TEN DAY RENEWAL TEST FOR DETERMINING ACUTE TOXICITY OF SEDIMENTS TO THE FRESHWATER AMPHIPOD *HYALELLA AZTECA* AND THE MIDGE *CHIRONOMUS TENTANS*

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1.0 SCOPE AND APPLICATION

This Standard Operating Procedure (SOP) describes general procedures used in conducting static, renewal, ten day solid phase freshwater toxicity tests. The objective of this procedure is to evaluate potential toxicity of sediment to two common freshwater benthic bioindicator species, a freshwater amphipod *Hyalella azteca* and a midge *Chironomus tentans*.

These are standard (i.e. typically applicable) operating procedures which are based on those adopted by the United States Environmental Protection Agency [U.S. EPA (1994)] for standardization of freshwater sediment test conditions. The testing conditions presented in U.S.EPA (1994) and herein should be strictly followed, however should these procedures contradict the above guidance, the EPA guidance document shall take precedence. Suggestions presented herein for the construction of testing chambers, etc. are provided as general guidance, therefore, other equally viable testing setups can be used contingent upon U.S. EPA/ERT/SERAS approval.

Mention of trade or commercial products does not constitute U.S.EPA endorsement or recommendation for use.

2.0 METHOD SUMMARY

This testing procedure involves exposing representative freshwater benthic invertebrates to contaminated sediments. Toxicity of the sediment is assessed by measuring mortality and sub-lethal growth endpoints.

3.0 SAMPLE PRESERVATION, CONTAINERS, HANDLING, and STORAGE

Sediment samples are maintained at 4^{o}C until the time of testing. At the time of test initiation, they are allowed to equilibrate at the recommended testing temperature.

4.0 INTERFERENCES AND PROBLEMS

This section is not applicable to the SOP

5.0 EQUIPMENT / APPARATUS

1. Test Organisms (either A or B)
   a. Juvenile amphipods [1 -2 millimeter (mm); 7-14 days old] acclimated to overlay water, quantity according to need
   b. Third instar midge larvae
2. Exposure Chambers: 400 milliliter (ml) high density polyethylene (HDPE) beakers (graduated in 25 ml increments), 0.1 mL nylon mesh, silicone adhesive3. Exchange lids for chambers (400 ml HDPE beakers, 100 mm petri plates (top or bottoms), 1/8 inch (in) diameter rigid plastic air hose, 1/8 in flexible plastic air hose)
3. Water quality meters: Temperature, pH, Dissolved Oxygen (DO), Salinity/Conductivity
4. 0.5-mm mesh sieve

5. Plastic tray
6. Dissecting microscope
7. Micrometer (graduated to 0.01 mm)
8. Forceps
9. 30 ml plastic cups
10. 10 ml plastic disposable pipets
11. Electronic balance [0.0001 gram (g) accuracy]
12. Plastic disposable spoons
13. Overlay water-moderately hard reconstituted water or substitute, depending on site conditions
14. Electric drill fitted with stainless steel mixer
15. Soldering iron
16. Decontamination equipment (brushes, distilled water, 10 % Nitric Acid, Acetone, soapy water)
17. 35 mm camera with 100 ASA slide film

6.0 REAGENTS

This section does not apply to the SOP.

7.0 PROCEDURE

7.1 Preparation

7.1.1 Calibrate meters for pH, salinity/conductivity, DO, and temperature measurements on a daily basis. Calibration should be done in accordance with the manufacturers instructions.

7.1.2 Prepare and label eight replicate exposure chambers (number of replicates may be adjusted, but 8 is recommended) for the control sediment, the reference sediment (if requested), and for each of the test sediments. Materials needed for each chamber include two 400 ml HDPE beakers, one 100 mm Petri plate top or bottom, one 150 mm length of rigid plastic tubing (air tube), one 50 mm length of rigid tubing (exchange tube), flexible air tubing, and two 20 mm square pieces of 1 mm nitex screen.

Construction of exposure chamber

7.1.2.1 Using a hot soldering iron, punch one hole in the first beaker at the desired water level, and another on the exact opposite side of the beaker as the previous hole.

