

***Chironomus tentans* Sediment Toxicity Test**

1.0 OBJECTIVE

Many anthropogenic contaminants in aquatic systems become bound to particles and may subsequently accumulate in sediments. Observed effects may be related to the presence of contaminants or to naturally occurring factors. To correctly interpret toxicity results, a number of sediment parameters should be analyzed, including chemistry, grain size, and TOC, as well as the following water quality parameters: dissolved oxygen, pH, conductivity, ammonia, alkalinity, hardness, and temperature.

In this procedure, sediment collected from field stations is divided into replicate test containers in the laboratory and covered with dilution water (U.S. EPA 2000). Ten larvae are placed into each replicate container and allowed to interact with the test sediments. After a 10-day exposure, the sediment is sieved to recover the larvae, and live animals are counted to determine the percentage that survived the exposure. Animals from each replicate are then dried and weighed, then ashed and reweighed. Sediment toxicity is characterized by the mean percent survival and growth (\pm standard deviation) for each sediment sample. This can be compared to the survival and growth observed in sediment from the larvae collection site (home sediment), or in sediment from reference sites presumed to have similar natural characteristics but low contaminant concentrations.

2.0 EQUIPMENT

The following equipment is necessary to conduct the toxicity test at the Marine Pollution Studies Laboratory at Granite Canyon (MPSL). The word "clean" here and throughout this procedure means that the item has been cleaned according to the MPSL glassware cleaning procedures outlined in a separate standard operating procedure (MPSL SOP 1.3).

2.1 Culture

- Large, clean culture tray to hold and acclimate *Chironomus*
- Air stones, tubing, and clean air system
- Granite Canyon well water ($23 \pm 1^\circ\text{C}$) for renewals
- Tetrafin® slurry for feeding
- *Chironomus tentans*, second or third instar (appx. 10 days old), supplied by Aquatic Biosystems (Fort Collins, CO)

2.2 Test Initiation

- Environmental chamber ($23 \pm 1^\circ\text{C}$, ambient laboratory illumination for 16 hours/day)
- Clean 300-mL glass beakers or 250-mL I-Chem jars (8 per sample)
- Clean air supply and air manifolds, with air tubing and plastic 100 μL micropipettor tips
- 400 μm screen strips to wrap around top of test beaker and affix with a rubber band or jar lid
- 30-mL disposable plastic cups (60) for reference toxicant test

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- 250-mL clean plastic volumetric flask and glass pipettes for reference toxicant dilutions
- Potassium chloride stock solution (20 g/L)
- Clean polypropylene spoons to scoop sediment into containers (one per sediment sample)
- Transfer pipettes for sorting larvae
- Hand counters
- Data sheets
- Gloves and appropriate safety gear (see MPSSL lab safety manual)
- Graduated pipettes (10 mL) and hand pipette pump or repeater for feeding Tetrafin slurry
- Plastic bins with 8 tubes for delivering renewal water into beakers (splitters)

2.3 Water Quality

- Meters, probes, spectrophotometer, digital titrator and standards for measuring pH, dissolved oxygen, hardness, alkalinity, ammonia, and conductivity
- Thermometers (glass spirit thermometer and continuously recording thermometer)
- Graduated pipettes (10 mL) and hand pipette pump for water quality sampling
- Water quality vials (30 mL glass)
- Gloves and appropriate safety gear (see MPSSL lab safety manual)

2.4 Dilution Water

In every step of this procedure, use Granite Canyon well water.

