posteriority: especially consider just	sal Fin origin above or slightly erior to pelvic fin origin
behind the head.	
Party and a grand a gr	with as a
- Contraction	
Main	Incomplete Lateral Line
Nuprial Tubercles	
Ferrale	Ovipositor

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Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan	N	05-31-22
Quality Assurance Officer	Jim Sumner	Un funse	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)	Lorimar Henning, Cindy Moore, Zach Thomas (NC DWR)	Updated procedure to include additional organism age requirements.
		Internal	Jim Sumner (ETS)	
05-31-22	5	External (NC DWR)	Lorimar Henning, Cindy Moore, Zach Thomas (NC DWR)	Provided clarification based on NC DWR comments.
		Internal	Jim Sumner (ETS)	

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.



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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</p>



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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.

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Exhibit AT40.1: Test Organism History Information Sheet.

	AC					
Date: 5-2-23	7		05-0	3.22 1115 J TEMP=	h	
Species: 1. M. bahia 2. M. bahia			pHs.u.	Ab 05.02-22 8.11 9.0	Ab 04.27.22 8.10 8.6	8.07 9.0
3. M. berylline Total Supplied: 1. 350 C			SALINITY Apt TOPAD TOTAL	22.9 0/350 ⁺	25.5	3/100+
1. 550 e e 2. 600 e e 3. 400 e e	500		0.ng Fe	d at 1120	, hatthe	
Brood Description 1. ECA 2. ECA 3. ECA						
Age: 1. "O" Days - 2. "G" Days - 3. 9 Days -	confected confected betw het ded betw	between (Jueen 04-3 Jueen 04-3)5 -01 no-əə c ns-əə c	Noon and C Noon and C Noon and	wn and C 64-27-22e 04-24-22e	05-02-02 0 1:30AU 11:30AU 2 1:30AU
Environmental Regime	Feeding:	Zooplankto Artemia NI	n	Photo:		
P.H.: 8,1 Comments:	Temp:	25°C TZarks.		Salinity:	25%) se	202 above
				-		
P	.O. Box 632 St.	WD-190365 & I Augustine,		(904) 829-2780)	

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Exhibit AT40.2: C. variegates, M. beryllina and A. bahia Culture Log.



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C. variegates, M. beryllina and A. bahia Culture Log

Test organism information:	the second second	Culture information:		
Organism source:	Aquatic Indicators, Inc.	Incubator number:	4	
Organism type:		Synthetic water type:	SaltSW	
Organism batch:		- All Alexander Sound Street		
Organism age upon receipt:	Note: Each batch of C. variegatus, M. beryllino and A. bahia are born within 24-hours of one another.			
Date and times organisms were born between:		A. Juno are junt within 24-hours of the another.		

and the state		Date Analyst Synthetic water batch	Activity						
Day Date Analys	water Feeding		Renewal Time	Number of living organisms received from	# Dead, Diseased, Fungused and Discarded	Tests initiated from organism batch			
				AM	PM	1.0	vendor	1 C	1
0		1.0		11		12.2	c = 1	1.1	
1	122				1Ê T		1		
2		a=0		-	10.0				
3					-				
4									
5					1				
6									
7			$ ==\rangle$	1230		12 22	==:		
8	1		z = 5	224	(=	2 - 2 -	z = 0		
9							1 - 1		
10									-
11					1				
12	1	1 = 2			1.2	12 22	$\mathbf{I} = \mathbf{I}$		
13	1								
14	0.000	i and					_		

Comments:	

SOP AT40-Revision 4-Exhibit AT40.2 SOP AT46-Revision 4-Exhibit AT46.2



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Approval

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

Document Revision History

Effective	Revision	Review	Evaluators	Revisions
Date	number	Туре		
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	Lance Ferrell	• The measurement of pH, DO, conductivity and salinity of each new, full-
		(NC DENR)	(NC DENR)	strength, undiluted sample was added.
				 The light intensity was amended to reflect that it is a <u>recommended</u>
		Internal	Jim Sumner (ETS)	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	Lorimar Henning, Cindy	Updated procedure to include additional organism age requirements.
		(NC DWR)	Moore, Zach Thomas	 Updated bench sheet (Exhibits AT18.2 and AT18.3) to include
			(NC DWR)	reporting limits, method numbers, meters and serial numbers used for
				chemical analyses.
		Internal	Jim Sumner (ETS)	

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.





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Quality Control

Acceptance Criteria: The test acceptance criterion is \geq 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 = $25.0 \pm 1.0^{\circ}$ 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



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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.



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- 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 $(25.0 \pm 1.0^{\circ}C)$ 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



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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.
 - 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.
 - 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.



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- 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.
- 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 μ L (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.



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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.

- 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.
- 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 $(25.0 \pm 1.0^{\circ}C)$ 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).



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- 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 \pm 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.

Confidential



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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.



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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 \geq 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 μ L Artemia nauplii. Organisms in each test cup are fed 200 μ L 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



Exhibit AT41.2: Pass/Fail Acute Toxicity Test Bench Sheet.

Client	Environ	mental Ch		821-R-02-0 c.	012, Meth	od 2007.0 NPDES #	NC0044	4806		
Facility	Atlantic	Beach WT	TP			Outfall	001 Carteret			
Project #		-				County				
Sample was not	aerated or treat		ise noted on this	90% form. The sample		Dilution preparation:	mL Sample	mL Dilution water	Total volun mL	
				wtion with salt syn			990	110	1100	
Hours	Date	Fee Time	ding Analyst	Test Initiation Time	or Termination Analyst	Location Incubator/Shelf	Bandomizing Template	Sample Number	Salt SW Bate	
0									1	
24				(1000		1	
Control	pH (S.U.) Dissolved o	oxygen (mg/L)		-		atch (Al Batch Ab ge (1 to 5 days ol		-		
Control Salt SW	Dissolved o *Salinity (p	and the second			A	ge (1 to 5 days of late organisms we rganisms were bo	ld): ere born: (tin om between is			
	Dissolved o *Salinity (p	(mg/L CaCO ₃)			A D O O	ge (1 to 5 days of bate organisms we rganisms were bo ot provided by su	ld): ere born: (tin prn between is upplier)	•		
	Dissolved o *Salinity (p *Alkalinity	(mg/L CaCO ₃)			A 0 0 0	ge (1 to 5 days of late organisms we rganisms were bo	ld): ere born: (tim om between is opplier) volume:	s < 0.25 mL		
Salt 5W	Dissolved o *Salinity (p *Alkalinity *Temperat pH (S.U.)	(mg/L CaCO ₃)			A 0 0 0	ge (1 to 5 days of Pate organisms we rganisms were be ot provided by su werage transfer v	ld): ere born: (tim om between is opplier) volume:	s < 0.25 mL pH (S.U.);	re (*0):	
	Dissolved o *Salinity (p *Alkalinity *Temperat pH (S.U.)	npt) (mg/L CaCO ₃) ture (°C) oxygen (mg/L)			A 0 0 0	ge (1 to 5 days of Pate organisms we rganisms were be ot provided by su werage transfer v	ld): ere born: (tim om between is opplier) volume:	s < 0.25 mL	re (°C):	
Salt SW Test	Dissolved d *Salinity (p *Alkalinity *Temperat pH (S.U.) Dissolved d	npt) (mg/L CaCO ₂) ture (°C) coxygen (mg/L)			A 0 0 0	ge (1 to 5 days of Pate organisms we rganisms were be ot provided by su werage transfer v	ld): ere born: (tim om between is opplier) volume:	s < 0.25 mL pH (S.U.);	re (°C);	
Sait SW Test Concentration	Dissolved o *Salinity (p *Alkalinity *Temperat pH (S.U.) Dissolved o *Salinity (p	npt) (mg/L CaCO ₂) ture (°C) coxygen (mg/L)			A 0 0 0	ge (1 to 5 days of Pate organisms we rganisms were be ot provided by su werage transfer v	ld): ere born: (tim om between is opplier) volume:	s < 0.25 mL pH (S.U.);	re (°C):	
Sait SW Test Concentration 100% (Salinity	Dissolved c *Salinity (p *Alkalinity PTemperat pH (S.U.) Dissolved c *Salinity (p #Temperat pH (S.U.)	npt) (mg/L CaCO ₂) ture (°C) coxygen (mg/L)			A 0 0 0	ge (1 to 5 days of Pate organisms we rganisms were be ot provided by su werage transfer v	ld): ere born: (tim om between is opplier) volume:	s < 0.25 mL pH (S.U.);	re ([°] C);	
Sait SW Test Concentration 100%	Dissolved c *Salinity (p *Alkalinity PTemperat pH (S.U.) Dissolved c *Salinity (p #Temperat pH (S.U.)	npt) (mg/L CaCO_) ture (°C) oxygen (mg/L) hure (°C) oxygen (mg/L)			A 0 0 0	ge (1 to 5 days of Pate organisms we rganisms were be ot provided by su werage transfer v	ld): ere born: (tim om between is opplier) volume:	s < 0.25 mL pH (S.U.);	re (°C):	
Sait SW Test Concentration 100% (Salinity	Dissolved c *Salinity (p *Alkalinity *Temperat pH (S.U.) Dissolved c *Salinity (p *Temperat pH (S.U.) Dissolved c	npt) (mg/L CaCO_) ture (°C) oxygen (mg/L) hure (°C) oxygen (mg/L)			A 0 0 0	ge (1 to 5 days of Pate organisms we rganisms were be ot provided by su werage transfer v	ld): ere born: (tim om between is opplier) volume:	s < 0.25 mL pH (S.U.);	re (°C):	
Sait SW 7est Contentration 100% (Satinity Adjunted)	Dissolved c *Salinity (p *Alkalinity *Temperat pH (S,U.) Dissolved c *Salinity (p *Temperat pH (S,U.) Dissolved c *Salinity (p pH (S,U.)	npt) (mg/L CaCO_) ture (°C) oxygen (mg/L) hure (°C) oxygen (mg/L)			A 0 0 0	ge (1 to 5 days of Pate organisms we rganisms were be ot provided by su werage transfer v	ld): ere born: (tim om between is opplier) volume:	s < 0.25 mL pH (S.U.);	re (°C);	
Sait SW Test Concentration 100% (Salinity	Dissolved c *Salinity (p *Alkalinity *Temperat pH (S,U.) Dissolved c *Salinity (p *Temperat pH (S,U.) Dissolved c *Salinity (p pH (S,U.)	pt) (mg/L CaCO ₂) (mg/L CaCO ₂) (mg/L CaCO ₂) (mg/L) (pt) (mg/L) (pt) (mg/L) (pt) (mg/L) (mg/L) (mg/L)				ge (1 to 5 days of hate organisms were be of provided by su werage transfer w rransfer bowl info	id); ere born: (tim on between is pipiler) volume: rrmation: esch day, perform	s < 0.25 mL pH (s.u.); Temperatur med pH, dissolved p	sygen and	
Sait SW 7est Contentration 100% (Satinity Adjunted)	Dissolved c *Salinity (p *Alkalinity Temperat pH (S,U.) Dissolved c *Salinity (p H (S,U.) Dissolved c *Salinity (p pH (S,U.) Dissolved c *Salinity (p	pt) (mg/L CaCO ₂) (mg/L CaCO ₂) (mg/L CaCO ₂) (mg/L) (pt) (mg/L) (pt) (mg/L) (pt) (mg/L) (mg/L)			A D D O O O O O O O O O O O O O O O O O	ge (1 to 5 days ol hate organisms we rganisms were bo of provided by su werage transfer v ransfer bowl info	id); ere born: (tim on between is pipiler) volume: rrmation: esch day, perform ents only. Tempe	s < 0.25 mL pH (S.U.); Temperatur Temperatur set pH, dissolved p	sygen and performed at	

Hours		Con	trol		Te	Test Concentration					
	Hours Replicate			Replicate Replicate					N. S. Same		
	A	В	c	D	E	F	G	н	Statistics:		
0	10	10	10	10	10	10	10	10	Method		
Initiation	10	10	10	10	10	10	10	10	t-Stat or		
2.2		1	1			1		1.1.1	Rank Sum		
24 Termination	1.1				1.1				1-Tailed Critical		
	Mean su	rvival:			Mean su	rvival:		1.2.1	PASS or FAIL		
ment code	s: d = dead, i	u = unhealt	hy, s = stres	sed							
ments:			_								

SOP AT41-Revision 4-Exhibit AT41.2



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Subject: Americamysis bahia Acute Toxicity Test, EPA 2007.0

