

STANDARD OPERATING PROCEDURE

Procedure for Assessing Chronic Residual Toxicity of a Ballast Water Treatment System to the Green Alga (*Selenastrum capricornutum*)

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RECORD OF AMENDMENTS:

No.	Date	Type	No.	Date	Type
1	8/22/11	Divided "Equipment" into two sections: "Equipment" and "Supplies". Added 125 mL or 300 mL Erlenmeyer flask test chambers. Updated "QAQC" and "Reference" sections.	7		
2			8		
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BACKGROUND

The Great Ships Initiative (GSI) is a regional effort devoted to ending the problem of ship-mediated invasive species in the Great Lakes-St. Lawrence Seaway System and globally. In support of that goal, the GSI has established superlative freshwater ballast treatment evaluation capabilities at three scales—bench, land-based, and on board ship. Each scale is dedicated to addressing specific evaluation objectives. These include:

GSI Bench-Scale Tests

- Range finding for effective treatment dose against diverse freshwater taxa and water quality conditions;
- Generation of freshwater relevant chemical degradation curves; and
- Estimation of residual toxicity given diverse freshwater taxa and water quality conditions.

GSI Land-Based Tests

- Pre-certification testing, i.e., operational and biological performance (including residual toxicity) status-testing given scale-up and a range of challenge conditions; and
- Certification/verification testing, i.e., formal assessment of performance against IMO and other discharge standards.

GSI Shipboard Tests

- Confirmation of biological and operational treatment performance as expected in the ship environment;
- U.S. Coast Guard Shipboard Technology Evaluation Program (STEP) testing;
- Shipboard type approval testing;
- Ship discharge monitoring; and
- Methods development.

The GSI awards its independent status-testing services to developers of ballast treatment systems and processes determined to be promising. GSI status-testing is performed at the scale appropriate to the state of development of the target treatment system. GSI has no involvement, intellectual or financial, in the mechanics, design or market success of the actual treatment systems it tests. To ensure that GSI tests are uncompromised by any real or perceived individual or team bias relative to test outcomes, GSI test activities are subject to rigorous quality assurance/quality control (QAQC) procedures and documentation. This attention to QAQC assures high quality and credible evaluation of GSI and its findings.

INTRODUCTION

This GSI Standard Operating Procedure (SOP) describes the procedure used to evaluate the chronic residual toxicity of whole effluent from a prospective ballast treatment system (BTS) to the green alga, *Selenastrum capricornutum* (Figure 1). This method is based on U.S. Environment Protection Agency (EPA) Method EPA-821-R-02-013 (2002), with the procedure applied to laboratory-based studies or semi-field studies using whole effluent. Regardless of application, chronic residual toxicity of treated water following neutralization of the active substance or a degradation/retention period is assessed.

In this procedure, *S. capricornutum* are exposed to whole effluent in a 96-hour, static, chronic toxicity test. Growth is assessed, and the no-observed effect concentration (NOEC), lowest-observed effect concentration (LOEC), and the concentration effecting the growth of 50% of the population (EC₅₀) may also be calculated if a dilution series is utilized. An appropriate statistical method may be used to determine the chronic residual toxicity of whole effluent from the BTS to *S. capricornutum*.

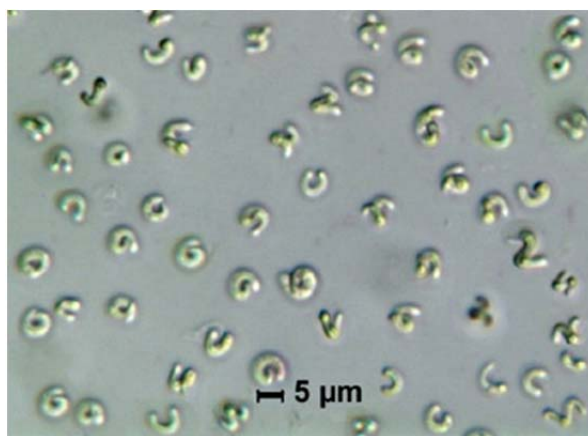


Figure 1. *Selenastrum capricornutum* are crescent or sickle-shaped cells that are longer than they are wide. Accessed from:

http://silicasecchidisk.conncoll.edu/LucidKeys/Carolina_Key/html/Selenastrum_Main.html (January 2010).

