

**STANDARD OPERATING PROCEDURE**  
**Bench-Scale Procedure for Culturing the Freshwater**  
**Amphipod *Hyaletta azteca***

Compiled By:

Signed: \_\_\_\_\_

Title: \_\_\_\_\_

Date: \_\_\_\_\_

Approved By:

Signed: \_\_\_\_\_

Title: \_\_\_\_\_

Date: \_\_\_\_\_

Cleared For Issue By:

Signed: \_\_\_\_\_

Title: \_\_\_\_\_

Date: \_\_\_\_\_

**RECORD OF AMENDMENTS:**

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**STANDARD OPERATING PROCEDURE**  
**Bench-Scale Procedure for Culturing the Freshwater**  
**Amphipod *Hyaella azteca***

**BACKGROUND**

The Great Ships Initiative (GSI) is a collaborative effort to end the problem of ship-mediated invasive species in the Great Lakes-St. Lawrence Seaway System through independent research and demonstration of environmental technology, financial incentives and consistent basin-wide harbor monitoring. To that end, the GSI has established a shore-based high-flow Research, Development and Technology Evaluation (RDTE) facility in Superior, Wisconsin to provide intensive testing services to vendors of ballast treatment prospects suitable to Seaway-sized vessels. Laboratory space within the University of Wisconsin-Superior (UW-S) and University of Minnesota-Duluth is utilized to meet GSI bench-scale test objectives, as well as for non-time sensitive analysis of samples from the shore-based and shipboard scale tests. The UW-S has space in several of their research labs dedicated to the GSI project. Bench-scale experiments are conducted in the university's Aquatic Toxicity Laboratory which maintains active cultures of zooplankton, phytoplankton, and aquatic invertebrates. The laboratory contains a series of mini-diluters for water-only acute and chronic toxicity tests and is equipped to run static, intermittent renewal, and flow-through tests. A variety of meters are available for monitoring water quality including conductivity, salinity, pH, dissolved oxygen, temperature, and select ions.

**INTRODUCTION**

This bench-scale procedure provides instructions for culturing the freshwater amphipod *Hyaella azteca*. This SOP is adapted from procedures that have been developed by the U.S. EPA. The 7- to 14-day old amphipods should be used to start a sediment test.

**EQUIPMENT LIST**

- Temperature controlled water bath
- Aeration source
- Counter
- ~14 x 14 cm gauze squares
- Controlled photoperiod lighting
- White panty hose
- 2 Liter Nalgene® beakers or glass beakers
- Ethanol (70%)
- 5 mm I.D., 18 cm long glass tubing or glass pipettes
- Illuminated desk magnifier

- Stainless steel counting pans
- *Selenastrum capricornutum* culture (Appendix 1)
- Moderate hard water (Appendix 2)
- YCT mixture (Appendix 3)
- Reference toxicant test (Appendix 4)

## PROCEDURE

1. Wash culture tanks with Liquinox®, rinse with tap water, refill tank with water, add approximately 2-5 mL bleach and allow tank to soak overnight in wet lab. Do not use bleach in the hatchery. After refilling tank with fresh water, add approximately 2-5 mL of a 5 % sodium thiosulfate solution and allow it to soak for a couple hours before rinsing with tap water. Refill tank with 8-10 L of moderately hard water, add food (see below) and allow tank to equilibrate to room temperature (overnight) before adding organisms. Handling tools can be made from 5 mm ID glass tubing cut into 6 inch sections and fire-polished at both ends, with a small pipette bulb attached. Small fire-polished pipettes can be used, as well. Water in the culture chambers is changed weekly. All handling pipettes must be stored in a 70 % ethanol solution.
2. Set up brood beakers using Nalgene® 2-L containers. Prepare substrate for *Hyalella azteca* by presoaking ~14 x 14 cm gauze squares or similar size cheese cloth (Johnson and Johnson sterile gauze sponges) in deionized water for 24 hours before use. Use only one square per brood beaker. Add one liter of fresh moderately-hard water with a hardness factor of 80-125 mg/L CaCO<sub>3</sub> (prepared according to Borgmann formula), and 10-15 mL of YCT per one L (standardized to 1800 mg suspended solids/L) and  $1.0 \times 10^8$  cells of *Selenastrum capricornutum*. The feeding rate for a 2 L beaker containing 1 L of water is 8 mL of algae. Algae feeding can be regulated as needed, so the water does not become too green.
3. Assign 50 randomly chosen *H. azteca* to each 2-L container (if neonate production has decreased, assign 60 adults/container). Aerate containers gently. Aggressive aeration leads to increased evaporative loss and "floaters" - animals trapped in the surface tension of the water. Cover beakers with a tightly stretched white panty hose (replace them when they become discolored or lose elasticity). The beakers should be placed in an area maintaining the beaker temperature at 23 °C + 1 (a water bath may be necessary if temperature fluctuates too much). The light intensity at the water surface below the panty hose should be 700-1000 Lux (65-95 ft/candle).
4. Mature *H. azteca*, (50 animals per 2-L beaker), are fed 10-15 mL YCT (blend YCT before using) per 2-L container upon initial set-up and at time of renewal. Beakers should receive 10-15 mL YCT on Tuesday, Thursday, and Saturday if they have not been renewed. Feed 8 mL of  $1.0 \times 10^8$  cells of *Selenastrum capricornutum* to each