Exhibit AT41.3: Acute Toxicity Test Bench Sheet. Page 1 of 2 ETS Acute LC50 Whole Effluent Toxicity Test, Species: Americamysis bahia EPA-821-R-02-012, Method 2007.0 Client Abac- DMRQA Project # **Dilution Preparation:** Sample was not aerated or treated unless otherwise noted on this Test concentrations (%) 6.25 12.5 25 50 100 form. Sample was warmed to $25.0\pm1.0^9 C$ in a warm water bath and then diluted to the test concentrations with salt synthetic 125 250 500 mL Sample 31.25 62.5 water. mL Dilution water 468.75 437.5 375 250 0 Total volume (mL) 500 500 500 500 500 Chemical Analyses: Hours 48 0 24 Concentration Analyst *Analyst identified for each day, performed pH and dissolved oxygen measurements only. Temperature and salinity performed at the time of test initiation or oH (S.U.) Dissolved oxygen (mg/L) 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 Control. *Salinity (ppt) SaltSW transcribed to this bench sheet. *Alkalinity (mg/L CaCO₃) *Temperature (°C) pH (S.U.) Dissolved oxygen (mg/L) 6.25% "Salinity (ppt) *Temperature (°C) pH (S.U.) Dissolved oxygen (mg/L) 12.5% *Salinity (ppt) *Temperature (°C) pH (S.U.) Dissolved oxygen (mg/L) 25% *Salinity (ppt) *Temperature (°C) pH (S.U.) Dissolved oxygen (mg/L) 50% *Salinity (ppt) *Temperature (°C) pH (S.U.) Dissolved oxygen (mg/L) *Salinity (ppt) 100% Alkalinity (mg/L CaCOz) Temperature (°C)

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SOF AT41-Revision 4-Exhibit AT41.3



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Subject: Americamysis bahia Acute Toxicity Test, EPA 2007.0

ent	Abac- DN	IRQA			_				
oject #	_	-		_	_			2.5	· · · · ·
Hours	Date	Fe	Analyst	Test Initiation Time	Analyst	Location Incubator/Shelf	Randomizing	Sample Number	SaltSW Batch
0	-	•						12.11	· · · · · ·
24						1 6		1 1	
48 Termination									1

Organism Source:	Aquatic Indicators, Inc
Batch (Al Batch Ab):	
Age (1 to 5 days old):	
Date organisms were born: (time organisms were born between is not provided by supplier)	1-07
Average transfer volume:	< 0.25 mL
Transfer bowl information:	рН (S.U.):
	Temperature (°C):

Survival Data (number of living organisms):

	Con	trol	6.2	5%	12.	5%	25	5%	50)%	10	0%
Hours	Repl	icate	Repl	icate	Rep	icate	Repl	icate	Repl	licate	Repl	icate
	A	в	c	D	E	F	G	н	1	4	к	L
0 Initiation	10	10	10	10	10	10	10	10	10	10	10	10
24			-									
48 Termination												
lean 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



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Subject: Americamysis bahia Acute Toxicity Test, EPA 2007.0

Exhibit AT41.4: Average Transfer Volume Log Sheet.

ETS				Page 1 of 1
	Larval Fi	ish Transfer Volume		
Analyst: J. Sumn	er	Species:	P. promelas	
Date: 12-05-1	7	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 3 11.4323 4 11.3821		11.4430	0.0473 0.1970 0.0513	
		11.6293		
		11.4334		
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 volu	me 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 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	Final Weight Medicine cup + 10 mL MHSW + 10 Larval Fish transferred	Transfer Volume Final - Initial Weight (g = mL)	
	(g)	(g)		
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 volu	ume to transfer 10 organisms (mL):	9.9546	

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SOP AT18-Revision 5-Exhibit AT18.4

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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	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



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Subject: Americamysis bahia Acute Reference Toxicity Test, EPA 2007.0

Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan	\mathcal{N}	09-01-19
Quality Assurance Officer	Jim Sumner	Uppe/unser	09-01-19

Document Revision History

Effective	Revision	Review	Evaluators	Revisions
Date	number	Туре		
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.



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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 \geq 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



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Subject: Americamysis bahia Acute Reference Toxicity Test, EPA 2007.0

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 (AbKCIAC) test number on the bench sheet.

B. Preparation of the Stock Solution.

 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



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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.
- 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.
- 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.

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

Table AT19.1: Test concentration, stock volumes, salt synthetic water volumes and finalvolumes for Americamysis bahiaKCl acute reference toxicant tests.

^{5.} Once all test concentrations have been prepared, follow the procedure described in SOP-AT41 for conducting *Americamysis bahia* Acute Toxicity Tests.



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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.

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Exhibit AT42.1: Americamysis bahia Acute Reference Toxicity Test Bench Sheet.

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Acute LC₅₀ Whole Effluent Toxicity Test, Species: <u>Americamysis bahia</u> EPA-821-R-02-012, Method 2007.0

Americamysis bahia Potassium Chloride Acute Reference Toxicant Test

AbKCIAC #

Dilution Preparation:

Test concentrations (mg/L KCI)	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 KCI into 2000 mL delonized water. This 50,000 mg/L KCI stock solution was used to prepare the concentrations evaluated for toxicity.

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Stock solution INSS #: **Chemical Analyses:** Hours 48 0 24 Concentration Analyst pH (S.U.) Dissolved oxygen (mg/L) Control, *Salinity (ppt) SaltSW *Alkalinity (mg/L CaCO₃) *Temperature (°C) pH (S.U.) Dissolved oxygen (mg/L) 250 mg/L *Salinity (ppt) *Temperature (°C) pH (S.U.) Dissolved oxygen (mg/L) 375 mg/L *Salinity (ppt) *Temperature (°C) pH (S.U.) Dissolved oxygen (mg/L) 500 mg/L *Salinity (ppt) *Temperature (°C) pH (S.U.) Dissolved oxygen (mg/L) 750 mg/L *Salinity (ppt) *Temperature (°C) pH (S.U.) Dissolved oxygen (mg/L) 1000 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.

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Subject: Americamysis bahia Acute Reference Toxicity Test, EPA 2007.0

ETS

Acute LC₅₀ Whole Effluent Toxicity Test, Species: <u>Americamysis bahia</u> EPA-821-R-02-012, Method 2007.0

Americamysis bahia Potassium Chloride Acute Reference Toxicant Test

AbKCIAC #

about .	Date	Feeding		Test Initiation	or Termination	Location	Randomizing	
Hours		Time	Analyst	Time	Analyst	Incubator/Shelf	Template	SaltSW Batch
0 Initiation					Sec. 27			
24		1	1		1		1	1000
48 Termination	1				1.			

Test Organism Information:

Organism Source:	Aquatic Indicators, Inc
Batch (Al 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:	рН (S.U.):
	Temperature (°C)

Survival Data (number of living organisms):

	Control		250 mg/L		375 mg/L		500 mg/L		750 mg/L		1000 mg/L	
Hours	Repl	icate	Repl	icate	Repl	icate	Repl	icate	Rep	icate	Repl	icate
	Α	B	c	D	E	F	G	н	Į.	1	к	ι
0 Initiation	10	10	10	10	10	10	10	10	10	10	10	10
24					p. 4							
48 Termination						11						
Mean Survival	-						-					

Comment codes: d = dead, u = unhealthy, s = stressed

Statistics:

Method	Comments:
Lower 95% confidence limit (mg KCl/L)	-33
Upper 95% confidence limit (mg KCl/L)	
48-hour LC ₅₀ (mg KCI/L)	

Test Reviewed by:

SOP AT42-Revision 3-Exhibit AT42.1



Exhibit AT42.2: Example of an Americamysis bahia Acute Reference Toxicant Control Chart.

	1.1.1.1	The second second	Lo	g ₁₀ Conversion	1			Anti-log	arithmic Values	(g/L KCI)		
Test number Test date	Test date	48-hour LC ₅₀ ToxCal Determination	48-hour LC ₅₀	our LC _{so} CT S		ст	Control Limits		Laboratory Calculated CV Warning Limits		10th Percentile CV Warning Limits	
		(g/L KCl)					CT - 25	CT + 25	CT - 2CV	CT + 2CV	CT - SA.10	CT + SA.1
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	0.0052	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

48-hour (C₆₀ = 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 LC₅₀ values. Note:

 $\mathbf{S} = \text{Standard deviation of the LC}_{S0} \text{ values}.$ Control Limits = Mean logarithmic LC_{S0} ± 2 standard deviations converted to anti-logarithmic values.

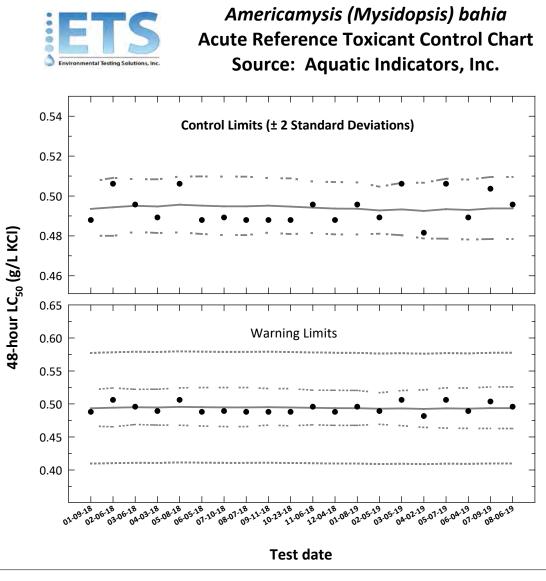
 Warning Limits = Mean logarithmic LG₀₀ ± 2CV or S_{A,10} converted to anti-logarithmic values.

 S_{A,10} = 5 standard deviation corresponding to the 10th percentile of CVs reported nationally by USEPA. (S_{A,10} = 0.17).

 CV = Coefficient of variation.



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•	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 $LC_{50} \pm 2$ standard deviations converted to anti-logarithmic values)
	Laboratory Warning Limits (mean logarithmic $LC_{50} \pm 2$ coefficent of variations converted to anti-logarithmic values)
	USEPA Warning Limits (mean logarithmic $LC_{50} \pm S_{A,10}$ converted to anti-logarithmic values,
	$S_{A,10} = 10^{th}$ percentile of CVs reported nationally by USEPA)





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Approval

Title	Name	Signature	Date
Laboratory Supervisor	Kelley E. Keenan	\mathcal{N}	09-01-19
Quality Assurance Officer	Jim Sumner	Un funse	09-01-19

Document Revision History

Effective	Revision	Review	Evaluators	Revisions
Date	number	Туре		
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	Lance Ferrell	• The measurement of pH, DO and conductivity and salinity of each new,
		(NC DENR)	(NC DENR)	full-strength, undiluted sample was added.
				• The light intensity was amended to reflect that it is a <u>recommended</u>
		Internal	Jim Sumner (ETS)	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 $\mu L)$ 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.

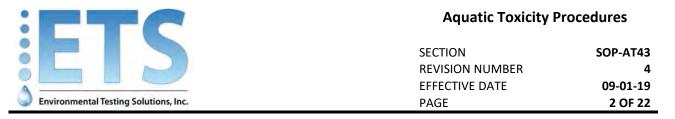
Scope and Application

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.



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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 16hour 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



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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



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- 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 $60 \pm 2^{\circ}$ C or 6-hours at $100 \pm 2^{\circ}$ 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.



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- a. The control/dilution water is salt synthetic water (SaltSW, SOP-AT1). SaltSW must have an alkalinity of $57 64 \text{ mg CaCO}_3/L$, hardness of 80 100 mg CaCO₃/L, and an initial pH of 6.5 8.5 S.U. (guidance range = 7.4 7.8 S.U.).
- 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 $(25.0 \pm 1.0^{\circ}C)$ 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.
 - 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.



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- 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



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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 μ L) 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



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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 2hours 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 $(25.0 \pm 1.0^{\circ}C)$ 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.



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- 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 μ L) of newlyhatched 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 \pm 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 preweighed 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.



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- 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 <u>minimum</u> 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.



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- 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.



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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.



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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 \geq 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:	\geq 80% control survival, control growth \geq 0.25 mg/surviving larvae



Exhibit AT20.2: Weekly Pimephales promelas Spawning / Egg Collection Log.

ETS

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Date	Time	Analyst	Total	Start Ha	tching	End Hatching		Comments
(Spawn date and Batch ID)		1.000	# Tiles	Date	Time	Date	Time	
07-28-19		()	1	1.000		_		Large stock: 1, 2, 3, 4, 5, 6 Removed larvae hatched prior to: Analyst
07-29-19]		-			1.53	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		1						Large stock: 1, 2, 3, 4, 5, 6 Removed larvae hatched prior to: Analyst
08-01-19		1 1	1		1		1.1.1	Large stock: 1, 2, 3, 4, 5, 6 Removed larvae hatched prior to: Analyst
08-02-19	1.5)			11.11	-	200	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

Weekly Pimephales promelas Spawning / Egg Collection Log

Egg Collection Source by Spawn Date:

Date													_				Tar	nkl	D (X lo	cat	ions	wh	ere	tile	s wit	th e	ggs	wer	re co	olled	ted	i)						١.,							1	_		
(Spawn date			- 4	Ą						В					(T.	2					E	11					F					G	1	÷	1				51	tock			
and Batch ID)	1	2	3	4	5	6	1	2	3	4	5	6	1	z	3	4	5	б	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	1											1												11	1																								Γ
07-29-19																																												1					
07-30-19		1	1							11		1									11		11	11																				1				\square	Γ
07-31-19	T İ	1	1				i.																		1																			Ĵ,				\square	Γ
08-01-19																									-													e l											
08-02-19			1						Ť					1										11.0	11							-	-											Τ					
08-03-19	i li		1						1			1			-				1			1	11																		T			1					5

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Exhibit AT20.3: Pimephales promelas Chronic Toxicity Test Bench Sheet.