DEFINITIONS

Active Substance: A substance or organism, including a virus or fungus that has a general or specific action on or against potentially invasive organisms (IMO, 2005).

Component: A mechanism that has general or specific action on or against potentially invasive organisms, or increases the effectiveness of an active substance.

Duluth-Superior Harbor Water (HW): Natural surface water collected from the Duluth-Superior Harbor of Lake Superior at a depth of approximately 3 m. Water may be filtered through a Whatman 934-AH glass microfiber filter (1.5 μm particle retention in liquid).

High Organic Content Laboratory Water (HOC-LW): Synthetic water created from laboratory water (LW) amended with organics and used as a surrogate in place of HW.

Laboratory Water (LW): City of Superior, Wisconsin municipal water that has been dechlorinated by passage through an activated carbon filter. Sodium sulfite may be added to remove remaining traces of chlorine. Note: Based on data from previous testing, background levels of chlorine from below the limit of detection (i.e., $\leq 3 \mu\text{g/L}$) to $10 \mu\text{g/L}$ are expected in dechlorinated LW.

Prospective Ballast Treatment System (BTS): A system containing an active substance and/or component that mechanically, physically, chemically, or biologically serves to remove, render harmless, or avoid the uptake or discharge of potentially invasive organisms within ballast water (IMO, 2005).

EQUIPMENT

- Centrifuge.
- Autoclave.
- Fluorometer (optional).
- Mechanical shaker.
- Meters: dissolved oxygen, pH, and specific conductivity.
- Microscope.
- Neubauer Hemocytometer.

SUPPLIES

- 125 mL or 300 mL borosilicate glass Erlenmeyer flasks.
- Centrifuge tubes.
- Coverslips, glass.
- Datasheets (can be prepared using Microsoft Access Database).
- Filtering apparatus.
- Filters, $0.45 \mu\text{m}$.
- Foam plugs.
- Graduated cylinders.
- Gridded Sedgewick-Rafter Cell.
- KimWipes®.
- Labels.
- Light meter.
- Liquinox® laboratory soap.
- Pipettes, adjustable volume.
- Sample containers.
- *Selenastrum capricornutum* culture (Purchased as American Type Culture Collection starter).

- Thermometer.
- Volumetric flasks.
- Volumetric pipettes.
- Wash bottles.
- 60 mL screw cap culture tubes with caps.

PROCEDURE

General

1. Conduct all test procedures in a vented work area, taking appropriate health and safety measures.
2. Ensure proper waste disposal before, during, and after test procedure.
3. Prepare and label sample collection containers, test chambers, and prepare data sheets to be used in the study. Ensure that the 125 mL or 300 mL Erlenmeyer flask test chambers are thoroughly cleaned by washing with Liquinox® detergent and rinsing well with tap water followed by distilled water. Sterilize the clean flasks by autoclaving at 121 °C for 15 minutes with foam plugs in place prior to use in the study.
4. Obtain *S. capricornutum* starter from American Type Culture Collection (ATCC Number 22662™; Manassas, VA). The algae starter will arrive frozen and can be stored in a freezer set at -70 °C for up to one week. To prepare the *S. capricornutum* starter for use, thaw the contents of the test tube and transfer to a sterile screw capped test tube containing 5 mL sterile EPA media (see Appendix 1 for information on preparation of EPA media). This starter may be stored in a refrigerator for up to one month. A stock culture should be prepared for use as inoculum 4-6 days prior to the initiation of the WET test using sterile techniques. To prepare, transfer 0.200 mL of the *S. capricornutum* culture with a sterile pipet into a 60 mL sterile screw-capped culture tube containing 50 mL sterile EPA media. Place the culture in an incubator set at 25 °C with continuous 55 % light for 4-6 days prior to initiation of the test. Shake the tubes twice daily (or once daily on the weekend).
5. Prepare the inoculum on the day of WET test initiation. Ensure that the volume of inoculum added (i.e., 0.5 mL to 3.5 mL per test flask) contains enough cells to provide an initial cell density of approximately 10,000 cells/mL ($\pm 10\%$) per test flask. Prepare the inoculum as follows:
 - a. Mix cells in the stock culture well and determine the cell density in the algae concentrate using a Neubauer Hemocytometer (Figure 2). The algae concentrate may need to be diluted prior to counting. A micropipette can be used to make a 1/20 dilution by pipetting 1 mL of algae into 19 mL of LW and mixing well. Add approximately 200 μ L of homogenized sample to the hemocytometer (the volume is not critical); i.e., approximately 100 μ L of sample should be added to the edge of the “V”-shaped notch on each chamber. Place a cover slip on the hemocytometer prior to