- container upon initial set-up. At 2-day intervals, feed 2-8 mL ( $1.0 \times 10^8$  cells). The 50 adults should be fully replaced every 3-5 months to insure reproductive success.
5. Since different age organisms are required for specific toxicity tests, the animals can be sorted into known age lots depending on test requirements. The general techniques for culturing *H. azteca* are as follows:
    - a) Using the aid of an illuminated desk magnifier, pour contents of an adult brood 2-L container into a collecting pan (stainless steel pan). Rinse the sides of the jar with a wash bottle of moderately hard water to free any *H. azteca* that may stick to the jar.
    - b) Free animals from the gauze by shaking the gauze back and forth underwater in the pan. Gently rinse the gauze with a wash bottle of moderately hard water to free remaining animals.
    - c) Set up a clean container and refill with one liter of fresh moderately hard water (fresh water should be the same temperature as original beaker water), 10-15 mL of YCT, 8 mL ( $1.0 \times 10^8$  cells) algae, and add new gauze substrate (gauze should be presoaked in deionized water for 24 hours).
    - d) Count adult *H. azteca* to keep track of brood stock survival, and count neonates to monitor reproduction (see Appendix 5 for form). Maintain survival and reproduction records on each jar. As reproduction drops, replace brood stock with new animals.
    - e) Return the adults to the container of fresh moderately hard water using the glass pipette. Add more adults to the container from the mass culture for a total of 50. Discharge individual adult *H. azteca* carefully, underwater, to avoid trapping air under the carapace, allowing adults to swim from the pipette without releasing the water. From this point, neonates of <1 to 7 day old can be collected.
    - f) Transfer the <1 to 7 day old neonates left in the counting pan to a clean 2-L beaker containing 2 L of moderately hard water using a pipette. Hold for one week before starting a toxicity test. Add no more than 200 neonates to the beaker and label it with a letter, date, approximate number and species. Allow the neonates to swim from the pipette without releasing the water. Upon completion of counting the neonates, empty the contents of the pan through a jar with a fine mesh bottom. Count the remaining neonates, record number on culture form, and release these neonates into the mass culture tanks. Feed the neonate container 10-15 mL of YCT and 16 mL ( $1.0 \times 10^8$  cells) of *Selenastrum capricornutum*. Add 1-2 mL of algae on the same days that the

adults are fed, if the neonate beaker is not light green in color. It is not necessary to feed with YCT. For testing, save numbers in excess of test requirements with approximately 200 per 2-L container. Record the number of neonates, date of set-up and age of organisms on the outside of the container. Set up neonate culture beaker as with brood stock. When the organisms are ~7-14 days old and are not used for the tests, count surviving neonates and record information on the form (Appendix 5).

- g) At 14 days, these neonate animals should be thinned to 50 per jar and allowed to mature for breeding purposes at this density. A good source of new brood stock are animals that have been matured to 21-28 days old but have not been used in testing. Mating pairs will become evident at about 24-27 days of age.
- h) Another source of new brood stock can be obtained from a mass culture of varying ages kept under near natural conditions. A mass culture can be maintained in a lightly-aerated aquarium (10 gallon) or 4-L Nalgene® beaker using cotton gauze as a substrate. Three-fourths of the moderately hard water should be exchanged weekly and small amounts of Selastrum (approximately 25-50 mL) and YCT (approximately 50-75 mL depending on organism density) added for an adequate food supply. When adding neonates to a freshly set-up mass culture tank, add 300-400 organisms. Culture may have to be thinned if overpopulation occurs within 2-3 weeks. The mass culture tanks and 4-L beakers should be fed 30-50 mL YCT on Tuesday, Thursday and Saturday. Algae feeding will vary, depending on water color. Record all mass culture information in blue hatchery notebook.
- i) Once a week, pipettes that are used for feeding YTC and algae should be rinsed thoroughly with water (and little soap), 10 % nitric acid, tap water rinse, and deionized water rinse.

## **QUALITY ASSURANCE/QUALITY CONTROL**

Reference toxicity tests must be performed to document organism sensitivity.

Lab performance is demonstrated by performing at least one reference toxicant test per month. A control chart is prepared for each combination of reference toxicants, test species, test conditions, and endpoints. The chart consists of a running plot for the 20 most recent values (LC50). End points are determined to see if they are within acceptable limits. The charts depict the central tendency of the mean value and the upper and lower control values are set as two standard deviations.

## REFERENCES

Borgmann, U. 1996. Systematic Analysis of Aqueous Ion Requirements of *Hyalella azteca*: A Standard Artificial Medium Including the Essential Bromide Ion. Arch. Environ. Contam. Toxicol. 0:1-8.

