

STANDARD OPERATING PROCEDURE

Procedure for Assessing Chronic Residual Toxicity of a Ballast Treatment System to the Fathead Minnow (*Pimephales promelas*)

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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 residual toxicity tests help estimate the effect that treated water (following neutralization of the active substance, a degradation period, or no treatment at all) may have on non-target organisms in the receiving system.

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 fathead minnow, *Pimephales promelas*. This method is based on US Environmental Protection Agency (EPA) Method EPA-821-R-02-013 (2002). The procedure outlined in this method can be applied to laboratory-based studies or semi-field studies using whole-effluent from a land-based test system. The objective of this method is to assess chronic residual toxicity of treated water from a prospective BTS following neutralization of the active substance or a degradation period.

In this method, newly hatched (≤ 24 hour old) *P. promelas* larvae are exposed to whole effluent in a 7-day, static-renewal chronic toxicity test. Survival and growth are assessed, and the no-observed effect concentration (NOEC), lowest-observed effect concentration (LOEC), and the concentration lethal to 50 % of the population (LC₅₀) are calculated. The chronic residual toxicity of whole effluent from a prospective BTS is assessed using an appropriate statistical method.

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.

High Organic Content Laboratory Water (HOC-LW): Synthetic water created from laboratory water (LW) that is 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 below the limit of detection ($\leq 3 \mu\text{g/L}$) to $10 \mu\text{g/L}$ 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).

EQUIPMENT LIST

- *Pimephales promelas* larvae (less than 24 hours old).
- Brine shrimp culture unit.
- Temperature-controlled environmental chamber.
- Sample containers.
- Water bath (temperature-controlled).
- 600 mL borosilicate glass beakers.
- Drying oven (50-105 °C range).
- Light meter.
- Light box.
- Pipettes, adjustable volume.
- Volumetric flasks.

- Graduated cylinders.
- Volumetric pipettes.
- Wide-bore glass droppers.
- Wash bottles.
- Thermometer.
- Meters: dissolved oxygen, pH, and specific conductivity.
- Analytical balance (capable of reading to 0.0001 g).
- Balance reference weights.
- Aluminum weigh pans.
- Labels (can be prepared using Microsoft Access Database).
- Datasheets (can be prepared using Microsoft Access Database).
- Dessicator.

PROCEDURE

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 test chambers are thoroughly cleaned and dry prior to use in the study.
4. Obtain newly hatched *P. promelas* larvae from a commercial supplier, such as Environmental Consulting and Testing (ECT, Superior, WI, USA). A record of the date the *P. promelas* eggs were laid and hatched and information on the sensitivity of contemporary fish to a reference toxicant should be provided by the supplier. Note: If desired, *P. promelas* larvae may be acclimated in 50 % culture water/50 % dilution water at test temperature prior to study initiation. Time and conditions of acclimation should be recorded in a study-specific laboratory notebook.
5. Measure dry weight on a subset of at least 20-80 organisms to provide an initial weight for growth determination; organisms to be used for initial dry weight should be divided into three replicate weigh pans. For dry weight determination, rinse larvae in deionized water to remove any debris. Transfer thoroughly rinsed larvae to a tared aluminum weigh pan and place in a drying oven set at 60 °C for a minimum of 24 hours. Once dry, transfer weigh pans to a desiccator and measure and record dry weight. Divide the final dry weight by the number of original larvae to determine the average individual dry weight and record on a data sheet.

Effluent Collection, Preservation, and Storage

1. Collect or prepare a sufficient volume of test material (i.e., approximately 18 L for entire test) to perform the outlined test method with daily renewal. The test material may

consist of test solution prepared in the laboratory or whole effluent collected from the GSI Land-Based Research, Development, Testing, and Evaluation (RDTE) Facility in Superior, WI. In addition, collect or prepare a sufficient volume of dilution water (i.e., approximately 31.5 L for entire test) to perform the outlined test method with daily renewal.

2. Follow the procedure below if test material will be prepared in the laboratory. Skip to Step 3 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 the entire duration of the study. Neutralization of the active substance will be conducted using a physical/chemical source added to the treated 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 water according to the type of treatment system being evaluated. In order to examine any effects of the neutralization method on *P. promelas* larvae, 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 receiving water, age the treated water in an environmental chamber set at 25 °C in the dark for an appropriate time period.
 - b. Store the 100 % effluent stock solution in the dark in a refrigerator set at 4 °C. Warm the stock solution to test temperature prior to preparing dilutions for renewal on subsequent days of the study.
3. Follow the procedure below if whole effluent will be collected from the GSI Land-Based RDTE Facility. Disregard this step if test material has already been prepared in the laboratory.
 - a. Collect a minimum of 18 L whole-effluent from Collection Tub #6 at the GSI Land-Based RDTE Facility upon discharge into receiving water. Prior to collection, the active substance in the treated water will be neutralized via a physical/chemical source or via aging by holding water in a land-based retention tank. Whole-effluent samples should be chilled immediately after collection and stored the dark in a refrigerator set at 4 °C until use in the study, then warmed to test temperature prior to dilution.