adding the sample. The algae are counted following the instructions described below in the section titled “Hemocytometer Counting Method to Determine Initial and Final Densities” and the final value is then multiplied by 20.

- b. Calculate the density of cells (cells/mL) in the algae concentrate using the equation:

$$\text{Density of Algal Cells} = \frac{\text{Average Number Algal Cells Counted in 1 Large Square}}{(0.0001 \text{ cm}^3 \text{ Total Volume Counted}) \left(1 \frac{\text{cm}^3}{\text{mL}}\right)}$$

- c. Calculate the required volume of inoculum as follows:

$$\text{Total Volume (mL) Inoculum Required} = \frac{\text{No. test flasks} * \text{Vol. test solution per flask} * 10,000 \text{ cells per mL}}{\text{Cell density in algae concentrate}}$$

- d. Dilute the algae concentrate as needed to obtain the appropriate inoculum cell density, and check the cell density in the final inoculum.
- e. The volume of the algal inoculum should be considered in calculating the dilution of the toxicant in the test flasks.
6. Collect or prepare a sufficient volume of dilution water to perform the outlined test method. If the dilution water is from a natural water source, such as HW, prepare dilution water by filtering through a 0.45 μm filter. Following filtration, add 1 mL of each of the stock nutrient solutions (see Appendix 1 for stock nutrient solution preparation method) per liter of dilution water.

Effluent Collection, Preservation, and Storage

1. Collect or prepare a sufficient volume of test material to perform the outlined static toxicity test method. The test material may consist of exposure solutions prepared in the laboratory or whole effluent collected from the GSI Land-Based Research, Development, Testing, and Evaluation (RDTE) facility in Superior, WI.
2. Follow the procedure below if test material will be prepared in the laboratory. Skip to Step 3 of this SOP if whole effluent will be collected from the GSI Land-Based RDTE facility.
 - a. Prepare a sufficient amount of the 100 % effluent stock solution for all control and treatment group replicates. Neutralization of the active substance will be conducted using a physical/chemical source added to the treated ballast water or via aging of the 100 % effluent stock solution to allow chemical dissipation to occur.
 - i. *Neutralization via physical/chemical source.* Neutralize the treated ballast water according to the type of BTS being evaluated. In order to examine any effects of the neutralization method on *S. capricornutum*, prepare a solution containing the neutralizing chemical or physical treatment only. The concentration of this solution should be the highest concentration that will be used for active substance neutralization; there is no need to prepare a

dilution series for this treatment.

- ii. *Neutralization via aging treated ballast water.* In order to simulate the holding time in ballast water tanks prior to release into the receiving water, age the treated ballast water in an environmental chamber set at 25 °C in the dark for an appropriate time period.
3. Follow the procedure below if whole effluent will be collected from the GSI Land-Based RDTE Facility.
 - a. Collect an appropriate volume of whole effluent from Sample Collection Tub #6 at the facility upon discharge into receiving water. Prior to collection, the active substance in the treated ballast water will be neutralized via a physical/chemical source or via aging by holding water in a 200 m³ retention tank. Whole effluent samples should be chilled immediately after collection and stored in the dark in a refrigerator set at 4 °C until use in the study. Samples should be warmed to test temperature prior to start of test procedure.