Cangelosi, A.A. 2006. RDTE Facility for the Great Ships Initiative (GSI) (OAR-SG-2006-20000364). Project Proposal to the National Oceanic and Atmospheric Administration/U.S. Fish and Wildlife Service.

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U.S. EPA. 1994. Methods for measuring the toxicity and bioaccumulation of sediment-associated contaminants with freshwater invertebrates. EPA 600/R-94/024. Office of Research and Development, Duluth, MN. Great Ships Initiative Standard Operating Protocols: <http://www.nemw.org/GSI/protocols.htm>.

## APPENDIX 1 PROCEDURES FOR CULTURING *SELENASTRUM CAPRICORNUTUM* AS FOOD FOR AQUATIC ORGANISMS

### INTRODUCTION

*Selenastrum capricornutum* is a small biconcave disk-shaped algal species that is often used as a food for cladocerans and other aquatic macroinvertebrates. In order to ensure a healthy culture, attention must be given to providing proper nutrition, illumination, and aeration, while maintaining aseptic conditions. At UW-Superior's Lake Superior Research Institute (LSRI) the algae is cultured in a modified Woods Hole MBL Medium (abbreviated as WMBL) at room temperature with a photoperiod of 16 hours light/8 hours dark.

### EQUIPMENT LIST

- Aeration source
- $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$
- Magnetic Stirrers
- $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$
- $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$
- 2.8 L wide-mouthed flasks

- $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$
- Autoclave
- $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$
- 4L Erlenmeyer flasks
- 4 L Brown glass jugs
- $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$
- Hemocytometer (Neubauer<sup>R</sup>)
- Detergent (Liquinox<sup>R</sup>)
- $\text{H}_3\text{BO}_3$
- 50 mL nalgene centrifuge
- Stir bars
- $\text{K}_2\text{HPO}_4$
- Tubes
- Deionized (DI) water
- $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$
- 5 mL pipettes
- Autoclave tape
- $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
- 2-Hole neoprene stoppers
- 1 L volumetric flasks
- $\text{NaHCO}_3$
- Glass tubing
- 1 L brown glass bottles
- $\text{NaNO}_3$
- Airline tubing
- $\text{Na}_2\text{EDTA}$
- Airstones/diffusers

## PROCEDURE

### 1. Preparation of Medium

The modified Woods Hole MBL medium is prepared in general accordance with the SOP written by the National Effluent Toxicity Assessment Center in June of 1990. This medium lacks the sodium silicate, vitamins and pH buffer used in the WMBL originally described by Nichols (1973), but includes boric acid and twice the amount of ferric chloride.

- a) Stock solutions are prepared in 1000-fold concentrations using the following amounts of nutrients. The stock solutions are made in 1 Liter volumetric

flasks using Millipore filtered dilution water. The chemicals are stored in the chemistry lab, McCaskill Room 141, in the gray chemical cabinet.

Stock Solution Number	Chemical	Amount
Solution No. 1	CaCl <sub>2</sub> 2H <sub>2</sub> O	36.76 g/L
	NaNO <sub>3</sub>	85.01 g/L
Solution No. 2	MgSO <sub>4</sub> 7H <sub>2</sub> O	36.97 g/L (or 18.06 g/L MgSO <sub>4</sub> ) <sup>1</sup>
Solution No. 3	NaHCO <sub>3</sub>	12.60 g/L
Solution No. 4	K <sub>2</sub> HPO <sub>4</sub>	8.71 g/L
Solution No. 5	Na <sub>2</sub> EDTA	4.36 g/L
Solution No. 6	CuSO <sub>4</sub> 5H <sub>2</sub> O	0.01 g/L
	CoCl <sub>2</sub> 6H <sub>2</sub> O	0.01 g/L
	ZnSO <sub>4</sub> 7H <sub>2</sub> O	0.022 g/L
	MnCl <sub>2</sub> 4H <sub>2</sub> O	0.018 g/L
	Na <sub>2</sub> MoO <sub>4</sub> 2H <sub>2</sub> O	0.006 g/L
	H <sub>3</sub> BO <sub>3</sub>	1.00 g/L
Solution No. 7	FeCl <sub>3</sub> 6H <sub>2</sub> O	3.15 g/L
Solution No. 8	Tris Buffer HCl	250 g/L

- b) Stock solutions are mixed well and then placed in labeled brown glass 1 L bottles and stored in the refrigerator in McCaskill Room 121. The labels should contain the formulation of the solution, the date made and the initials of the person making them. These solutions are stable for at least a year if they do not become contaminated.
- c) The medium is prepared by adding 1 mL of Solutions No. 1, 2, 3, 4, 5 and 6 and 2 mL of Solution No. 7 for every 1 L of Millipore filtered water. A separate sterile 5 mL pipette is used for measuring each stock solution. It is critical that every solution be added in the correct proportion, so a label is placed on every container of solution and used to record the addition of each individual stock solution.
- d) The prepared modified WMBL medium is placed in labeled culture flasks or 4 L brown glass jugs and autoclaved for 45 minutes at 121 °C. Prior to autoclaving (*write autoclave usage on the form posted by the instrument*), a piece of indicator tape is placed on each container of medium. The tape remains white until it is heated. The word "Autoclaved" appears in black on the tape when the medium has been sterilized.
- e) After autoclaving, the containers are covered and the medium is allowed to cool to room temperature before use.