2.5 Test Maintenance

- Thermometer (glass spirit) for daily temperature check
- Graduated pipettes (10 mL) and hand pipette pump for feeding Tetrafin slurry and sampling water quality

2.6 Test Termination

- Toxic 400- μ m screens to sieve larvae at test termination
- Toxic glass tubes with squeeze bulbs and screens for collecting larvae
- Pre-weighed foil packets incised with numbers, empty box with dividers sufficient to contain all foil packets, small sieve, tweezers, drying oven, muffle furnace, crucibles, crucible tongs, desiccator, and balance
- Data sheets and clipboards
- Pens (and towel to dry hands for writing)
- Gloves and appropriate safety gear (see MPSSL lab safety manual)

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3.0 EXPERIMENTAL DESIGN

Sediment toxicity tests can be used as screening tools or as part of more comprehensive studies to assess sediment quality. Careful consideration must be given to site characteristics, reference site selection, field replication, choice of synoptic measures, seasonal factors, comprehensive planning and peer review to determine that study designs are adequate to meet program objectives.

This laboratory toxicity test consists of eight replicate test containers for each sediment sample. Containers are arranged per site in groups of 8, and each receives ten larvae. The quality of test animals and testing conditions is determined through concurrent testing of reference toxicants (positive controls) and formulated and/or reference sediments (negative controls). Testing of reference sites is recommended to demonstrate the suitability of test sediments in the absence of toxic contaminant concentrations. Dissolved oxygen, pH, conductivity, ammonia, alkalinity, and hardness are measured at the beginning and end of the exposure. Old dissolved oxygen is measured daily. Temperature is measured daily by hand and measured continuously by a temperature logger. The photoperiod for the test is 16 hours light: 8 hours dark, and the temperature is $23 \pm 1^\circ\text{C}$.

4.0 PREPARATION OF SEDIMENTS FOR TESTING

Label test beakers as indicated on the loading sheet generated for the test. Remove test sediments from refrigerated storage and place samples on the lab bench prepared for distributing sediment into test beakers. Carry a small number of samples at a time to avoid injury and possible loss of samples to breakage. Minimize sample exposure to sunlight (never leave samples in direct sunlight), and schedule loading times to avoid prolonged sample exposure to temperatures above 23°C . Do not sieve, freeze, or allow test sediments to dry prior to testing. Remove large objects such as sticks or clams with forceps, and note their presence on the data sheet.

Using a separate clean polypropylene spoon for each sample, re-homogenize (stir) the sediment in the sample jar to thoroughly mix overlying water back into the sediment. Spoon 100 mL of sediment into each of the eight test containers. Leave the spoon in the sample jar so it won't be used for other samples. Arrange the test beakers in numerical order in water tables in the constant temperature room. Place prepared splitter over corresponding beakers. Add 1.6L well water to each splitter.

5.0 CONTROLS

5.1 Sand and Reference Controls (Negative Controls)

Prior to test initiation, prepare sand (clean, kiln-dried sand, #60, RMC Pacific Materials, Monterey, CA, U.S.) for use as control. Using a clean polypropylene spoon, distribute the sand into eight test beakers. If a reference site is to be used as an additional control, collect 1L sediment from a predetermined site and place in refrigerated storage until it is loaded into test containers.

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5.2 Reference Toxicant Tests (Positive Controls)

For cultured organisms, conduct a concurrent reference toxicant at least monthly. The reference toxicant test is a 96-hour exposure using a water-only dilution series of potassium chloride, and provides data on the relative sensitivity of cultured organisms.

Reagent grade potassium chloride (KCl) should be used as the reference toxicant for *Chironomus* tests, unless another toxicant is specified by the Regional Water Quality Control Board or other appropriate regulatory agency. Prepare a 20 g/L KCl stock solution by adding 20.0 g of reagent grade KCl to a final volume of one liter of well water in a plastic volumetric flask. Cap tightly and mix thoroughly.

Reference toxicant solutions should be ten replicates of 0, 2.5, 5, 10 and 20 g/L KCl. Other concentrations may be added between these if greater precision is desired for quality control chart purposes. Prepare 250 milliliters of each concentration by adding 0, 15.63, 31.25, 62.5, 125, and 250 mL respectively to a 250 milliliter plastic volumetric flask and fill with culture water. Aliquot each concentration to randomly numbered test containers as indicated on the random number sheet. Start with the control solutions and progress to the highest concentration to minimize contamination. Equilibrate the reference toxicant test containers in the constant temperature room.

