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STANDARD OPERATING PROCEDURE
Procedure for Assessing Dose Effectiveness of a Ballast Treatment System Using *Selenastrum capricornutum*

Compiled By -

Name: Euan Reavie & Kelsey Prihoda

Title: GSI Senior Algae/Phytoplankton Scientist & GSI Assistant QAQC Officer

Date: June 4, 2009

Approved By -

Name: Nicole Mays

Title: GSI Senior QAQC Officer

Date: June 4, 2009

Cleared For Issue By -

Name: Allegra Cangelosi

Title: GSI Principal Investigator

Date: June 4, 2009

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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, GSI has established research capabilities at three scales—bench, land-based, and shipboard. Each scale is dedicated to addressing specific evaluation objectives, with protocols as consistent with IMO and federal requirements as practicable. Developers of ballast treatment systems apply for GSI research services online, and awards are offered based on an objective review process. GSI incubation/testing will allow meritorious ballast treatment systems to progress as rapidly as possible to an approval-ready and market-ready condition.

GSI bench-scale tests take place year-round at the University of Wisconsin-Superior's Lake Superior Research Institute (LSRI) in Superior, Wisconsin. The LSRI is amply equipped with staff expertise and resources to conduct the tests, and has a long history of successfully undertaking similar tests.

The overarching goals of GSI bench-scale testing are to explore dose-effectiveness, chemical degradation, residual toxicity, and sensitivity to challenge conditions of a proposed ballast treatment method about which little is known. To that end, the tests are "range-finding" missions, to determine the optimal treatment dose/intensity that would maximize effectiveness and minimize residual toxicity. Findings help treatment developers better design an effective system and/or to move to the next stage of treatment evaluation. The tests are also a form of trouble-shooting to encounter possible problems with the proposed treatment in advance of more extensive and larger scale tests.

GSI bench-scale dose effectiveness tests help determine the range of concentrations of an active substance/component of a prospective treatment that is harmful to a variety of robust freshwater zooplankton, algae and bacteria known to be relatively resilient to stressors. Dose effectiveness test results are expressed as percent survival, percent mortality, or percent hatch. They may also be expressed in terms of a series of absolute quantifications: LC_{99}, i.e., the experimentally derived concentration of an active substance estimated to kill 99 percent of the test population following 24 or 48 hours of continuous exposure; No Observed Effect Concentration (NOEC), i.e., the highest concentration of an active substance shown to have no significantly adverse effect on the test population compared to controls; and Lowest Observed Effect Concentration (LOEC), i.e., the lowest concentration of an active substance known to have a significantly adverse effect on the test population compared to controls.

INTRODUCTION

This GSI Standard Operating Procedure (SOP) describes the method used to evaluate the dose effectiveness of an active substance/component of a prospective ballast treatment system (BTS) by measuring mortality of the freshwater algal species *Selenastrum capricornutum*. In this procedure *S. capricornutum* are acutely exposed to various doses of the active substance/component. Following 0, 24, and 48 hours of exposure, the *S. capricornutum* cells are stained with SYTOX® Green stock solution and the number of live and dead cells within a known area are counted. The dose effectiveness of the active substance/component is determined using an appropriate statistical method.

DEFINITIONS

Active Substance: A substance or organism, including a virus of 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): Water collected at a depth of 3 m from the Duluth-Superior Harbor of Lake Superior (may or may not be filtered).

High Organic Content Lab Water (HOC-LW): Synthetic water created from laboratory water with the addition of tannic and humic acid to mimic Duluth-Superior Harbor water.

Laboratory Water (LW): City of Superior, Wisconsin municipal water that has been dechlorinated by passage through an activated charcoal column. Note: Based on data from previous testing, background levels of chlorine from 5-10 μg/L are expected in dechlorinated laboratory water.

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).

***Selenastrum capricornutum*:** A species of freshwater green algae that is commonly used as a model organism in aquatic toxicology studies. *S. capricornutum* cells are typically cresent-shaped and are longer than they are wide. The cells may also be twisted or “s”shaped.