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Chronic Whole Effluent Toxicity Test (EPA-821-R-02-013 Method 1000.0) Species: <u>Pimephales promelas</u>

Client: <u>City of Chattanooga, Moccasin Bend WWTP</u> NPDES #: <u>TN 0024210</u> Project #: _____

County: <u>Hamilton</u> Outfall #: <u>001</u> Permit Limit: <u>5.5%</u>

Dilution prepara	tion infor	Comments:					
Dilution prep (%)	1.38	2.8	5.5	11	22		
Effluent volume (mL)	20.7	42	82.5	165	330	1	
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.	Date / Time in oven:
	Temperature = °C	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 initiatio or term	on, renewal, ination	Sample numbers used	MHSW batch used
		Time	Analyst	Time	Analyst	Time	Analyst	And shares that I	Contraction in the
0	05-07-19	1		100			10.00	P	1 · · · · · · · · · · · · · · · · · · ·
1	05-08-19								
2	05-09-19						1		
3	05-10-19				· · · · · · · · · · · · · · · · · · ·		· · · · · · ·	h-a-	
4	05-11-19								-
5	05-12-19			1					
6	05-13-19								-
7	05-14-19			(

Control information:	Acceptance criteria	Summary of test endpoints:				
% Mortality:	≤ 20%	7-day LC50 (%)				
Average weight per initial larvae:	-	NOEC (%)				
Average weight per surviving larvae:	≥ 0.25mg/larvae	LOEC (%)				
		ChV (%)				
		IC25 (%)				

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			recusii	Dente	WWT		1.5				te: <u>05-</u>	07-15	
	_	-			-	Survivo		A	h Date	7	-		-
Day	1	A	CON	C	D	E	1.3 F	8% G	н	1	2.8	8% K	1
0		10	10	10	10	10	10	10	10	10	10	10	10
1									-	11			
2				1 11 1				1.1.1		1.4	h		
3		Ē		1.5.1				1					
4								151		1	1		
5								111		2.1			
6				1 1 1									
7								177				-	
A = Pan weight (mg) Tray color code:: Analyst: Date:		Î			1	Ĩ					Ĩ		
B = Pan + Larvae weight (mg) Analyst: Date:													
C = Larvae weight (mg) = B – A		1											
Analyst:			-							1.1			1
Weight per initial number of I = C / Initial number of larvae	arvae (mg)												
Analyst:		bi h	1 1							Į., ,			
Average weight per initial number of larvae (mg)	Percent reduction from control (%)		3										

<u>Comment codes</u>: 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:

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Species: <u>Pimephales promelas</u> Client: <u>City of Chattanooga, Moccasin Bend WWTP</u>

Survival and Growth Data Day 5.5% 11% 22% м N 0 Ρ R т U ٧ w Q s х 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 Percent number of larvae (mg) 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.

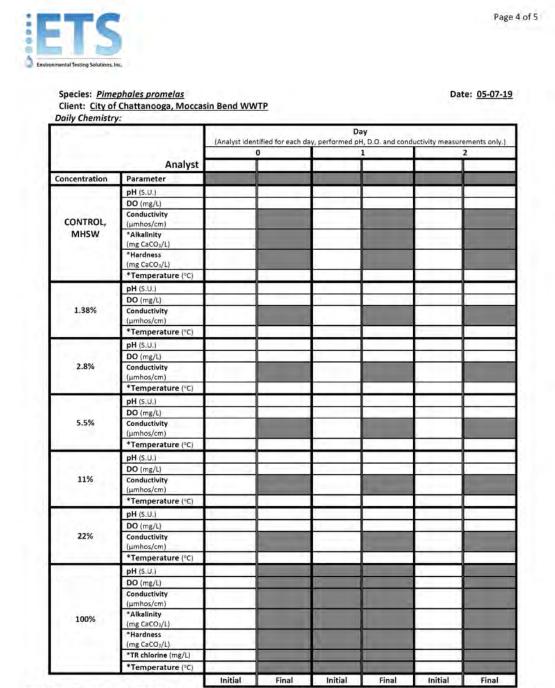
Comments:		
		TÎ

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	Client: City of Chattan	iouga, wiot	Lasin Denu	VVVVIE			Date	: 05-07-19	
			(Analist ident	ified for each da		ay	du atinitu manci	romonte only V	
		-	Analyst Ident	-	1		5	1	6
	Analyst				-		5 	-	0
C			-		_	-		-	-
Concen- tration	Parameter					1000			
	pH (S.U.)							1 C	
	DO (mg/L)		1	1 mar - 1 mar - 1				Q	
	Conductivity		1		1				1
CONTROL	(µmhos/cm) *Alkalinity		-		-		-		-
	(mg CaCO ₁ /L)				1				
	*Hardness			1	1	L	1	$Y = \pm i$	
	(mg CaCO ₁ /L) *Temperature (°C)							· · · · · · · · · · · · · · · · · · ·	
	pH (S.U.)		-	-					
	DO (mg/L)		0		-		-		
1.38%	Conductivity				A DESIGNATION OF		1		1
	(µmhos/cm)		-						
	*Temperature (°C)						1	- 1 - Hill	
	pH (S.U.)		A second second						
1.2.2	DO (mg/L)								
2.8%	Conductivity			1	1		Par		C. Sec.
	(µmhos/cm) *Temperature (°C)		-	-	-	-			-
	pH (S.U.)				-		-	-	-
	DO (mg/L)		1					1	-
5.5%	Conductivity				-	-	1	-	
	(µmhos/cm)		1		I provide the second			1	
	*Temperature (°C)			1)					
	pH (S.U.)			1			r		-
	DO (mg/L)		P					(
11%	Conductivity								
	(µmhos/cm) *Temperature (°C)				A DESCRIPTION OF	-		-	
	the second second second second second second second second second second second second second second second s				-	-			
	pH (S.U.) DO (mg/L)				-				
22%	Conductivity		-	-	S		1	-	
	(µmhos/cm)				-			· ·	1
	*Temperature (°C)		C S					1.1	C
	pH (S.U.)		5		(Internet in the second second second second second second second second second second second second second se	lan mend		14	
	DO (mg/L)		-	1	Terrare and the second		1	Territoria de la competitiva	in the second
	Conductivity		1		1	100 C	1.0	1000	1
	(µmhos/cm) *Alkalinity	_			-	-	-	1	
100%	"Alkalinity (mg CaCO ₃ /L)		1		1	The second second		1	
	*Hardness				1 -	the second is	A DESCRIPTION OF	1.1	
	(mg CaCO ₃ /L) *TR chlorine (mg/L)		-		-	-	-	-	-
	the second second second second second second second second second second second second second second second se		-		-	-		-	-
	*Temperature (°C)	Initial	Final	Initial	-	Initial	Final	Initial	

*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: _______.

Confidential

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Exhibit AT20.4: Average Transfer Volume Log Sheet.

ETS				B1-(1
- Overland and address of		Larval Fish Transfer Volume		Page 1 of 1
Analyst: J.	Sumner	Species:	P. promelas	
Date: 1	2-05-17	Source / Batch:	Spawn date: 11-29-17	_
Ambient temperature: 2	4.3°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 volu	me 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 10 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	Final Weight Medicine cup + 10 mL MHSW + 10 Larval Fish transferred	Transfer Volume Final - Initial Weight (g = mL)
	(g)	(g)	
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 vol	ume 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

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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.

study periorn	neu using	T minuo	w per	rep
Analyst:	J. Sum	ner		

naiyst:	J. Sumner
Date:	08-23-08

		1	Larvae removed,	Weight loss
			killed, and returned	-
			to pan.	
Replicate	Initial Pan Weight	Pan + Larvae weight	Pan + Larvae weight	
	(mg)	(mg)	(mg)	(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 =

Using forceps, sever the spinal cord of the larvae on the pan. Larvae never removed from pan.

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a.



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Study to determine the amount of moisture lost during weighing.

Study performed using 1 minnow per replicate.

Analyst: J. Sumner

	The second s
Date:	08-23-08

			Pan + Larvae	Weight loss
			reweighed	
			after 3-5 seconds.	
Replicate	Initial Pan Weight	Pan + Larvae weight	Pan + Larvae weight	
	(mg)	(mg)	(mg)	(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.
- 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.



Document Revision History

Revision Date	Surveillance number	Surveillance Type	Evaluators	Revisions
12-01-00				Original document
06-01-11	Not	Internal	Jim Sumner (ETS)	 Updated references and exhibits.
	applicable.			
11-01-14	Not	Internal	Jim Sumner (ETS)	 Updated exhibits during document review.
	applicable.			



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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 = $26.0 \pm 1.0^{\circ}$ C, photoperiod of 16hour 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



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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



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- 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).

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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.



Exhibit AT44.1: Americamysis bahia Chronic Reference Toxicant Test Benchsheet.



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Potassium Chloride Chronic Reference Toxicant Test (EPA-821-R-02-014, Method 1007.0) Species: Americanysis (Mysidopsis) bahia

AbKCICR Test Number: 126

Dilution preparat	tion info	Comments:						
KCl Stock INSS number: INSS					A second s			
Stock preparation:	50 g KCVI Deionized	.: Dissolve : water	50 g KCl in	1-1				
Dilution prep (mg/L)	250	375	500	750	1000	0.00		
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 in	formati	on:				Test information:		
Organism age:						Randomizing template:	1	
Date and times organisms were born between:		1				Incubator number and shelf location:		
Organism source:		AI Batel	1 Ab:			Artemia CHM number:	CHM780	
	1.1				Drying information for weight determination:			
Transfer bowl information:		pH = S.U.				Date / Time in oven:		
		Tempera	ture =	"C		Initial oven temperature:		
Average transfer volume:		0.1271 n	nL		-	Date / Time out of oven:		
						Final oven temperature:	3	
						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					
1	10-08-14							
2	10-09-14				1		-	
3	10-10-14			1	1	A	· · · · · · ·	
4	10-11-14				1			
5	10-12-14				11-11-11			
6	10-13-14							
7	10-14-14	1	0	A Designation	1. Transfer	1		

Control information:	Acceptance criteria	Summary of test endpoints:
% Mortality:	≤ 20%	7-day LC ₅₀
Average weight per initial shrimp:	1	NOEC
Average weight per surviving shrimp:	≥ 0.20 mg/shrimp	LOEC
		ChV
		IC25

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Survival and Growth Data CONTROL 250 mg KCl/L L M Day P A В C E H J N O D F G I K 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 tac # females with developing invalue ortidacts **P** makes A = Par weight (mg) Tray color code:: Analyst: Date: B - Pan + Shring weight Analyst: Date: C = Shrimp weight (mg) -8-A Hand calculatest. Analyst: Weight per initial number of shrimp (ing) = C / Initial number of of calculated. abretz Average weight per initial Average weight per initial Percent reduction from control number of shrimp (mg) number of shrimp (mg) (%)

AbKClCR Test Number: <u>126</u>

<u>Comment codes</u>: 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



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AbKCICR Test Number: 126

Day	375 mg KCVL									500 mg KCI/L						
	Q	R	s	Т	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				111	1.11	1.11	111	11	10.2							
1					1	11		11.1								
3				1 :	1.11			1.11								
4								1.1.								
5				1.11				110		1.1	1.0	11-01			1	1
б	1			1	1					1	1	1.2.1	112			
7	-			-	-	-			-					-		
# finaks with app in broad asc																
# females with developing ova in oviduets # immature females																
# makes								-						-		
A = Pas weight (mg) Tray color code::					17	1	18	F								
Analyst: Date:			÷.,		1.1	1.	1.00	5.1	1.1				1.1		1.1	1
B = Pan + Shrimp weight (mg) Analyst: Dute:					1		i Ei									
C = Shrimp weight (mg) = B = A				1.77	17			111			100		177			1
Hund cakulaied. Analyst:			_		1.1.1											
Weight per initial number of shrinep (ing) = C / Initial pumber of shrinep													Γ			
Hund calculated. Analyst:																
-	Averag number	e weight of shrim	per initial p (mg)	1	Percent (%)	t reductio	n from co	ntrol	Averag numbe	e weight r of shrin	per initial np (mg)		Percent (%)	t reductio	n from co	ntrol