7.1.2.2 Using a silicone adhesive, attach a piece of nitex screen over each hole, making sure to not clog th

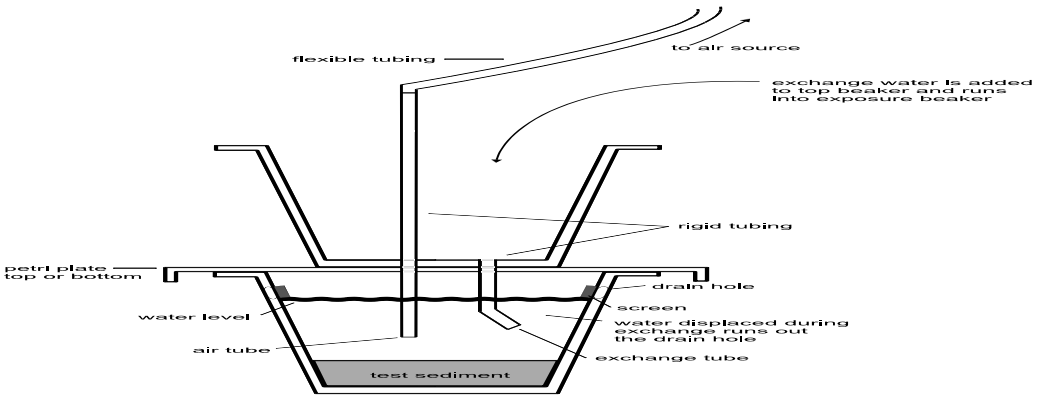
Construction of exchange lid

7.1.2.3 Using the silicone adhesive, attach a second beaker to the center of the disposable plastic Petri plate.

7.1.2.4 Using the hot soldering iron, punch two holes, side by side, but not touching through the Petri plate and attached beaker.

7.1.2.5 Push the longer piece of rigid plastic tubing (air tube) through one hole. Put a 45 degree bend in the other tube (exchange tube) about half way along its length. The air tube should stick up out of the second beaker and an airline is attached. The exchange tube is pushed through until it is flush with the bottom of the beaker. During the test, this allows all exchange water to flow out of the exchange chamber and into the test chamber. Little or no standing water should be held in the exchange chamber after an exchange is completed. The water that is displaced in the test chamber due to the addition of the exchange water simply runs out the screen covered holes in the first beaker and into a water bath. If requested, the exchange water can be easily collected by placing a short piece of rigid tubing into the drain holes; the draining water is then collected in a smaller beaker.

7.1.2.6 Refer to the following figure for a cross-section of the example exposure chamber.



7.1.3 Thoroughly mix each sediment with an electric drill fitted with a stainless steel mixing blade in order to ensure sample homogeneity.

7.1.4 Measure 100 cubic centimeters (cm^3) of the appropriate sediment into each replicate exposure chamber. Smooth the sediment with a plastic spoon or similar implement.

7.1.5 Monitor the temperature of the overlay water prior to any further preparation. Adjust and

record the temperature as necessary to 23±1° C.

7.1.6 Carefully introduce the overlay water into each exposure chamber by pouring slowly down the inside wall of the chamber in order to minimize disturbance of the sediment. Fill each chamber to the 275-mL mark with the overlay water.

7.1.7 Run a length of air tubing from a valve on an aeration manifold or air pump to each exposure chamber. Fit the free end of the tube to an air tube on an exposure chamber lid. Set the exchange chamber on top of the test chamber, and adjust the air tube position so that the tip rests approximately 2 cm above the sediment surface. Additionally, adjust the exchange tube so that an addition of water is unlikely to disturb the test sediment in the test chamber.

7.1.8 When the sediment has settled, monitor the temperature, dissolved oxygen, pH, conductivity, alkalinity and hardness in every treatment.

7.1.9 If dissolved oxygen drops below 40% any time during the test (which happens in most cases), adjust the rate of air flow to approximate 100 bubbles per minute and gently aerate the chamber for the remainder of the testing period.

