

STANDARD OPERATING PROCEDURE
Bench-Scale Procedure for Culturing the Freshwater
Rotifer *Branchionus calyciflorus*

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
Rotifer *Branchionus calyciflorus*

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 rotifer *Branchionus calyciflorus*. The rotifer can be obtained from cysts purchased from Florida Aqua Farms Inc. Continuous cultures are kept to produce cysts and live individuals for testing purposes. Population densities are monitored three times per week, along with temperature, dissolved oxygen, and pH.

EQUIPMENT LIST

- *B. calyciflorus* cysts
- One-Liter culture flasks
- Five-Gallon tank
- Aeration source
- Dissecting scope with counting wheel
- Petri® dishes
- Separatory funnels
- *Selenastrum capricornutum* (see Appendix 1)

- Moderately hard rotifer water (see Appendix 2)

PROCEDURE

1. Conduct procedure in a vented work area, taking appropriate health and safety measures.
2. Initiate cultures by placing cysts in a Petri® dish with approximately 25 mL of moderately hard rotifer water. Cover Petri® dish and place in a temperature-controlled chamber set at 25 °C for a 24 hour photoperiod. After 24 hours add 250 µL of the green algae, *Selenastrum capricornutum* (1.0×10^8 cells/mL), and incubate at the same settings for another 24 hours.
3. Check to see if organisms have hatched using 10-25X magnification. If successful hatching has occurred, empty the contents of the Petri® dish into a one-Liter culture flask filled with 0.5 L of moderately hard rotifer water. Feed the flask 1.0 mL of algae after adding the organisms and again at 24 hrs.
4. After 48 hours increase the volume of water to 1.0 L using fresh culture water, and feed rotifers 1.0 mL of algae (1.0×10^8 cells/mL) daily per 1.0 L of water. Note: feeding rates are based on healthy a population. If the water does not clear in 24 hours, do not continue feeding.
5. Maintain a high population density in the flasks (> 4 animals/mL). Sexual reproduction is induced when the population densities are 5-10 animals/mL. If the population becomes too dense (> 15 animals/mL) the water quality decreases causing the population to crash.
6. When cysts are needed, transfer 1.0 L of water containing sexually reproducing animals and place the water in a separatory funnel. Feed the culture at the same rate as the flasks.
7. After 1-2 days, remove the cysts from the bottom of the funnel by opening the stop-cock, and allowing a small amount of water containing the cysts to flow through. Drain the water into a small flask or other appropriate container. Cysts should be dried and stored in a cool dark area for later use.
8. Check population density by gently inverting the flask 3-4 times or stirring the tank to evenly distribute the animals throughout the container. Take a small sample of water from the container with a calibrated pipette, and place the sample in a counting wheel and evenly distribute the water around the wheel.

9. Count the number of *B. calyciflorus*, and record the number in the culture log (Appendix 3 and 4). Note if males (indicating sexual reproduction) are observed, but do not include them in the count.
10. To thin an active population, replace a portion of the water with new culture water (moderately hard rotifer water). The portion of water replaced should be based on the current population density and the desired population (i.e., if population density is 10 animals/mL, sieve 600 mL to achieve a density of four animals/mL). Use the following calculation: $1 - (\text{desired population}/\text{current population}) * 100 = \text{percent to replace}$.
11. If contaminants are observed, pass the entire contents of the flasks or tank through a 100 μm sieve, rinse with the rotifer water, then place the animals in a small culture dish for holding as the container is being cleaned. After the tank is cleaned, add the new rotifer water, animals, and algae.
12. If more culture flasks are needed transfer half the water from an existing culture flask to a new culture flask, and fill to 1.0 L. All flasks should be labeled with the organism name, date, and a sequenced number.
13. A separate tank should be maintained at a low population density (<4 animals/mL) to be used if population crashes occur or if broods are set up.

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

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.

Gilbert JJ. 1963. Contact chemoreception, mating behavior, and sexual isolation in the rotifer genus *Brachionus*. *Journal of Experimental Biology* 40: 625-641.

Great Ships Initiative Standard Operating Protocols: <http://www.nemw.org/GSI/protocols.htm>.

Great Ships Initiative website: www.greatshipsinitiative.org.

Hoff FH, Snell TW. 1987. Plankton Culture Manual (5th ed.). Florida Aqua Farms Inc, Dade City, FL.

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^R)
- Detergent (Liquinox^R)
- H_3BO_3
- 50 mL nalgene centrifuge

- Stir bars
- K_2HPO_4
- Tubes
- Deionized (DI) water
- $MnCl_2 \cdot 4H_2O$
- 5 mL pipettes
- Autoclave tape
- $MgSO_4 \cdot 7H_2O$
- 2-Hole neoprene stoppers
- 1 L volumetric flasks
- $NaHCO_3$
- Glass tubing
- 1 L brown glass bottles
- $NaNO_3$
- Airline tubing
- Na_2EDTA
- 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	$CaCl_2 \cdot 2H_2O$	36.76 g/L
	$NaNO_3$	85.01 g/L
Solution No. 2	$MgSO_4 \cdot 7H_2O$	36.97 g/L (or 18.06 g/L $MgSO_4$) ¹
Solution No. 3	$NaHCO_3$	12.60 g/L
Solution No. 4	K_2HPO_4	8.71 g/L
Solution No. 5	Na_2EDTA	4.36 g/L

¹ Note if $MgSO_4$ is used instead of the hydrated salt, 18.06 g of salt must be added to each liter of stock solution.

