

**STANDARD OPERATING PROCEDURE**  
**Procedure for Assessing Chronic Residual Toxicity of a Ballast Treatment System to *Ceriodaphnia dubia***

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## **STANDARD OPERATING PROCEDURE**

### **Procedure for Assessing Chronic Residual Toxicity of a Ballast Treatment System to *Ceriodaphnia dubia***

#### **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 *Ceriodaphnia dubia*. This method is based on U.S. Environmental Protection Agency (EPA) Method EPA-821-R-02-013 (2002). The procedure outlined here can be applied to laboratory-based studies or semi-field studies using whole-effluent from a land-based test system. Regardless of application, chronic residual toxicity of treated water following neutralization of the active substance or a degradation period will be assessed.

In this test method, neonate ( $\leq 48$  hours) *C. dubia* are exposed to whole effluent in an 8-day,

static-renewal chronic toxicity test. Survival and reproduction are assessed using this method, and the no-observed effect concentration (NOEC), lowest-observed effect concentration (LOEC), and the concentration lethal to 50 % of the population (LC<sub>50</sub>) is calculated. The chronic residual toxicity of whole effluent from a prospective treatment system 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).

**Brood Stock:** The *Ceriodaphnia dubia* that are cultured to produce test organisms through reproduction.

***Ceriodaphnia dubia* Neonate:** A newly hatched, freely swimming daphnid that is less than 48 hours old.

**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) 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

- *Ceriodaphnia dubia* and algal culture units.
- YCT.
- Sample containers.
- Environmental chamber.
- 30-ml borosilicate glass beakers or disposable polystyrene cups.
- Light meter.
- Lighted magnifying lens.

- Dissecting microscope.
- Microscope with 10X, 45X, and 100X objective lenses.
- Boards to hold test chambers.
- Light box.
- Volumetric flasks.
- Graduated cylinders.
- Pipettes, adjustable volume.
- Volumetric pipettes.
- Disposable polyethylene pipettes.
- Glass droppers.
- Wash bottles.
- Thermometer.
- Meters: dissolved oxygen, pH, and specific conductivity.
- Labels (can be prepared using Microsoft Access Database).
- Datasheets (can be prepared using Microsoft Access Database).

## **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 the test chambers are thoroughly cleaned and dry prior to use in the study.
4. Obtain neonate *C. dubia* from healthy brood stock. Monthly reference toxicity testing should be conducted in order to determine the health/sensitivity of the organisms used in the test. Use only neonates from adults in at least their third brood.
5. Acclimate neonate *C. dubia* for approximately 24 hours at test temperature by transferring <24 hour old neonates from brood boards to 50 % culture water (i.e., hard reconstituted water)/50 % test water. Combine organisms from multiple brood boards into a single container to allow for complete randomization prior to the start of the test.

### **Effluent Collection, Preservation, and Storage**

1. Collect or prepare a sufficient volume of test material (i.e., approximately 8 L for entire test) to perform the outlined test method with daily renewal. 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 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 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 treatment system being evaluated. In order to examine any effects of the neutralization method on *C. dubia* neonates, prepare a control 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 ballast 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 following Step 2 of this SOP.
  - a. Collect a minimum of 8 L whole-effluent from Tub #6 at the Facility upon discharge into receiving water. Prior to discharge, the active substance in the treated water will be neutralized via a physical/chemical source or via chemical degradation by holding water in a 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 procedures 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 % 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 if used) in the 100 % effluent stock solution on Day 0 to determine the extent of the active substance degradation. Measure and record the temperature, dissolved oxygen, pH, and

conductivity of all stock solutions prepared on Day 0 (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 stock solutions on Day 0, unless the treatment system is expected to have an effect on these parameters.