7.2 Test Initiation

7.2.1 Obtain test organisms from the culture in pre-counted groups of 10 each in 30-mL cups - the exact number per chamber will vary with test design. Examine each set to validate the count and assess the health of the organisms

7.2.2 Randomly assign one cup to each exposure chamber until all cups are assigned. Suspend each cup in the assigned chamber and allow 10 minutes for temperature equilibration.

7.2.3 Introduce the organisms to the chambers by carefully dipping and then inverting the cups in the test medium. Swirl the contents from each cup as it is lifted from the medium. Check each cup carefully after stocking to ensure that no organisms have adhered to its surface.

7.2.4 Record the initiation time and the initial live count for each chamber.

7.2.5 Examine each test chamber regularly over the first two hours post-initiation. Replace any organisms which remain at the surface during this period.

7.3 Monitoring and Maintenance

7.3.1 The following operations are to be conducted at each successive 24-hour interval following test initiation:

7.3.1.1 Observations: mortality and behavior

7.3.1.2 Monitoring of physical and chemical parameters: temperature and dissolved oxygen

7.3.1.3 Feeding:
a. Yeast-cerophyll-trout chow (YCT) food (1.5 ml daily) for amphipods
b. Tetrafin goldfish food suspension (1.5 ml daily) for midges (4.0 mg dry solids/ 1.5 ml culture water)

7.3.1.4 Daily water exchanges: two volume additions per day should be made to the test chambers by either continuous or intermittent flow.

7.3.2 Examine each chamber and record the numbers of dead and moribund organisms and of floating or actively swimming individuals.

7.3.3 Measure and record alkalinity, conductivity, pH, and ammonia at test initiation and termination.

7.4 Test Termination

7.4.1 Upon completion of the full exposure period, the test is terminated.

7.4.2 Perform final behavioral observations for each test chamber.

7.4.3 Record the temperature, pH, dissolved oxygen and conductivity for each test chamber.

7.4.4 Measure the alkalinity and hardness for a composite sample taken from each test chamber in a replicate series.

7.4.5 Working with one chamber at a time, carefully pour off all but 1 cm of the overlay water, using a sieve to retain any floating or swimming organisms. Using a disposable pipet, transfer any organisms to a 30-mL cup containing clean overlay water.

7.4.6 Agitate the upper layer of sediment and remaining overlay water in the chamber to create a slurry. Pour the slurry through the sieve. Most organisms will be found in this upper layer.

7.4.7 Fill a plastic tray with clean overlay water and set the sieve into the tray. Gently agitate the sieve to allow as much sediment as possible to pass through the mesh. Using a disposable pipet, transfer any live organisms to the 30-mL cup.

7.4.8 Using clean overlay water, continue working with the remaining sediment, examining small portions at a time, until the chamber is empty.

7.4.9 Determine and record the final live count.

7.4.10 Repeat steps 3.4.5 through 3.4.9 for each test chamber.

7.4.11 If deemed appropriate, characterization of organism size or growth may be determined from measurement of organism weights and/or lengths.

7.4.12 For *C. tentans* determine weight measurements as follows:

7.4.12.1 Calibrate the electric balance

7.4.12.2 Air dry each replicate group at room temperature for 12 to 24 hrs or to constant weight..

7.4.12.3 Tare a section of weigh paper and weigh each group to the nearest 0.0001 g.

7.4.13 Determine *C. tentans* head capsule width measurements as follows:

7.4.13.1 Anesthetize the organisms by placing them in a 30-mL cup containing a small quantity of 3 percent Magnesium Chloride ($MgCl_2$) or carbonated water. Organisms may also be preserved in 30% methanol for later measurement. Place an organism on the micrometer slide and place the slide onto the microscope stage. Working with a forceps or dissecting needle, position the organism so that it lies laterally parallel with the graduated scale, dorsal side up.