SYTOX® Green Nucleic Acid Stain: A nucleic acid stain that penetrates cells with compromised plasma membranes (i.e., dead cells) but does not enter live cells, allowing the nucleic acids of dead cells to fluoresce bright green upon excitation with a 450-490 nm source.

EQUIPMENT LIST

- Aeration source.
- Dilution water (i.e., LW or HOC-LW)
- 125 mL or 250 mL Erlenmeyer flasks.
- Inoculum of *Selenastrum capricornutum.*
- Light source.
- Mechanical shaker.
- Temperature-controlled incubator.
- Light meter.
- Thermometer.
- Dissolved oxygen meter.
- Conductivity meter.
- Hardness and alkalinity titration equipment and reagents.
- pH meter.
- Analytical instrumentation needed to measure active substance concentration.
- 10-mL Test tubes.
- Foam plugs.
- Hemocytometer and rectangular coverslips.
- Upright microscope equipped with 40X objective lens, epifluorescence, able to excite samples at 450-490 nm.
- Data collection forms (see Appendix 1 and 2).
- Tally counter for live/dead counts.
- SYTOX® Green stock solution (e.g., 5 μL SYTOX® mixed with 1 mL deionized water).

PROCEDURE

1. Conduct procedure in a vented work area, taking appropriate health and safety measures.

2. Obtain *Selenastrum capricornutum* from in-house cultures by following the procedure outlined in GSI/SOP/BS/RA/C/4. Use *S. capricornutum* harvested from a four to seven day old stock culture. The algae should be concentrated to a density of at least 1 x 10^8 cells/mL prior to use in the study. Record algae culture data in the algae culture log book and/or in a test system-specific laboratory notebook.

3. Prepare the inoculum no more than two to three hours prior to the beginning the test. Ensure that each milliliter of inoculum contains enough cells to provide an initial cell density of approximately 200,000 cells/mL in the exposure solutions.

4. Prepare at least three replicates for each treatment group in the study by following the procedure outlined in GSI/SOP/BS/RA/DE/6 for tests conducted using large volume influent and effluent tanks or GSI/SOP/BS/RA/DE/7 for tests conducted using

exposure solutions prepared in beakers.

5. Collect water chemistry samples directly from the replicate flasks at 0, 24, and 48 hours. At 0 hours, water chemistry samples may be taken directly from control and treatment stock solutions instead of from replicate flasks. Note: Water chemistry parameters and number of replicates measured at each time period will vary depending on the active substance/component being tested. Parameters to be measured may include active substance(s) concentration, total residual oxidants, organic carbon, percent transmittance at 254 nm, temperature, conductivity, pH, dissolved oxygen, hardness, and alkalinity. Only measure hardness and alkalinity in the control group(s) and the highest dose treatment group unless the ballast treatment is expected to have an impact on these parameters. The replicates may be composited at 48 hours to provide enough sample volume for analysis.

6. Document water chemistry results on water chemistry data collections forms (see Appendix 1 for an example data form).

7. Incubate test chambers in complete darkness at 25 ± 2 °C and shake continuously on a mechanical shaker set at 100 rpm. Randomly rotate flask positions each day to minimize possible spatial differences in temperature. Plug flasks with foam plugs to prevent contamination and to allow for gas exchange.

8. Remove a 1 mL subsample from each replicate flask at each time point in the study (0, 24, and 48 hours) and place each subsample in a 10 mL test tube for SYTOX® Green staining. Note: Any overhead/incoming light should be limited during cell staining and counting to protect the stain from photodegrading and to minimize glare while counting.

9. Add 5 μl SYTOX® Green stain stock solution to each 1 mL subsample and mix well. Allow the sample to incubate at room temperature at low light or in the dark for 5-10 minutes. Stained cells should be counted within two hours.

10. Mix the sample well to ensure homogeneity prior to adding a subsample of stained cells to the hemocytometer for counting. Up to 1 mL of sample can be added to the hemocytometer, however, the volume is not critical. Immediately place a cover slip on top of the sample in order to spread out the cells for counting.

11. Count cells at 400X magnification (40X objective, 10X ocular). Use brightfield microscopy to orient and focus the sample at the desired starting point on the hemocytometer. Use brightfield on the lowest power tolerable so that the natural (red) and SYTOX® (green) fluorescence from *S. capricornutum* cells does not become fatigued prior to counting.

12. With the fluorescence on, begin counting at the first transect. Cells that are alive are red under fluorescence; cells that are dead emit bright green fluorescence from the

nuclei and/or contents. Count all the cells (alive and dead) within each transect. If a cell is on a transect line, but is more than halfway in that transect it needs to be counted. Typically, five transects will be counted consisting of 12 squares each. Additional rows should be counted if fewer than 100 cells are counted in the initial five transects. Record the total number or grid cells counted so that the number of alive/dead cells per volume may be calculated. Record the number of alive and dead *S. capricornutum* cells counted in each sample on a data collection form (see Appendix 2 for an example data collection form).

13. Calculate the number of alive/dead cells per volume using the following conversion:

$$1 \text{ square} = 0.00625 \text{ mm}^3 \text{ or } 0.00000625 \text{ cm}^3 \text{ (mL)}.$$

$$\textit{Number of alive/dead cells per volume} = \frac{\textit{(Number alive or dead cells)}}{\textit{(Number squares counted)(0.00000625 mL)}}.$$

14. 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.

15. Repeat steps 7-14 for each sample at every time point in the study. Terminate the study 48 hours after exposure or when complete mortality has occurred in all treatments containing the active substance/component, whichever comes first.

STATISTICAL ANALYSIS

1. Analyze data according to ASTM Standard E1847-96 (2004).

2. Use an appropriate toxicity data analysis software, such as Toxcalc® Toxicity Data Analysis Software (Tidepool Scientific Software, McKinleyville, CA, USA) for statistical analysis.

3. Generate and report the mean percent survival (± 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, EC_{99}, and EC_{100} values. For all other treatments, generate and report the significant difference ($p<0.05$) between control and treatment groups and follow steps 4-6 below.

4. Use an appropriate Analysis of Variance (ANOVA) model to compare means across control and treatment groups.

5. 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.

6. Use residual plots to determine how well the statistical model fits the data set.

QUALITY ASSURANCE/QUALITY CONTROL

1. Conduct all quality assurance/quality control procedures according to the GSI/QAPP/1 - Quality Assurance Project Plan (QAPP) for Great Ships Initiative Bench-Scale and Land-Based Biological Tests (2009). Analyze data to ensure that all applicable data quality criteria are met.

2. Ensure at least 10 % of samples are counted by a suitably qualified second operator.

3. Follow all procedures outlined in this SOP. Any deviations known ahead of time must be approved by the GSI Lead Investigator for Bench-Scale Studies. Any deviations made during the experiment must be recorded and also approved by the GSI Lead Investigator for Bench-Scale Studies as soon as practicable.

4. Record data on data forms or in test system-specific laboratory notebooks. Store data forms in a three-ring binder, and also ensure hard copies are scanned and stored electronically.

DATA STORAGE AND ARCHIVING

1. Store and archive data according to GSI/QAPP/1 - Quality Assurance Project Plan (QAPP) for Great Ships Initiative Bench-Scale and Land-Based Biological Tests (2009).

2. Archive all hard- and electronic-copies of data and records generated for a period of five years.

REFERENCES AND RELATED DOCUMENTS

ASTM (2004). Standard Guide For Conducting 96-h Toxicity Tests With Microalgae. E 1218 – 90 (Reapproved 2004).

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

Cangelosi AA (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.

Fleming K (2004). State of Wisconsin Aquatic Life Toxicity Testing Methods Manual, 2nd edition, Wisconsin Department of Natural Resources, Bureau of Watershed Management. P.O. Box 7921, Madison, WI. 53707.

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

Great Ships Initiative website: www.greatshipsinitiative.org.

GSI/SOP/BS/RA/C/4 – Procedure for Culturing *Selenastrum Capricornutum* as Food for Aquatic Organisms.

GSI/SOP/BS/RA/DE/6 – Dose-Effectiveness Procedure for Exposing Test Organisms to a Ballast Water Treatment System Using 1000-L Influent and Effluent Tanks (2009).

GSI/SOP/BS/RA/DE/7 – Dose-Effectiveness Procedure for Exposing Test Organisms to an Active Substance (2009).

GSI/QAPP/1 - Quality Assurance Project Plan (QAPP) for Great Ships Initiative Bench-Scale and Land-Based Biological Tests (2009).

International Maritime Organization (IMO) (2005). Guidelines for Approval of Ballast Water Management Systems (G8) Adopted by Resolution MEPC.125 (53). London, England.

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

APPENDIX 1

EXAMPLE OF WATER CHEMISTRY DATA COLLECTION FORM (created using Microsoft Access Database)

0 hr Water Chemistry

Environmental Chamber/Water Bath Temperature Check

Measured Temp. (°C)	Time

Test Start Date: 4/6/2009
Analysis Date/Time:
Analyst:

TEST ID: CS-1-EF-SC

Sample ID	Temp (°C)	DO (mg/L)	pH	Conductivity (µs/cm)	Hardness (mg/l as CaCO3)	Alkalinity (mg/l as CaCO3)	Chemical Analysis Sample Collected By:
Meter Number							
L-D25-C0-S0-EFF-1				NA	NA	NA	
L-D25-C0-S0-EFF-2				NA	NA	NA	
L-D25-C0-S0-EFF-3				NA	NA	NA	

Test Start Date: 4/6/2009
Analysis Date/Time:
Analyst:

TEST ID: CS-2-EF-SC

Sample ID	Temp (°C)	DO (mg/L)	pH	Conductivity (µs/cm)	Hardness (mg/l as CaCO3)	Alkalinity (mg/l as CaCO3)	Chemical Analysis Sample Collected By:
Meter Number							
L-D25-C0-S100-EFF-1				NA	NA	NA	
L-D25-C0-S100-EFF-2				NA	NA	NA	
L-D25-C0-S100-EFF-3				NA	NA	NA	

C- Analysis to be completed on composite of all replicates.
NA-No analysis performed at this time.
Test Start Date: 4/6/2009

APPENDIX 2

EXAMPLE OF ORGANISM SURVIVAL DATA COLLECTION FORM (created using Microsoft Access Database)

0 hr_Algae Counts

Test Start Date: 4/6/2009
Analysis Date/Time:
Analyst:

TEST ID: CS-1-EF-SC

Sample ID	TIME	# LIVE	# DEAD
L-D25-C0-S0-EFF-1			
L-D25-C0-S0-EFF-2			
L-D25-C0-S0-EFF-3			

Test Start Date: 4/6/2009
Analysis Date/Time:
Analyst:

TEST ID: CS-2-EF-SC

Sample ID	TIME	# LIVE	# DEAD
L-D25-C0-S100-EFF-1			
L-D25-C0-S100-EFF-2			
L-D25-C0-S100-EFF-3			

Test Start Date: 4/6/2009
Analysis Date/Time:
Analyst:

TEST ID: CS-3-EF-SC

Sample ID	TIME	# LIVE	# DEAD
L-D25-C0.1-S0-EFF-1			
L-D25-C0.1-S0-EFF-2			
L-D25-C0.1-S0-EFF-3			

Test Start Date: 4/6/2009
Analysis Date/Time:
Analyst:

TEST ID: CS-4-EF-SC

Sample ID	TIME	# LIVE	# DEAD
L-D25-C0.1-S100-EFF-1			
L-D25-C0.1-S100-EFF-2			
L-D25-C0.1-S100-EFF-3			
L-D25-C0.1-S100-INF-1			

Test Start Date:4/6/2009