Survival and Growth Data

<u>Comment codes</u>: 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:

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Subject: Americamysis bahia Chronic Reference Toxicity Test, EPA 1007.0



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Survival and Growth Data 750 mg KCl/L Day 1000 mg KCl/L JJ KK LL MM NN GG HH П 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 aggr in brood tac # females with developing ava in oridacts # immature & make f in A = Pan weight mag Tray color code:: Anabat B - Pan + Shring weight ight (mg) -Hand calculated Weight per initial number £. f shrimp (mg) C / Initial number of Hand calculated. Percent reduction from control Percent reduction from control Average weight per initial Average weight per initial number of shrimp (mg) (%) number of shrimp (mg) (%)

<u>Comment codes</u>: 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:

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ETS

Daily Chemistry:

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1.5	Complex 11	(Analyze id	Day Analyst identified for each day, performed pH, D,O, and salinity measurements o						
Conc.	Parameter)	uny, periormen	1	2			
1	Analyst	1	3			N			
CONTROL	pH (S.U.)		1			1 mm			
	DO (mg/L)				1	· · · · · · · · · · · · · · · · · · ·	-		
	Salinity (ppt)						÷		
	*Alkalinity (mg CaCO ₃ /L)			-					
	*Temperature (°C)								
100	pH (S.U.)					2			
250 mg	DO (mg/L)				· · · · ·	[]			
KC1/L	Salinity (ppt)	to an error f	and the second second	·					
	*Temperature (°C)	· · · · · · · · · · ·							
375 mg KCVL	pH (S.U.)		1						
	DO (mg/L)		-			1			
	Salinity (ppt)				1				
	*Temperature (°C)				1				
A	pH (S.U.)	· · · · · ·)	(/	A	11 14	21	14		
500 mg	DO (mg/L)		-	· · · · · · · · · · · · · · · · · · ·		2			
KCI/L	Salinity (ppt)		1		1	· · · · · · · · · · · · · · · · · · ·	· · · · ·		
	*Temperature (°C)								
1	pH (S.U.)		-		· · · · · · · · ·	1			
750 mg	DO (mg/L)								
KCUL	Salinity (ppt)					1			
	*Temperature (°C)		· · · · · · · · · · · · · · · · · · ·			P	-		
Acres 1	pH (S.U.)	J	h			Ť			
1000 mg	DO (mg/L)								
KC1/L	Salinity (ppt)			· · · · · · · · · ·		. · · · · · · · · · · · · · · · · · · ·			
	*Temperature (°C)				1	1	-		
		Initial	Final	Initial	Final	Initial	Final		

AbKCICR Test Number: 126

*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:



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			(Analyze id	antified for each	day, parformer	ay ipH, D.O. and s	alinity measure	ment: only)	
Conc.	Parameter	1	3		4		S		6
	Analyst	C		Sec. 1. 1	-	Processing Con-	· · · · · · · · · · · · · · · · · · ·	· · · · · · · · · · · · · · · · · · ·	
CONTROL	pH (S.U.)	1	t	1 mar 1	·	÷	T		1
	DO (mg/L)					1	· · · · · ·	10-1- P	×
	Salinity (ppt)	L		1	,	1	1		-
	*Alkalinity (mg CaCO ₁ /L)	1				(1 T)			
	*Temperature ("C)		1	100		17 L		· · · · ·	11
	pH (S.U.)					1			Y
250 mg	DO (mg/L)				1				1
KCIL	Salinity (ppt)	-			1. ····································				2.
	*Temperature (°C)	i57		J	1	ò c			
375 mg KCl/L	pH (S.U.)	1.000	Compared and	(1.00	1		Carlow Street of	Pre-
	DO (mg/L)	F		1	1	3(1
	Salinity (ppt)				1		÷.		-
	*Temperature (°C)	c	- Carrow 1	/		0	A		
-	pH (S.U.)			11.000	1	1			
500 mg	DO (mg/L)				-	1			1
KCI/L	Salinity (ppt)				t; ==/				1
2112	*Temperature (°C)				X				-
Contraction of the	pH (S.U.)	1	-		1	2			1
750 mg	DO (mg/L)								
KC1/L	Salinity (ppt)	C		1	· · · · · · · · · · · · · · · · · · ·	12		· · · · · · · · · · · · · · · · · · ·	-
	*Temperature ("C)	÷			· · · · ·	4.		i	
	pH (S.U.)				· · · · · · · · · · · · · · · · · · ·				
1000 mg	DO (mg/L)	÷				1			
KCUL	Salinity (ppt)					1			÷
	*Temperature (°C)	1.00	Section 1	a star and	terms and	1.11		1	1.000
		Initial	Final	Initial	Final	Initial	Final	Initial	Final

AbKClCR Test Number: 126

*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:



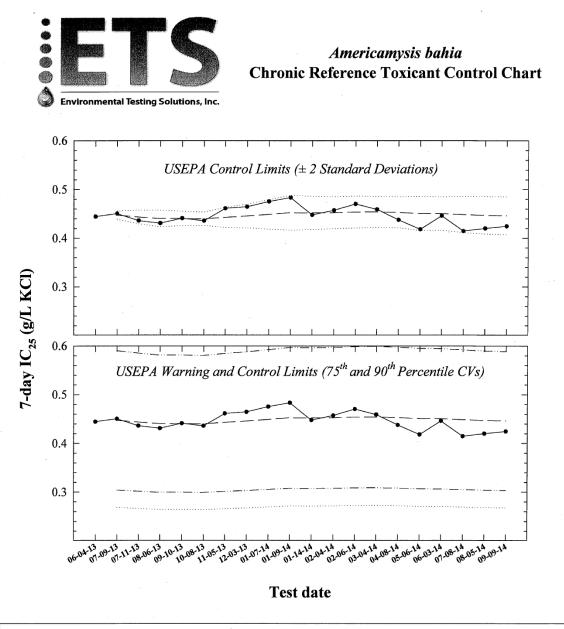
Exhibit AT44.2: Example Americamysis bahia Chronic Reference Toxicant Control Chart.

))			nericamys							
				Chr	onic Refe	rence Tox	icant Co	ntrol Cha	rt				
		-											
Environ	mental Testi	ng Solutions,	Inc										
est number	Test date	7-day IC ₂₅	СТ	S	Contro	ol Limit	S _{A.75}	Warnir	ng Limit	S _{A.90}	Contro	ol Limit	CV
		(g KCl/L)	(g KCl/L)		CT - 2S	CT + 2S		CT - S _{A.75}	$CT + S_{A.75}$		CT - S _{A.90}	CT + S _{A.90}	
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:	7.110	= 7-day 25% in	hibition conc	antration A	a actimation of	the concentrat	ion of notace	inn ablarida ti	hat would again	250/ #2	duction in Ame	uio autoria ano	with four th
Note:	/-a IC ₂₅ =	test populatio		entration. Ai	resumation of	the concentrat	ion of potass		hat would cause	e a 23% ie	duction in Ame	ricamysis gio	wui ioi u
	CT -	Central tende		7)									
		 Standard dev 											
				20	r ==th								
		Standard dev			•								
		Standard dev				entile CV. (S	$A_{.90} = 0.40$						
	CV =	Coefficient of	variation of t	the IC25 value	es.								



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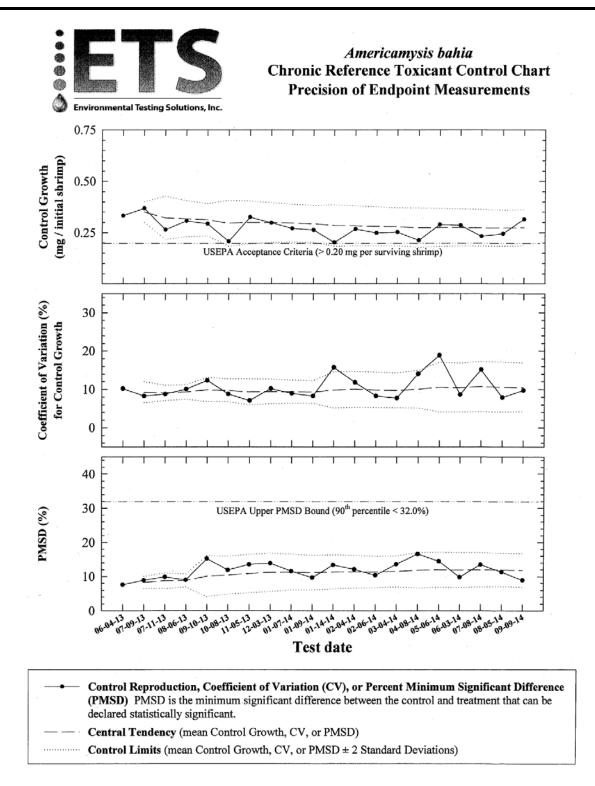
7-day IC₂₅ = 25% inhibition concentration. An estimation of the concentration of potassium chloride that would cause a 25% reduction in *Americamysis* growth for the test population.
 Central Tendency (mean IC₂₅)
 Warning Limits (mean IC₂₅ ± S_{A.75})
 Control Limits (mean IC₂₅ ± S_{A.90} or 2 Standard Deviations)

	Aquatic Toxicity	Procedures
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Environmental Testing Solutions, Inc.	DATE REVISION DATE	12-01-00 11-01-14

			A	mericamysis l	bahia				
			Chronic	Reference To	oxicant D	ata			
Environment	al Testing Solu	tions, Inc.							
`est number	Test date	Control Survival	Control Mean Growth	СТ	CV	СТ	MSD	PMSD	СТ
		(%)	(mg/shrimp)	for Control Growth (mg/shrimp)	(%)	for Control Growth CV (%)		(%)	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 13	02-04-14	100 100	0.268	0.283	11.8	10.1 9.9	0.03	12.1 10.4	11.4
13	03-04-14	100	0.249	0.281	8.3 7.7	9.9	0.03	10.4	11.3 11.5
14	04-08-14	100	0.233	0.279	14.1	10.1	0.03	16.6	11.5
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 =		variation for control						
				SEPA (10 th percentile)					
				SEPA (90 th percentile)	= 28%				
			ificant Difference						
	PMSD =	PMSD is a mea	•	rence on. The PMSD is the n a whole effluent toxici	•	nt difference betweer	the contro	and treatme	ent that can be
				y USEPA (10 th percent	-				
				y USEPA (90 th percent					
	CT -			wowth, CV, or PMSD)					

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.







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REVISION DATE	11-01-14

Subject: Taxonomic Identification of Americamysis bahia

Document Revision History

Revision Date	Surveillance number	Surveillance Type	Evaluators	Revisions
12-01-00		2.5.50		Original document
06-01-11	Not	Internal	Jim Sumner (ETS)	Exhibit AT45.2 revised for the key taxonomic characteristics and to
	applicable.			provide a more efficient logsheet.
11-01-14	Not	Internal	Jim Sumner (ETS)	 Updated exhibits during document review.
	applicable.			



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11-01-14

Subject: Taxonomic Identification of Americamysis bahia

Purpose

To verify the genus and species of *Americanysis 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.



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Subject: Taxonomic Identification of Americamysis bahia

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 *Americanysis 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.



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ON DATE 1	1-01-14

Subject: Taxonomic Identification of Americamysis bahia

- 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.



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PAGE	5 OF 7
DATE	12-01-00
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Subject: Taxonomic Identification of Americamysis bahia

Exhibit AT45.1: Supplier Certification of Americamysis bahia



P.O. BOX 632 ST. AUGUSTINE, FL 32085 (904) 829-2780

ORGANISM DOCUMENTATION

January 1, 2013

Aquatic Indicators currently produces two marine organisms: <u>Mysidopsis bahia</u> (aka <u>Americamysis bahia</u>) and <u>Menidia beryllina</u>.

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 \pm 1 C Photo period - 16 hours light, 8 hours dark

SPECIES HISTORY AND IDENTIFICATION:

<u>Mysidopsis bahia</u> - 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.