3. Add 15 mL of the appropriate exposure solution to ten replicate 30 mL borosilicate glass beakers or disposable polystyrene cups.
4. Delicately transfer one *C. dubia* neonate to each test chamber using a 2 mm glass or polyethylene pipette. Release the organisms under the surface of the water so that air is not trapped under the carapace. Discard any organisms that are dropped or injured. The amount of water transferred when adding neonates should be kept to a minimum to avoid dilution of the test solutions.
5. Record the number of *C. dubia* neonates added, time added, and the initials of the responsible individual on the data recording form (see Appendix 2 for example test set-up data recording form). A separate individual must confirm that there is one *C. dubia* neonate in at least 10 % of the test chambers and record this information under 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. Exposure solutions should be 22-28 °C throughout the duration of the study. Measure the temperature of the environmental chamber daily and record on the water chemistry data sheet.
7. Prepare new exposure solutions daily using the previously prepared/collected 100 % effluent stock solution (warm to test temperature prior to use) and following the procedure outlined in Step 1 above (Test Procedure section).
8. Transfer the test organisms to new exposure solution daily following Step 4 in the Test Procedure section of this SOP. Count and record the number of surviving adults and the number of young produced (see Appendix 3 for survival and reproduction example data sheet). A separate individual must confirm the survival of the adult *C. dubia* and number of young in at least 10 % of the test chambers and record this information under the “QA Count” column of the data sheet.
9. If the active substance was detected on Day 0 in the 100 % 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.
10. Measure and record (see Appendix 1 for water chemistry example data sheets) the following routine physical/chemical parameters during the test:
  - a. Temperature and pH at the end of each 24-hour non-renewal period in at least one

- replicate from the control, low, middle, and high concentrations.
- b. Conductivity and dissolved oxygen in each newly created stock solution from the control, low, middle, and high concentrations.
  - c. Hardness and alkalinity in a composite of the replicates from the dilution water control and 100 % whole effluent treatments on Day 8 (or test termination), unless the treatment system is expected to have an effect on these parameters.
11. Feed the test organisms daily. Food is added to freshly renewed exposure solutions immediately before or immediately after the adults are transferred. Each feeding consists of 0.1 mL Yeast-Cereal Leaves-Trout Chow suspension (YCT) and 0.1 mL *Selenastrum capricornutum* concentrate/15 mL exposure solution (to provide  $2\text{-}2.3 \times 10^5$  cells/mL) The amount of YCT and algae added to each test chamber, time of feeding, and initials of responsible individual should be recorded on that day's survival and reproduction data sheet.
  12. Terminate the test when 60 % of the control organisms have produced their third brood, or at the end of 8 days, whichever occurs first. Any animal not producing young should be examined to determine if it is a male. The endpoint of this test method is survival and reproduction.
  13. For test results to be acceptable there must be at least 80 % survival in the control treatment and 60 % of surviving control adults must have at least three broods with an average total number of at least 15 young per adult.

## 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 number of young produced per adult ( $\pm$  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 EC<sub>50</sub> 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 neonates and number of young 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.
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 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>.

Great Ships Initiative website: [www.greatshipsinitiative.org](http://www.greatshipsinitiative.org).

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.

United States Environmental Protection Agency (2002). Daphnid, *Ceriodaphnia dubia*, Survival and Reproduction Test Method 1002.0 from Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, 4<sup>th</sup> edition. EPA-821-R-02-013.

