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STANDARD OPERATING PROCEDURE
Procedure for Zooplankton Sample Analysis

Compiled By -

Name: Mary Balcer

Title: GSI Senior Zooplankton Scientist

Date: July 20, 2009

Approved By -

Name: Nicole Mays

Title: GSI Senior Quality Systems Officer

Date: July 20, 2009

Cleared For Issue By -

Name: Allegra Cangelosi

Title: GSI Principal Investigator & Director

Date: July 20, 2009

RECORD OF AMENDMENTS:

No.	Date	Type	No.	Date	Type
1	02/21/2011	Changed nomenclature from "rotifers" to "microzooplankton", and from "crustaceans" to "macrozooplankton". Added equipment to "Equipment List". Added ¶3-5 to "Sample Preparation" procedure. Formatted SOP to describe the analysis procedure for intake and control discharge samples separately from the analysis procedure for treatment discharge samples. Also, separated the microzooplankton and macrozooplankton analysis procedures within each section. Added "Procedure for Zooplankton Analysis Data Entry". Added text to "QA/QC", including PSC and RPD formulas. Added Appendix 2.	7		
2	05/20/2011	Added text to the macrozooplankton definition. Added Figure 1. Edited ¶ I.2.	8		
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6			12		

STANDARD OPERATING PROCEDURE
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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 international 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.

GSI awards its independent status-testing services to candidate systems only if technical and programmatic criteria are met. Decisions are based on third party technical assessments as well as GSI Advisory Committee programmatic input. Testing services are currently offered at no cost to the developer with the exception of transportation and system installation/removal costs. Instead, tests are supported by general project funds which derive from federal and state agency grants, Great Lakes port contributions, and in-kind contributions by local governments and universities.

GSI has no involvement, intellectual or financial, in the mechanics, design or market success of the actual treatment systems it tests. To ensure GSI remains completely independent and is uncompromised by any real or perceived individual or project bias, GSI subjects itself to rigorous quality management policies and procedures. In addition, GSI test activities are subject to rigorous QAQC procedures and documentation. This attention to quality management and QAQC assures the high quality and credible evaluation of both GSI and its findings.

INTRODUCTION

This GSI Standard Operating Procedure (SOP) describes analysis of zooplankton samples collected at the GSI Land-Based Research, Development, Testing, and Evaluation (RDTE) Facility. Based on the International Maritime Organization's Convention (IMO, 2004) and the United States Environmental Protection Agency, Environmental Technology Verification Program's Generic Protocol (NSF International, 2010), this category refers to organisms equal to or greater than 50 μm in minimum dimension, typically dominated in the Duluth-Superior Harbor of Lake Superior by rotifers, dreissenid veligers, copepod nauplii, cladocerans, and copepod copepodites. Sample assessments comprise determinations of the number of live and dead organisms present. Samples may also be archived for size measurements.

DEFINITIONS

Microzooplankton: The smaller-sized taxa groups of zooplankton within the $\geq$ 50 μm size class, including rotifers, copepod nauplii, and veligers. Microzooplankton are analyzed in a Sedgewick-Rafter Counting Chamber by examination under a compound microscope at a magnification of 40X to 100X.

Macrozooplankton: The larger-sized taxa groups of zooplankton within the $\geq$ 50 μm size class, including, copepods, cladocerans, and macroinvertebrates. Macrozooplankton are typically analyzed in a Ward's Counting Wheel at a magnification of 20 to 30X using a dissecting microscope, but may also be analyzed simultaneously with microzooplankton in a Sedgewick-Rafter Counting Chamber by examination under a compound microscope.

EQUIPMENT LIST

- Coolers with ice packs
- Filtration funnels and vacuum source
- 1 5, and 10 mL Henson-Stempel pipettes
- Sedgewick rafter slides with grid and cover slips for microzooplankton
- Ward's or Serpentine counting chambers for macrozooplankton
- Dissecting and compound microscopes
- Digital thermometer
- Digital camera with microscope adaptor
- Computer with software for measuring digital images
- Eyepiece micrometer and stage micrometer
- Folsom plankton splitter and bull's-eye level

SUPPLIES

- 1 L cod ends for plankton net
- 2 to 10 L containers for holding concentrated plankton samples
- 1 L containers for holding "filtered" harbor/sample water for use in processing samples
- Filters