<u>Menidia beryllina</u> - 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:

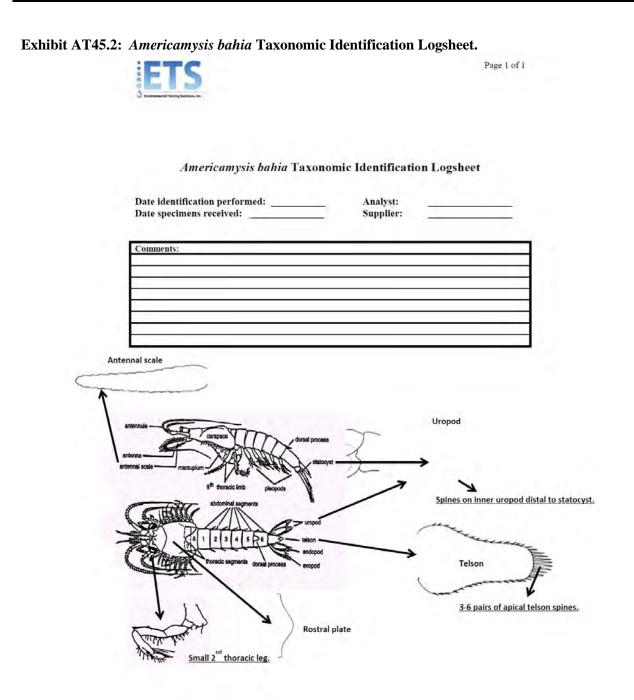
Middaugh, D. P., M. J. Hemmer and L. R. Goodman. 1987. EPA. Methods for Spawning, Culturing and Conducting Toxicity-Tests with Early Life Stages of Four Atherinid Fishes: The Inland Silverside, *Menidia beryllina*, Atlantic silverside, *M. menidia*, Tidewater Silverside, *M. peninsulae* and California grunion, *Leuresthes tenuis.* p4.

Preserved samples of our culture organisms (5% formalin) are available upon request, as are monthly Standard Reference Toxicant tests.

Raymond A. Lewis



Subject: Taxonomic Identification of Americamysis bahia



SOP AT45 - Exhibit AT45.2, revision 11-01-14

	Aquatic Toxicity Procedures	
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Environmental Testing Solutions, Inc.	REVISION DATE	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. 09.09.14 1005 from TEMP = 24.8°C HATCH 09-02-14 Total Supplied: HATCH 04.01.1 1. 700 @ 1 day + 500 @ 6 days 2. 120 Ab 09-07-14 | Ab 09-02-14 MB 08-30-14 3. 7.79 7.17 7.74 PH **Brood Description:** 10.4 10.2 9.2 D0(312) 1. CCA 21.9 22.0 SALINITY (PP+) 2. EBA 21.6 1120 3. °/ 500+ 0/ 700+ appear healthy ANA Age: 1. see above 2. 9 days - HATCH 08.30-14 Allongo Fed at 1010. M 3. Photo: L D 8 Zooplankton Feeding: Environmental Artemia NH Regime р.н.: Х Salinity: 20% Temp: 25°C Comments: Danks



Document Revision History

Revision	Surveillance	Surveillance	Evaluators	Revisions
Date	number	Туре		
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.



SOP-AT46
2 OF 6
04-01-09
11-01-14

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 ³/₄ 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 $25.0 \pm 1.0^{\circ}$ 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 $25.0 \pm 1.0^{\circ}$ C 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.

	Aquatic Toxici	
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Environmental Testing Solutions, Inc.	REVISION DATE	11-01-14

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 9-8-14 Date Species: 1. M. bahia 2. M. beryllina 3. 09.09.14 1005 Jun TEMP = 24.8°C HATCH 09-02-14 Total Supplied: HATCH OF 01.1 1. 700 @ 1 day + 500 @ 6 days 2. 120 Ab 09-57-14 | Ab 09-52-14 | Mb 05-30-14 з. 7.79 7.17 7.74 PH **Brood Description:** 10.4 10.2 9.2 DO(312) 1. CCA 21.9 22.0 SALINITY (PPT) 2. ERA 21.6 1,20t 3. appear healthy °/ 500+ 0/ 700+ Age: 1. see above 2. 9 days - HATCH 08.31-14 Fed at 1010. 3. D 8 Photo: L Environmental Feeding: Zooplankton Artemia NH Regime Р.Н.: Х, Salinity: Oloo Temp: 25°C Comments: Danks



Subject: Acclimation and Maintenance of Menidia beryllina Cultures

Exhibit AT46.2: P. promelas, C. variegates, M. beryllina, and A. bahia Culture Log.



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P. promelas, C.	variegates, M	I. beryllina,	and A.	bahia
	Culture	Log		

Test organism information:	Culture information:			
Organism source:	Incubator number:	4		
Organism type:	Synthetic water type:			
Organism batch:	A share the second of the	and the second second second second second		
Organism age upon receipt:	Note: Suppliers of C. variegatus, M. A. bahta do not provide the time that			
Date and times organisms were born between:	A. banka do not provide the time that organ were born between.			

		1.4.4.1	Section Street	Activity							
Day Date	Day	Date	W	Synthetic water batch	water	Fee Ti	ding me	Renewal Time	Number of living organisms received from vendor	# Dead, Diseased, Fungused and Discarded	Tests initiated from organism batch
	1.1			AM	PM		venuor				
0 Any received)				1	2-1						
1				1							
2						1000					
3											
4). 		1		·			
5	1.1				1-1	[]	· · · · · · · · ·	[]			
6		1.00			1	1.	·	1			
7	1	1									
8		1.000			1.1		1				
9		1.000	· · · · · ·		1			1			
10		1.201	10.00	12.2	1						
11	1000	1	[1		1			
12											
13				1	1	1	*	1			
14			-	1		1	*				

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



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REVISION DATE	11-01-14
DATE	12-01-00

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 <u>recommended</u> 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.



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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 = $25.0 \pm 1.0^{\circ}$ C, photoperiod of 16hour 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



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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



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(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 $(25.0 \pm 1.0^{\circ}\text{C})$ 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-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 $25.0 \pm 1.0^{\circ}$ C before the test organisms are placed in the test cups. Warm the test cups in a warm water bath or temperaturecontrolled 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.**



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- 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 $25.0 \pm 1.0^{\circ}$ 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 $25.0 \pm 1.0^{\circ}$ 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 \pm 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.



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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 \pm 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 $\ge 90\%$ 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.



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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:	$25.0 \pm 1.0^{\circ}$ 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:	None
Age of test organisms (days old):	9 to 14 days old, \leq 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:	<i>Artemia nauplii</i> made available while holding prior to test initiation (2 to 5-hours prior to initiation).
Control / Dilution water:	Salt synthetic water $(25.0 \pm 2.0 \text{ ppt})$
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:	\geq 90% control survival



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.

					-02-012,			: <u>Menidia beryllina</u> 16.0
lient	_			1000	1. A		NP	DES #
Facility							Ou	ıtfall
Project #	-						Co	unty
Dilution P	reparation:							
est concent	trations (%)	6.25	12.5	25	50	100		pple was not aerated or treated unless otherwise noted on this
nL Sample		31.25	62.5	125	250	500		n. Sample was warmed to 25.0 ± 1.0°C in a warm water bath an n diluted to the test concentrations with salt synthetic water.
nL Dilution	water	468.75	437.5	375	250	0	C., .	
rotal volum	e (mL)	500	500	500	500	500		
Chemical I	Analyses:		E	_	Hours	-		Í.
				0	24	4	8	
Concentration	Anoryse					11		Lange to be a set as
	pH (S.U.)				1			*Analyst identified for each day, performed pH and dissolved oxygen measurements only. Temperature and
	Dissolved axy	gen (mg/L)			1		0.01	salinity performed at the time of test initiation or termination by the analyst performing the toxicity test.
Control, SaltSW	*Salinity (ppt)	*Salinity (ppt)			U.	1		Alkalinity and total residual chlorine performed by the analysts identified on the test specific bench sheets and
	*Alkalinity (mg/L CaCO ₃) *Temperature (°C)			1		1		transcribed to this bench sheet.
				-		1		
1.1	pH (S.U.)			-	1	1		
	Dissolved oxygen (mg/L)				1	1	1	
6.25%	*Salinity (ppt)			-				
	*Temperature (°C)					-	-	2 N N
-	pH (S.U.)					1	-	
	Dissolved axy	gen (mg/L)	1	_		1	-	
12,5%	*Salinity (ppt)			-	1	1	-	
	*Temperature	(°C)						
	pH (S.U.)						-	
	Dissolved axy	gen (mg/L)		-		-	-	
25%	*Salinity (ppt)			_	-	-	-	
	*Temperature	(°C)			-	1		
-	pH (S.U.)	11		-		-	-	
	Dissolved axy	gen (mg/L)	-	-	-	-	-	
50%	*Salinity (ppt)	0.000			1	-	- 1	
		*Temperature (°C)				-		
-	pH (S.U.)	1.7		- 1	-	-		2
	Dissolved axy	gen (mg/L)		-		-	-	
100%	*Salinity (ppt)	A A REAL				-	-	
100%	*Total residua	I chlorine tr	ne/L1	-	-	-	-	
	*Temperature	N	5-1			-	-	C



ETS

Aquatic Toxicity Procedures

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12,329 (1)	Date	a la san la	Feeding		Test Initiation	or Termination	Locati	ion	Randomizing		1.44
Hours	Date	Time	Ant	ilyst	Time	Analyst	Incubator	/Shelf	Template	Sample Number	SalisWi
0 primarizar		•	1				1.1	1			1.1
24		1	1	1	_	-				-	
48 Termination	vete fed in hok	ding 2 to 5 hos	rs prior to test	Initiation. To	est organisms v	ere not fed duri	ing the lest.		-	-	
Test Organ											
Organism Sou	rce:		Aquatic	Indicators,	inc.				species of < 0.4 14 day old M. be	0 g/L at 25.0°C has	been docun
Batch (Al Bate	th Mb):		1.0								
Age (9 to 14 d	ays old):	-	1								
Date organism organisms we provided by st	re born bet		1.5		- 1						
Average trans		:	0.2542	mL							
Transfer bow	informatio	in:	pH (S.U.	.):							
Transfer bow			Temper	ature (°C):							
rvival Data	(number Con	of living of liv	Temper organisms 6.2	ature (°C): ;): 25%		5%	255 Partic			0%	100
	(number Con Repl	of living of Itrol	Temper organisms 6.2 Repl	ature (°C): ;): 1.5% licate	Rep	licate	Replic	ate	Rep	licate	Replic
rvival Data	(number Con	of living of liv	Temper organisms 6.2	ature (°C): ;): 25%		COMP.	- ×.			$C_{M} = 1$	
rvival Data	(number Con Repl	of living of Itrol	Temper organisms 6.2 Repl	ature (°C): ;): 1.5% licate	Rep	licate	Replic	ate	Rep	licate	Replic
Hours	(number Con Repl A	of living of liv	Temper organisms 6.2 Repl C	icate	Rep E	icate F	Replic G	H	Rep	licate J	Replic K
Hours 0 Initiation	(number Con Repl A	of living of liv	Temper organisms 6.2 Repl C	icate	Rep E	icate F	Replic G	H	Rep	licate J	Replic K
Hours 0 netitation 24 48	(number Con Repl A	of living of liv	Temper organisms 6.2 Repl C	icate	Rep E	icate F	Replic G	H	Rep	licate J	Replic K
Hours 0 milation 24 48 Termination	(number Con Repi A 10	of living of trol B 10	Temper organisms 6.2 Repl C 10	ature (°C): 5% icate D 10	Rep E	icate F	Replic G	H	Rep	licate J	Replic K
Hours Hours 0 miliation 24 48 Ternination ean Survival ment codes: Statistics:	(number Con Repi A 10	of living of trol B 10	Temper organisms 6.2 Repl C 10	ature (°C): 5% icate D 10	Rep E	icate F	Replic G	H	Rep	licate J	Replic K
Hours Hours 0 nitiation 24 48 Termination tean Survival ment codes: Statistics: Method	(number Con Repi A 10	of living o itrol icate B 10 = unbealth	Temper organisms 6.2 Repl C 10	ature (°C): 5% icate D 10	Rep E	icate F	Replic G	H	Rep	licate J	Replic K
Hours Hours 0 Initiation 24 48 Termination ean Survival nment codes: Statistics: Method Upper 95% co	(number Con Repl A 10 d = dead, u	of living of itrol icate B 10 = unhealth	Temper organisms 6.2 Repl C 10	ature (°C): 5% icate D 10	Rep E	icate F	Replic G	H	Rep	licate J	Replic K
Hours Hours 0 nitiation 24 48 Termination tean Survival ment codes: Statistics: Method	(number Con Repl A 10 d = dead, u	of living of itrol icate B 10 = unhealth	Temper organisms 6.2 Repl C 10	ature (°C): 5% icate D 10	Rep E	icate F	Replic G	H	Rep	licate J	Replic K

Acute LC₅₀ Whole Effluent Toxicity Test, Species: <u>Menidia beryllina</u> EPA-821-R-02-012, Method 2006.0



Exhibit AT47.4: Average Transfer Volume Logsheet.