<sup>1</sup> Note if MgSO<sub>4</sub> is used instead of the hydrated salt, 18.06 g of salt must be added to each liter of stock solution.

## 2. Preparation of Culture Flasks

Several sterile 2.8 L wide-mouthed Erlenmeyer flasks are used for growing starter cultures of *Selenastrum capricornutum*, while batch cultures are grown in 4 L Erlenmeyer flasks.

- a) Each flask is covered with a two-hole neoprene stopper. A short piece of glass tubing is inserted in one hole to serve as a vent. The other hole contains a longer piece of glass tubing that extends to the bottom of the flask. An air diffuser is attached to the bottom end of the glass tubing.
- b) All glassware is cleaned with Liquinox laboratory detergent and rinsed at least three times in dechlorinated lab water prior to use.
- c) The flasks can be autoclaved empty or can be filled approximately 2/3 full with prepared medium before autoclaving. For convenience, fill a 4 L flask/jug up to 3.5 L and 2.8 L flask up to 2.5 L.
- d) A teflon stir bar is placed in each 2.8 L flask. A neoprene stopper containing the glass tubing and air diffuser is set on top of each flask. The stopper is placed at an angle on the flask to allow for escape of pressure as the medium is autoclaved as directed above.
- e) In order to maintain sterility of the cultures, the mouths of all flasks and jugs of medium are flamed every time the covers are removed and again before recovering. Stoppers, glass tubes, pipettes and airstones should not be placed on unsterile surfaces. Pipettes should be autoclaved after each use.

## 3. Culture Inoculation and Renewal

Starter cultures of *Selenastrum capricornutum* are initially purchased from Carolina Biological Supply. Using sterile techniques, a single tube of algae is added to 2.5 L of WMBL in a 2.8 L flask. The flask is labeled with the name of the alga, the number of the starter culture, and the date started.

- a) The starter cultures are placed on a magnetic stirrer and an airline is attached to the glass tube containing the air diffuser. Enough aeration and stirring should be maintained so that the alga do not begin to settle on the sides or bottom of the flask. Aeration should be checked at least twice daily.
- b) The cultures are maintained at room temperature (20 to 25 °C) and illuminated with wide spectrum and Grow-Lux fluorescent bulbs with a photoperiod of 16 hours light/8 hours dark.

- c) When the starter cultures begin to grow exponentially and appear dark green (usually 5 to 7 days for a culture started from a tube of algae) the density of the algae should be determined daily by the methods described below.
- d) When the density of a starter culture reaches  $3 \times 10^6$  cells per mL, sterile techniques should be used to transfer 1 L of the starter culture to 3 L of WMBL in a 4 L flask. Fresh sterile WMBL should be used to refill the starter culture flask. The new batch culture should be labeled, an airline attached, placed in a lighted area and allowed to grow for 2 or 3 days.
- e) Each week 1 L of the starter culture should be transferred to a clean 2.8 L flask with 1.5 L of sterile WMBL. The rest of the old starter can then be harvested. As long as the starter cultures remain uncontaminated, they can be used to start several batch *Selenastrum capricornutum* cultures. The starter culture should be harvested or discarded when contamination is noted.
- f) Starter and batch cultures should be counted daily once they enter the exponential growth phase. Starter cultures should never be allowed to exceed  $5 \times 10^6$  cells per mL and batch cultures should be harvested as soon as they exceed 5 to  $7 \times 10^6$  cells per mL. These densities can often be reached in 3 or 4 days under ideal conditions. Although we have been able to obtain densities of  $1 \times 10^7$  cells/mL, we have found that these cultures can "crash" overnight.

#### 4. Harvesting *Selenastrum capricornutum*

The method of harvesting *Selenastrum capricornutum* will depend on the protocol used for culturing and testing the aquatic organisms to which it will be fed. Some organisms are more sensitive, and require that the *Selenastrum capricornutum* be concentrated to remove the WMBL medium and then placed in an appropriate dilution water.

- a) If the algae require concentration, the mature *Selenastrum capricornutum* cultures should be placed in 50 mL Nalgene centrifuge tubes and centrifuged for 10 minutes at the highest speed setting on the International Centrifuge (desired speed of 2000 rpm). The supernatant is then discarded. At this point, more of the algal culture can be added to each tube and centrifugation continued until a sizeable pellet of algae is formed in the bottom of each tube. Diluent water or medium should be used to resuspend the algae and wash it from the centrifuge into a clean container. A minimal amount of water should be used in this process (less than 1/4 the original amount of algal culture which was centrifuged).