Test Procedure

1. Prepare exposure solutions using 100 % effluent prepared or collected following the above procedure for effluent collection and storage. The exposure solutions are created

using a 0.5 dilution series (i.e., 0, 6.25, 12.5, 25, 50, and 100 % test material/whole effluent) or other appropriate dilution scheme for a minimum of five test concentrations and one control. Dilutions of the whole effluent are made with LW, HOC-LW, HW or other appropriate dilution water type.

2. Measure the concentration of the BTS active substance (and the neutralizing chemical(s) if used) in the 100 % effluent stock solution to determine the extent of the active substance degradation. Measure and record the temperature, dissolved oxygen, pH, and conductivity of the exposure solutions (see Appendix 1 for example Day 0 stock solution water chemistry data sheet). Alkalinity and hardness is measured in the dilution water control and the 100 % effluent concentrations, unless the treatment system is expected to have an effect on these parameters.
3. Add 250 mL of the appropriate exposure solution to four replicate 600 mL borosilicate glass beakers (test chambers) for each treatment and control group.
4. Delicately transfer newly-hatched *P. promelas* larvae, one or two at a time, to each test chamber using a large-bore glass pipette until each test chamber has 15 larvae. Discard any organisms that are dropped or injured. The amount of water transferred when adding larvae should be kept to a minimum to avoid dilution of the test solutions.
5. Record the number of *P. promelas* added, time of addition, and initials of responsible individual on the survival data recording form (see Appendix 2 for example survival data sheet). A separate individual must verify in at least 10 % of the test chambers that 15 *P. promelas* were added and record that information in the "QA Count" column of the data sheet.
6. Maintain test organisms in an environmental chamber set at 25 °C with a 16:8 light:dark photoperiod, and exposure solutions should be 22-28 °C throughout the duration of the study. Measure and record the temperature of the environmental chamber daily on the water chemistry data sheet.
7. Feed the test organisms twice daily at 6-hour intervals. Larvae are fed approximately 0.1 g of a concentrated solution of less than 24-hour old brine shrimp (*Artemia* spp.). The brine shrimp should be rinsed with fresh water to remove salinity prior to feeding. Larval fish are not fed during the final 12 hours of the test.
8. Prepare new exposure solutions daily using the previously prepared 100 % effluent stock solution (warm to test temperature prior to use) and following the procedure outlined in Step 1 above (Test Procedure section).
9. Remove uneaten and dead brine shrimp (*Artemia* sp.), dead fish larvae, and other debris from the bottom of the test chambers with a large-bore glass pipette prior to daily renewal of test solutions. Siphon water from the top of the test chambers, leaving 15-20 % of the old test solution in each test chamber without siphoning any surviving fish, and add new

test solution to 250 mL by slowly pouring down the side of the test chamber.

10. Count and record the number of surviving larvae and discard the dead larvae (see Appendix 2 for example survival data sheet). Whenever possible, a separate individual must confirm the survival of the *P. promelas* in at least 10 % of the test chambers and record this information under the “QA Count” column of the data sheet.
11. If the active substance was detected on Day 0 in the 100 % effluent stock solution, measure and record the concentration of the BTS active substance(s) and, if used, neutralizing chemical(s) in each newly created exposure stock solution daily, or until all stocks are below the limit of detection.
12. Measure and record (see Appendix 1 for water chemistry example data sheet) the following routine physical/chemical parameters during the test:
 - a. Temperature, pH, and dissolved oxygen at the end of each 24-hour non-renewal period in at least one replicate from the control, low, middle, and high concentrations (see Appendix 1 for water chemistry example data sheet). In addition, measure and record temperature, pH, and dissolved oxygen in each newly created exposure stock solution daily. Note: Dissolved oxygen should not fall below 4.0 mg/L during the test.
 - b. Temperature should also be continuously measured or measured and recorded daily in the environmental chamber.
 - c. Conductivity in each newly created exposure solution prepared from the control, low, middle, and high concentrations.
 - d. Alkalinity and hardness on Day 7 (or test termination) in at least one replicate from the dilution water control and 100 % treatment solutions, unless these parameters are expected to be affected by the BTS.
13. Terminate the test after 7 days of exposure. At test termination, remove dead larvae and count and record surviving larvae. A second individual must confirm the survival count in at least 10 % of the test chambers, and record that information in the “QA Count” column of the data sheet (see Appendix 2). For dry weight determination, surviving larvae from each replicate are pooled and rinsed in deionized water to remove any debris. Transfer thoroughly rinsed larvae to a tared aluminum weigh boat and place in a drying oven set at 60 °C for a minimum of 24 hours. Once dry, transfer weigh boats to a desiccator and measure and record dry weight. For each test chamber, divide the final dry weight by the number of surviving larvae in the test chamber to determine the average individual dry weight and record on a data sheet. For the controls, also calculate the mean weight per surviving fish in the test chamber to evaluate if weights met test acceptability criteria.
14. For test results to be acceptable there must be at least 80 % survival in the control and average dry weight per surviving organism in the control must be at least 0.25 mg.