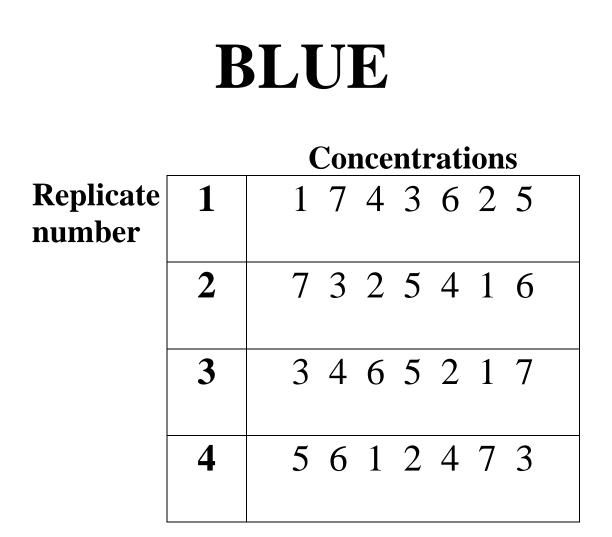
Pimephales promelas are used as surrogate organisms to determine the average transfer volume.

Environmental Testing Solutions, Inc.			Page 1 o
	Larval I	Fish Transfer Volume	
	J. Sumner 02-12-14		P. promelas
Ambient temperature:		Wet Weight of 10 Larvae (g):	ATOX Batch Pp 02-03-14
			00205
Estimate transfer v	olume, where minnows	are allowed to swim from	the pipette into the test ves
Numerically label 10 me			
Add 10 mL MHSW to ea	ich of the 10 cups. e weight of each cup containir	ng MHSW/	
			AT53 for vertebrate acute toxicity te
		swim from the pipette into the N	
	e weight of each cup containir		
Determine each transfe	r volume and average transfe	er volume.	
Replicate	Initial Weight	Final Weight	Transfer
Number	Medicine cup +	Final Weight Medicine cup +	Volume
	10 mL MHSW	10 mL MHSW	Final - Initial Weight
		+ 10 Larval Fish transferred	(g = mL)
	(g)	(g)	
1	11.3533	11.6989	0.3456
2	11.3337	11.4850	0.1513
3	11.3607	11.6976	0.3369
5	11.2950 11.2945	11.5662 11.5634	0.2712 0.2689
6	11.3349	11.3360	0.2689
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 ne to transfer 10 organisms (mL):	0.2008 0.2542
Estimate transfer v	olume, where the minn	ows are transferred with N	AHSW into the test vessels.
Numerically label 10 me			
Measure and record the			
	nL MHSW to each of the 10 c		AT53 for vertebrate acute toxicity te
		swim from the pipette into the N	
	e weight of each cup containir		
Determine each transfe	r volume and average transfe	er volume.	
Replicate	Initial Weight	Final Weight	Transfer
Number	Medicine cup	Medicine cup +	Volume
	····-	10 mL MHSW	Final - Initial Weight
		+ 10 Larval Fish transferred	(g = mL)
	(g)	(g)	
	1.7092	11.6989	9.9897
1	1.6929	11.4850 11.6976	9.7921
2			9.9884 9.8934
2 3	1.7092		
2 3 4	1.7092 1.6728	11.5662	
2 3	1.7092 1.6728 1.6967	11.5662 11.5634	9.8667
2 3 4 5	1.7092 1.6728	11.5662	
2 3 4 5 6 7 8	1.7092 1.6728 1.6967 1.6740 1.7015 1.6870	11.5662 11.5634 11.3360 11.7630 11.7335	9.8667 9.6620 10.0615 10.0465
2 3 4 5 6 7 8 9	1.7092 1.6728 1.6967 1.6740 1.7015 1.6870 1.7009	11.5662 11.5634 11.3360 11.7630 11.7335 11.4930	9.8667 9.6620 10.0615 10.0465 9.7921
2 3 4 5 6 7 8	1.7092 1.6728 1.6967 1.6740 1.7015 1.6870 1.7009 1.7152	11.5662 11.5634 11.3360 11.7630 11.7335	9.8667 9.6620 10.0615 10.0465 9.7921 9.8177

SOP AT18 - Exhibit AT18.5, SOP AT47 - Exhibit AT47.5, SOP AT53 - Exhibit AT53.5, revision 11-01-14

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Exhibit AT47.5: Randomization Templates.



Random number seeds 10 through 13.



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.



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 = $25.0 \pm 1.0^{\circ}$ C, photoperiod of 16hour 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



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Subject: Menidia beryllina Acute Reference Toxicity Test, EPA 2006.0

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.



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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



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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.

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Exhibit AT48.1: Menidia beryllina Acute Reference Toxicity Test Benchsheet.

	-	_
		-

Acute LC₅₀ Whole Effluent Toxicity Test, Species: <u>Menidia beryllina</u> EPA-821-R-02-012, Method 2006.0

Menidia berylli	ina Potassium	Chloride Acute	Reference	Toxicant	Test
-----------------	---------------	-----------------------	-----------	----------	------

MbKCIAC #

Dilution Preparation:

Test concentrations (mg/LKCI)	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
Totai volume (mL)	500	500	500	500	500

A stock solution was prepared by diluting 100 g KCI into 2000 mL Milli-Q water. This 30,000 mg/L KCI stock solution was used to prepare the concentrations evaluated for toxicity.

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Stock solution INSS #:

Chemical A	nalyses:		Hours	
		0	24	48
Concentration	Analyst			1000
Control, Saltsw	pH (5.U.)	1	1):
	Dissolved oxygen (mg/L)	1	1	-
	*Salinity (ppt)	1	1.2.2.2.1	
Sansw	*Alkalinity (mg/L CaCO _s)	1	1	58
	*Temperature (°C)	1		
1.1	pH (S.U.)	I I	/	
	Dissolved oxygen (mg/L)			
1000 mg/L	*Salinity (ppt)	1 La T		1
	*Temperature (°C)	1		
	pH (S.U.)	1		
	Dissolved oxygen (mg/L)	1	1	1
1250 mg/L	*Salinity (ppt)		12	1
	*Temperature (°C)			
	pH (S.U.)	1 1	1	1
	Dissolved oxygen (mg/L)	· · · · · · ·		1
1500 mg/L	*Salinity (ppt)		18	ie 1
	*Temperature (°C)	(i = 4)		T
	pH (S.U.)	f Le sul 6		
1750 mg/L	Dissolved oxygen (mg/L)	1 L	0.000)
	*Salinity (ppt)		12	ù
	*Temperature (°C)			
	pH (S.U.)	1		
	Dissolved oxygen (mg/L)			1
2000 mg/L	*Salinity (ppt)	1.	() · · · · · · · · · · · · · · · · · ·	10
	*Temperature (°C)		1 1	

*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.

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Subject: Menidia beryllina Acute Reference Toxicity Test, EPA 2006.0

Mar. 1. 19	

Acute LC₅₀ Whole Effluent Toxicity Test, Species: <u>Menidia beryllina</u> EPA-821-R-02-012, Method 2006.0

Menidia beryllina Potassium Chloride Acute Reference Toxicant Test

MbKCIAC #

		Feeding		Test initiation	or Termination	Location	Rendemiting	SaltSW Betch
Hours	Date	Time	Analyst	Time	Analyst	Incubetor/Shelf	Templata	SHILS W GHILL
0 vitedon			1200	1000		10.1	1	
24		1				1		
48	-	-	-	1.550	1	1	-	

Test Organism Information:

Organism Source:	Aquatic Indicators, Inc.		
Batch (Al 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 34 day old AL berylling .

Survival Data (number of living organisms):

2.041	Con	trol	1000	mg/L	1250	mg/L	1500	mg/L	1750	mg/L	2000	mg/L
Hours	Repl	icate	Repl	eplicate Replica		Replicate Replicate		Replicate		Replicate		
	A	в	с	D	E	F	G	н	4	1	ĸ	U.
0 Initiation	10	10	10	10	10	10	10	10	10	10	10	10
24				1.1								1.1
48 Termination					Ü.							
Mean Survival		-				-					12.00	-

Comment codes: d = dead, u = unhealthy, bs = bent spines, s = stressed

Statistics:

Method	Comments:
Upper 95% confidence limit	
Lower 95% confidence limit	
48-hour LC ₅₀	

Reviewed by

SOP AT48 - Exhibit AT48.1, revision 11-01-14

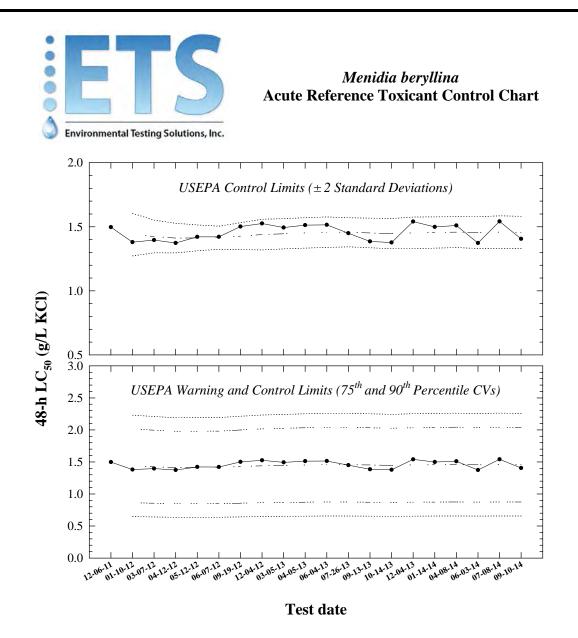


Exhibit AT48.2: Example of a *Menidia beryllina* Acute Reference Toxicant Control Chart.

					Λ	1enidia be	ryllina						
		M		Ac	ute Refer	ence Toxic	ant Cor	ntrol Chart	ţ				
A		~											
Environm	ental Testing	Solutions, Inc	1										
'est number	Test date	48-h LC ₅₀	CT	S		ol Limits	S _{A.75}	Warnin	,	S _{A.90}	Contro		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 1	12.06.11	1.50											
1 2	12-06-11 01-10-12	1.50	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	1.38	1.44	0.08	1.27	1.55	0.58	0.85	1.99	0.79	0.63	2.23	0.00
4	04-12-12	1.40	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	1.30	1.55	0.57	0.85	1.98	0.78	0.64	2.19	0.04
6	06-07-12	1.42	1.41	0.05	1.31	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	1.55	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
37 .	40.1.7.0	40.1				1	C			5000 6.1			
Note:					n estimate of t	the concentration	on of potass	ium chloride w	nch is lethal to	50% of the	e test organisms	in 48-hours.	
			ency (mean LC	507									
			viation of the L										
	S _{A.75} =	Standard dev	iation corresp	onding to the	e the 75 th perc	entile CV. SA.	75 = 0.40, a	as determined b	y USEPA for t	he method	and endpoint.		
								as determined b				int.	
			f variation of th				,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,		,		in mapo		
	5.	Coefficient O		~ ~~ ₅₀ valu									







48-hour LC₅₀ = 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 LC₅₀)
 Warning Limits (mean LC₅₀ ± S_{A.75})
 Control Limits (mean LC₅₀ ± S_{A.90} or 2 Standard Deviations)

Graphs generated from associated excel spreadsheet. Excel spreadsheet entered by: J. Sumner Reviewed by: _____



SOP-AT49
1 OF 19
12-01-00
11-01-14

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)	Lance Ferrell (NC DENR)	• Section B.2.a and Exhibit AT49.1 amended to indicate that 9 to 11 day old larvae are used to initiate chronic <i>Menidia</i> tests.
		Internal	Jim Sumner (ETS)	
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)	Lance Ferrell (NC DENR)	 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
		Internal	Jim Sumner (ETS)	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.



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12-01-00
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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 = $25.0 \pm 1.0^{\circ}$ C, photoperiod of 16hour 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



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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 $60 \pm 2^{\circ}$ C or 6-hours at $100 \pm 2^{\circ}$ C.



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- 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 $(25.0 \pm 1.0^{\circ}C)$ of the test concentrations. This may be accomplished by placing the test beakers into a temperature-controlled incubator.



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- 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.



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- 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 $25.0 \pm 1.0^{\circ}$ 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 $25.0 \pm 1.0^{\circ}$ 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 $(150 \ \mu l)$ 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.



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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 2hours 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 \pm 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 newlyhatched 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 \pm 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.



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- 4. Obtain the appropriately labeled 20-ml glass beakers or spot plates containing preweighed 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 $60 \pm 2^{\circ}$ C or 6-hours at $100 \pm 2^{\circ}$ 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.



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- 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	bervllina	chronic	toxicity test	t acceptability criteria.
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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.



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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:	$25.0 \pm 1.0^{\circ}$ 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 \leq 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:	\geq 80% control survival, control growth \geq 0.50 mg/surviving larvae

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Exhibit AT49.2: Menidia beryllina Chronic Toxicity Test Benchsheet.