- 50, 100, 500, and 1000 mL graduated cylinders
- 25, 50, 100, 250, 500, and 1000 mL beakers
- Forceps
- Probes
- Wash bottles with filtered sample (Harbor) water
- 10 % Lugol's preservative solution in a calibrated dispenser
- 5 % Lugol's solution in a dropper bottle
- Diluted solution of lab detergent (Liquinox) in wash bottle
- 50 % acetic acid (v/v) in a dropper bottle
- Fine tipped pipettes
- Paper towels
- Calculator
- 20 μm mesh concentrating cups
- 60 and 125 mL brown bottles to archive samples
- Containers for waste solutions
- Tally counters

PROCEDURES

I. Sample Preparation

1. Collect intake and discharge zooplankton samples in sample collection containers as described in *GSI/SOP/LB/RA/SC/1 – Procedure for Collecting Biological Sample Water via In-Line Sample Ports* and *GSI/SOP/LB/RA/SC/6 – Procedure for Zooplankton Sample Collection*, and as outlined in the Test/Quality Assurance Plan (TQAP) or Test Plan.

2. Ensure sample collection containers are appropriately sized to assure that the density of zooplankton in the container is no greater than 10,000 X ambient density. In general, 1 to 2 L collection containers are suitable for up to 10 m^3 of filtrate. Add filtered sample water to ensure that collection bottles are full.

3. Store samples in coolers and deliver to the onsite mobile laboratory as soon as possible.

4. Measure and record the temperature of the sample as soon as it arrives in the laboratory (see Appendix 1). Maintain this sample temperature within +/- 3 °C during the analysis period by placing the sample and dilution water in a cooler if ambient air temperature is different from the water temperature.

5. Prepare dilution and rinse water by passing the water that has been filtered by the plankton net during the zooplankton collection procedure through a 10 μm or smaller membrane filter to remove small organisms which will not be included in this analysis.

II. Analysis of Organisms in Pre-Treatment Intake and Control Discharge Samples

Note: *The density of live organisms in pre-treatment intake samples and control discharge samples will generally be very high, (i.e., >1,000,000 live organisms/m^3 in control intake samples and >1,000 live organisms/m^3 in control discharge samples) necessitating the examination of only a subsample of the sample concentrate that was collected.*

Procedure for Microzooplankton Analysis:

1. Adjustment of Sample Volume

a. Estimate the density of microzooplankton including rotifers, dreissenid veligers and copepod nauplii by stirring the sample in a figure "8" motion and withdrawing a 1 mL subsample with a Henson-Stempel pipette. Place the subsample in a Sedgewick Rafter counting chamber and examine under a compound microscope at a magnification of 40X. Count the number of organisms in one row of the slide and then calculate the density of organisms in the 1 mL subsample by multiplying the number of organisms per row by the number of rows in the chamber.

b. Adjust the sample volume to ensure there are between 100 and 200 organisms present in a single 1 mL subsample. If the sample is too concentrated, remove an aliquot (e.g., 10 to 100 mL) of the original sample with a 5 or 10 mL Henson-Stempel pipette and dilute with filtered Harbor water measured from a graduated cylinder. If the sample is too dilute, concentrate a subsample through a 20 μm mesh to obtain a suitable density. Record the volume of the aliquot and the dilution or concentration factors on the data sheet (Appendix 1).

2. Enumeration of Microzooplankton

a. To determine the actual density of live and dead microzooplankton in the sample, mix the sample thoroughly by stirring in a figure "8" motion, and then use a Henson-Stempel pipette to remove a 1 mL subsample from the original sample jar or prepared aliquot. Place the subsample in a Sedgewick Rafter cell with a coverslip and examine the entire slide under a compound microscope at 40X.

b. Examine the entire slide, counting only dead microzooplankton at this time. Dead organisms are those which show no signs of movement or heartbeat. Identify the rotifers to major taxonomic group and record the number of dead organisms of each group on the data sheet (Appendix 1).

c. Carefully slide the cover of the slide open and add 1 or 2 drops of 50 % acetic acid solution to the slide. Let the slide sit for a few minutes in order to kill all

organisms, then count the total number of organisms of each group on the slide. Record this number on the data sheet.

d. Continue to count up to four slides or at least 300 organisms, recording the values for each slide separately.

e. Complete analyses within two hours of sample collection

f. Rinse the sample from the slide into a labeled brown storage bottle.

g. Rinse the slides and cover slips well and dry with a paper towel prior to reuse.

h. Calculate the number of live organisms by subtracting the number of dead organisms in the original count from the number of total organisms on the slide.

Procedure for Macrozooplankton Analysis: Adjustment of Sample Volume

1. Adjustment of Sample Volume

a. Estimate the density of macrozooplankton including crustacean zooplankton in the sample by stirring the sample in a figure "8" motion and extracting a 1 mL subsample from the original sample.

b. Examine the subsample in a Ward's or Serpentine counting chamber and count the number of crustaceans in the subsample. Use this density estimate to determine whether the original sample will need to be concentrated or diluted to obtain approximately 100 to 200 organisms per 1 to 5 mL subsample. If the sample volume is adjusted, record the aliquot size and dilution or concentration factors on the data sheet (Appendix 1).

2. Enumeration of Macrozooplankton

a. To determine densities of live and dead macrozooplankton, mix the sample thoroughly by stirring in a figure "8" motion, and then use a Henson-Stempel pipette to transfer 1 to 5 mL of the sample to a counting chamber. Select a sample volume that will ensure that there are between 100 and 200 organisms in the chamber. Record the volume used on the data sheet.

b. Initially determine the density of dead macrozooplankton in the chamber. Carefully examine the macrozooplankton and identify to major taxonomic group. Count those organisms with no reactive movement or heartbeat as dead. After each chamber is counted, record the number of dead organisms of each major taxonomic group on the data sheet (Appendix 1).

c. Add a few drops of diluted Lugol's (5% solution) to the chamber to kill the organisms and some diluted lab detergent to minimize the occurrence of

floating organisms. Let the chamber sit for a few minutes to ensure that all organisms are dead.

d. Examine the counting chamber again and count the total number of organisms in each taxonomic group in the chamber. Record this information on the data sheet (Appendix 1).

e. Continue to count up to four subsamples until approximately 300 - 400 total organisms have been counted. Be sure to count the entire chamber for each subsample and not stop until a total of 300 is reached.

f. Complete analyses within two hours of sample collection.

g. Rinse the counted samples into the labeled brown storage bottle.

h. Rinse the counting chambers well and dry with paper towels prior to reuse.

i. Calculate the number of live organisms in the subsample by subtracting the number of dead animals in the original count from the number of total organisms in the chamber

Final Measurement of Sample Volume and Sample Storage

1. Measure the volume of sample left in the original sample container after all the subsamples have been removed and counted. If a subsample has been taken from a larger container, be sure to measure the volume of both the subsample and the remaining sample.

2. Calculate the total volume of the original sample by adding these values to the total volume of sample that has been removed for the rotifer and crustacean analyses. Record on the data sheet in Appendix 1.

3. Concentrate each sample to approximately 40 mL to 100 mL and add to the labeled, 60 mL or 125 mL brown storage bottle and preserve with 10 mL of Lugol's (10 % solution).

III. Analysis of Organisms in Post-Treatment Intake and Treatment Discharge Samples

Note: *If the treatment method is effective, there will generally be very few live organisms present in the post-treatment intake and treatment discharge samples (ideally < 10 live organisms/m^3) and the equivalent of an entire cubic meter of discharge water should be examined for the presence of live animals.*

Subsample Preparation:

1. Split the plankton sample in half, with half to be examined for macrozooplankton and the other half examined for both micro- and macrozooplankton (both micro- and macrozooplankton can be quantified using a compound microscope at 40X, while only macrozooplankton can be quantified using a dissecting microscope; see Figure 1). Stir the plankton sample carefully and then pour it into the Folsom plankton splitter, which has been leveled using the bull's eye level. Gently stir the sample in the splitter, and then split into halves. Concentrate each subsample through a 20 μm mesh filter to approximately 500 mL.

2. Examine 1 mL of the concentrated split sample on a Sedgewick-Rafter slide (micro- and macrozooplankton; Figure 1) or in a counting chamber (macrozooplankton; Figure 1). If more than two live organisms are observed in the counting chambers, continue the analysis as directed above in Section II for pre-treatment intake/control discharge samples.

3. If the density of live organisms is less than 2 per mL, and there is not a lot of detritus in the sample, continue to concentrate the subsamples down to 10 to 100 mL, depending on the amount of detritus. Take care not to concentrate the sample to a level where the detritus will obscure the living organisms during the analyses.

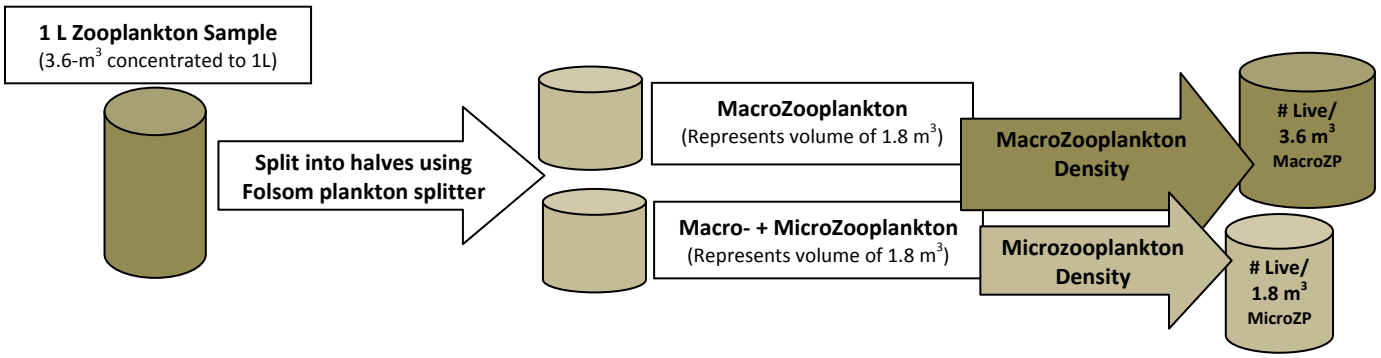

Figure 1. Process for Analysis of Post-Treatment Intake and Treatment Discharge Zooplankton Samples. Sample volumes of up to 3.6 m^3 may be collected in each sample collection tub. The sample is concentrated to a 1-L cod end and split for analysis of macro- and microzooplankton (first half) and macrozooplankton only (second half).

Microzooplankton and Macrozooplankton Analysis:

1. Mix the concentrated subsample thoroughly by stirring in a figure "8" motion, and then use a Henson-Stempel pipette to remove a 1 mL subsample. Place the subsample in a Sedgewick rafter cell with a coverslip and examine the entire slide under a compound microscope at 40X.

2. Record the presence of all live organisms (macro- and/or microzooplankton) on the data sheet (Appendix 1).

3. Ensure that a second observer verifies each live organism and that the information is recorded on a separate data sheet (see Appendix 2)

4. Capture and save a digital image of each small organism (i.e., those < 75 μm) showing the minimum visible dimension so that measurements can be made to verify that the minimum dimension was greater than 50 μm.

5. Continue to examine 1 mL at a time until the entire subsample has been analyzed, or until two hours have elapsed from the time of sample collection. Note: Additional analysts may be employed to analyze a larger volume of discharge water.

6. Measure and record the volume of subsample analyzed if the entire subsample was not analyzed (Appendix 1). Measure and record the volume of subsample remaining (Appendix 1). The total sample volume is the sum of these two volumes.

7. Examine additional sample water from another sample collection tub if the entire subsample was analyzed in less than two hours. This will increase the accuracy of the estimates of live density.

Macrozooplankton Analysis:

1. Mix the concentrated subsample thoroughly by stirring in a figure "8" motion. Use a Henson-Stempel pipette to transfer a 1 to 5 mL subsample to a counting chamber. Examine the entire chamber on a dissecting microscope.

2. Record the presence of any live organisms on the data sheet (Appendix 1).

3. Ensure that a second observer verifies each live organism and that the information is recorded on a separate data sheet (Appendix 2).

4. Continue to examine 1 mL at a time until the entire subsample has been analyzed, or until two hours have elapsed from the time of sample collection. Note: Additional analysts may be employed to analyze a larger volume of discharge water.

5. Measure and record the volume of subsample analyzed if the entire subsample was not analyzed (Appendix 1). Measure and record the volume of subsample remaining (Appendix 1). The total sample volume is the sum of these two volumes.

6. Examine additional sample water from another sample collection tub if the entire subsample was analyzed in less than two hours. This will increase the accuracy of the estimates of live density.

Note: When calculating total density of macrozooplankton in the post-treatment intake or treatment discharge samples, be sure to add the density of live macrozooplankton counted in both the micro- and the macrozooplankton counts (Figure 1).

IV. Determination of Zooplankton Size Distributions

Note: *Use a 35 µm mesh net to collect and concentrate the zooplankton samples. Organisms smaller than 50 µm in minimum dimension should pass through the net, assuring that the majority of the zooplankton in the sample are greater than 50 µm. Samples will be archived so that measurements can be taken later* ***if necessary*** *to determine the actual size distribution of the zooplankton taxa that may have individuals smaller than 50 µm in minimum visible dimension.*

1. If measurements of certain taxa are necessary, conduct these measurements at a magnification of 100X using the eyepiece micrometer of the microscope or, take digital images of the organisms at a magnification of 100X and determine minimum visible dimensions from the images using a calibrated software program.

2. Calculate the density of live organisms in each size category (> or < 50 µm in minimum dimension) by multiplying the density of live organisms in the sample by the proportion of organisms in each size category.

V. Procedure for Zooplankton Analysis Data Entry

1. Enter zooplankton analysis data as soon as possible following analysis activities into the GSI Zooplankton Database following *GSI/SOP/G/RA/DM/1 – Procedure for Data Entry, Data Quality Control, and Database Management*.

2. For final calculations of zooplankton density, do not enter zooplankton that are on the list of potential facility contamination sources, and should be categorically excluded from the reported zooplankton density (Appendix 3). The zooplankton on this list are counted, and reported on the zooplankton analysis datasheet (Appendix 1), but they are not entered into the GSI Zooplankton Database and are not counted for reporting purposes.

3. Check data entered into the GSI Zooplankton Database for accuracy against the original, raw data, keeping in mind those organisms that are potential facility contamination sources and were not entered into the GSI Zooplankton Database (Appendix 3).

QUALITY ASSURANCE/QUALITY CONTROL (QA/QC)

1. Conduct all QA/QC procedures according to *GSI/QAQC/QAPP/LB/1 - Quality Assurance Project Plan for Great Ships Initiative (GSI) Land-Based Tests (2011).*

2. Follow all procedures outlined in this SOP. Any deviations known ahead of time must be approved by the GSI PI and communicated to the GSI Senior QAQC Officer. Any deviations made during the experiment must be recorded, communicated to the GSI Senior QAQC Officer, and also approved by the GSI PI as soon as practicable.

3. Record data on data collection forms or in specific laboratory notebooks. All instrument data output and data forms must be stored in a project-specific three-ring binder. Ensure hard copies of all raw data (e.g., data output and data collection forms) collected during treatment technology performance evaluation/certification testing are scanned and stored electronically on the LSRI secured Local Area Network (LAN). The requirement for a backup, electronic copy of raw data is implemented only during treatment technology certification testing at the GSI Land-Based RDTE Facility.

4. Ensure that a second analyst counts at least 10 % of control and treatment intake, and control discharge samples in duplicate to provide consistency and replicability of assessment methods and taxonomy. If an experimental trial has less than 10 samples, at least one sample from each trial should be analyzed in duplicate.

a. Perform QA analysis on one of the microzooplankton subsamples/slides and one of the macrozooplankton subsamples/counting chambers for the selected QA sample. Samples are counted while still in the original counter chambers.

5. For treatment discharge samples, a second analyst must examine at least one of every ten microzooplankton slides and at least one of every ten macrozooplankton counting chambers to ensure that all live organisms have been enumerated.

6. To meet QAQC standards, densities of dead and total organisms and measurement data calculated by each analyst must have at least 90 % similarity (PSC) and less than 20 % relative percent difference (RPD). Percent similarity and relative percent difference are calculated using the following formulas:

$$\boldsymbol{PSC = \left(1 - 0.5 \sum_{i=1}^{K} |a_i - b_i|\right) \times 100\%}$$

where:

where a_i **and** b_i **= the relative proportions of species *i* in the sample found by operator *A* and *B*, respectively**

$$\mathbf{RPD} = \left(\frac{(|x_1 - x_2|)}{\frac{x_1 + x_2}{2}}\right) * \mathbf{100}\ \%$$

where:

x_1**= sample result**
x_2 **= duplicate sample result**

DATA STORAGE AND ARCHIVING

1. Store and archive original, raw data according to *GSI/QAQC/QAPP/LB/1 - Quality*

Assurance Project Plan for Great Ships Initiative (GSI) Land-Based Tests (2011).

2. Store and archive electronic data (e.g., scanned copies of original, raw data and/or data entered for analysis) by posting the data distribution files to the LSRI LAN in an organized hierarchical folder system such that the entire LSRI-GSI staff are able to recognize and access the data. Distribute data files via email, when needed, to the applicable GSI team members who do not have access to the LSRI LAN.

3. Archive all hard- and electronic-copies of data and records for a period of at least years.

REFERENCES AND RELATED DOCUMENTS

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

GSI/FORM/LB/A/4 - Zooplankton Identification Datasheet – Live/Dead Count

GSI/FORM/LB/A/5 - Zooplankton Verification of Live Organisms

GSI/QAQC/QMP/1 – Great Ships Initiative Quality Management Plan (Revision 2; 2011).

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

GSI/SOP/G/RA/DM/1 – Procedure for Data Entry, Data Quality Control, and Database Management.

GSI/SOP/G/RA/SC/2 – Procedure for Labeling Samples Collected at the GSI Land-Based RDTE Facility.

GSI/SOP/LB/G/O/1 – Procedure for Operating the GSI Land-Based RDTE Facility.

GSI/SOP/LB/RA/SC/1 – Procedure for Collecting Biological Sample Water via In-Line Sample Ports.

GSI/SOP/LB/RA/SC/6 – Procedure for Zooplankton Sample Collection.

GSI/SOP/LB/RA/SC/7 – Procedure for Preparing Lugol's Solution.

Great Ships Initiative website: www.greatshipsinitiative.org.

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

IMO (2004). International Convention for the Control and Management of Ships Ballast Water and Sediments. As adopted by consensus at a Diplomatic Conference at IMO, London, England, February 13 2004.

NSF International (September, 2010). Generic Protocol for the Verification of Ballast Water Treatment Technology. Version 5.1. EPA/600/R-10/146. Produced for the USEPA, Environmental Technology Verification Program in Conjunction with U.S. Coast Guard, Environmental Standards Division and U.S. Naval Research Laboratory, Center for Corrosion Science and Engineering. NSF International, Ann Arbor, Michigan.

Appendix 1. Zooplankton Identification Datasheet – Live/Dead Count (GSI/FORM/LB/A/4)

Zooplankton Identification Datasheet – Live/Dead Count

Sample Identification _____ Date Collected _____ Time Sample Received _____

Test Scenario _____ Time Sample Completed _____

Water Temp _____ Container Volume _____

Vol. Rotifer Aliquot (mL) _____ Vol. Crustacean Aliquot _____ Rotifer Analyzed by _____

Diluted to (mL) _____ Concentrated to (mL) _____ Add. mL live rotifers ___

Subsample size (mL) _____ Subsample size (mL) _____ Crustacean analyzed by _____

Total mL counted rotifers _____ Total mL counted Crust. _____ Add mL live crustaceans ____

ORGANISM	Subsample 1			Subsample 2			Subsample 3			Subsample 4 or QA		
	#Dead	Total	Live	#Dead	Total	Live	#Dead	Total	Live	#Dead	Total	Live
MicroZooplankton												
Rotifers												
Keratella												
Polyarthra												
Synchaeta/ Conochilus												
Other												
Dreissenid (zm)												
Nauplii												
MacroZooplankton												
Cladocerans												
Daphnia												
Bosmina												
Chydoridae												
Holopedium												
Other Cladocerans												
Copepods												
Other Organisms												

Additional Notes:

Appendix 2. Zooplankton - Verification of Live Organisms (GSI/FORM/LB/A/5)

Appendix 2. Documentation of Live Zooplankton found During Treatment Discharge

Sample Identification _____Date Collected _____

Macrozooplankton or Microzooplankton count (circle) Total volume analyzed from sample _____

	Name of organisms found	Identified by	Confirmed by	Photo # if applicable	Minimum length
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					
15					
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30					

Notes:

APPENDIX 3. List of Zooplankton Categorically Excluded as Facility Contamination Sources

The following zooplankton are excluded as facility contamination sources:

1. Nematodes
2. Lecanid Rotifers (including Lecane and Monostyla)
3. Bdelloid Rotifers
4. Harpacticoid copepods