All tests (sample and reference toxicant) must use organisms from the same culture. They must be handled in the same way and delivered to the test containers at the same time.

6.0 TEST INITIATION

6.1 Larvae Acclimation

Order larvae to arrive between two and seven days before test initiation. Larvae must be between second and third instar at test initiation with at least 50% of larvae at third instar, approximately 7 days post hatch. Place the larvae in a culture tray containing well water at a temperature that varies by no more than 3 °C from transport conditions. Acclimate the larvae to test temperature and conductivity. Hold larvae at test temperature for 48 hours prior to initiating sediment testing. Remove any dead or moribund animals. Make sure water in the tray is constantly aerated. Check the larvae daily, and monitor the health of larvae by observing appearance. If more than 5% of the larvae appear unhealthy during the 48 hours prior to the test, reschedule the test and immediately arrange for another shipment. Renew the culture every other day before the test with dilution water, and feed 10 mL of 4 g/L Tetrafin slurry daily.

6.2 Larvae Loading

Using a clean transfer pipette with the tip removed, transfer the larvae in their tubes from the culture tray into the test containers. Only transfer animals that are healthy and have built tubes. Replace injured or stressed larvae. Continue until each container has 10 animals for sediment or 1 animal for reference toxicant. Maintain water

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temperature ($23^{\circ}\text{C} \pm 1$) by sorting animals in the constant temperature room where the test is being conducted. Save 20 larvae for developmental stage determination. Place larvae onto a pre-weighed pan and place in 60°C oven overnight. Place in desiccator to cool for one hour and weigh to 0.0001 g. Dry weight should be 0.08 to 0.23 mg/individual for second to third instar. (U.S. EPA 2000)

7.0 MONITORING AND RENEWAL

Measure temperature, dissolved oxygen, pH, conductivity, ammonia, alkalinity, and hardness in the overlying water from each sediment sample at the beginning and end of the test. Also measure dissolved oxygen and temperature daily in each 10-day sediment test. If dissolved oxygen falls below 2.5 mg/L, begin aeration on all replicates of that sample. Temperature should be constantly monitored in reference toxicant and sediment tests. To collect a water quality sample from an exposure chamber, use a clean graduated 10-mL pipette to remove the sample from within 1 cm of the sediment surface. Sample as close to the sediment as possible without disturbing the sediment or drawing fine particles into the pipette. Deliver the sample into water quality containers that are pre-labeled with the sample number. Rinse the pipette twice between each sample. Repeat this sampling procedure as necessary during the test, using extreme caution not to pull up any larvae in the process. If larvae are pulled from a test beaker, note this and the beaker number on the data sheet.

The test is renewed twice per day, once by hand and once automatically. The automatic renewal takes place during the night. After collecting the daily water quality sample, add 1.6L of water to each splitter. Use heated water from dilution manifold. After water runs through splitter, feed each replicate 1.5 mL of 4 g/L Tetrafin slurry per day. Food should be thoroughly mixed before aliquots are taken. Watch for any bacterial or fungal matting on surface of sediment as that can be a sign of overfeeding. If this occurs suspend feeding for one or more days. Reference toxicant tests are fed 0.25 mL on day 0 and day 2.

8.0 TERMINATING THE TOXICITY TEST

After 4 days of exposure, larvae survival in the reference toxicant test is determined, and final test temperature recorded. After 10 days of sediment exposure, water quality samples are collected, and larvae are removed from the sediment exposures to determine survival and growth.

8.1 Preparation for Sieving

Before sieving, make sure the data sheet is ready on a clipboard, with a towel and pen next to it, in a place where it will be very difficult to go to the next beaker without remembering to write down your last count. Put on a lab coat, apron, boots, and gloves. Clear the water table of any old mud, if necessary, check water flows, and carefully examine your 400- μm screen to make sure there are no holes ($>400\mu\text{m}$).