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County: Glynn

Outfall #: 001

Chronic Whole Effluent Toxicity Test (EPA-821-R-02-014, Method 1006.0) Species: <u>Menidia beryllina</u>

Client: <u>Pinova, Inc., Brunswick Plant</u> NPDES #: <u>GA0003735</u> Project #: _____

 Dilution preparation information:

 Dilution prep (%)
 6.25
 12.5
 Comments: 25 50 100 Effluent volume (mL) 125 250 500 1000 2000 1750 1875 1500 1000 Diluent volume (mL) 0 Total volume (mL) 2000 2000 2000 2000 2000

Test organism informatio	on:	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 = 5.0	Date / Time in oven:
	Temperature = °C	Initial oven temperature:
Average transfer volume:	0.2542 mL	Date / Time out of oven:
	1.7.2.40 http://	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		11.				i		
1	10-15-14								
2	10-16-14					·	1		
3	10-17-14				1			1	
4	10-18-14						1 - 1		1
5	10-19-14	1000				1	1		
6	10-20-14				-		1		
7	10-21-14	-	1 0		2			1	

Control information:	Acceptance criteria	Summary of test endpoints:	
% Mortality:	≤20%	7-day LC ₅₀	
Average weight per initial larvae:	Second states and the	NOEC	
Average weight per surviving larvae:	≥ 0.50 mg/larvae	LOEC	
Constraint and the second second second second second second second second second second second second second s	and the second second second second second second second second second second second second second second second	ChV	
		IC ₂₅	

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Species: Menidia beryllina Client: Pinova, Inc.

Date: 10-14-14

		-	100	_	-	Surviv	al and		h Data	_	-	-	
Day		1.077		IROL	-	1000		5%		1. 10. 1		5%	_
		A	В	C	D	Ε	F	G	H	1	J	K	L
0		10	10	10	10	10	10	10	10	10	10	10	10
-1			1				11						
2					-		1.44	-			1		
3			1.00			1111	111			1		111	
4					-1		1.0	1	The			111	
5		1211					1				1	111	
6					T I								
7		1					1			1	·	-	
A = Pan weight (mg) Tray color code:: Analyst: Date:		T											
B = Pan + Larvae weight (/ Analyst: Date:	mg)	Ĩ											
C = Larvae weight (mg) = B - A Hand calculated. Analyst:													
Weight per initial number = C / Initial number of larv Hand calculated. Analyst:	of larvae (mg) ae												
Average weight per initial number of larvae (mg)	Percent reduction from control (%)												

Comments:						
(†						

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Species: <u>Menidia beryllina</u> Client: <u>Pinova, Inc.</u>

		-		-		Surviv	al and		h Data	1			-
Day			25	%			.50	%	100		10	0%	
		М	N	0	P	Q	R	5	T	U	V	W	X
0		10	10	10	10	10	10	10	10	10	10	10	10
1		1000	1.1		i []	1	14.1.1	1.1.1	A	·	-	1.1.1	1 · · · 1
3				1.1		2.1		1.7					1.1
3										1			
4					F	-						1111	
5				1	111	21		111				1.1.1	1.1
6			121				1.1	1					1 2
7			-			ir ² 1		i in		• = :		1.1.1	
A = Pan weight (mg) Tray color code:: Analyst: Date:		T				1						1Th	ħŷ
B = Pan + Larvae weight (m Analyst: Date:	And an and a second sec			Ē		Π.							111
C = Larvae weight (mg) = B – A Hand calculated. Analyst:													X
Weight per initial number of = C / Initial number of larva	f larvae (mg) e												
Hand calculated. Analyst:										4	_		40
Average weight per initial number of larvae (mg)	Percent reduction from control (%)												

Comments.	
	-

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							P:
wieided festing Solutions.	WC.						
Species:	Menidia beryllina						
Client: P	inova, Inc.					Date:	10-14-14
Daily Chem	istry:						
		Analas	et identified for	D	W mdnH DO m	nd salinity meas	
)		nea pri, D.O. a 1		2
	Analyst		(1	1	
Concentration	Parameter	1	1			1	
	pH (S.U.)		K		1	12.2.2.1	
	DO (mg/L)						
CONTROL	Salinity (ppt)					12 = 1 i	
CONTROL	*Alkalinity			1.000		1.000	
	(mg CaCO ₃ /L)					-	
	*Temperature (°C)				-	1	
6.25%	pH (S.U.)						
	DO (mg/L)			-		_	
	Salinity (ppt) *Temperature ("C)		-	-			
-		-	-	-	-	-	
12.5%	pH (S.U.) DO (mg/L)			_		_	
	Salinity (ppt)		-	-			
	*Temperature (°C)		-			· · · · · · · · · · · · · · · · · · ·	
-	pH (S.U.)	-	-	-	-		-
	DO (mg/L)	-		-			
25%	Salinity (ppt)			-		1	
	*Temperature ("C)		-	-			
	pH (S.U.)		· · · · · ·			· · · · · · · · · · · · · · · · · · ·	
14.1	DO (mg/L)				11		
50%	Salinity (ppt)			-			
	*Temperature (°C)				-	· · · · · · · · · · · · · · · · · · ·	
	pH (S.U.)						
100%	DO (mg/L)	1					
100%	Salinity (ppt)						
	*Temperature ("C)						
1	pH (S.U.)			-			
	DO (mg/L)				2		
100%	Conductivity (umhos/em)						
(Not salted-up)	Salinity (ppt)		1			V	
saited-up)	*Alkalinity		1	-	-	1	
	(mg CaCO ₀ /L)	·	-				
	*TR chlorine (mg/L)	1.1				A	
	*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: _________

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Species: <u>Menidia beryllina</u> Client: <u>Pinova, Inc.</u>

			(Analust id	antified for each	D day performe	ay ipH, D.O. and s	alinity measure	mants on h	
			(Analyst to	entimed for each		pH, D.O. and s			5
	Analyst	1	· · · · ·	· · · · · · · · · · · · · · · · · · ·			· · · · · ·	P	
Conc.	Parameter					1	-	-	
	pH (S.U.)					-	-	200 - 11 Y	-
	DO (mg/L)	1	1	· · · · ·	1			· · · · · · · · · · · · · · · · · · ·	
المتصدد	Salinity (ppt)		1			C		-	-
CONTROL	*Alkalinity (mg CaCO ₃ /L)	112.71	-			17.21		121	
	*Temperature (°C)								
	pH (S.U.)	10 C	E		1			·	
6.25%	DO (mg/L)		I	·= =: :		1)===	0 ····································	
0.25%	Salinity (ppt)								
	*Temperature (°C)		1)	Q	
	pH (S.U.)		· · · · · · · · · · · · · · · · · · ·				·	l	-
12.5%	DO (mg/L)	1	Y	Y			1	1	
	Salinity (ppt)		1	· · · · · ·		1.1		·	
	*Temperature (°C)		· · · · · · · · · · · · · · · · · · ·	č=- 1	· · · · · ·	· · · · · · · · · · · · · · · · · · ·	· · · · · · · · · · · · · · · · · · ·		
25%	pH (S.U.)	C	(Second Se	1	A	0	la contra c	
	DO (mg/L)		10					1.1.1.1	
	Salinity (ppt)		· · · · · · · · · · · · · · · · · · ·					0	
	*Temperature (°C)			C	1	Sec	1	in	
	pH (S.U.)	I	J	S		1)	Ne	
	DO (mg/L)			1			1	1	1.1.1
50%	Salinity (ppt)		Y				1	15	-
	*Temperature (°C)	1	1				1	E	
	pH (S.U.)	1. m	· · · · · ·		1	A second second	1.1	100 million (1997)	
	DO (mg/L)		1		1	1		1	1.1
100%	Salinity (ppt)						1		
	*Temperature (°C)				h			1	
	pH (S.U.)		1					-	-
	DO (mg/L)								-
100% (Not	Conductivity (µmhos/cm)		-	1.01					
	Salinity (ppt)		1	· · · · · · · · · · · · · · · · · · ·				-	
salted-up)	*Alkalinity (mg CaCO ₁ /L)								
	*TR chlorine (mg/L)	1	-			1	1	1	-
	*Temperature (°C)	-	-	provide a support de		A DECEMBER OF THE OWNER OWNER OF THE OWNER	and the second s	Seat and the second second	And Manager

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Confidential

Date: 10-14-14

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Exhibit AT49.3: Average Transfer Volume by Medicine Cup for each Analyst.

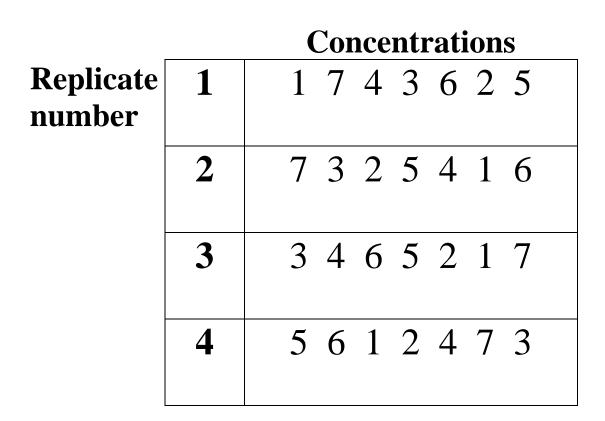
Pimephales promelas are used as surrogate organisms to determine the average transfer volume.

	Larval Fig	sh Transfer Volume	Page 1 o
		sh fransfer volume	
Analyst:	J. Sumner	Species:	P. promelas
	02-12-14		ATOX Batch Pp 02-03-14
Ambient temperature:	24.1°C	Wet Weight of 10 Larvae (g):	0.0209
		re allowed to swim from	the pipette into the test vess
Numerically label 10 me Add 10 mL MHSW to ea			
	e weight of each cup containing		
	ch cup, following procedures ide manner that allows them to sw		AT53 for vertebrate acute toxicity tes
	e weight of each cup containing		insw contained in each cup.
	r volume and average transfer		
Replicate	Initial Weight	Final Weight	Transfer Volume
Number	Medicine cup + 10 mL MHSW	Medicine cup + 10 mL MHSW	Final - Initial Weight
		+ 10 Larval Fish transferred	(g = mL)
	(g)	(g)	
2	11.3533	11.6989	0.3456
3	11.3337 11.3607	11.4850 11.6976	0.1513 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 Average volume	11.5329 to transfer 10 organisms (mL):	0.2008
Estimate transfer v	olume, where the minno	ws are transferred with I	MHSW into the test vessels.
Numerically label 10 me	edicine cups.		
Measure and record the	e weight of each cup.		
	nL MHSW to each of the 10 cup		
	ch cup, following procedures ide manner that allows them to sw		AT53 for vertebrate acute toxicity tes
	weight of each cup containing		
Determine each transfe	r volume and average transfer v	volume.	
Replicate	Initial Weight	Final Weight	Transfer
Number	Medicine cup	Medicine cup + 10 mL MHSW	Volume Final - Initial Weight
		10 mL MHSW + 10 Larval Fish transferred	(g = mL)
	(g)	(g)	18
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 1.7015	11.3360 11.7630	9.6620
8	1.6870	11.7335	10.0615
9	1.7009	11.4930	9.7921
10	1.7152	11.5329	9.8177

	Aquatic Toxicity Procedures			
	SECTION PAGE	SOP-AT49 19 OF 19		
Environmental Testing Solutions, Inc.	DATE REVISION DATE	12-01-00 11-01-14		

Exhibit AT49.4: Randomization Templates.

BLUE



Random number seeds 10 through 13.



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PAGE	1 OF 14
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REVISION DATE	11-01-14

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.



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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 = $25.0 \pm 1.0^{\circ}$ C, photoperiod of 16hour 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



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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 (CuSO₄, 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 CuSO₄ (SOP-G10). Place approximately 400 ml of Milli-Q water in a 500-ml volumetric flask. Add the CuSO₄ to the flask, dissolve the CuSO₄ 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.



Exhibit AT50.1: Menidia beryllina Chronic Reference Toxicity Test Benchsheet.