7.4.13.2 Determine head capsule width to the nearest 0.01 mm for each organism recovered.

7.4.13.3 Repeat for each replicate.

7.4.14 Determine *H. azteca* length measurements as follows:

7.4.14.1 Anesthetize the organisms by placing them in a 30-mL cup containing a small quantity of 3 percent Magnesium chloride or carbonated water. Organisms may also be preserved in 30% methanol for later measurement. Place an organism on the micrometer slide and place the slide onto the microscope stage. Working with a forceps or dissecting needle, position the organism so that it lies laterally parallel with the graduated scale, dorsal side up.

7.4.14.2 Determine to the nearest 0.01 mm the overall length, measuring along the dorsal surface from the base of the antennae to the base of the third uropod.

7.4.14.3 Repeat for each replicate.

8.0 CALCULATIONS (STATISTICAL ANALYSIS

Data analysis is completed by strictly following U. S. EPA analysis guidelines (U.S. EPA, 1994). In most cases, the assumptions of normality and homoscedasticity for using one way analysis of variance models are met. In these cases, straight one way analysis of variance models are fit followed by Dunnett's multiple comparisons tests. Any breakdowns in the classic model such as deviations from normality, heteroscedasticity of error variances or unequal replication, etc. are addressed by the following diagram which is taken directly from the EPA guidance (U. S. EPA 1994).

9.0 QUALITY ASSURANCE/QUALITY CONTROL

QA/QC requirements for this test are outlined in U.S. EPA (1994).

10.0 DATA VALIDATION

Analyzed data are compared against the laboratory data sheets. Survival data are compared against QA/QC minimum acceptable survival.

11.0 HEALTH AND SAFETY

The level of health and safety should be identical to that observed under field conditions in the presence of ample laboratory ventilation. In cases where ventilation is inadequate, the Health and Safety group should be consulted.

12.0 REFERENCE

U.S. EPA (1994) Methods for Measuring the Toxicity and Bioaccumulation of Sediment-associated Contaminants with Freshwater Invertebrates. EPA/600/R-94/024

13.0 APPENDIX

As a convenience to the reader, U.S. EPA (1994) has been included as an appendix.

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TABLE 1. Recommended test conditions for *Hyalella azteca* and *Chironomus tentans* chronic toxicity test

1. Test type:	Whole sediment static, renewal, 10 day
2. Temperature:	23.0 1□C
3. Light quality:	Ambient laboratory illumination
4. Light intensity:	500-1000 lux
5. Photoperiod:	16 hours light, 8 hours dark
6. Test chamber:	300 ml beaker
7. Sediment Volume	100 mL
8. Overlying Water Volume	175 mL
9. Renewal of overlying water	2 volume additions/day; continuous or termittent
10. Age of test organisms:	H. azteca: 7-14 days old C. tentans: third instar
11. Number of organisms/container:	10
12. Number of replicate chambers/treatment	Varies; minimum of 8 recommended
13. Feeding:	*H. azteca*: YCT food, fed 1.5 mL daily to each chamber *C. tentans*: Tetrafin goldfish food, fed 1.5 ml daily to each test chamber (1.5 ml contains 4.0 mg of suspended flakes)
14. Aeration:	None unless DO concentration falls below 40% saturation then <100 bubbles per minute
15. Overlying Water:	Culture water, well water, surface water, site water, or reconstituted
16. Test chamber cleaning:	If screens become clogged during the test, gently brush outside of screen to permit water flow/exchange
17. Overlying water quality:	Hardness, alkalinity, conductivity, pH, and

	ammonia at the beginning and end of a test. Temperature and dissolved oxygen daily.
18. Test duration:	14 days
19. Effects measured:	*H. azteca*: survival and length *C. tentans*: survival and dry weight
20. Test Acceptability:	*H. azteca*: 80% control survival* *C. tentans*: 70 % control survival*

*Note: This testing protocol differs from the standard EPA 1994 guidance only in test duration (i.e. 14 vs. 10 days). Adherence to all other testing requirements summarized in EPA/600/R-94/024 "Methods for Measuring the Toxicity and Bioaccumulation of Sediment-associated Contaminants with Freshwater Invertebrates" is required.