## **APPENDIX 1**

### **EXAMPLE DATA RECORDING FORMS FOR WATER CHEMISTRY PARAMETERS MEASURED IN C. DUBIA 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 (µs/cm)	Hardness (mg/l as CaCO3)	Alkalinity (mg/l as CaCO3)	Chemical Analysis Sample Collected By:
Meter #							
CL-4-CRT-CD							
<b>FH-LT-25-0-0-S</b> OHS							
CL-4-CRT-CD							
<b>FH-LT-25-0-2.5-S</b> OHS							
CL-4-CRT-CD							
<b>FH-LT-25-0-25-S</b> OHS							
CL-4-CRT-CD							
<b>FH-LT-25-3-2.5-S</b> OHS							
CL-4-CRT-CD							
<b>FH-LT-25-3-25-S</b> OHS							
CL-4-CRT-CD							
<b>L-LT-25-0-0-S</b> OHS							
CL-4-CRT-CD							
<b>L-LT-25-0-2.5-S</b> OHS							
CL-4-CRT-CD							
<b>L-LT-25-0-25-S</b> OHS							
CL-4-CRT-CD							
<b>L-LT-25-3-2.5-S</b> OHS							
CL-4-CRT-CD							
<b>L-LT-25-3-25-S</b> 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				
<b>FHLT-25-0-0-6</b> 24HRS				
CL-4-CRT-CD				
<b>FHLT-25-0-0-4</b> 24HRS				
CL-4-CRT-CD				
<b>FHLT-25-0-0-2</b> 24HRS				
CL-4-CRT-CD				
<b>HLLT-25-0-2.5-2</b> 24HRS				
CL-4-CRT-CD				
<b>HLLT-25-0-2.5-4</b> 24HRS				
CL-4-CRT-CD				
<b>HLLT-25-0-2.5-6</b> 24HRS				
CL-4-CRT-CD				
<b>HLLT-25-0-2.5-2</b> 24HRS				
CL-4-CRT-CD				
<b>HLLT-25-0-2.5-4</b> 24HRS				
CL-4-CRT-CD				
<b>HLLT-25-0-2.5-6</b> 24HRS				
CL-4-CRT-CD				
<b>HLLT-25-3-2.5-2</b> 24HRS				
CL-4-CRT-CD				
<b>HLLT-25-3-2.5-4</b> 24HRS				
CL-4-CRT-CD				
<b>HLLT-25-3-2.5-6</b> 24HRS				
CL-4-CRT-CD				
<b>HLLT-25-3-2.5-2</b> 24HRS				
CL-4-CRT-CD				
<b>HLLT-25-3-2.5-4</b> 24HRS				
CL-4-CRT-CD				
<b>HLLT-25-3-2.5-6</b> 24HRS				
CL-4-CRT-CD				
<b>L-LT-25-0-0-4</b> 24HRS				

CL-4-CRT-CD

Test Start: 8/12/2008

Day: 1

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## **APPENDIX 2**

### **EXAMPLE DATA RECORDING FORM FOR ADDITION OF TEST ORGANISMS (*C. dubia*) IN CHRONIC RESIDUAL TOXICITY TEST**

# Day 0 \_Addition of Organisms

Date: \_\_\_\_\_

Organisms fed: \_\_\_\_\_ mL YTC \_\_\_\_\_ mL Algae Time: \_\_\_\_\_ By: \_\_\_\_\_

Test ID **OS-1-EF-RE**

Sample ID	# Organisms Added	Time Organisms Added	Organisms Counted By	QA Count By	Comments:
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-O100-S100-EFF-					
L-DK-25-O100-S100-EFF-					
L-DK-25-O100-S100-EFF-					
L-DK-25-O100-S100-EFF-					
L-DK-25-O100-S100-EFF-					
L-DK-25-O100-S100-EFF-					
L-DK-25-O100-S100-EFF-					
L-DK-25-O100-S100-EFF-					
L-DK-25-O100-S100-EFF-					

## **APPENDIX 3**

### **EXAMPLE DATA RECORDING FORM FOR SURVIVAL AND REPRODUCTION OF *C. dubia* IN CHRONIC RESIDUAL TOXICITY TEST**

**Survival of Organisms**

Test ID: **CL-4 CRT-CD**

Day: **1**

Date:

Organisms fed: \_\_\_\_\_ mL YTC \_\_\_\_\_ mL Algae Time: \_\_\_\_\_ By: \_\_\_\_\_

Sample ID	# Organisms Algae	# Young	Organisms Counted By	QA Count By	Comments
FH-LT-25-0-0-1					
FH-LT-25-0-0-2					
FH-LT-25-0-0-3					
FH-LT-25-0-0-4					
FH-LT-25-0-0-5					
FH-LT-25-0-0-6					
FH-LT-25-0-0-7					
FH-LT-25-0-0-8					
FH-LT-25-0-0-9					
FH-LT-25-0-0-10					
FH-LT-25-0-2.5-1					
FH-LT-25-0-2.5-2					
FH-LT-25-0-2.5-3					
FH-LT-25-0-2.5-4					
FH-LT-25-0-2.5-5					
FH-LT-25-0-2.5-6					
FH-LT-25-0-2.5-7					
FH-LT-25-0-2.5-8					
FH-LT-25-0-2.5-9					
FH-LT-25-0-2.5-10					
FH-LT-25-0-25-1					
FH-LT-25-0-25-2					
FH-LT-25-0-25-3					
FH-LT-25-0-25-4					
FH-LT-25-0-25-5					
FH-LT-25-0-25-6					
FH-LT-25-0-25-7					