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Copper Sulfate Chronic Reference Toxicant Test (EPA-821-R-02-014, Method 1006.0) Species: <u>Menidia beryllina</u>

MbCuCR Test Number: 102

Dilution prepara	Comments:					
Cu Stock INSS nut	nber:	INSS				
Stock preparation	100 mg Ci Dissolve 0		0, in 500-mL			
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	
Diluent volume (mL)	1999.5	1999.0	1998.0	1996.0	1990.0	
Total volume (mL)	2000	2000	2000	2000	2000	

Test organism informatio	on:	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	
	1.000		Drying information for weight determination:		
Transfer bowl information:	pH =	S.U.	Date / Time in oven:		
	Temperature =	°C	Initial oven temperature:	1	
Average transfer volume:	0.2542 mL		Date / Time out of oven:		
	a second a second		Final oven temperature:		
			Total drying time:	-	

Daily feeding and renewal information:

Day Date	Date	Morning	feeding	Afternoo	n feeding	Test initiation or term	Salt SW batch used	
12.4		Time	Analyst	Time	Analyst	Time	Analyst	1.11
0	10-14-14		· · · · · · · ·	-			1000	-
1	10-15-14						1	
2	10-16-14				1	C	· · · · ·	
3	10-17-14	1.1.1						1.00
4	10-18-14		1					
5	10-19-14	1.1.1					-	-
6	10-20-14					· · · · · · · · · · · · · · · · · · ·		1
7	10-21-14		1		1			-

Control information:	Acceptance criteria	Summary of test endpoints:		
% Mortality:	≤ 20%	7-day LC ₅₀		
Average weight per initial larvae:	the second second second second second second second second second second second second second second second se	NOEC		
Average weight per surviving larvae:	≥ 0.50 mg/larvae	LOEC		
		ChV		
		IC25		

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Species: Menidia beryllina

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MbCuCR Test Number: 102

		-	0.01	mor	-				h Data				-
Day	·			TROL			0.025 mg Cu/L			_	0.050 mg Cu/L		
		A	В	C	D	E	F	G	H	I	J	K	L
.0		10	10	10	10	10	10	10	10	10	10	10	10
1		41			1.1		-		-			-	
2					* - 1			-		-	1		1-1-1
3		1.1.1			5.4		1.2.27				100		1.5
4					111								
5					11						1111		
6		· * ·	-	1				1					1 1
7													-
A = Pan weight (mg) Tray color code:: Analyst: Date:													
B = Pan + Larvae weight (n Analyst: Date:	A	111											
C = Larvae weight (mg) = B Hand calculated. Analyst:	I-A					-							
Weight per initial number o = C / Initial number of larv:	of larvae (mg) ae												
Hand calculated. Analyst:	-	11											
Average weight per initial number of larvae (mg)	Percent reduction from control (%)												

 $\frac{Comment\ codes}{c}:\ c=clear,\ d=dead,\ fg=fungus,\ k=killed,\ m=missing,\ sk=sick,\ sm=unusually\ small,\ lg=unusually\ large,\ d&r=decanted\ and\ returned,\ w=wounded.$

Comments:		
		_

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Species: Menidia beryllina

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MbCuCR Test Number: 102

					1	Survis	al and	Growt	h Data				
Day		1.57	0,100 n	ng Cu/L			0.200 n	ng Cu/L			0,500 n	ng Cu/L	
		М	N	0	P	Q	R	5	T	U	V	W	X
0		10	10	10	10	10	10	10	10	10	10	10	10
1		11		1		171	1					1111	1.1
2		12.1	127			1	11.1	-		1	1 1	1111	
3							-						
4			124		. 1		1.			21	1. 11	112	
5			111	-	1			-					
6				1	- 1						1.11		-
.7		1.1					1.1.1				1		
A = Pan weight (mg) Tray color code:: Analyst: Date:		1											
B = Pan + Larvae weight (n Analyst: Date:	ng)	11					111						
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 (%)	1											

Comments:	
	7 25 (200) 1 4 3 3 (202) 4 4 4

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DATE	12-01-00
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MbCuCR Test Number: 102

Subject: Menidia beryllina Chronic Reference Toxicity Test, EPA 1006.0

	_	-			
Ξ.	No.			×.	
2			1.0		

Species: Menidia beryllina

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		(Analyst id	entified for each	D: day performed		alinity measurem	ents only)
		(runiy)riu		uny, performed	1	dataty measured	2
	Analyst	10 m	e	1		y	
Conc.	Parameter		1	1			
1.000	pH (S.U.)			L		11	1
	DO (mg/L)		1				
CONTROL	Salinity (ppt)		1				1
CONTROL	*Alkalinity (mg CaCO ₃ /L)	Alter and		1.1		X = 1	
	*Temperature ("C)		11			· · · · · · · · · · · · · · · · · · ·	-
	pH (S.U.)		1	-	1		
0.025 mg Cu/L	DO (mg/L)		×				1
	Salinity (ppt)	1				1	-
	*Temperature (°C)		i		·	1	
_	pH (S.U.)	1	· · · · · · · · · · · · · · · · · · ·		(· · · · · · · · · · · · · · · · · · ·	-
0.050	DO (mg/L)) — — ·	1		-	1.1	-
mg Cu/L	Salinity (ppt)		· · · · · ·				
1. A. 1. A.	*Temperature ("C)	P		· · · · · · · · · · · · · · · · · · ·	1.		
1000	pH (S.U.)	1.000	Provide States	1	A	1	
0.100	DO (mg/L)	0	1 8			1	1
mg Cu/L	Salinity (ppt)	2	N	1			
C	*Temperature (°C)				C		
	pH (S.U.)	1					
0.200	DO (mg/L)		1. The second se	1		1	
mg Cu/L	Salinity (ppt)	ta - mill	1			1	
	*Temperature ("C)		1 1 1		1	1	1
1.1.1.1.1.1	pH (S.U.)						-
0.500	DO (mg/L)						
mg Cu/L	Salinity (ppt)	0	4	1	1	1	1
1.00	*Temperature (°C)	i and a state			A	11	1
		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:

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Species: Menidia beryllina

MbCuCR Test Number: 102

		ic	(Analyst id	entified for each	day, performe	ay d pH, D.O. and s	alinity measure	ments only.)	
		>	3		1		5	and the second se	5
	Analyst	1.00	· · · · · · · · · · · · · · · · · · ·	1	((m. 11.)			
Conc.	Parameter	Sec. 1	1	1	1	1.0	1	1	Y
	pH (S.U.)	N	7		A second second second	No. Annual State of	j	·	· · · · · ·
	DO (mg/L)			· · · · · · · · · · · · · · · · · · ·					÷
CONTROL	Salinity (ppt)	· · · · · · · · · · · · · · · · · · ·	-				-		· · · · ·
CONTROL	*Alkalinity	10.000				· · · · ·			
	(mg CaCO ₃ /L)	1		· · · · · · · · · · · · · · · · · · ·	-	A			
	*Temperature (°C)	· · · · ·		A	· · ·		C	. · · · · · · · · · · · · · · · · · · ·	-
	pH (S.U.)	10	1 - 1	1	1	P		11	2 h
0.025 mg Cu/L	DO (mg/L)					1			j.,
	Salinity (ppt)	2	11						
	*Temperature (°C)		1		1	10 m m	S - 1	1	-
ALC: NOT	pH (S.U.)	· · · · · · · · · · · · · · · · · · ·	1	1	1.00		2	* p	1.00
0.050	DO (mg/L)	S		1	1	·	1		-
mg Cu/L	Salinity (ppt)								
The second	*Temperature ("C)	0 30	1	()		$h = -\lambda$		4.	. · · · · · · · · · · · · · · · · · · ·
	pH (S.U.)	,	$c = -\lambda$		7	t = -25	Y	1	
0.100	DO (mg/L)	1	-		· · · ·			1 p	
mg Cu/L	Salinity (ppt)	· · · · · · · · ·	· · · · · · · ·	· · · · · · · · · · · · · · · · · · ·	· · · · · · · ·				
	*Temperature ("C)	0	1		C		<u> </u>	1	100 million
	pH (S.U.)	01	S	1	1	17 i 1	· · · · · · · · · · · · · · · · · · ·	· · · · · · · · · · ·	21 ·····
0.200	DO (mg/L)							· · · · · · · · · ·	-
mg Cu/L	Salinity (ppt)	0	1 1	1	1		t	1	·
	*Temperature (°C)		-						1.
	pH (S.U.)	h	-			-		f	
0.500	DO (mg/L)	· · · · · ·			1	1	r		
mg Cu/L	Salinity (ppt)	1	1		-		1		·
	*Temperature ("C)	In survey of	A received	in the second se	-	Sec. 1		-	·
		Initial	Final	Initial	Final	Initial	Final	Initial	Final

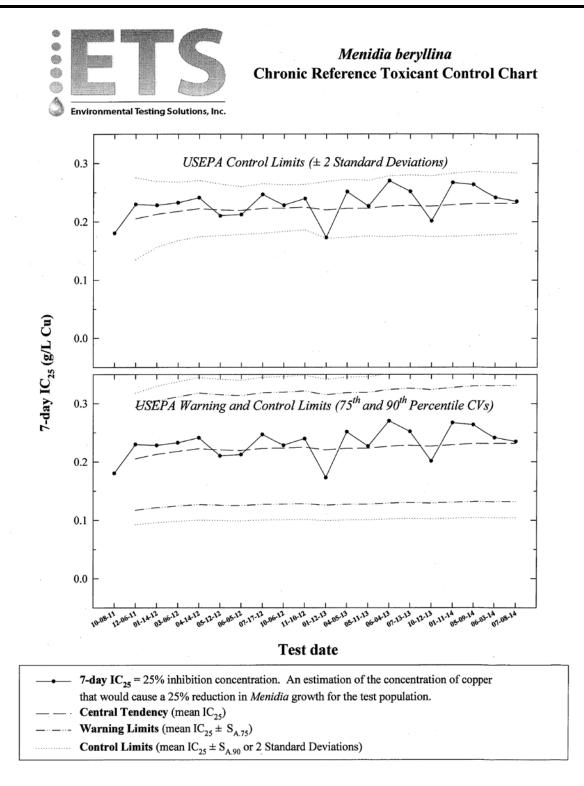
*Temperatures performed at the time of test initiation, renewal or termination by the analyst identified in the Daily Renewal Information table located on Page 1. Alkalinity performed by the analyst identified on the bench sheet specific for this analysis and transcribed to this bench sheet by:



Exhibit AT50.2: Example of a *Menidia beryllina* Chronic Reference Toxicant Control Chart.

						lenidia be							
	1			Chro	onic Refei	ence Toxi	cant Co	ntrol Chai	rt				
) Environ	nmental Testi	Calution	100										
Enviror	nmental lesti	ng Solutions	, Inc.										
					State and	IISEDA		USI	EPA		US	EPA	
est number	Test date	7-day IC ₂₅	СТ	S	Control		S _{A.75}	Warnin		S _{A.90}		l Limits	CV
est number	Test date		(mg/L Cu)	3	CT - 2S	CT + 2S	SA.75		-	SA.90		CT + S _{A.90}	CV.
		(mg/L Cu)	(mg/L Cu)		CI - 28	CI + 25		CT - S _{A.75}	$C1 + S_{A.75}$		CI - S _{A.90}	$C1 + S_{A.90}$	
1	10-08-11	0.180											
2	12-06-11	0.180	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	0.230	0.21	0.04	0.15	0.28	0.09	0.12	0.29	0.11	0.09	0.32	0.17
4	03-06-12	0.223	0.21	0.03	0.10	0.27	0.09	0.12	0.30	0.12	0.10	0.33	0.13
5	04-14-12	0.233	0.22	0.02	0.17	0.27	0.10	0.12	0.31	0.12	0.10	0.34	0.12
6	05-12-12	0.241	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	0.210	0.22	0.02	0.18	0.26	0.09	0.13	0.32	0.12	0.10	0.34	0.09
8	07-17-12	0.212	0.22	0.02	0.18	0.20	0.10	0.13	0.32	0.12	0.10	0.34	0.10
9	10-06-12	0.247	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% i	nhibition conce	ntration. An	estimation of	the concentrati	on of coppe	r that would c	ause a 25% rec	luction in M	<i>lenidia</i> growtl	n for the	
		test population	on.										
	CT =	Central tende	ency (mean IC	25).									
	S =	= Standard dev	viation of the IO	225 values.									
	USEPA Contr			25									
		Standard dev	0	onding to the	the 75 th perce	entile CV (S.	0.43						
			-	-	-								
		Standard dev				entile CV. $(S_A$	90 = 0.55						
	CV =	Coefficient o	f variation of th	e IC ₂₅ value	s.								





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)		Menidia bery	lina				
			Chronic	Reference To	oxicant D	ata			
Environmenta	al Testing Solu	tions, Inc.							
est number	Test date	Control Survival	Control Mean Growth	СТ	CV	СТ	MSD	PMSD	СТ
		(%)	(mg/larvae)	for Control Growth (mg/larvae)	(%)	for Control Growth CV (%)		(%)	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 bou	nd determined by U	SEPA (10 th percentile)	= 4.4%.				
		Upper CV bou	nd determined by U	SEPA (90 th percentile)	= 18%				
	MSD =	Minimum Sign	ificant Difference						
	PMSD =		um Significant Diffe						
		PMSD is a me	asure of test precisio	on. The PMSD is the r	ninimum percei	nt difference between	n the contro	and treatme	ent that can be
		declared statis	tically significant in	a whole effluent toxici	ty test.				
				y USEPA (10 th percent					
		Upper PMSD	bound determined b	y USEPA (90 th percent	ile) = 28% .				
	CT =	Central Tenda	ncy (mean Control C	rowth, CV, or PMSD)					