Test Procedure

1. Prepare exposure solutions using 100 % effluent prepared or collected following the above procedure for effluent collection and storage. Depending on the test objectives, a 0.5-dilution series may be prepared. At a minimum, exposure solutions must consist of 0 % and 100 % test material/whole effluent and a performance control (e.g., EPA Nutrient Media, see Appendix 1). If dilution water is from a natural source, such as HW, it must be filtered through a 0.45 µm filter before use.
2. Measure the concentration of the BTS active substance (and the neutralizing chemical(s)) in the 0 % control, 100 % whole effluent, and performance control stock solution, and in any other dilutions used. Measure and record the temperature, dissolved oxygen, pH, conductivity, alkalinity, and hardness of the exposure stock solutions. Alkalinity and hardness should be measured in the dilution water control and the 100 % effluent concentrations, unless the BTS is expected to have an effect on these parameters (Appendix 2).
3. Add 150 mL of the appropriate exposure solution to four replicate 300 mL Erlenmeyer flasks (test flasks) plus one chemistry flask with foam plugs for each treatment and control group. (If 125 mL flasks are being used, add 50 mL of the appropriate exposure solution.)
4. Begin the test period by adding the calculated volume of homogenized, prepared inoculum to each test flask. Count and record the cell density in three of the test solutions within two hours of the inoculation to confirm the initial cell density by following the “Sedgewick-Rafter Counting Procedure to Determine Initial Cell Density” outlined below.

Sedgewick-Rafter Counting Procedure to Determine Initial Cell Density

1. Lay the gridded Sedgewick-Rafter cell (Figure 3) on a flat surface with the coverslip placed diagonally across it.

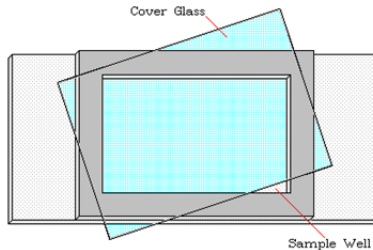


Figure 3. Sedgewick-Rafter counting cell and proper alignment of the cover glass. A 1-mL sample should be added to the sample well. Accessed from: <http://el.erdc.usace.army.mil/zebra/zmis/image/moni0018.gif> in January 2010.

2. Mix the sample thoroughly.
3. Remove two, 550 to 600 μL aliquots using a pipette and transfer it to the Sedgewick-Rafter cell, being careful to avoid getting bubbles under the coverslip. The coverslip should align itself parallel with the counting cell via capillary action.
4. Allow the counting cell to stand on a level surface for a minimum of 24 minutes for the algae to settle.
5. Count the algae using the following technique:
 - a. Count cells until a minimum of 100 cells have been counted, keeping track of the number of squares which have been counted. Avoid counting cells in the squares immediately adjacent to the edges of the slide.

- b. Calculate the cell density as follows:

$$\text{Initial } S. \text{ capricornutum density} = \text{Number of cells counted} / \text{Number of squares counted} \times 1000$$

6. Ensure that a second individual verifies the initial cell density in at least one of the test solutions and records the information as “QA Count Results” on the data sheet (see Appendix 2).
7. Maintain the test at ambient room temperature. Exposure solutions should be 22–28 °C throughout the duration of the study. Measure and record the ambient air temperature daily in the “chemistry” flask of each control and treatment. Test flasks should be continuously mixed using a shaker table set at 100 rpm. Continuous illumination should be maintained with light

levels at approximately $86 \pm 9 \mu\text{E}/\text{m}^2/\text{s}$ ($400 \pm 40 \text{ ft-c}$). Measure and record light levels using a light meter prior to the start of the study.