- b) The harvested algae should be counted and refrigerated until used, preferably within a few weeks.

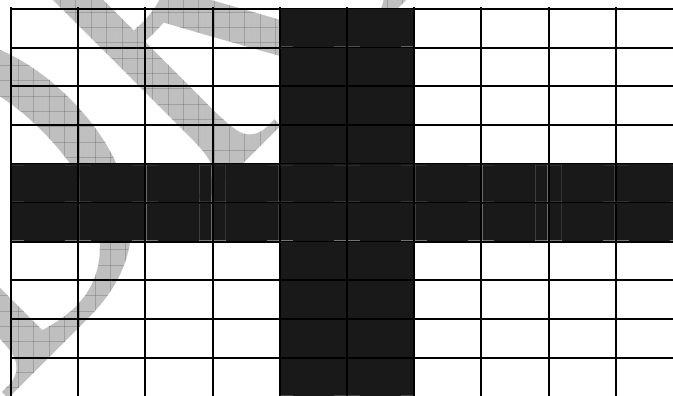
5. Counting *Selenastrum capricornutum*

A Neubauer Hemocytometer should be used to determine the density of algal cells in the culture. Follow the directions supplied by the manufacturer. Batch cultures that have not been centrifuged can be counted directly. A sterile pipette is used to fill the hemocytometer. The number of algal cells contained in the square regions of the diagram that are clear are counted. Each of these regions is 1 mm square and 0.1 mm deep. Therefore the volume of the algal suspension in each square is 0.1 mm<sup>3</sup>.

- a) The density of the algae is determined by the following formula:

$$\text{Number of algal cells/mL} = \frac{(\text{total \# algal cells counted in 4 squares})}{(4 \text{ squares counted} \times 0.1 \text{ mm}^3 \text{ volume of each square})} \times 1,000,000 \text{ mm}^3/\text{mL}$$

- b) Algae that have been centrifuged and concentrated may need to be diluted prior to counting. A Thoma pipette that is designed for counting human white blood cells can be used for this purpose. The pipette is filled to the 0.5 mark with concentrated algae and then filled with water to the 11 mark. The pipette has been calibrated to produce a 1/20 dilution using this technique. The pipette should be shaken well and the tip streaked twice on a clean laboratory tissue prior to filling the hemocytometer. The algae are counted as indicated above and the final value is then multiplied by 20.



- c) After the density of the algae has been determined, it can be diluted to a standard concentration if required by the feeding protocol. Otherwise the harvested algae can be labeled and refrigerated until needed.

#### 6. Record Keeping

- a) All data should be recorded in a hardbound notebook dedicated to algal culture. Data should include the date that cultures were started, renewed, counted, and harvested as well as information on preparation of medium, signs of contamination, etc.

#### **REFERENCES**

Nichols, H.W. 1973. Growth Media-Freshwater. pp. 8-24 In: *Handbook of Phycological Methods, Culture Methods and Growth Measurements*. J.R. Stein (ed.). University Press, Cambridge.



**Autoclave Cleaning Log**

**Drain, rinse, sponge out, and fill with TAP water. Clean each week when used heavily, every ten uses when not.**

Number	Date	Initials (XXX)	Number of Runs
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**APPENDIX 2**  
**PROCEDURES FOR PREPARING MODERATELY HARD WATER FOR USE**  
**USE IN HYALELLA AZTECA CULTURING AND HARD WATER FOR USE**  
**IN DAPHNIA MAGNA CULTURING**

## INTRODUCTION

Water used to culture *Hyalella azteca* and *Daphnia magna* should maintain a hardness range of 80-125 mg/L CaCO<sub>3</sub> and 110-180 mg/L CaCO<sub>3</sub>, respectively. To obtain the desired hardness range, high quality deionized water should be used with the specified amounts of calcium sulfate, calcium chloride, magnesium sulfate, sodium bicarbonate, sodium bromide, and potassium chloride. The water chemistry parameters consisting of hardness, alkalinity, pH, and conductivity should be measured on each batch of prepared water. Acceptable criteria for these water quality parameters should be: pH = 6.0 - 9.0 for both the hard and moderately hard water; alkalinity = 42.0 - 56 mg/L CaCO<sub>3</sub> (moderately hard water) and 95.0 - 125 mg/L CaCO<sub>3</sub> (hard water); conductivity = 300 - 500 mhos/cm (moderately hard water) and 350 - 550 mhos/cm (hard water).