STATISTICAL ANALYSIS

1. Analyze data according to ASTM Standard E1847-96 (2004).
2. Use an appropriate toxicity data analysis software, such as Comprehensive Environmental Toxicity Information System (CETIS, Tidepool Scientific Software, McKinleyville, CA, USA) for statistical analysis.
3. Generate and report the mean percent survival and mean weight (\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.
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. 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.
3. 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.
4. Ensure a second operator counts the number of surviving larvae in at least 10 % of the test chambers.
5. Conduct reference toxicity tests to determine organism sensitivity. Perform these tests concurrently with the procedure described above or at least once per month.

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

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. Northeast-Midwest Institute, Washington, D.C.

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

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

United States Environmental Protection Agency (2002). Fathead Minnow, *Pimephales promelas*, Larval Survival and Growth Test Method 1000.0 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

EXAMPLE DATA RECORDING FORMS FOR WATER CHEMISTRY PARAMETERS DURING FATHEAD MINNOW CHRONIC RESIDUAL TOXICITY TEST

DAY 0 Stock Water Chemistry

Environmental Chamber/Water Bath Temperature Check

Measured Temp. (°C)	Time

Test Start Date	8/12/2008
Analysis Date:	
Analyst:	

Sample ID	Temp (°C)	DO (mg/L)	pH	Conductivity (us/cm)	Hardness (mg/L as CaCO3)	Alkalinity (mg/L as CaCO3)	Chemical Analysis Sample Collected By:
Meter #							
CL-4-CRT-CD							
FM-LT-25-0-0-S OHS							
CL-4-CRT-CD							
FM-LT-25-0-2.5-S OHS							
CL-4-CRT-CD							
FM-LT-25-0-25-S OHS							
CL-4-CRT-CD							
FM-LT-25-3-2.5-S OHS							
CL-4-CRT-CD							
FM-LT-25-3-25-S OHS							
CL-4-CRT-CD							
L-LT-25-0-0-S OHS							
CL-4-CRT-CD							
L-LT-25-0-2.5-S OHS							
CL-4-CRT-CD							
L-LT-25-0-25-S OHS							
CL-4-CRT-CD							
L-LT-25-3-2.5-S OHS							
CL-4-CRT-CD							
L-LT-25-3-25-S OHS							

DAY 1 Water Chemistry

Environmental Chamber/Water Bath Temperature Check:

Measured Temp. (°C)	Time

Test Start Date	8/12/2008
Analysis Date:	
Analyst:	

Sample ID	Temp (°C)	DO (mg/L)	pH	Comments:
Meter #				
CL-4-CRT-CD				
FHLT-25-0-0-6 2MHRS				
CL-4-CRT-CD				
FHLT-25-0-0-4 2MHRS				
CL-4-CRT-CD				
FHLT-25-0-0-2 2MHRS				
CL-4-CRT-CD				
HILT-25-0-2.5-2 2MHRS				
CL-4-CRT-CD				
HILT-25-0-2.5-4 2MHRS				
CL-4-CRT-CD				
HILT-25-0-2.5-6 2MHRS				
CL-4-CRT-CD				
HILT-25-0-2.5-2 2MHRS				
CL-4-CRT-CD				
HILT-25-0-2.5-4 2MHRS				
CL-4-CRT-CD				
HILT-25-0-2.5-6 2MHRS				
CL-4-CRT-CD				
HILT-25-3-2.5-2 2MHRS				
CL-4-CRT-CD				
HILT-25-3-2.5-4 2MHRS				
CL-4-CRT-CD				
HILT-25-3-2.5-6 2MHRS				
CL-4-CRT-CD				
HILT-25-3-2.5-2 2MHRS				
CL-4-CRT-CD				
HILT-25-3-2.5-4 2MHRS				
CL-4-CRT-CD				
HILT-25-3-2.5-6 2MHRS				
CL-4-CRT-CD				
L-LT-25-0-0-4 2MHRS				

APPENDIX 2

EXAMPLE DATA RECORDING FORM FOR SURVIVAL OF THE FATHEAD MINNOW DURING A CHRONIC RESIDUAL TOXICITY TEST

Organism Survival

Sample ID	# Organisms Added	Time Organisms Added	Organisms Counted By	QA Count By	# Alive at 2 hours	# Alive at 24 hours	# Alive at 2 days	# Alive at 3 days	# Alive at 4 days	# Alive at 5 days	# Alive at 6 days	# Alive at 7 days
Test ID OU-1-RT-PP												
L2-LT-25-INF-1												
L2-LT-25-INF-2												
L2-LT-25-INF-3												
L2-LT-25-EFF-1												
L2-LT-25-EFF-2												
L2-LT-25-EFF-3												
Test ID OU-2-RT-PP												
CW2-LT-25-INF-1												
CW2-LT-25-INF-2												
CW2-LT-25-INF-3												
CW2-LT-25-EFF-1												
CW2-LT-25-EFF-2												
CW2-LT-25-EFF-3												