8. If the BTS active substance(s) is above the limit of quantification in the 100 % test material/whole effluent stock solution, then the concentration of the BTS active substance(s) and neutralizing chemical (if used) must be measured in the “chemistry” replicate from all treatment groups (including dilution water and performance controls) every 24 hours until all exposure solutions are below the limit of quantification.
9. Measure and record temperature and pH every 24 hours in the “chemistry” replicate from each control and treatment group (Appendix 2).
10. Terminate the test 96 hours after initiation. The algal growth in each flask is measured using a manual microscope counting method (see the below section entitled “Hemocytometer Counting Method to Determine Final Densities”). If algal growth will not be determined immediately, store test solutions in a refrigerator set at 4 °C. Ensure that a second individual verifies the cell concentration in at least 10 % of the replicates and records the data as a “QA Count” on the data sheet (see Appendix 3).
11. For test results to be acceptable the algal cell density in the control flasks must exceed 1×10^6 cells/mL at test termination and not vary more than 20 % among replicates.

Hemocytometer Counting Method to Determine Final Cell Densities

1. Count 40-80 cells per replicate for the initial cell density (Hour 0) determination. For the final cell density determination count at least 100-200 cells per replicate, whenever possible. To determine counting bias, duplicate samples from at least 10 % of the test chambers should be counted.
2. A Neubauer Hemocytometer (Figure 2) should be used to determine the concentration of algae cells in each replicate.
3. Mix each sample well using a vortex mixer to avoid clumping of the algae.

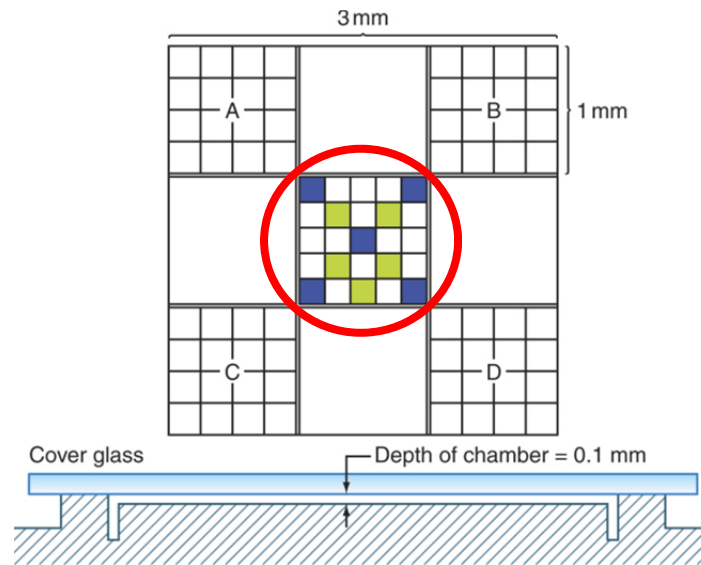


Figure 2. Diagram of the grid on a hemocytometer. The hemocytometer consists of nine large (1 mm^2) squares divided into smaller squares. The nine larger squares have an area of 1 mm^2 (100 nL). The large corner squares (A, B, C, and D) are divided into 16 small squares, each having an area of 0.0625 mm^2 (6.25 nL). Each of the 25 squares within the central square (circled in red) is 0.04 mm^2 , or 4 nL , and is comprised of 16 smaller squares (0.0025 mm^2 or 0.25 nL).

4. Add approximately $200 \mu\text{L}$ of homogenized sample to the hemocytometer equipped with a cover slip (the volume is not critical); i.e., approximately $100 \mu\text{L}$ of sample should be added to the edge of the “V”-shaped notch on each chamber.
5. Allow the cells to settle on the counting chamber for at least one minute prior to counting.
6. Count cells using a compound microscope at 200X magnification (20X objective, 10X ocular), using the PH1 setting and the #1 green filter.
7. To determine stock culture cell density for inoculum preparation, count *S. capricornutum* cells within the central square (see Figure 2, area circled in red). The central square consists of five rows of five small squares each. Depending on the density of the algae stock culture, select one of the five rows or one of the 25 small squares within the central square to be counted. Count one row/small square of the central square, and continue randomly selecting rows/small squares until 100-200 cells total are counted. Record the total number of cells counted in a treatment-system specific laboratory notebook. Calculate the stock culture cell density using the calculation in Step 9 below.
8. To determine final cell density at test termination, count *S. capricornutum* cells within the central square (see Figure 2, area circled in red), using the top and bottom grid of the hemocytometer if needed. Count one row of the central square, and continue randomly selecting rows until 100-200 cells total are counted. Whenever possible, use both the top and bottom grid of the hemocytometer to avoid counting cells from only one area of the hemocytometer. If a cell is on the edge of the 1 mm square area, but is more than halfway in

the large square, it must be counted. Record the total number of cells counted on the algae WET test datasheet (see Appendix 3 for an example datasheet). The central square contains a volume of 10^{-4} mL.