Solution No. 6	CuSO ₄ 5H ₂ O	0.01 g/L
	CoCl ₂ 6H ₂ O	0.01 g/L
	ZnSO ₄ 7H ₂ O	0.022 g/L
	MnCl ₂ 4H ₂ O	0.018 g/L
	Na ₂ MoO ₄ 2H ₂ O	0.006 g/L
	H ₃ BO ₃	1.00 g/L
Solution No. 7	FeCl ₃ 6H ₂ 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.

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 3×10^6 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 5×10^6 cells per mL and batch cultures should be harvested as soon as they exceed 5 to 7×10^6 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 1×10^7 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.

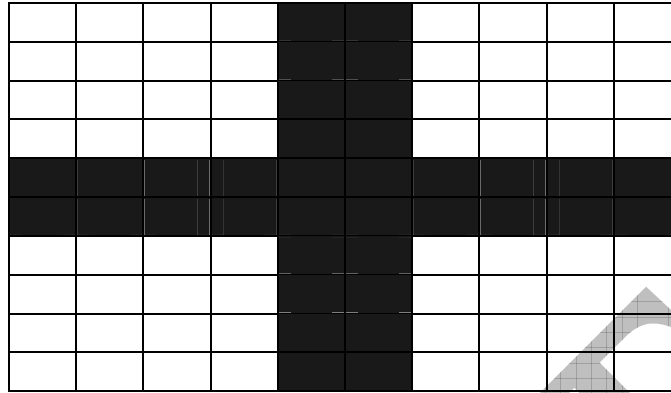
5. Counting *Selenastrum capricornutum*

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^3 .

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

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

- 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
1.			
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APPENDIX 2

PROCEDURES FOR PREPARING MODERATELY HARD WATER FOR USE IN *BRACHIONUS CALYCIFLORUS* CULTURING

INTRODUCTION

Water used to culture *Brachionus calyciflorus* should maintain a hardness range of 80–100 mg/L and alkalinity range of 60-70 mg/L CaCO₃. To obtain the desired hardness range, high quality deionized water should be used with specified amounts of calcium sulfate, magnesium sulfate, sodium bicarbonate, and potassium chloride. The water chemistry parameters consisting of hardness, alkalinity, pH, and conductivity should be measured on each batch of prepared water.

EQUIPMENT LIST

- Deionized water (Milli-Q)
- Calcium sulfate
- Magnesium sulfate
- Sodium bicarbonate
- Potassium chloride
- Stirring plate
- Circulation pump
- Aeration source
- Nalgene® carboy
- Fisherbrand® tape

PROCEDURES

To prepare 50 L of reconstituted fresh water for *Brachionus calyciflorus* cultures, use the reagent grade chemicals as follows:

1. Place about 35 L of deionized (Milli-Q) water in a properly cleaned carboy.
2. Add 3.0 g of CaSO₄·2H₂O to a 2-L aliquot of deionized water and mix (e.g., on a stir plate) for 30 min or until all the salts dissolve.
3. Add 3.0 g of MgSO₄, 4.8 g of NaHCO₃, and 0.2 g of KCl to a second 2-L aliquot of deionized water and mix for 30 min or until all the salts are dissolved.
4. Pour the two aliquots containing the dissolved salts into 35 L of deionized water and fill the carboy to 50 L with deionized water. Solutions should be prepared at least monthly. Record the date of preparation and the initials of the preparer on Fisherbrand® tape attached to the side of the carboy. Also record the preparation information on the *Water Chemistry Summary for Rotifer Culture Water* and *Rotifer Culture Water Data* forms.

5. Circulate the solution for at least 24 hr before use. The solution should be continuously aerated after preparation is complete.

REFERENCES

Hoff FH, Snell TW. 1987. *Plankton Culture Manual* (5th ed.). Florida Aqua Farms Inc, Dade City, FL.

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APPENDIX 3 – FORM: POPULATION CHECKS OF THE ROTIFERS

APPENDIX 4 – FORM: ROTIFER WEEKLY CHECKLIST

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Population Checks of the Rotifers

Algae Batch Code: _____

Exp. Date: _____

Cell Count (cells/mL): _____

Week Dates: _____

Day	Flask ID	D.O. (mg/L)	Temp (°C)	pH	Rep 1	Rep 2	Rep 3	Average of Reps (rep1 + rep2 + rep3) / 3	Sample Size of Rep (mL)	#/mL (Average of reps) / (Sample Size)	Portion of water removed	Fed Flasks	Other Organism (y/n) (if yes how many?)	Cysts (y/n)	Males (y/n)
<i>Example</i>					<i>10</i>	<i>11</i>	<i>12</i>	<i>11</i>	<i>2mL</i>	<i>5.5</i>					
Monday															
Wednesday															
Friday															

Comments: _____

Rotifer Weekly Checklist

Date: _____

Daily Care	Mon	Tues	Wed	Thur	Fri	Sat	Sun
Water Chemistry (temp, pH, D.O.)							
Renewal (if needed to control pop.)							
Count Population							
Algae Feeding (only as needed)							

Date: _____

Daily Care	Mon	Tues	Wed	Thur	Fri	Sat	Sun
Water Chemistry (temp, pH, D.O.)							
Renewal (if needed to control pop.)							
Count Population							
Algae Feeding (only as needed)							

Date: _____

Daily Care	Mon	Tues	Wed	Thur	Fri	Sat	Sun
Water Chemistry (temp, pH, D.O.)							
Renewal (if needed to control pop.)							
Count Population							
Algae Feeding (only as needed)							