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8.2 Sieving, Recovering Larvae, and Recording Data

Pour the overlying water into the sieve and rinse out the surficial layer of sediment. The majority of surviving larvae will be in this layer. If ten animals are recovered, the remaining contents of the container can be discarded. If fewer than ten larvae are recovered, rinse the remaining contents of the beaker onto the sieve, hosing down the beaker walls to remove all particles. Spray water over the mud in the screen to wash particles away from the remaining larvae. Break up any clods or mats, and continue spraying until all the fine sediment particles are removed. Using forceps hold larva and rinse to remove any tube remnants and mucus. Use a forceps to transfer larvae to a pre-weighed foil packet incised with a number. Continue this procedure until you are certain that you have recovered every larva. Count the number of live larvae you have collected, and record that number immediately on the survival and weight data sheets. Missing animals and animals that do not respond to a probe are considered dead. Foil packets should be placed in a box with enumerated divisions, such as an empty scintillation vial box, to minimize confusion or loss of foil packets. Care should be taken to ensure the incised number remains legible on the foil packets as ashing will remove numbers written in ink. Carry the box in a covered tray to the drying oven and dry them overnight at 60°C. After 24 hours, cool the larvae in a desiccator for at least one hour, weigh to the nearest 0.0001 g, and record the weights on the data sheet. Foil packets then are placed by sediment site into crucibles and ashed in a muffle furnace at 550°C for 2 hours. Foil packets are then cooled and reweighed to the nearest 0.0001 g.

Take the completed data sheet to the office for data entry and analysis. Notify the data analyst that the data has arrived.

9.0 DATA HANDLING

Immediately after test termination, check the data sheet to determine whether sand and reference sediment controls have acceptable survival ($\geq 70\%$). Larvae must have average ash free dry weight of 0.48 mg or greater in the controls. Tests with temperature, conductivity, or dissolved oxygen measurements outside the specified ranges, may be considered conditionally acceptable based on the project officer's best professional judgment. Overlying water quality parameters must not vary by more than 50% during the test.

10.0 REFERENCES

U.S. Environmental Protection Agency. 2000. Methods for measuring the toxicity and bioaccumulation of sediment-associated contaminants with freshwater invertebrates. EPA 600/R-99/064. Office of Research and Development. Washington, DC.

11.0 TEST SUMMARY

Species	<i>Chironomus tentans</i>
Test Duration	10 days

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Endpoint	survival, ash free dry weight (AFDW)
Renewals	twice daily with dilution water (1.6L per 8 beakers)
Organism Source	Aquatic Biosystems (Fort Collins, CO)
Age of Test Organisms	second or third instar (appx. 10 days post-hatch)
Acclimation	48 hours
Conductivity	Salinity up to 12‰
Dissolved Oxygen	2.5 mg/L required – aerate if below
Temperature	23 ± 1°C daily mean required 23 ± 3°C instantaneous required
Overlying water	Granite Canyon well water
Light intensity	ambient laboratory illumination (10-20 $\mu\text{E}/\text{m}^2/\text{s}$)
Photoperiod	16 hour light: 8 hour dark
Replication	8 (samples), 10 (reference toxicant)
Containers	300-mL glass beakers or 250 mL I-Chem jars (sediment), 25-mL plastic Solo cups (reference toxicant)
Volume	100 mL sediment topped with dilution water (samples), 20 mL (reference toxicant)
Loading	10 larvae per container
Feeding	1.5 mL Tetrafin slurry daily (samples), or 1 mL on day 0 and 2 (reference toxicant)
Overlying Water Quality	pH, dissolved oxygen, temperature, conductivity, ammonia, alkalinity, hardness 10-day test - dissolved oxygen (daily)
Reference Toxicant	potassium chloride (KCl)
Daily Monitoring:	water renewal, dissolved oxygen, temperature, feeding
Acceptability Criteria:	mean survival in controls $\geq 70\%$, 0.48 mg or greater AFDW in control