- Calculate *S. capricornutum* cell density using the equations below:

a. If small squares within the central square were counted:

$$\text{Inoculum Cell Density} = \frac{\bar{X}_{\text{cells per small square}} \times 5 \text{ small squares per row} \times 5 \text{ rows per central square}}{10^{-4} \text{ mL}}$$

b. If rows within the central square were counted:

$$\text{Inoculum or Final Cell Density} = \frac{\bar{X}_{\text{cells per row}} \times 5 \text{ rows per central square}}{10^{-4} \text{ mL}}$$

- Thoroughly wash and dry the hemocytometer and coverslip (using a lint-free tissue such as a Kimwipe[®]) after each sample is counted to avoid cross-contamination. Dispose of unanalyzed samples by dumping down the drain and rinsing with tap water.

STATISTICAL ANALYSIS

- Analyze data according to ASTM Standard E1847-96 (2004).
- Use an appropriate toxicity data analysis software, such as Comprehensive Environmental Toxicity Information System (CETIS, Tidepool Scientific Software, McKinleyville, CA, USA) for statistical analysis.
- Generate and report the mean number of cells per volume (\pm standard deviation or standard error) for each control and treatment group. For treatments where a dose-response relationship exists (i.e., those involving active substances), generate and report NOEC, LOEC, and EC₅₀ values. For all other treatments, generate and report significant difference ($p < 0.05$) between control and treatment groups.
- Use an appropriate Analysis of Variance (ANOVA) model to compare means across control and treatment groups.
- Test data normality and homogeneity of variance using an appropriate statistical method. If data normality and homogeneity of variance assumptions are not met, use an appropriate data transformation method and re-test the assumptions.
- Use residual plots to determine how well the statistical model fits the data set.

QUALITY ASSURANCE/QUALITY CONTROL

- Conduct all QAQC procedures according to *GSI/QAQC/QAPP/LB/1 - Quality Assurance Project Plan for Great Ships Initiative (GSI) Land-Based Tests (2011)*.
- Analyze data to ensure that all applicable data quality objectives are met.

3. Perform a second (QA) count on at least one of the samples analyzed to determine initial (Hour 0) cell density and at least 10 % of the samples analyzed to determine final (Hour 96) cell density in order to quantify operator bias. The same area of the Sedgewick-Rafter Cell or hemocytometer must be counted, and the person performing the QA count must not know the results of the first count. The first and second count must have a Relative Percent Difference (RPD) of less than 10 %.
4. Analyze duplicate samples from at least 10 % of the test chambers (during analysis of final cell density only) to quantify within-sample precision. The duplicate samples must have a RPD of less than 20 %.
5. Follow all procedures outlined in this SOP. Any deviations known ahead of time must be approved by the GSI Principal Investigator and Director. Deviations must also be communicated to a GSI Senior QAQC Officer and recorded on *GSI/FORM/G/1 - GSI Standard Operating Procedure Deviation Form*.
6. Record data on data collection forms or in specific laboratory notebooks. Ensure hard copies of all raw data collected are scanned and stored electronically on the LSRI secured Local Area Network (LAN), as well as GSI Sharepoint.

DATA STORAGE AND ARCHIVING

1. Store and archive data according to *GSI/QAQC/QAPP/LB/1 - Quality Assurance Project Plan for Great Ships Initiative (GSI) Land-Based Tests (2011)*.
2. Archive all hard- and electronic-copies of data and records generated for a period of at least seven years.

REFERENCES

ASTM (2004). Standard Practice for Statistical Analysis of Toxicity Tests Conducted Under ASTM Guidelines. E1847-96 (Reapproved 2003).