## EQUIPMENT LIST

- Deionized water (Milli-Q)
- Calcium Chloride (CaCl 2H<sub>2</sub>O)
- Magnesium Sulfate (MgSO<sub>4</sub>)
- Sodium Bicarbonate (NaCHO<sub>3</sub>)
- Sodium Bromide (NaBr)
- Potassium Chloride (KCl)
- Aeration Source
- Stirring Plate

## PROCEDURE

To prepare 100 L of reconstituted fresh water for the *Hyalella azteca* culture, use the reagent grade chemicals as follows:

1. Place about 75 L of deionized (Milli-Q) water in a properly cleaned container.
2. Add 0.105 g of NaBr and 14.7 g of CaCl 2H<sub>2</sub>O to a 2-L aliquot of deionized water and mix (e.g., on a stir plate) for 30 min or until the salts dissolve.
3. Add 3 g of MgSO<sub>4</sub>, 8.4 g of NaCHO<sub>3</sub>, and 0.37 g KCl to a second 2-L aliquot of deionized water and mix on a stir plate for 30 min.
4. Pour the two 2-L aliquots containing the dissolved salts into the 75 L of deionized water and fill the carboy to 100 L with deionized water.

5. Recirculate the mixture for at least 24 hr before use by placing the short segment of tygon tubing on the carboy nozzle (which has been cocked open) and the long segment inserted into the carboy through the top cover. Plug the pump in and check to make sure water is circulating.
6. Before adding animals to the freshly prepared culture water, check the pH, hardness, alkalinity, and conductivity to assure they meet the water quality parameter criteria. Record the measured values on their respective bench sheets.

To prepare 50 L of reconstituted fresh water using a modified version of the above recipe for the *Daphnia magna* culture, use the reagent grade chemicals as follows:

1. Place about 35 L of deionized water in a properly cleaned container.
2. Add 6.0 g of  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$  to a 2-L aliquot of deionized water and mix (e.g., on a stir plate) for 30 min or until the salts dissolve.
3. Add 6.0 g of  $\text{MgSO}_4$ , 9.6 g of  $\text{NaHCO}_3$ , and 0.4 g  $\text{KCl}$  to a second 2-L aliquot of deionized water and mix on a stir plate for 30 min.
4. Pour the two aliquots containing the dissolved salts into the 35 L of deionized water and fill the carboy to 50 L with deionized water. Solutions should be prepared at least monthly.
5. Aerate the mixture for at least 24 hours before use.
6. Before adding animals to the freshly prepared culture water, check the pH, hardness, alkalinity, and conductivity to assure they meet the water quality parameter criteria. Record the measured values on their respective bench sheets.

## REFERENCES

American Society for Testing and Materials (ASTM). 1993. Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates and Amphibians. Annual Book of ASTM Standards. 11.04. 456-475.

Borgmann, U. 1996. Systematic Analysis of Aqueous Ion Requirements of *Hyalella azteca*: A Standard Artificial Medium Including the Essential Bromide Ion. Arch. Environ. Contam. Toxicol. 0:1-8.

### APPENDIX 3

## YEAST/CEREAL LEAVES/TROUT FOOD (YCT) PREPARATION FOR AQUATIC INVERTEBRATES

### INTRODUCTION

The following is a description of the YCT (Yeast-Cereal-Trout food) preparation. YCT is used in combination with the algae, *Selenastrum capricornutum*, as the diet for *Ceriodaphnia*.

YCT is composed of yeast at a concentration of 5 g/L, Cereal leaves at 5 g/L, and fermented trout food at 5 g/L. These three ingredients are combined in equal volumes (1:1:1) to form the final product.

### EQUIPMENT LIST

- Fleishman's® Active Dry Yeast
- Cereal leaves (Pines wheat grass powder)
- Distilled/deionized water
- 1 L Erlenmeyer flasks
- Trout Chow Pellets (Starter or No. 1 Pellets)
- Balance
- Stir bar/stir plate assembly
- Nitex® 110 mesh
- 1 gallon Nalgene® jug with stopper
- Aeration source
- Plastic wrap
- Fermentation chamber
- Water bath
- Freezer
- Graduated cylinder
- Oven
- Aluminum weighing pans
- 5 mL pipette
- Food preparation notebook

### PROCEDURE

Each ingredient is made up in the following manner:

#### 1. Trout Food

This ingredient must be prepared at least one week in advance, as it must ferment before using. It is best to make a supply ahead of time and freeze it in small batches.

However, after the YCT is made, the solution cannot be refrozen. Careful planning is needed to avoid being short of this ingredient.

- a) Weigh 10 g of trout chow pellets (1/8" pellets work well).
- b) Add 2 L of deionized water to fermentation chamber.
- c) Place pellets in fermentation chamber and aerate, gently rolling the pellets to prevent settling.
- d) Cover with glass or plastic lid to decrease evaporation.
- e) Label the container with the date the fermentation process is complete (one week from starting date).
- f) Keep the water level at 2 L by replacing evaporated deionized water each day.
- g) After one week, shut the air off, let settle for one hour, and filter supernatant through a fine screen placed in a funnel (Nitex® 110 mesh).

## 2. Cereal Leaves (Pines Wheat Grass Powder)

- a) To be prepared 24 hr before the trout chow is finished fermenting.
- b) Weigh 12.5 g cereal leaves.
- c) Combine cereal leaves and 2.5 L deionized water in an Erlenmeyer flask.
- d) Insert a clean large stir bar, and stir for 24 hours at medium speed.
- e) After 24 hours, remove from stir plate and let settle for one hour. Pour off supernatant.
- f) Discard the excess and particulates on filter.
- g) For reordering the material, logon to [www.wheatgrass.com](http://www.wheatgrass.com).

## 3. Yeast

To be made the same day as when all ingredients for the YCT are combined.

- a) Add 10 gm Fleishman's® active dry yeast to 2 L deionized water.
- b) Place on stir plate until the yeast is well dispersed.

- c) Combine the yeast suspension immediately (do not allow to settle) with equal volumes of supernatant from the trout chow and cereal leaves.

3. Preparation of YCT

The batch size of YCT may vary, depending upon usage. Generally, 6.5 L batches are made. Batches are made which are used for only two weeks and new batches should be prepared a day or two in advance to insure a continuous supply.

- a) Each ingredient should be measured out in equal volumes.
- b) Combine all equal volumes into large container and mix well. Split the total volume into two 4 L beakers.
- c) Suspended solid's level must be measured and adjusted to 1800 mg/L before the food is fed.

4. Suspended Solids Monitoring

Solids are monitored on each batch of YCT and adjusted to a constant measure (1800 mg/L) before feeding to keep feeding volumes and food levels consistent.

- a) Label and number six weighing pans. Weigh pans and record the weights. (Label as A1, A2, A3, B1, B2, B3).
- b) Stir YCT solution well; it is important that the solution be uniform so as to get a good representative sample.
- c) Label the two 4-L beakers, A and B and measure three 10-mL samples from each beaker, stirring well between samples and place in oven-dried pans (these must also be labeled according to which 4-L beaker they came from, e.g., A1, A2, A3, B1, B2, B3).
- d) Place pans containing YCT in oven and dry completely (24 hr) at 100 °C.
- e) Weigh pans again and subtract weight of pan alone to get weight of solids in YCT.
- f) Convert this figure to mg/L and divide by 1800 mg/L to get the dilution factor.
- g) Multiply the volume of YCT by the dilution factor to get the final volume and dilute to this final volume. For example:

Pre-weight (g)	Post-weight (g)	Difference	Average
----------------	-----------------	------------	---------

(over dried and pan alone)	(pan and 10 mL YCT dried)	(YCT alone)	
A1 1.0016	1.0277	0.0261	0.0260
A2 1.0063	1.0320	0.0257	
A3 0.9996	1.0258	0.0262	
B1 0.9973	1.0227	0.0254	0.0251
B2 0.9973	1.0220	0.0247	
B3 0.9960	1.0212	0.0252	
			Total average = 0.02555

Then, (0.02555) multiplied by (1000) g in 10 mL = 25.55 mg in a 10 mL sample. To find the mg/L, divide 25.55 mg/.01 L = 2555 mg/L.

$$\text{Total Suspended Solids Dilution Factor} = \frac{2555 \text{ mg/L}}{1800 \text{ mg/L}} = 1.4$$

This number (1.4) is multiplied by the volume of YCT prepared to determine final volume obtained after dilution to dilute up to. The batch volume is (6.5 L) multiplied by the dilution factor (1.4) = 9.1 L. Then, 9.1 - 6.5 = 2.6 L. To correct for the TDS, 2.6 L of deionized water should be added to the A and B combined batches.

- h) After dilution, take three additional 10-mL YCT samples from the container and place in oven-dried pans (C1, C2, C3). Dry samples for 24 hours at 100 °C to confirm suspended solids.
- i) Record suspended solids information and mark the solids level of the YCT on the container.
- j) Acceptable solids levels are between 1700 and 1900 mg/L (1800 is preferable). If solids levels are above 1900 mg/L, make another dilution.
- k) Blend and shake well before feeding as freezing it causes chunking.
- l) YCT is used for only two weeks once it has been thawed and algae can be used for up to three weeks.

**APPENDIX 4**  
**CONDUCTING A REFERENCE TOXICANT TEST**  
**WITH HYALELLA AZTECA (Nebeker Strain)**

## INTRODUCTION

Toxicity tests that are accurate and reproducible must be conducted with careful technique and with test animals of standard high quality. A reference toxicity test is conducted with a toxicant that has an expected toxicity range to a species of test organism. This is a method of assessing the overall condition of a group of test organisms.

## EQUIPMENT LIST

- 1 - 1.0 L volumetric flask
- 6 - 500 mL Erlenmeyer flask
- 1 - 200 mL graduated cylinder
- 24 - 100 mL beakers
- Reagent grade KCl
- Analytical balance
- Nytex® screen disks
- Plastic light lens
- Conductivity meter
- Temperature controlled chamber

## PROCEDURE

Note: Weigh 30 g of reagent grade potassium chloride (KCl). Add to a 500 mL volumetric flask and dilute to volume with moderately-hard (80-100 mg/L CaCO<sub>3</sub>) water. This provides a 60,000 mg/L KCl Super stock. (Super stock should be remade every six months).

1. Pipette 20 mL of the 60,000 mg/L KCl Super stock solution into a 1 L volumetric flask. Dilute to volume with moderately-hard water. This produces a 1200 mg/L KCl stock solution.
2. Make six exposure solutions of 400 mL each having 100, 50, 25, 12.5, 6.25 and 0 % of the 1200 mg/L KCl stock solution.
3. Measure conductivity of each of the exposure solutions, beginning with the control and working up to the highest concentration.
4. Add about 80 mL of an exposure solution to four 100 mL beakers and label. Do this for each exposure and control solution (24 beakers total).
5. Add a piece of Nytex® screen to each beaker.

6. Add three test organisms to each beaker (one to each beaker, then repeat three times). The organisms should be 0-7 or 7-14 days old, depending on the ages needed for the definitive test.
7. Cover beakers with a translucent shade (e.g., plastic light lens) and place in a 23.0 C temperature-controlled environment which has about 16 hr light and 8 hr dark daily. Note: The trays must be rotated at 24 hours due to the temperature differential in the front of the chamber versus the back of the chamber.
8. Count survival at 48 hours. Control survival should be at least 90 % for the test to be acceptable. Death is defined as lack of any movement when viewing an organism with a microscope.
9. Conduct a Spearman-Kärber calculation of an LC50.
10. Calculate a new mean LC50 and standard deviation

(use the sample statistic  $\frac{(X_1 - )^2}{n-1}$ )

Data Form

Name of Researcher \_\_\_\_\_ Species *H. azteca*

Beaker #/Date of Beaker \_\_\_\_\_

Set-Up \_\_\_\_\_

Date of Test \_\_\_\_\_ Organism Age (D) \_\_\_\_\_

Toxicant \_\_\_\_\_ Purity and Lot No. \_\_\_\_\_

Concentration of 100% Stock (mg/L) \_\_\_\_\_ LC501 (mg/L) \_\_\_\_\_

Treatment	Control	6.25%	12.5%	25%	50%	100%
Conductivity ( mhos/cm)						
Temperature (C)						
Original No. of Organisms						
Number Dead at 48 hr						

Preparation of Exposure Solutions

Treatment	Control	6.25%	12.5%	25%	50%	100%
mL of Stock <sup>2</sup>	0	25	50	100	200	400
mL of Dilution Water <sup>3</sup>	400	375	350	300	200	0
Nominal KCl Concentration (mg/L)	0	75	150	300	600	1200

- 1 The LC50 generally averages 360 - 400 mg/L. The average lower/upper control limits are: 310 - 490 mg/L, while the average lower/upper warning limits are 325 - 430 mg/L. If the test endpoints are outside of these ranges, contact the Lab Manager immediately.
- 2 Stock is prepared with moderately-hard water at a KCl concentration of 1200 mg/L.
- 3 Dilution water is moderately-hard water.

**APPENDIX 5**

**EVALUATION FORM FOR THE HEALTH AND REPRODUCTION  
OF A *HYALELLA AZTECA* CULTURE**

Culture Record  
Culture Renewal

Date: \_\_\_\_\_ YTC: \_\_\_\_\_  
 Algae: \_\_\_\_\_ Batch Date: \_\_\_\_\_  
 Batch: \_\_\_\_\_ Feeding Rate: \_\_\_\_\_  
 Feeding Rate: \_\_\_\_\_ Cell Count (cells/mL): \_\_\_\_\_  
 Temp(C)/Chamber #: \_\_\_\_\_ Exp. Date: \_\_\_\_\_  
 pH/Chamber #: \_\_\_\_\_ Dissolved Oxygen (mg/L)/Chamber #: \_\_\_\_\_

Culture Chamber	# Adults	# Pairs	Approx. # Young	# Young Saved	Adults # Added	Total Adults

Total # Young Saved: \_\_\_\_\_ Age: \_\_\_\_\_ days  
 Survival of last week's 0-7-d-olds: \_\_\_\_\_ %  
 \*Record culture chamber number.

Date: \_\_\_\_\_ Temperature\*: \_\_\_\_\_

Feeding (mL YCT/algae): #1 \_\_\_\_\_ #2 \_\_\_\_\_ #3 \_\_\_\_\_ #4 \_\_\_\_\_ #5 \_\_\_\_\_ #6 \_\_\_\_\_  
 #7 \_\_\_\_\_ #8 \_\_\_\_\_ #9 \_\_\_\_\_

Date: \_\_\_\_\_ Temperature\*: \_\_\_\_\_

Feeding (mL YCT/algae): #1 \_\_\_\_\_ #2 \_\_\_\_\_ #3 \_\_\_\_\_ #4 \_\_\_\_\_ #5 \_\_\_\_\_ #6 \_\_\_\_\_  
 #7 \_\_\_\_\_ #8 \_\_\_\_\_ #9 \_\_\_\_\_

Comments: