

STANDARD OPERATING PROCEDURE
Procedure for Assessing Dose-Effectiveness of a
Ballast Treatment System Using Cysts of the Freshwater
Rotifer *Brachionus calyciflorus*

Compiled By -

Name: Matt TenEyck &
Kelsey Prihoda

Title: GSI Senior Zooplankton Scientist &
GSI Assistant QAQC Officer

Date: May 28, 2009

Approved By -

Name: Nicole Mays

Title: GSI Senior QAQC Officer

Date: May 28, 2009

Cleared For Issue By -

Name: Allegra Cangelosi

Title: GSI Principal Investigator

Date: May 28, 2009

RECORD OF AMENDMENTS:

No.	Date	Type	No.	Date	Type
1			7		
2			8		
3			9		
4			10		
5			11		
6			12		

STANDARD OPERATING PROCEDURE
Procedure for Assessing Dose-Effectiveness of a
Ballast Treatment System Using Cysts of the Freshwater
Rotifer *Brachionus 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, 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: LC₉₉, 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 resting egg (cyst) hatching of the freshwater rotifer *Brachionus calyciflorus*. In this procedure, rotifer cysts are acutely exposed to various doses of the active substance/component. Following exposure, the cysts are retained in the treated water if the BTS is proposed to treat on ballast intake, or transferred to untreated water (i.e., laboratory water) if the BTS is proposed to treat on ballast discharge. The percentage of neonate hatching is measured after 48 hours, and the dose effectiveness of the active substance/component determined using an appropriate statistical method. Note: dose effectiveness testing is conducted using two exposure methods—cysts may be exposed using large volume influent and effluent tanks (see GSI/SOP/BS/RA/DE/6) or in beakers (see GSI/SOP/BS/RA/DE/7).

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): 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 Laboratory Water (HOC-LW): Synthetic water created from laboratory water (LW) with the addition of tannic and humic acid. Used as a surrogate in place of Duluth-Superior Harbor water.

Laboratory Water (LW): City of Superior, Wisconsin municipal water that has been dechlorinated by passage through an activated carbon filter. Note: Based on data from previous testing, background levels of chlorine from 5-10 $\mu\text{g/L}$ 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).

Rotifer Cyst: A dormant rotifer embryo, also referred to as a resting egg, that is enclosed in an envelope and is resistant to extreme environmental conditions (ASTM E1440-91, 2004).

Rotifer Neonate: A newly hatched, freely swimming rotifer (ASTM E1440-91, 2004).

EQUIPMENT LIST

- *Brachionus calyciflorus* cysts.
- Timer.
- Dilution water (e.g., dechlorinated laboratory water).
- Temperature-controlled environmental chamber.
- Specific conductance meter.
- Hardness/Alkalinity titration materials.
- Pasteur pipette with syringe attachment.
- Dissolved oxygen meter.
- pH Meter.
- Partial immersion thermometer.
- 12-Well polystyrene tissue cell culture plate.
- Microscope with 10-15X magnification.
- Labels (can be prepared using Microsoft Access Database).
- Datasheets (can be prepared using Microsoft Access Database).

PROCEDURE

1. Conduct procedure in a vented work area, taking appropriate health and safety measures.
2. Ensure proper waste disposal before, during, and after test procedure.
3. Obtain rotifer cysts from a commercial supplier, such as Aquatic Eco-Systems, Inc. (Apopka, Florida, USA). Note: Rotifer cysts can be stored in a freezer set at -20 °C for up to one month prior to beginning the test.
4. Establish a homogenous population of rotifer cysts prior to the start of each test by weighing the contents of each vial received from the supplier, recording the weights of the cysts in a study-specific notebook, and combining all vials into one large vial. Gently shake/rotate the vial until a homogenous population is achieved, and weigh a subsample of the rotifer cysts approximately equal to the mean weight of the contents of the individual vials weighed previously. Number the vial that the subsample is weighed into, and record the weight of each subsample in a study-specific notebook.
5. Prepare and label sample collection containers and cell culture plates, and prepare data sheets to be used in the study.
6. Collect water chemistry samples from control and treatment solutions prior to organism exposure ("Time Zero" water chemistry samples). Follow the procedure outlined in GSI/SOP/BS/RA/DE/6 for tests conducted using the large-scale, flow-through exposure system. For tests conducted using exposure solutions in beakers, follow the sample collection procedure outlined in GSI/SOP/BS/RA/DE/7.
7. Document the water chemistry results on water chemistry data collection forms (see

Appendix 1 for an example data form). Note: Water chemistry parameters measured may include active substance 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 two control groups and the highest dose treatment group for each water type (i.e., LW, HW, and/or HOC-LW) unless the ballast treatment is expected to have an impact on these parameters.

8. Prepare rotifer cysts, previously homogenized in step 3, by adding a small amount of LW to the vial containing the cysts. Shake the capped vial until the cysts are thoroughly hydrated. Be sure to record the vial number used in a study-specific notebook.
9. Expose rotifer cysts to the active substance/component following the procedure outlined in GSI/SOP/BS/RA/DE/6 for tests conducted using the large-scale, flow-through exposure system or GSI/SOP/BS/RA/DE/7 for tests conducted using exposure solutions in beakers.
10. Record the approximate cyst exposure time in a study-specific laboratory notebook or on data collection forms in a three-ring binder. Exposure time is defined as the time period from when the cysts are added to the active substance/component to when the test is terminated or when the cysts are transferred to LW.
11. Add 2.0 mL of test solution/EFF water or 2.0 mL of LW, depending on whether the ballast treatment is intended to treat on intake or discharge, to eight replicate wells of a 12-well cell culture plate (four empty wells may be filled to provide additional volume for water chemistry but not counted). Note: There will be a separate cell culture plate for each control/treatment group in the study.
12. Use a microscope equipped with at least 10-15X magnification and transfer 20 randomly selected rotifer cysts into each replicate well. A Pasteur pipette topped with a syringe is used to transfer cysts into each well by slowly raising the plunger on the syringe and carefully drawing up rotifer cysts into the pipette. Minimize the volume of water carried over with the cysts. A second individual will count the number of cysts added to at least 10 % of the wells in order to minimize counting bias. Note: Do not include rotifer cysts that appear to be non-viable in the total count (i.e., empty resting egg case, light brown color).
13. Maintain test organisms in an environmental chamber set at 25 °C with constant lighting to induce neonate hatching. Record the temperature of the environmental chamber daily on the water chemistry data collection form.
14. Count the number of neonates hatched in each replicate under a microscope 24 and 48 hours after test initiation. A second individual will count the number of neonates in at least 10 % of the wells in order to minimize counting bias.
15. Collect water chemistry data after neonates have been counted at test termination. Note: Solutions from the replicate wells may need to be combined into a composite sample to

have sufficient volume to measure all parameters. Record data on water chemistry data collection forms.

16. Terminate the test 48 hours after test initiation. In LW, a typical hatching rate of 50-60 % is common after 48 hours. Record data on the organism survival data collection form (see Appendix 2 for an example data sheet).

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 cyst hatching (\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, EC₉₉, and EC₁₀₀ values. For all other treatments, generate and report significant difference ($p < 0.05$) between control and treatment groups.
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 objectives are met.
2. Ensure a second operator counts the number of neonates in at least 10 % of samples.
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 specific laboratory notebooks. Store data forms in a three-ring binder, and also ensure hard copies are scanned and stored electronically.

5. Conduct reference toxicity tests to determine organism sensitivity. Perform these tests concurrently with the procedure described above or at least once per month.
6. Prepare a quality control chart consisting of a running plot of at least the 20 most recent values (EC_{50}). Determine end points to see if they are within acceptable limits. The control chart depicts the central tendency of the mean value and the upper and lower control values are set as two standard deviations from the mean. Analyze the sensitivity of the batch of rotifer cysts that was used for the study in comparison with historical data.

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 Acute Toxicity Test With The Rotifer Brachionus. E 1440 – 91 (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.

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

Great Ships Initiative website: www.greatshipsinitiative.org.

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.

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:	1/27/2009
Analysis Date:	
Analyst:	

TEST ID: OS-1-EF-RE

Sample ID	Temp (°C)	pH (mg/L)	pH	Conductivity (µs/cm)	Hardness (mg/L as CaCO3)	Alkalinity (mg/L as CaCO3)	Chemical Analysis Sample Collected By:
Meter Number							
OS-1-EF-RE L-DK-25-0- INF-1_0hr							
OS-1-EF-RE L-DK-25-0- EFF-1_0hr							

Test Start Date:	1/27/2009
Analysis Date:	
Analyst:	

TEST ID: OS-2-EF-RE

Sample ID	Temp (°C)	pH (mg/L)	pH	Conductivity (µs/cm)	Hardness (mg/L as CaCO3)	Alkalinity (mg/L as CaCO3)	Chemical Analysis Sample Collected By:
Meter Number							
OS-2-EF-RE L-DK-25- O100-S100-EFF-1_0hr							

0 hr Water Chemistry	Environmental Chamber/Water Bath Temperature Check:	Measured Temp. (°C)	Time

Test Start Date:	1/27/2009
Analysis Date:	
Analyst:	

TEST ID: OS-1-EF-RE

Sample ID	Temp (°C)	pH (mg/L)	pH	Conductivity (µs/cm)	Hardness (mg/L as CaCO3)	Alkalinity (mg/L as CaCO3)	Chemical Analysis Sample Collected By:
Meter Number							
OS-1-EF-RE L-DK-25-0- INF-1_0hr							
OS-1-EF-RE L-DK-25-0- EFF-1_0hr							

Test Start Date:	1/27/2009
Analysis Date:	
Analyst:	

TEST ID: OS-2-EF-RE

Sample ID	Temp (°C)	pH (mg/L)	pH	Conductivity (µs/cm)	Hardness (mg/L as CaCO3)	Alkalinity (mg/L as CaCO3)	Chemical Analysis Sample Collected By:
Meter Number							
OS-2-EF-RE L-DK-25- O100-S100-EFF-1_0hr							

Test Start Date:	1/27/2009
Analysis Date:	
Analyst:	

TEST ID: OS-3-EF-RE

Sample ID	Temp (°C)	pH (mg/L)	pH	Conductivity (µs/cm)	Hardness (mg/L as CaCO3)	Alkalinity (mg/L as CaCO3)	Chemical Analysis Sample Collected By:
Meter Number							
OS-3-EF-RE L-DK-25- O100-S50-EFF-1_0hr							

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

Organism Survival

Sample ID	# Organisms Added	Time Organisms Added	Organisms Counted By	QA Count By	# Hatched at 2 hours	# Hatched at 24 hours	# Hatched at 48 hours
Test ID OS-1-EF-RE							
L-DK-25-0-EFF-1							
L-DK-25-0-EFF-2							
L-DK-25-0-EFF-3							
L-DK-25-0-EFF-4							
L-DK-25-0-EFF-5							
L-DK-25-0-EFF-6							
L-DK-25-0-EFF-7							
L-DK-25-0-EFF-8							
L-DK-25-0-INF-1							
Test ID OS-2-EF-RE							
L-DK-25-O100-S100-EFF-1							
L-DK-25-O100-S100-EFF-2							
L-DK-25-O100-S100-EFF-3							
L-DK-25-O100-S100-EFF-4							
L-DK-25-O100-S100-EFF-5							
L-DK-25-O100-S100-EFF-6							
L-DK-25-O100-S100-EFF-7							
L-DK-25-O100-S100-EFF-8							
Test ID OS-3-EF-RE							
L-DK-25-O100-S50-EFF-1							
L-DK-25-O100-S50-EFF-2							
L-DK-25-O100-S50-EFF-3							
L-DK-25-O100-S50-EFF-4							
L-DK-25-O100-S50-EFF-5							
L-DK-25-O100-S50-EFF-6							
L-DK-25-O100-S50-EFF-7							
L-DK-25-O100-S50-EFF-8							