GSI/FORM/G/1 - GSI Standard Operating Procedure Deviation Form

GSI/LB/QAQC/EHSP/1 - Great Ships Initiative Environmental, Health and Safety Plan (2011).

GSI/QAQC/QMP/1 – Great Ships Initiative Quality Management Plan (2011).

GSI/QAQC/QAPP/LB/1 - Quality Assurance Project Plan for Great Ships Initiative (GSI) Land-Based Tests (2011).

GSI/SOP/LB/G/S/1 - Procedure for Ensuring Worker Health and Safety at the GSI Land-Based RDTE Facility.

Great Ships Initiative website: www.greatshipsinitiative.org.

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

United States Environmental Protection Agency (2002). Green Alga, *Selenastrum capricornutum*, Growth Test Method 1003.0 *from* Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, 4th edition. EPA-821-R-02-013.

APPENDIX 1

PREPARATION OF EPA ALGAL CULTURE MEDIA

EQUIPMENT LIST

- Volumetric flasks
- Volumetric pipets
- Deionized water
- Laboratory Precision or Analytical Balance
- $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$
- $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$
- NaNO_3
- $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
- K_2HPO_4
- NaHCO_3
- H_3BO_3
- $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$
- ZnCl_2
- $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$
- $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$
- $\text{Na}_2\text{MoO}_4 \cdot 2 \text{H}_2\text{O}$
- $\text{CuCl}_2 \cdot 2 \text{H}_2\text{O}$
- $\text{Na}_2\text{EDTA} \cdot 2 \text{H}_2\text{O}$
- Na_2SeO_4
- Filter apparatus with 0.22 μm filter

PROCEDURE

1. Prepare each macronutrient stock solution, micronutrient super stock solution and micronutrient stock solution quantitatively in 500 mL glass volumetric flasks using deionized water according to Table 1.
2. Weigh each macronutrient and micronutrient separately on an analytical balance and record the mass of each nutrient in an appropriate laboratory balance notebook (see *GSI/SOP/BS/RA/GL/1-Procedure for Verification of Laboratory Balances*, for balance accuracy verification procedure). **Read the label on the bottle to be absolutely sure that you have the correct chemical including that the number of waters of hydration is correct.**
3. Quantitatively transfer, using deionized water, the appropriate macronutrient into each appropriately labeled volumetric flask and dilute to volume using deionized water. Mix the solution thoroughly by inverting the flask to dissolve each nutrient. Stock solutions can be stored in the refrigerator. The label should contain the formulation of the solution, the date made, and the initials of the preparer. These solutions are stable for at least one year if they do not become contaminated.
4. Prior to using the stock solutions in WET testing, they should be sterile filtered using a 0.22 μm filter.

5. Add 1 mL each of Macronutrient Stock Solutions A, B, C and D and 1 mL of Micronutrient Stock Solution to one liter of each test water (i.e. LW, filtered HW or 100 % effluent).
6. If the EPA media is to be used for starter cultures or inoculum preparation, after adding the 1 mL of each Macronutrient Stock Solutions A, B, C and D and 1 mL of Micronutrient Stock Solution to 1 L of deionized water, the pH should be adjusted to 7.5 ± 0.1 using 0.1 N NaOH or HCl. The pH adjusted media may be sterilized by autoclaving within culture vessels or filtering with a 0.22 μg filter.

Table 1. EPA nutrient media macronutrient and micronutrient stock solution components.

MACRONUTRIENT STOCK SOLUTIONS				
Stock Solution 1	Macronutrient	Amount Dissolved in 500 mL of DI water	Concentration in Medium (mg/L)	Notes
A.	MgCl ₂ ·6H ₂ O	6.08 g	12.2	Add 1 mL to Medium
	CaCl ₂ ·2H ₂ O	2.20 g	4.41	
	NaNO ₃	12.75 g	25.5	
B.	MgSO ₄ ·7H ₂ O	7.35 g	14.7	Add 1 mL to Medium
C.	K ₂ HPO ₄	0.522 g	1.044	Add 1 mL to Medium
D.	NaHCO ₃	7.50 g	15.0	Add 1 mL to Medium
MICRONUTRIENT STOCK SOLUTION				
Stock Solution 2	Micronutrient	Amount Dissolved in 500 mL of DI water	Concentration in Medium (μg/L)	Notes
	H ₃ BO ₃	92.8 mg	185.0	Add 1 mL to Medium
	MnCl ₂ ·4H ₂ O	208.0 mg	416.0	
	ZnCl ₂	1.64 mg ¹	3.27	
	FeCl ₃ ·6H ₂ O	79.9 mg	1.43	
	CoCl ₂ ·6H ₂ O	0.714 mg ²	0.012	
	Na ₂ MoO ₄ ·2 H ₂ O	3.63 mg ³	7.26	
	CuCl ₂ ·2 H ₂ O	0.006 mg ⁴	160.0	
	Na ₂ EDTA·2 H ₂ O	150.0 mg	300.0	
Na ₂ SeO ₄	1.196 mg ⁵	2.39		

¹ ZnCl₂ – Weigh out 164 mg and dilute to 100 mL. Add 1 mL of this solution to Stock 2, micronutrients.² CoCl₂·6H₂O – Weigh out 71.4 mg and dilute to 100 mL. Add 1 mL of this solution to Stock 2, micronutrients.³ Na₂MoO₄·2 H₂O – Weigh out 36.6 mg and dilute to 10 mL. Add 1 mL of this solution to Stock 2, micronutrients.⁴ CuCl₂·2 H₂O – Weigh out 60.0 mg and dilute to 1000 mL. Take 1 mL of this solution and dilute to 10 mL. Take 1 mL of the second dilution and add to Stock 2, micronutrients.⁵ Na₂SeO₄ – Weigh out 119.6 mg and dilute to 100 mL. Add 1 mL of this solution to Stock 2, micronutrients.

APPENDIX 2

EXAMPLE DATA RECORDING FORMS FOR WATER CHEMISTRY PARAMETERS MEASURED IN S.CAPRICORNUTUM CHRONIC RESIDUAL TOXICITY TEST

- **Day 0** Exposure Stock Solution Water Chemistry (Record Data and Initial after Completion of Water Chemistry):

Treatment	Water Temp. (°C)	pH	DO (mg/L)	Conductivity (µS/cm)	Hardness (mg/L CaCO ₃)	Alkalinity (mg/L CaCO ₃)
Measured By/ Meter No.	/	/	/	/	/	/
Reference Control						
0%						
100%						

DAY 1 CHEMISTRY DATA TABLE

TEMPERATURE METER NO: _____ PH METER NO: _____

MEASURED BY: _____

Treatment – Rep.	Water Temp. (°C)	pH
Reference - CHEM		
0% - CHEM		
100% - CHEM		

COMMENTS/OBSERVATIONS ON TEST DAY 1:

APPENDIX 3

EXAMPLE DATA RECORDING FORMS FOR INITIAL AND FINAL CELL COUNTS OF TEST ORGANISMS (*SELENASTRUM CAPRICORNUTUM*) IN CHRONIC RESIDUAL TOXICITY TEST

Sample ID	Count 1	Count 2	Count 3	Count 4	QA Counts			
					Count 1	Count 2	Count 3	Count 4
YEAR-BWTS-TRIAL-WET-SC W0-3								
YEAR-BWTS-TRIAL-WET-SC W0-4								
YEAR-BWTS-TRIAL-WET-SC W100-1								
YEAR-BWTS-TRIAL-WET-SC W100-2								
YEAR-BWTS-TRIAL-WET-SC W100-3								
YEAR-BWTS-TRIAL-WET-SC W100-4								
YEAR-BWTS-TRIAL-WET-SC SCW-1								
YEAR-BWTS-TRIAL-WET-SC SCW-2								
YEAR-BWTS-TRIAL-WET-SC SCW-3								
YEAR-BWTS-TRIAL-WET-SC SCW-4								

COMMENTS/OBSERVATIONS ON TEST TERMINATION: