Final Report of the Land-Based, Freshwater Testing of the AlfaWall AB PureBallast® Ballast Water Treatment System

March 17, 2011

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EXECUTIVE SUMMARY

The Great Ships Initiative (GSI) provides independent, no-cost performance verification testing services to developers of ballast treatment systems and processes at a purpose-built, land-based ballast treatment test facility located in the Duluth-Superior Harbor of Lake Superior (Superior, WI). GSI test protocols are consistent with the requirements of the International Maritime Organization's International Convention for the Control and Management of Ships Ballast Water and Sediments (IMO, 2004) and the United States Environmental Protection Agency's (USEPA's), Environmental Technology Verification Program (ETV; NSF, 2010). GSI procedures, methods, materials and findings are also publicly accessible on the GSI website (www.greatshipsinitiative.org).

In August through October 2010, GSI conducted freshwater, land-based tests on three versions of the AlfaWall PureBallast® ballast water treatment system (BWTS). One version (hereafter referred to as v.1) of the PureBallast® BWTS received Type Approval by Det Norske Veritas (DNV) on behalf of the Norwegian Administration in June of 2008, following successful land-based testing at the Norwegian Institute of Water Research (NIVA). The second version (v.2), designed to conserve power relative to the first, was still undergoing IMO certification testing, and had completed successful land-based tests at NIVA immediately prior to testing at GSI during early summer 2010. The third version was a hybrid of versions 1 and 2, hereafter referred to as version 3 (v.3). The BWTS v.3 combined the 40 μm filtration of PureBallast® BWTS v.2 with the advanced oxidation system of PureBallast® BWTS v.1.

The GSI Test Plan for the AlfaWall PureBallast® BWTS, hereafter referred to as the GSI Test Plan, called for evaluation the PureBallast® BWTS v.2 consistent with IMO G8 and G9 guidelines for its ability to: (a) successfully treat ballast water without interruption, (b) meet IMO D-2 discharge standards after a five day holding time, and (c) discharge water after the five day retention period that is environmentally benign (i.e., no residual toxicity). Additional research and development testing of v.1 was also planned. However, the PureBallast® BWTS (both v.1 and v.2) encountered mechanical filter failures such that no valid trials (i.e. meeting IMO and ETV threshold conditions) were completed. Instead, GSI tested the hybrid version of the AlfaWall BWTS (v.3) under a set of GSI source water conditions less challenging than those required by IMO and ETV, strictly for research and development purposes. As an addition to the research and development trials of the PureBallast® BWTS v.3 at the GSI Land-Based RDTE Facility, a set of observations on living organisms in sample water 24 hours post discharge from treatment and control retention tanks was incorporated into the revised test protocol to detect any delayed effects of the BWTS.

The PureBallast® BWTS v.3 performed without interruption during the first two trials under less challenging conditions than required by IMO and ETV. During the third and final trial, the PureBallast® BWTS v.3 encountered a filter failure, and the trial was stopped and restarted under ambient Duluth-Superior Harbor conditions. No residual toxicity was detected in the discharge waters of the PureBallast® BWTS v.3. The BWTS did not effectively reduce live organism densities in the two regulated size classes despite the fact that ambient densities were well below IMO and ETV testing intake thresholds. Part of the problem likely resided with filter

ineffectiveness given filamentous algal forms in Duluth-Superior Harbor water. At the same time, very low ambient UV transmittance of Duluth-Superior Harbor water (naturally caused by tannins) during these tests likely impeded effectiveness of the advanced oxidation system. These two conditions also likely account for discrepancies between performance outcomes at GSI versus NIVA. Globally, the risk of very low UV transmittance conditions is not unique to Duluth-Superior Harbor, but it is relatively rare and can be anticipated in advance. Thus, this problem could be mitigated with management practices such as open ocean BWE in combination with treatment. Conditions present in Duluth-Superior Harbor likely leading to filter malfunction, on the other hand, may be relatively common to many fresh water and brackish water harbors.

ACKNOWLEDGMENTS

The authors would like to express our sincere gratitude to the Great Ships Initiative (GSI) Advisory Committee which provides invaluable input to the GSI Principal Investigator. We also wish to thank the ten United States and Canadian Great Lakes Ports which launched the GSI, and the Great Lakes Protection Fund which supported the initial scoping exercise. We sincerely thank the United States Department of Transportation, Maritime Administration, and National Oceanic and Atmospheric Administration for their substantial financial and in-kind support for the construction of the state-of-the-art GSI land-based testing facility. We thank the United States and Canadian St. Lawrence Seaway organizations, the Legislative Citizens Commission on Minnesota Resources, the University of Wisconsin-Superior, and the City of Superior for their active financial and/or in-kind support for GSI operations.

1.0. INTRODUCTION

In September and October 2010, the Great Ships Initiative (GSI) conducted land-based tests on three versions of the AlfaWall AB PureBallast® BWTS (i.e., v.1, v.2, and v.3). The GSI Test Plan (Appendix 1) called for evaluation of the PureBallast® BWTS v.1 and v.2 consistent with International Maritime Organization (IMO) G8 and G9 guidelines for their ability to: (a) successfully treat ballast water without interruption, (b) meet IMO D-2 discharge standards after a five day holding time, and (c) discharge water after the five day retention period that is environmentally benign (i.e., no residual toxicity) pursuant to United States Environmental Protection Agency (USEPA) water quality criteria. However, the PureBallast® BWTS (both v.1 and v.2) encountered mechanical filter failures such that no valid trials (i.e. meeting IMO and ETV threshold conditions) were completed. Instead, GSI tested a hybrid version of the AlfaWall BWTS (v.3) under a set of GSI source water conditions less challenging than those required by IMO and ETV, strictly for research and development purposes.

1.1. 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, GSI has established superlative freshwater ballast treatment evaluation capabilities at three scales—bench, land-based, and on board ship.

GSI awards its independent status-testing services to developers of ballast water treatment systems (BWTSs) and processes determined to be promising. GSI status-testing is performed at the scale appropriate to the state of development of the target treatment system, with the goal of facilitating the rapid progression of meritorious BWTSs through the research, development, and approval processes to a market-ready condition.

GSI has no involvement, intellectual or financial, in the mechanics, design or market success of the actual treatment systems it tests. To ensure that GSI tests are uncompromised by any real or perceived individual or team bias relative to test outcomes, GSI test activities are subject to rigorous quality assurance and quality control (QAQC) procedures and documentation (GSI, 2010a and 2010b). This QAQC attention also assures high quality and credible evaluation findings.

GSI has worked to standardize and calibrate its protocols to evaluate the performance of BWTSs with IMO guidelines, USEPA Environmental Technology Verification (ETV) protocol, and other test facilities. GSI test protocols are as consistent with the requirements of the IMO Convention for the Control and Management of Ships Ballast Water and Sediments (IMO, 2004) and United States federal requirements (NSF, 2010) as practicable. In particular, GSI testing directly supports IMO G8 and G9 evaluations. GSI procedures, methods, materials and findings are also not proprietary, and are accessible on GSI's public website: www.greatshipsinitiative.org.

1.2. The AlfaWall AB PureBallast® Ballast Water Treatment System

AlfaWall AB of Tumba, Sweden, is a joint venture company of Alfa Laval AB and Wallenius Water AB. Together these two companies have developed the PureBallast® BWTS. The PureBallast® BWTS involves filtration using a 40 or 50 μm screen filter, implemented during ballast uptake operations only, followed by a patented advanced oxidation treatment (Wallenius AOT™) involving ultraviolet (UV) radiation and a catalyst. The Wallenius AOT™ is the main stage of treatment and is applied during both ballasting and deballasting. The PureBallast® BWTS AOT can be scaled by connecting one to ten components in parallel to achieve flow rates between 250 and 2500 m^3/hr; the capacity of each component is 250 m^3/hr.

The original PureBallast® BWTS version (hereafter referred to as v.1) received Type Approval Certification by Det Norske Veritas (DNV) on behalf of the Norwegian Administration on June 27, 2008. This version entailed filtration at 50 μm and AOT using 20 UV lamps per one AOT reactor. The filter is cleaned using automatic back flushing, and is bypassed during deballasting operations. The PureBallast® BWTS v.1 is commercially available and to date there have been approximately 80 systems sold to a large variety of ship owners and for many different types of vessels, e.g., car carriers, Ro-Ros, container carriers, bulk carriers, general cargo carriers, drilling vessels, supply vessels, LPG tankers, bitumen tankers, etc.

The second more energy efficient version (v.2) was undergoing IMO Type Approval evaluation at the time of the GSI testing reported here, and the GSI tests were to become part of the system's land-based testing portfolio (see Test Plan in Appendix 1). The same prototype unit subjected to evaluation at GSI had been subjected to land-based testing in salt and brackish water at the NIVA test facility in Norway immediately prior to shipment to GSI. This version, modified from the PureBallast® BWTS v.1 to enhance energy efficiency, entailed automatic backflush filtration during ballasting at either 40 μm or 50 μm, combined with 12 lamps per one AOT reactor. Like the PureBallast® BWTS v.1, the filtration system is bypassed during deballasting.

The third version (v.3), ultimately subjected to the GSI testing reported here, is a hybrid of versions 1 and 2. This version combined the 40 μm automatic backflushing filter of PureBallast® BWTS v.2 with the 20-lamp AOT reactor of PureBallast® BWTS v.1. Again, filtration was performed during uptake only.

1.3. Treatment Performance Requirements in Regulation D-2

The International Convention for the Control and Management of Ships Ballast Water and Sediments was adopted by consensus at a Diplomatic Conference at IMO in London on February 13, 2004. Annex D-2 of the Convention relates to ballast water performance standards for ships conducting ballast water management, including use of a BWTS to effectively treat the ballast water. The regulation states that ships conducting ballast water management shall discharge:

- Less than 10 viable organisms per m^3 greater than or equal to 50 μm in minimum dimension;

- Less than 10 viable organisms per mL less than 50 μm in minimum dimension and greater than or equal to 10 μm in minimum dimension; and
- Discharge of the indicator microbes shall not exceed the specified concentrations. The indicator microbes, as a human health standard, include, but are not be limited to:
  - Toxicogenic *Vibrio cholerae* (O1 and O139) with less than 1 colony forming unit (cfu) per 100 mL or less than 1 cfu per 1 gram (wet weight) zooplankton samples;
  - *Escherichia coli* - less than 250 cfu per 100 mL;
  - Intestinal *Enterococci* - less than 100 cfu per 100 mL.

1.4. Relationship of GSI Testing to G8 and G9 Requirements in IMO Convention

The fundamental approach of GSI is to conduct independent, scientifically-sound, rigorous, and quality assured evaluations of BWTSs. At the same time, GSI seeks immediate relevance of its freshwater, land-based testing to regulatory processes such as those outlined in the IMO Convention and those under development domestically in the United States and Canada. To that end, GSI protocols are rooted in the essential features of the IMO G8 guidelines for testing, and the USEPA ETV protocols. All aspects of the testing infrastructure (e.g. flow rate, retention tank size, sample size, sample collection, analysis equipment and data logging) are directly consistent with these requirements. GSI also formally partners with the Maryland-based Maritime Environmental Resource Center (MERC), and other test facilities to assure that GSI freshwater land-based testing can be directly complemented by comparable brackish/salt water testing.

With respect to physical/chemical and biological characteristics of the intake stream, GSI is fortunate in that its feed water source (i.e., the Duluth-Superior Harbor of Lake Superior) naturally meets many of the IMO G8 and USEPA ETV requirements for intake organism densities and physical/chemical conditions during the testing season (June to October, see Table 1). For those parameters that often do not naturally meet the IMO G8 and USEPA ETV requirements (i.e., total suspended solids, mineral matter, particulate organic carbon, and phytoplankton), GSI has the ability to augment intake water to achieve recommended IMO/ETV parameter levels (Table 1). IMO and ETV consistent tests at GSI tests are only conducted when parameters that may occasionally fall below the challenge water requirements (i.e., zooplankton and heterotrophic bacteria) are sufficiently high. In addition, GSI will not conduct tests involving a UV system when DOCs, which are naturally high in Duluth-Superior Harbor, exceed 20 mg/L, though no ceiling is indicated in IMO or ETV guidelines. GSI conducts and documents frequent monitoring of water chemistry and biology to predict valid run conditions for GSI, IMO G8 and USEPA ETV performance evaluation/certification test trials.

Table 1. Comparison of USEPA ETV and IMO G8 Recommended Challenge Conditions to Ranges of Various Physical/Chemical and Biological Parameters in Ambient Water from Duluth-Superior Harbor (June – October).

Parameter	USEPA ETV[1]	Recommended IMO G8[2]	Duluth/Superior Harbor Ambient Ranges
Temperature (^oC)	4 – 35	–	4 - 30
Salinity (ppt)	< 1	Two salinities, >10 ppt difference	0 – 1
Total Suspended Solids (mg/L)	Min. 24	> 50	< 1 – 40
Mineral Matter (mg/L)	Min. 20	No Requirement	<1- 40
Particulate Organic Carbon (mg/L)	Min. 4	> 5	< 0.1 – 3
Dissolved Organic Carbon (mg/L)	Min. 6	> 5	6 – 30
Transmittance at 254 nm (%)[b]	No Requirement	No Requirement	14.0 – 68.5
Zooplankton (> 50 $\mu m/m^3$)	Min. 100,000	> 100,000	100,000 - 3,000,000
Phytoplankton (10 - 50 $\mu m/mL$)	Min. 1000	> 1,000	25 – 4,500
Heterotrophic Bacteria (MPN[a]/mL)	Min. 1000	> 10,000	100 - 10,000

[a]MPN = Most Probable Number
[b]Measured on filtered Duluth-Superior Harbor water samples (May 2009 to October 2010).

2.0. METHODS

The following section describes how each physical, chemical and biological parameter and variable was sampled and analyzed during the PureBallast® BWTS trials at GSI. Additional details on GSI's standard operating procedures (SOPs) can be found at www.greatshipsinitiative.org. All SOPs relevant to the PureBallast® tests, as amended, also are listed by analysis category in Appendix 3. Any deviations from these SOPs during the performance of the PureBallast® tests were minor and did not affect data quality. More detail on these deviations is available upon request.

1 U.S. Environmental Protection Agency (USEPA), Environmental Technology Verification Program. Generic Protocol for the Verification of Ballast Water Treatment Technologies. Version 5.1. September, 2010.
2 IMO MEPC 57, Annex 3: Revised Guidelines for Approval of Ballast Water Management Systems (G8). April 4, 2008.

2.1. Experimental Design and Set-Up

The GSI Test Plan (Appendix 1) for tests on PureBallast® v.1 and v.2 was consistent with IMO G8 and USEPA ETV requirements for land-based testing in freshwater. As such, the GSI test facility was fully validated and prepared to conduct two consecutive sets of five, five-day tests (starting with PureBallast® v.2) contrasting treated discharge and control discharge meeting IMO and ETV testing challenge condition thresholds and quality assurance rigors. GSI began with intake water amended with total suspended solids (TSS), particulate organic carbon (POC), and phytoplankton to assure that all tests met challenge water conditions in the IMO G8 guidelines throughout the trial series (see Appendix 1 for GSI Test Plan and Appendix 2 for details on the PureBallast® v.1 and v.2 commissioning trials). However, due to filter failures during the PureBallast® v.2 commissioning (see Appendix 2), the GSI tests were conducted on a PureBallast® v.3, using challenge water augmented with TSS and phytoplankton but at lower concentrations (see §2.1.2., Table 4) than described in the original GSI Test Plan (Appendix 1). The GSI PureBallast® Test Plan (Appendix 1) was revised and down-sized to a set of three, 48-hour trials with the goal of research and development testing rather than IMO land-based certification testing.

The research and development performance evaluation of the PureBallast® BWTS v.3 involved physical, chemical, and biological characterization of water upon ballasting (uptake/intake of water), as well as, enumeration, sizing, and live/dead analysis of organisms in control and treated discharge water after a two-day, in-tank holding time. In addition, to detect any delayed mortality effects associated with the AOT, live/dead analysis of zooplankton was conducted after holding control and treatment discharge water in collection tubs overnight, with analyses conducted the next morning. The objective of the performance evaluation trials was not to compare the treatment discharge to the IMO or ETV standards, but to compare the control and treatment discharge in order to gauge the relative effectiveness of the PureBallast® BWTS v.3. Table 2 shows the schedule of the three tests, including the sequence of intake operations (simultaneous control and treatment) and discharge operations (sequential, treatment then control).

Table 2. Timing of Intake and Discharge Operations, and Sample Collection Times for the Three PureBallast® v.3 Ballast Water Treatment System Trials at the GSI Land-Based Facility in 2010.

Trial	GSI Test ID	Treatment	Timing of Operation					
			Intake		Discharge		Delayed Mortality Sample Drain	
A	10-A3-1	Treatment	27 September	12:17-13:03	29 September	10:03-10:48	30 September	08:15-08:39
		Control				12:38-13:24		09:45-10:08
B	10-A3-2	Treatment	28 September	10:19-11:06	30 September	10:31-11:15	01 October	08:30-08:52
		Control				12:48-13:34		10:00-10:25
C	10-A3-3	Treatment	01 October	10:45-11:06; 11:55-12:19	03 October	10:14-10:58	04 October	09:00-09:23
		Control				12:03-12:49		10:15-10:39

2.1.1. The GSI Land-Based Research, Development, Testing, and Evaluation (RDTE) Facility

GSI tests reported here evaluated the biological efficacy of PureBallast® BWTS at GSI's purpose-built, Land-Based Research, Development, Testing, and Evaluation (RDTE) Ballast Treatment Test Facility located in Superior, WI in the Duluth-Superior Harbor of Lake Superior (Figures 1-3). Key features of the facility include:

- Four x 200 m^3 matched retention tanks with internal agitation for experimental water;
- Matched control and treatment intake flows up to 341 m^3/hour;
- Highly automated flow and pressure control, monitoring and data logging;
- A freshwater estuary with plentiful aquatic life as a water intake source;
- Capacity to augment intake water to intensify challenge conditions;
- Semi-automated and validated facility sanitation prior to trials;
- High quality in-line or in-tank sampling and/or spiking;
- On-site laboratory space for live analysis of organisms ≥10 and <50 μm and ≥ 50 μm size classes;
- Capacity to test treatment systems that operate on intake, discharge, in-tank, or combinations thereof;
- Off-site whole effluent toxicity (WET) testing; and
- Easy plug-in connections for treatment systems.

Figure 1. Location of the GSI Land-Based RDTE Facility in Superior, Wisconsin.

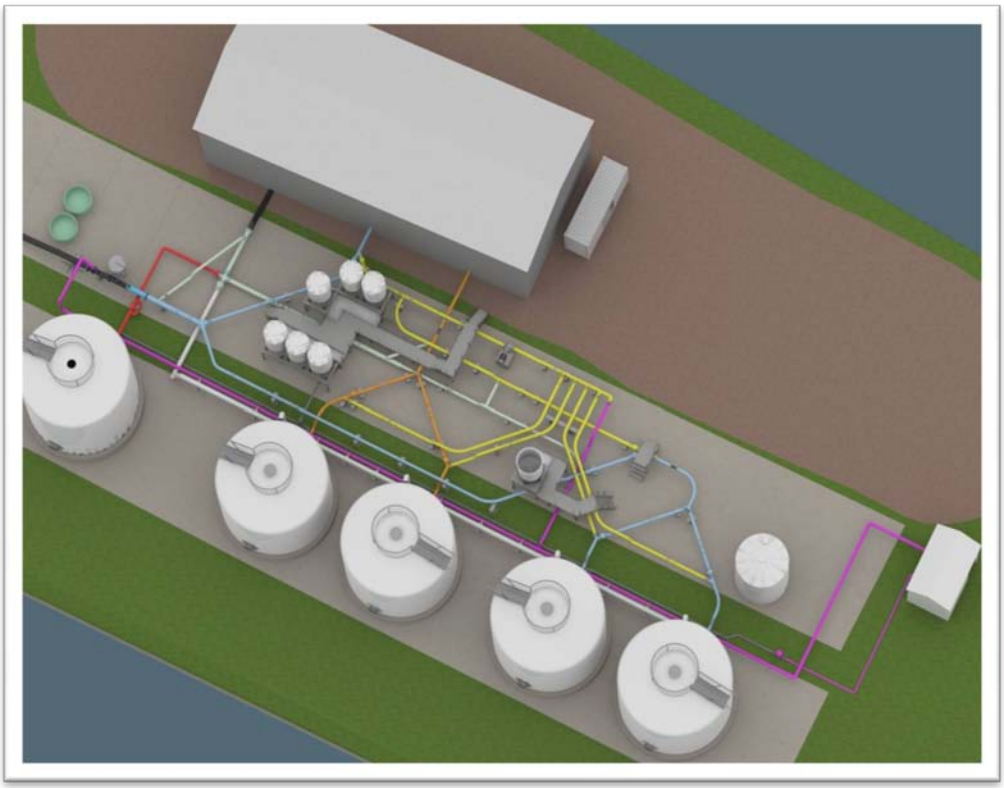

Figure 2. Computer-Generated Rendering of the GSI Land-Based RDTE Facility.

Figure 3. Photo of the GSI Land-Based RDTE Facility.

GSI's Land-Based RDTE Facility draws raw intake water from Duluth-Superior Harbor at rates from 400 m^3/hr to 680 m^3/hr. This main flow of intake water can be augmented with solids and/or phytoplankton just prior to being split into control and treatment tracks (see injection points A and B; Figure 4).

A Y-split in the intake piping simultaneously channels one half of the flow (200 m^3/hr to 340 m^3/hr) to a treatment track and the other half (also 200 m^3/hr to 340 m^3/hr) to a matched control track (Figure 4). The treatment track directs water through the experimental BWTS and into a 200 m^3 cylindrical treatment retention tank (Figure 4). The control track by-passes the treatment system and channels water directly into a matched control retention tank (Figure 4).

After a retention period, water is discharged sequentially from the treatment and control retention tanks at 200 m^3/hr to 340 m^3/hr. The water is directed either back to the harbor, to a 260 m^3 wastewater storage tank for subsequent discharge to the sewer, neutralization, or circulated to a second set of matched facility retention tanks (Figure 4).

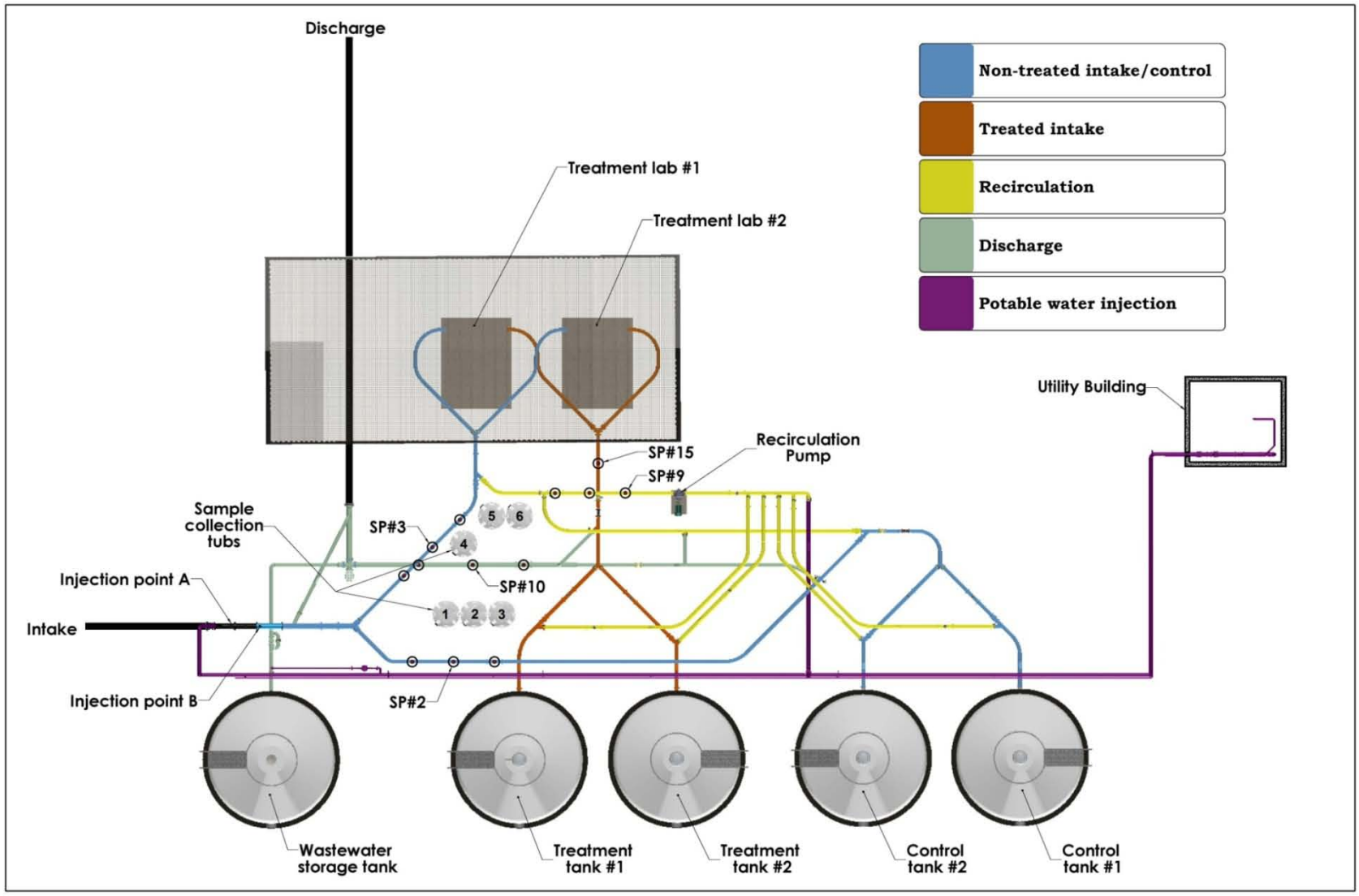

Figure 4. Simplified Schematic of the GSI Land-Based RDTE Facility Showing Location of Sample Points, Sample Collection Tubs, Injection Points, Retention Tanks, and Treatment and Control Tracks. Note: Main intake and discharge lines are coded black.

Water is sampled continuously throughout ballasting operations (i.e., intake, recirculation or discharge) through in-line sample points (SPs). Intake sampling takes place at paired intake sample points (SP#2 and SP#3) on the control and treatment tracks, respectively, and immediate post-treatment sampling occurs at SP#15 (Figure 4). Typically, discharge biological sampling is conducted at SP#9, with samples for water quality analysis collected at SP#10 (Figure 4), although these can be reversed as required by test design. All these SPs, with the exception of SP#15, consist of three identical sample ports spaced at regular intervals in a length of straight pipe consistent with IMO guidelines. One sample port is used at SP#15. Each port is fitted with a center-located, elbow-shaped pitot tube (90^{o}) which samples the water (Figure 5). This pitot design is based on a design developed and validated analytically by the U.S. Naval Research Laboratory in Key West, Florida. The design and lay-out of these replicate sample ports was also validated empirically at GSI, and shown to produce equivalent, representative and unbiased samples of water flow. Other SPs (with single sample ports), not shown in Figure 4, are used for facility calibration experiments.

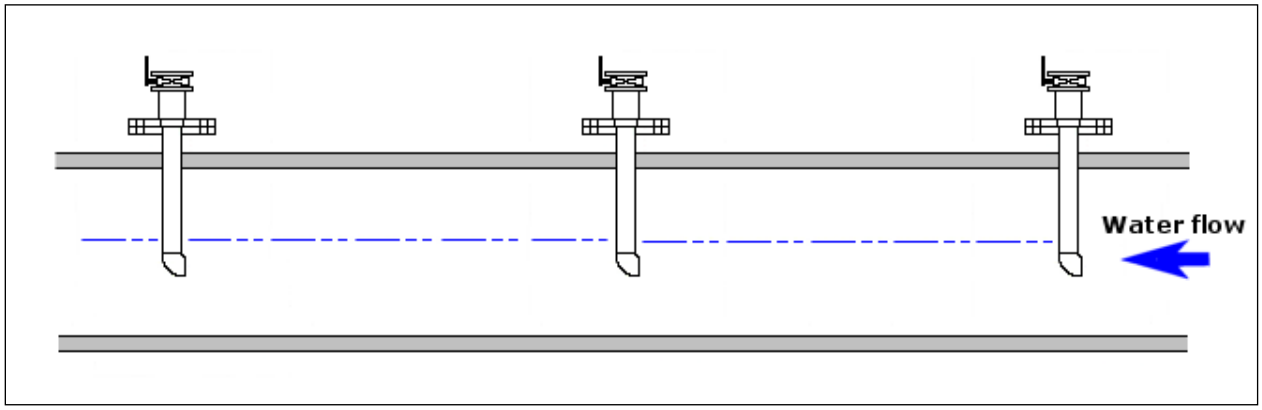

Figure 5. Simplified Schematic of a Sample Point (SP), Showing the Three Sample Ports.

Sample water drawn by sample ports is transferred simultaneously and continuously throughout ballasting operations (intake, recirculation or discharge) from the sample ports to replicate 3.8-m^3 sample collection tubs via clean 3.8-cm (internal diameter; ID) flexible hoses and automated flow-controlled pneumatic diaphragm valves. The sample collection tubs, pictured in Figure 4, connect to the sample ports in the arrangement detailed in Table 3. Though the same tubs serve as collection mechanisms for sample flow from more than one pitot, only one such pitot is used at a time during any given sample collection event. The naming convention for an individual pitot is: “SP number” plus “sample port letter”. Sample collection tubs are labeled numerically 1-6.

Table 3. Intake and Discharge Sample Points (SPs) and their Corresponding Sample Port Pitots and Sample Collection Tubs.

	INTAKE							DISCHARGE					
	SP#2			SP#3			SP#15	SP#9			SP#10		
Sample Port Pitot	a	b	c	a	b	c	a	a	b	c	a	b	c
Sample Collection Tub	1	2	3	4	5	6	6	3 & 6	2 & 5	1 & 4	3 & 6	2 & 5	1 & 4

An on-site mobile field laboratory (Figure 6) and stationary laboratory (Figure 7) provide space to support time sensitive analyses associated with the GSI land-based tests, including live analysis of phytoplankton and zooplankton. The laboratories are climate-controlled, and have enough bench space to allow for simultaneous analysis of samples by multiple personnel.

Figure 6. The GSI Mobile Field Laboratory.

Figure 7. The GSI Stationary Laboratory.

2.1.2 Challenge Conditions and Injection Procedures

The GSI Test Plan (Appendix 1) called for use of ambient harbor water and organism assemblages amended with Fine Arizona Test Dust (ISO 12103-1, A2; nominal 0-80 μm particle size; Powder Technology Inc.; Burnsville, MN, USA), Micromate (Micronized Humate Product for Liquid Suspension; Mesa Verde Resources; Placitas, NM) and concentrated algae harvested from the Duluth-Superior Harbor to assure IMO-consistent concentrations of TSS, POC, and live phytoplankton. Due to PureBallast® BWTS v.1 and v.2 filter failures during the commissioning period, target challenge conditions were revised downward and the use of Micromate to augment POC was discontinued. Revised target levels for the PureBallast® BWTS v.3 performance evaluation appears in Table 4.

During the PureBallast® BWTS v.3 performance evaluation, ambient Duluth-Superior Harbor water conditions were employed as the physical/chemical challenge conditions, except that Fine Arizona Test Dust was added to the facility intake water to achieve 25 mg/L TSS (half the IMO required level) for all the trials (Table 4). Mineral matter, defined as the difference between TSS and POC, was also augmented through the addition of Fine Arizona Test Dust (Table 4). The solids injection procedure is detailed in *GSI/SOP/LB/G/O/5 – Procedure for Injecting Organisms and Solids into the GSI Land-Based RDTE Facility*. The Fine Test Dust was sterilized at the Lake Superior Research Institute (LSRI) of the University of Wisconsin-Superior prior to injection by baking in an oven at 190 °C for one hour. TSS were measured frequently in Duluth-Superior Harbor during the 2010 testing season, which allowed close approximation of the ambient TSS on the day of each test trial. The weight of Fine Test Dust to be used in the Solids Injection System (SIS) tank was determined based on recent measurements in order to augment

the intake water to achieve the desired intake concentration of TSS. The SIS tank was filled with harbor water, sterile Fine Test Dust was poured into the SIS tank slowly to prevent clumping, and the dust was mixed for a minimum of 20 minutes prior to the start of the trial. The test dust mixture was injected into the intake water for the entire duration of the fill at a constant rate using a peristaltic pump located at Injection Point A (Figure 4).

Biological challenge conditions were also largely ambient, except that organism densities in the smaller of the two plankton size classes (i.e., ≥ 10 μm and < 50 μm) were augmented to achieve a density of 1000 cells/mL on intake (Table 4). The solids and phytoplankton injection systems are kept separate to reduce the risk of interference. The phytoplankton injection procedure is detailed in *GSI/SOP/LB/G/O/5 – Procedure for Injecting Organisms and Solids into the GSI Land-Based RDTE Facility*. One to two days prior to the test trial, phytoplankton from the Duluth-Superior Harbor was collected and concentrated using 50- to 80-μm plankton nets towed from an outboard-powered boat. The concentrated phytoplankton was stored at the GSI Land-Based RDTE Facility in holding ponds equipped with aeration systems for less than 48 hours. Prior to injection, holding pond water containing concentrated phytoplankton was mixed, sampled, and analyzed for viable cell density. In addition, a sample of Duluth-Superior Harbor water was collected to determine the ambient viable cell density. Based on the density of cells in the holding ponds and ambient intake water, the volume of phytoplankton concentrate that was needed to achieve the desired density in intake water was calculated. This volume was added to the Organism Pressure Injection System (OPIS) vessel. The OPIS vessel was pressurized to 25 psi greater than the target system pressure. The phytoplankton concentrate was added at a constant rate to the intake water via the pressure differential for the entire duration of the intake procedure via Injection Point B (Figure 4). A static mixer, installed in the main intake line just downstream of the two injection systems (SIS and OPIS) and prior to the main system "Y split" (Figure 4), ensured that the concentrations of these additives were equivalent in the control and treatment tracks of the facility. Gentle agitators installed in the control and treatment retention tanks ensured that live organisms, especially less motile organisms that may settle to the bottom of the tank during the retention period, were accounted for to the greatest extent possible in the discharge water analysis (see *GSI/SOP/LB/G/O/7 – Procedure for Maintaining Solids Suspension in the GSI Land-Based RDTE Facility's Retention Tanks*).

Table 4. Target Physical, Chemical, and Biological Challenge Water Conditions for the PureBallast® BWTS v.3 Performance Evaluation in Comparison to USEPA ETV and IMO G8 Recommended Challenge Conditions.

Parameter	DRAFT U.S. EPA ETV[3]	Recommended IMO G8[4]	Target Values for PureBallast® v.3 Challenge Water
Temperature (^oC)	4 – 35	–	4 - 30
Salinity (ppt)	< 1	Two salinities, >10 ppt difference	0 - 1
Total Suspended Solids (mg/L)	Min. 24	> 50	≥ 25 (Amended)
Particulate Organic Carbon (mg/L)	Min. 4	> 5	<0.1 – 3
Dissolved Organic Carbon (mg/L)	Min. 6	> 5	6 – 30
Mineral Matter (mg/L)	Min. 20	--	≥20 (Amended)
Zooplankton (> 50 μm/m^3)	Min. 100,000	> 100,000	>100,000
Phytoplankton (≥ 10 to < 50 μm/mL)	Min. 1000	> 1,000	>1,000 (Amended)
Heterotrophic Bacteria (MPN/mL)	Min. 1000	> 10,000	75 - 10,000

2.1.3. Preventing Cross Contamination

To minimize potential cross contamination of the treatment discharge water between trials, prior to the first trial and after each test trial, the interior of the retention tanks were cleaned according to *GSI/SOP/LB/G/O/3 – Procedure for Cleaning and Verifying Cleanliness of the Retention Tanks and Piping at the GSI Land-Based RDTE Facility*. After each intake and discharge operation, the sampling equipment (sample collection tubs, drain spout hose and nozzle, plankton nets, etc.) was also cleaned according to *GSI/SOPLB/G/O/4 – Procedure for Cleaning Sampling Equipment at the GSI Land-Based RDTE Facility*. The GSI facility lines were flushed with city-supplied potable water. The flushing was undertaken after each facility intake and prior to each discharge operation. After flushing, the thoroughness of the cleaning process was checked by partially filling a randomly selected treatment sample collection tub with

3 USEPA, Environmental Technology Verification Program. Generic Protocol for the Verification of Ballast Water Treatment Technologies. Version 5.1. September, 2010.
4 IMO MEPC 57, Annex 3: Revised Guidelines for Approval of Ballast Water Management Systems (G8). April 4, 2008.

0.5 m^3 of additional potable water, draining that water through a verified-clean plankton net, and examining the filtrate for evidence of residual organisms. The facility was deemed clean only if the rinse water was completely free of live Duluth-Superior Harbor zooplankton visible with a compound microscope at a magnification of 40X to 100X (see *GSI/SOP/LB/G/O/3*). Nets and other sample collection equipment were likewise validated for cleanliness prior to each sampling operation (see *GSI/SOP/LB/G/O/4*).

2.2. Water Quality Analysis

2.2.1. Total Suspended Solids (TSS), including Mineral Matter (MM)

During each trial, samples for TSS analysis were collected during intake and discharge as follows:

- On intake, three 1 L whole water samples were collected from the pre-treatment line (SP #3, Figure 4) at approximately 10, 25, and 40 minutes after the start of the intake procedure.
- On discharge, three 1 L whole water treatment samples were collected at approximately 10, 25, and 40 minutes after the start of the discharge procedure (SP #9, Figure 4). In addition, three 1 L whole water control samples were collected at approximately 10, 25, and 40 minutes after the start of the procedure (SP #9, Figure 4).

Samples were collected in-line rather than from the sample collection tubs to avoid settling of suspended solids. This approach ensured a more accurate measurement of solids and organic carbon in the intake water.

Sample analysis was conducted according to *GSI/SOP/BS/RA/C/8, v.1 – Procedure for Analyzing Total Suspended Solids (TSS)*. The samples were vacuum filtered through pre-washed, dried, and pre-weighed Whatman 934-AH glass fiber filters. After the sample was filtered it was dried in an oven and brought to constant weight. TSS values were determined based on the weight of particulates on the filter and the volume of water filtered.

Quality control measures consisted of collecting and analyzing approximately 10 % of the total number of samples collected from all three trials in duplicate. A TSS reference standard (QCI, 711, ULTRA Scientific) was analyzed on multiple occasions along with TSS samples to confirm the accuracy of the data being generated.

Mineral matter is defined as the difference between TSS and particulate organic matter (measured as POC). Therefore, MM concentrations were determined in each sample collected during these trials on intake following analysis of TSS, and the determination of POC as calculated from the NPOC and DOC concentrations (see §2.2.2.).

2.2.2. Non-Purgeable Organic Carbon (NPOC) and Dissolved Organic Carbon (DOC), and Determination of Particulate Organic Carbon (POC) Concentrations

During these trials, samples for NPOC, DOC, and POC analysis were collected immediately after TSS sample collection during intake only as follows:

- Three, 125 mL whole water samples were collected in glass bottles from the pre-treatment line (SP #3) at approximately 10, 25, and 40 minutes after the start of the operation.

In these tests, NPOC was measured as a surrogate for total organic carbon (TOC), though it may be a slight underestimate of TOC. The analytical instrument used to measure NPOC purges the sample with air to remove inorganic carbon before measuring organic carbon levels in the sample. Thus, the NPOC analysis does not incorporate any volatile organic carbon which may be present in the sample.

Sample analysis was conducted according to *GSI/SOP/BS/RA/C/3, v.1 – Procedures for Measuring Organic Carbon in Aqueous Samples*. Upon arrival at LSRI, an aliquot of the 125 mL sample was filtered through a Whatman GF/F filter and acidified with hydrochloric acid for analysis of DOC. The remaining portion of the sample was acidified with hydrochloric acid and analyzed for NPOC. A Shimadzu Total Organic Carbon Analyzer (Model TOC-5050A; Shimadzu Scientific Instruments, Inc.; Columbia, MD) was employed for analysis of both NPOC and DOC. Concentrations of NPOC and DOC were determined based on a calibration curve developed on the instrument using organic carbon standards prepared from potassium hydrogen phthalate. Reported POC concentrations were determined as the difference between the NPOC and DOC values for a given sample.

Quality control measures consisted of collecting and analyzing approximately 10 % of the samples in duplicate from all organic carbon samples collected during the three trials. A reference standard (#516 Demand, Environmental Resource Associates) was analyzed daily to confirm the accuracy of the data being generated.

2.2.3. Percent Transmittance (%T)

An aliquot of the filtered portion of each sample collected for TSS analysis was analyzed to determine percent transmittance. Sample analysis was conducted according to *GSI/SOP/BS/RA/C/4 – Procedure for Determining Percent Transmittance (%T) of Light in Water at 254 nm*. A spectrophotometer set at 254 nm was used to measure %T of the filtered samples. Deionized water was used as a reference to adjust the spectrophotometer to 100%T, and each filtered sample was measured in a pre-rinsed sample cuvette.

2.2.4. Water Quality Measurements using YSI Multiparameter Water Quality Sondes

Water quality was measured during each trial using calibrated YSI Multiparameter Water Quality Sondes (YSI 6600 V2-4 Sondes; YSI Incorporated; Yellow Springs, OH, USA). The sondes were calibrated prior to each test trial following *GSI/SOP/LB/G/C/4 - Procedure for Calibration, Deployment, and Storage of YSI Multiparameter Water Quality Sondes*. The YSI sondes have multiple probes that are able to measure dissolved oxygen, specific conductivity, salinity, temperature, pH, turbidity, and total chlorophyll. Water quality parameters were measured from approximately 1 L samples of water from each sample collection tub sampled on intake and discharge. Samples were taken immediately following collection of phytoplankton and microbial samples, and each measurement was recorded on pre-printed datasheets. In addition, water quality parameters in the control and treatment retention tanks were measured at mid-depth every 15 minutes during the two-day holding time. Prior to discharge of the respective tanks, the sondes were removed and taken to the mobile laboratory where the data were later downloaded as test files to a laptop computer using EcoWatch® for Windows® Software (v.3.18, 14 April 2006; YSI Incorporated); the files were then translated to MS Excel files, which were stored on a laptop computer in the mobile laboratory and later uploaded to the GSI SharePoint intranet website.

2.3. Viable Organism Analysis

During these trials, sample water for analysis of viable organisms was simultaneously collected from replicate sample ports into identical 3.8 m^3 collection tubs during each intake, treatment discharge, and control discharge operation (retention tank discharge was sequential, treatment then control). Volumes retained were always greater than IMO guideline volumes. The water in each collection tub constituted an independent, time-integrated replicate sample of the 200 m^3 experimental water mass.

2.3.1. Organisms ≥ 50 µm in Minimum Dimension

2.3.1.1. Sample Collection

During the intake operation for each trial (i.e. the filling of the treatment and control 200 m^3 retention tanks), the following time-integrated sample volumes were collected by continuous flow from the intake lines simultaneously:

- Two 2 m^3 sample from the pre-treatment intake line (i.e., Tubs #4 and #5, Figure 4),
- Two 2 m^3 sample from the control intake line (i.e., Tubs #1 and #2, Figure 4), and
- One 2 m^3 sample from the immediate post-treatment intake line (i.e., Tub #6, Figure 4).

During trial discharges the following time-integrated sample volumes were collected by continuous flow:

- Two samples of 2 m^3 each (total volume 4 m^3) were collected from the treatment discharge (i.e., Tubs #4 and #6, Figure 4),
- One 2 m^3 sample was collected from the control discharge (i.e., Tub #1, Figure 4),
- One sample of 2 m^3 was collected from the treatment discharge (i.e., Tub #5, Figure 4) and held overnight for delayed mortality assessment, and
- One 2 m^3 sample was collected from the control discharge (i.e., Tub #2, Figure 4) and held overnight for delayed mortality assessment.

Flow control valves and system logic ensured that sample flow rates were equivalent and proportional to intake and discharge flow rates throughout each operation. Immediately after filling, the phytoplankton and microbial whole-water samples were collected and sonde readings recorded, followed by the zooplankton sample collection. The zooplankton samples were collected by draining the remaining sample volumes (i.e., 2 m^3 minus 5 L of rinse/sonde water and the 1 L phytoplankton and microbial samples) from the sample collection tubs and concentrating through 35 μm (50 μm diagonal dimension) plankton nets into 1 L cod-ends for microscopic examination. See *GSI/SOP/LB/RA/SC/6 - Procedure for Zooplankton Sample Collection*. On intake and discharge, the zooplankton sample collection order was sequential. On intake, the Tub #6 post-treatment sample was collected first, followed by the Tub #4 pre-treatment sample, and then the Tub #1 control. Sample water in Tub #5 and #2 was not concentrated but held as a back-up sample if a replacement was needed due to operational errors in concentration and analysis of water from Tub #1 and #4, respectively. On discharge, treatment samples were collected from Tub #4 and then Tub #6, with Tub #5 collected the following morning for delayed mortality assessment. Control samples were collected from Tub #1, with Tub #2 collected the following morning for delayed mortality assessment.

2.3.1.2. Live/Dead and Size Analysis

All live/dead analysis was conducted according to *GSI/SOP/LB/RA/SA/2 - Procedure for Zooplankton Sample Analysis*, and took place within two hours of collecting and concentrating the individual samples. Microzooplankton (e.g., rotifers, copepod nauplii, and dreissenid veligers) and macrozooplankton (e.g., copepods, cladocerans, and insect larvae.), all generally greater than or equal to 50 μm in minimum dimension, were analyzed simultaneously by separate taxonomists. Microzooplankton subsamples were analyzed in a Sedgewick-Rafter counting chamber by examination under a compound microscope at a magnification of 40X to 100X. Macrozooplankton were analyzed in a Ward's Counting Wheel at a magnification of 20 to 30X using a dissecting microscope. Due to high densities, quantification of zooplankton in intake and control discharge samples required analysis of sub-samples and extrapolation to number per cubic meter. For these samples, a subsample was removed for analysis using a Henson-Stempel pipette. The dead organisms (i.e., those organisms that did not move or respond to stimuli) were enumerated, then all organisms in the sample were killed by adding 50% (v/v) acetic acid solution (for microzooplankton) or Lugol's solution (for macrozooplankton) to the counting chamber/wheel and the total number of organisms was enumerated. The number of live organisms was quantified by subtracting the number of dead organisms from the total number of organisms in the counting chamber/wheel. The post-treatment intake and treatment discharge samples had lower densities allowing for analysis of a greater proportion of the sample.

Therefore, the post-treatment intake and treatment discharge samples were split in half using a Folsom Plankton Splitter. Half of the sample was analyzed for macrozooplankton and the other half was examined for microzooplankton. Only live organisms were enumerated using standard movement and response to stimuli techniques.

Statistical analysis of organisms in the ≥ 50 μm size class was conducted for each trial using SigmaStat, version 3.5 (Systat Software, Inc.; Chicago, IL USA). The density data were not normally distributed, therefore, the data from post-treatment intake, control discharge, treatment discharge, and the control and treatment discharge after 24 hours were log-transformed to achieve a normal distribution and equal variance (ASTM, 2004; Eaton *et al*., 2005; USEPA, 2002). A one-way analysis of variance was used to determine the differences in the mean values among the treatment groups, and the Holm-Sidak method was used to perform pair-wise comparisons between each treatment group. A paired t-test was used to compare the control discharge density to the treatment discharge density. In all cases α=0.050.

Quality assurance measures during these trials included live/dead analysis of one intake and one discharge sample by two separate taxonomists over the course of the three trials. The average percent similarity of taxonomic identification (live organisms only) and the average relative percent difference of the number of live organisms counted were calculated for all second analyses.

2.3.2. Organisms ≥ 10 and <50 μm in Minimum Dimension

2.3.2.1. Sample Collection

The following whole-water samples were collected during intake for each trial for analysis of live organisms ranging in size from ≥ 10 to < 50 μm in minimum dimension:

- One 1 L sample was collected immediately after filling from the pre-treatment sample collection tub (i.e., Tub #4, Figure 4),
- One 1 L sample was collected from the immediate post-treatment sample collection tub (i.e., Tub #6, Figure 4), and
- One 1 L sample was collected from the control sample collection tub (i.e., Tub #1, Figure 4) and archived.

During discharge for each trial:

- Three 1 L samples were collected, one from each of the three treatment sample collection tubs (i.e., Tubs #4-#6, Figure 4), and
- One 1 L sample was collected from the control sample collection tub (i.e., Tub #1, Figure 4).

The three, 1 L treatment discharge samples were composited for analysis. Analysis of all samples occurred on-site within 1.5 hours of sample collection, with samples stored in coolers during the interim. Prior to analysis, samples were concentrated through 10 μm mesh plankton

netting and stored in a 25 mL sample container. See *GSI/SOP/LB/RA/SC/3 - Procedure for Algae/Small Protozoa Sample Collection.*

2.3.2.2. Sample Analysis

All sample analyses were conducted according to *GSI/SOP/LB/RA/SA/1 - Procedure for Algae/Small Protozoan Sample Analysis.* A 2.0 mL subsample of the concentrated sample was transferred to a 5 mL sample container, with 5 μL of fluorescein diacetate (FDA) viability stain stock solution added. The subsample was then allowed to incubate in the dark for 5 minutes. The 2.0 mL incubated sample was mixed and 1.1 mL was immediately transferred to a Sedgwick-Rafter cell, covered and placed on the stage of a compound microscope that was set for simultaneous observation using brightfield and epifluorescence. At least two horizontal transects were counted (an area known to reflect greater than 1 mL of original sample water), aiming for at least 100 entities (i.e., unicellular organism, colony or filament) counted. If time permitted, additional transects were counted to increase statistical power. Single cell entities and cells comprising colonial and filamentous entities were characterized as follows: alive = cells showing obvious green fluorescence from cell contents; dead = cells showing no or very little evidence of green fluorescence from cell contents; and ambiguous = cells or entities that cannot be clearly identified as alive or dead (were uncommon). Records were kept of transect lengths and widths so that the total counted area and volume analyzed could be calculated later.

Entities less than 10 μm in all visible dimensions or greater than 50 μm in minimum visible dimension were not counted. Counting and measurement of all other entities followed standard procedures for individuals (length and width), colonies (e.g., number of cells, cell length and width) and filaments (e.g., number of cells, cell length and width or total filament length if cells could not be discerned). The remaining concentrated sample in the 25 mL container was archived using a preservative (formalin or Lugol's) for long-term storage.

Statistical analysis for the $\geq$ 10- and < 50 μm size class for the three trials was conducted using SigmaStat, version 3.5 (Systat Software, Inc.; Chicago, IL USA). A one-way analysis of variance was used to determine the differences in the mean values among the treatment groups. A paired t-test was used to compare the control discharge density to the treatment discharge density. In all cases α=0.050.

Quality assurance measures included analysis of a portion of the samples by two separate taxonomists using a dual-headed compound microscope (i.e., both taxonomists analyzed the same sample at the same time) and/or subsample analysis of a portion of the samples collected by a single taxonomist (i.e., one taxonomist analyzed two separate aliquots from one sample) over the three trials. The average percent similarity of taxonomic identification and the average relative percent difference of the number of live organisms counted were calculated for all second analyses.

2.3.3. Organisms < 10 μm

Control and treatment samples for these trials were collected and analyzed for heterotrophic bacteria and three specific indicator organisms for waterborne pathogens: total coliform bacteria, *E. coli*, and enterococci.

2.3.3.1. Sample Collection

One liter whole water samples were collected as follows:

- On intake, three samples were collected immediately after filling and collection of the 1 L phytoplankton sample from the pre-treatment sample collection tub (Tub #4, Figure 4), and three were collected from the post-treatment sample collection tub (Tub #6, Figure 4).
- On discharge, three samples were collected from the control sample collection tub (Tub #1, Figure 4) and three were collected from each of three treatment sample collection tubs (one each from Tubs #4-#6, Figure 4).

All samples were collected according to *GSI/SOP/LB/RA/SC/4 – Procedure for Microbial Sample Collection*, and were immediately transported in an insulated cooler to LSRI and analyzed as individual replicates.

2.3.3.2. Sample Analysis

Viable heterotrophic bacteria were enumerated according to *GSI/SOP/BS/RA/MA/1 – Procedure for Quantifying Heterotrophic Plate Counts (HPCs) using IDEXX's SimPlate® for HPC Method*. This method utilizes the IDEXX SimPlate® for HPC Method (IDEXX Laboratories, Inc.; Westbrook, Maine), which is based on IDEXX Laboratories' patented multiple enzyme technology.

The abundance of *E. coli* (*GSI/SOP/BS/RA/MA/4 - Procedure for the Detection and Enumeration of Total Coliforms and E. coli Using IDEXX's Colilert®*) and enterococci (*GSI/SOP/BS/RA/MA/3 - Procedure for the Detection and Enumeration of Enterococcus using Enterolert™*) were determined using Quanti-Tray/2000® with Colilert® and Enterolert™, respectively, which are both based on IDEXX's patented Defined Substrate Technology (DST®; IDEXX Laboratories, Inc.; Westbrook, Maine).

Statistical analysis for the < 10 μm size class for the three trials was conducted using SigmaStat, version 3.5 (Systat Software, Inc.; Chicago, IL USA). A paired t-test was used to compare the control discharge density to the treatment discharge density. In all cases α=0.050.

Quality control samples analyzed for each intake and discharge operation included a media blank and a positive control for *E. coli*/total coliforms and *Enterococcus spp.*, and a media blank for heterotrophic bacteria. Quality assurance measures included analysis of at least 10 % of the samples in duplicate from the total number of samples collected over the three trials. The

average relative percent difference of all duplicates analyzed during the test trials was calculated separately for *E. coli*, *Enterococcus spp*., and heterotrophic bacteria.

2.4. Whole Effluent Toxicity (WET) Analysis

GSI conducted whole effluent toxicity (WET) testing for a single trial of the PureBallast®, v.3 BWTS. The WET test trial was conducted after the three trials in the biological performance evaluation were completed (i.e., biological performance evaluation trials ended 04 October 2010, and treatment discharge water was collected for WET testing 08 October 2010 from a trial conducted 06 to 08 October 2010). These chronic toxicity evaluations involved three freshwater species as arrayed in Table 5.

Whole effluent toxicity of treatment discharge water was determined using standard USEPA procedures (USEPA, 2002). A 19 L whole water sample was collected following the treatment discharge operation from a treatment sample collection tub in a 19 L, high-density, polyethylene container. The WET test sample was immediately transported to LSRI and was used upon arrival to set up the WET tests. Following initial set up of the tests (described below), the remaining sample water was stored at 4 °C in the dark to retain as much of the initial water quality/chemistry properties as possible, and used as a source of renewal water (once warmed to 25 °C) each day throughout the bioassay. Filtered (i.e., using a Whatman 934-AH Glass Microfiber Filter, 1.5 μm particle retention in liquid) Duluth-Superior Harbor water served as the control. Treatment groups consisted of 0 % treatment discharge water (i.e., all control water), 100 % treatment discharge water (i.e., no control water), and a performance control (i.e., culture water or algae growth media as appropriate). All tests were conducted in temperature-controlled incubators, water baths, or at ambient room temperature following the species specific SOPs listed in Table 5. Differences in mean percent survival, mean dry weight values (for *P. promelas*), mean cell density (for *S. capricornutum*), and mean number of young per female (for *C. dubia*) between the 0 % and 100 % treatment discharge groups were analyzed using SigmaStat, version 3.5 (Systat Software, Inc.; Chicago, IL USA) for statistical significance at α=0.050 using a One-Way Analysis of Variance and a post hoc statistical comparison.

WET tests were initiated with healthy, vigorous organisms. To determine the overall health of the test organisms, reference toxicant tests were performed with the cladoceran, *Ceriodaphnia dubia*, and the minnow, *Pimephales promelas*, prior to the start of each definitive test or at least once per month. In addition, a performance (reference) control was used for all species tested. The performance control consists of the normal culturing conditions for each species, providing the test organisms with the optimal environment for survival, growth, and reproduction. Therefore, the performance control along with the reference toxicant tests, provided verification of the health of the test organisms. To determine the validity of the WET tests, percent survival, dry weights of survivors, mean cell density for algae, and mean number of young per female for the cladocerans in the controls were compared to the test acceptability criteria published in the USEPA's *Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms* (4^{th} edition, 2002). Class I weights were used as a check for the accuracy of the laboratory balance. Daily or weekly calibration of test meters ensured optimal performance. The *P. promelas* drying process was verified by re-weighing a percentage of fish after they had been dried for an additional length of time in the oven.

Table 5. Standard Operating Procedures Relative to Whole Effluent Toxicity (WET) Testing.

GSI SOP Code	Test Type	Test Species	Test Endpoint
GSI/SOP/BS/RA/WET/1	Chronic	Cladoceran (*Ceriodaphnia dubia)*	Survival and Reproduction
GSI/SOP/BS/RA/WET/2	Chronic	Fathead Minnow (*Pimephales promelas)*	Survival and Growth *(growth measured via dry weight)*
GSI/SOP/BS/RA/WET/3	Chronic	Green Alga (*Selenastrum capricornutum)*	Growth *(measured via direct counts of density)*

2.5. Data Management

2.5.1. Data Recording

All biological and chemical data were recorded by hand (using indelible ink) on pre-printed data collection forms and/or in bound laboratory notebooks that are uniquely-identified and were specific to the PureBallast® BWTS tests (i.e., v.1, v.2, and v.3 data were recorded in the sample notebook and forms were stored in the same binder). The types of biological and chemical data collected include: sample collection data (e.g., date, time, and location of collected samples), water quality and chemistry analysis data (e.g., TSS, DOC, and MM concentration), microbial analysis data (e.g., sample preparation, incubation, and direct counts), phytoplankton analysis data (e.g., number of live and number of dead entities), zooplankton analysis data (e.g., sample concentration; number of dead, total, and live organisms), and WET test data (e.g., test set up, direct counts, and test take down). The data that were recorded on pre-printed data collection forms were secured in uniquely-identified three ring binders, specific to the type of data and to the treatment technology. Biological and chemical data that were recorded by hand were entered into either a MS Access Database that was designed, developed, and is maintained by the GSI Database Manager, or the data were entered into a MS Excel Spreadsheet (see *GSI/SOP/G/RA/DM/1 – Procedure for Data Entry, Data Quality Control, and Database Management*).

All electronic data files are stored on the LSRI's secured Local Area Network (LAN) that can be accessed only by relevant GSI personnel. The GSI Database Manager is the single point of control for access to the LSRI LAN. The LSRI LAN is automatically backed up every 24 hours. The electronic data files are also stored on the GSI's internal SharePoint website, which acts as a secondary data backup/storage mechanism. All original raw data are stored in a climate-controlled, secure archive room at the LSRI for five years after this report is finalized.

In-tank water quality data (e.g., temperature, pH, dissolved oxygen, specific conductivity, salinity, turbidity, and chlorophyll-a) was measured every fifteen minutes during each retention period and automatically recorded in a text file, which is later translated to a Microsoft (MS) Excel spreadsheet. Facility data (e.g., flow rates and pressure measurements) were electronically recorded every five seconds during intake and discharge. This data was exported to MS Excel

for subsequent analysis, and is stored by AMI Engineers on a secure network, as well as on GSI SharePoint for addition storage and archiving.

A percentage of data that was recorded by hand and entered into MS Access or MS Excel was verified against the original raw data, this also included verification of formulas/calculations (i.e., hand-calculation of data) done using MS Access or MS Excel. The percentage of verified raw data generally depends on the amount of raw data that was generated, and for the PureBallast®, v.3 test ranged from 10 % to 100 % of the original raw data. Data validation is additionally detailed in Section 7 of the GSI Quality Assurance Project Plan (QAPP) for Land-Based Tests (GSI, 2010a). This section also details the acceptable values, where appropriate, for the following quality objectives: accuracy, precision, completeness, comparability, representativeness, and sensitivity.

Following the completion of PureBallast®, v.3 BWTS trials, a thorough review of all data sheets and laboratory notebooks was completed to ensure compliance with the documentation procedures outlined in all relevant GSI SOPs and in the GSI land-based QAPP (GSI, 2010a). A Technical Systems Audit Checklist (TSA) was completed during observation of sample collection and analysis activities, and during the data review. A QAQC Log Book was used to document any additional data verification and validation activities. The TSA checklist and QAQC log book were scanned to electronic format and posted to the GSI SharePoint website.

2.5.2. Data Processing and Storage

All original datasheets were stored in three-ring binders, each with a unique identification code specific to the PureBallast® BWTS tests. All log books were also given a unique identification code specific to the PureBallast® BWTS tests. At least one backup copy (i.e., an electronic copy stored on the GSI SharePoint website) was made of all completed datasheets, and in some cases additional hardcopies were also made. The raw data is in the custody of the appropriate GSI Senior Staff Member, and will be archived by the GSI Senior QAQC Officer at LSRI for a period of at least five years.

A dedicated database designed using the Microsoft Access software suite was used to store, manage and process phytoplankton and zooplankton data. Microsoft Excel was used in conjunction with the database to create various dataset formats for subsequent analysis. Microsoft Excel was also used to store, manage, and process microbial, water chemistry, water quality, and whole effluent toxicity data. Database entry and maintenance was the responsibility of the GSI database management staff. Regular checks for data entry errors were conducted by comparing database records and Excel spreadsheets with the original paper data sheets. This was a manual inspection process and though rather time consuming, was an essential procedure for discovering errors. After examination and quality assurance analysis, the data distribution files from the Access database were posted to LSRI's Local Area Network (LAN) in an organized hierarchical folder system. A backup of the database is also made regularly to avoid any loss of data following computer/electronic glitches. Files were also posted to GSI's SharePoint website to provide a secondary data backup/storage mechanism.

3.0. RESULTS

Three test trials were completed with the GSI facility operating effectively. During one trial (Trial C), the BWTS filter became clogged. This trial was temporarily halted, the filter cleaned and restarted. Results from the three trials and the WET test are presented below.

3.1. Challenge Conditions

3.1.1. Operational Conditions

The PureBallast®, v.3 BWTS encountered similar operating conditions during all three trials (Table 6). The GSI facility and the BWTS operated at a flow rate of 500 m3/hour (or 250 m^3/hour for the control and treatment tracks) for a fill duration of approximately 45 minutes at pressures ranging from 3.1 to 3.6 bars. The system performed continuously during Trial A and Trial B, with filter backwash cycles every 40 to 48 seconds. The flow rate for the treatment track was set to an average of 250 m^3/hour to accommodate the frequent and rapid backwash cycles. During Trial C, the filter operated with a backwash cycle every 40 seconds for the first 20 minutes of flow and then became clogged. In order to salvage BWTS performance data from the trial, the flow was stopped, the facility monitors and BWTS were reset, and the flow was restarted, now without the injection of Arizona Fine Test Dust or phytoplankton. Consequently, Trial C was completed following a brief interruption, and the TSS concentration and phytoplankton density on intake was less than for the previous two trials.

Table 6. Operational Parameters Measured During Intake Operations of the Completed Test Trials of the PureBallast®, v. 3 Ballast Water Treatment System.

Trial	Filter	Backwash Cycle Duration	Fill Duration (min)	Flow Rate (m^3/h)	Pressure (bar)	Engineering Comments
A	40 μm mesh	48 sec	45	250	3.2-3.5	System performed continuously.
B	40 μm mesh	40 sec	45	250	3.1-3.6	System performed continuously.
C	40 μm mesh	40 sec prior to clog; then 8.7 min after restart	45	250	3.3-3.4	Filter clogged 20 min into fill. Fill was stopped, system reset, and fill restarted without injection of Fine Test Dust or phytoplankton.

3.1.2. Physical/Chemical Conditions

A summary of actual physical/chemical conditions of intake and discharge water (where measured) along with the minimum target concentrations appear in Table 7. Overall the TSS and

MM averaged 19.3 mg/L and 19.2 mg/L on intake, respectively. The concentrations for TSS and MM were ≥ 20 mg/L during Trial A and ≥ 25 mg/L in Trial B. Due to the interruption in Trial C noted above, less than the minimum target concentration of 25 mg/L TSS and 20 mg/L MM was achieved for that trial. All other parameters measured were not augmented (i.e., ambient Duluth-Superior Harbor water), and these parameters remained consistent during all three trials.

Table 7. Average Concentration (± Std. Dev.) of Total Suspended Solids (TSS), Non-Purgeable Organic Carbon (NPOC), Particulate Organic Carbon (POC), Dissolved Organic Carbon (DOC), Mineral Matter (MM), and Percent Transmittance (%T) in Challenge Water During Three Trials of the PureBallast®, v.3 Ballast Water Treatment System.

Parameter	Target Concentration	Sample	Trial A	Trial B	Trial C	Summary (*n*=3)
TSS (mg/L)	≥ 25	Intake	20.6 ± 0.8	25.4 ± 0.2	11.9 ± 11.9	**19.3 ± 6.8**
	--	Discharge	10.1 ± 1.6	10.4 ± 1.9	14.0 ± 10.5	**11.5 ± 2.2**
NPOC (mg/L)	Ambient	Intake	19.4 ± 0.1	18.9 ± 0.1	19.2 ± 0.1	**19.2 ± 0.3**
DOC (mg/L)	Ambient	Intake	18.7 ± 0.2	18.8 ± 0.2	19.1 ± 0.0	**18.9 ± 0.2**
POC (mg/L)	Ambient	Intake	0.7 ± 0.3	0.2 ± 0.3	0.1 ± 0.0	**0.3 ± 0.3**
MM (mg/L)	≥ 20	Intake	19.9 ± 1.1	25.3 ± 0.1	12.5 ± 15.2	**19.2 ± 6.4**
%T (254 nm)	Ambient	Intake	15.6 ± 0.1	16.3 ± 0.2	15.9 ± 0.1	**15.9 ± 0.4**
	--	Discharge	15.7 ± 0.4	16.1 ± 0.2	16.1 ± 0.1	**16.0 ± 0.2**

3.1.3. In-Tank Water Quality

Table 8 summarizes the water quality measured in the retention tanks during the two day holding period for each trial. The water quality in the control and treatment retention tanks was very similar during all three trials, with the exception of chlorophyll and dissolved oxygen. The average chlorophyll concentration in the control retention tanks during the three trials was 11.0 μg/L, as compared to the treatment retention tanks with an average of 8.8 μg/L. These water quality data are supported by the biological data, which shows a reduction of phytoplankton density in the treatment as compared to the control discharge. The dissolved oxygen concentration in the treatment retention tanks was higher during the three trials, perhaps due to decreased density of organisms and associated respiration and oxygen demand in the treatment as compared to the control. The average dissolved oxygen concentration in the control retention tanks was 9.21 mg/L (87.6 % saturation), while the treatment tanks had an average of 9.70 mg/L (92.5 % saturation) over all three trials. Again, the water quality data supports the biological data as this increase in dissolved oxygen concentration is coupled with a decrease in organisms in the treatment. There was a decrease in turbidity during Trial C in both the treatment and control retention tanks when compared to the previous two trials. This was due to the termination of the solids injection after the filter clogged.

Table 8. Retention Tank Water Quality (Average ± Std. Dev.) During Trials of the PureBallast® v.3 Ballast Water Treatment System.

Parameter	Retention Tank	Trial A	Trial B	Trial C	Summary (*n*=3)
Temperature (°C)	Control	13.12 ± 0.07	13.38 ± 0.16	12.64 ± 0.41	**13.05 ± 0.38**
	Treatment	13.32 ± 0.07	13.50 ± 0.13	12.73 ± 0.45	**13.18 ± 0.40**
Specific Conductivity (mS/cm)	Control	0.248 ± 0.000	0.249 ± 0.001	0.254 ± 0.000	**0.250 ± 0.003**
	Treatment	0.249 ± 0.000	0.249 ± 0.000	0.252 ± 0.000	**0.250 ± 0.002**
Salinity (ppt)	Control	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	**0.1 ± 0.0**
	Treatment	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	**0.1 ± 0.0**
pH	Control	7.8 ± 0.0	7.8 ± 0.0	7.8 ± 0.0	**7.8 ± 0.0**
	Treatment	7.7 ± 0.0	7.7 ± 0.0	7.7 ± 0.0	**7.7 ± 0.0**
Turbidity (NTU)	Control	9.6 ± 0.6	9.3 ± 0.6	5.8 ± 0.3	**8.2 ± 2.1**
	Treatment	9.0 ± 0.7	9.0 ± 0.8	5.0 ± 0.3	**7.7 ± 2.3**
Chlorophyll (μg/L)	Control	11.8 ± 0.5	11.2 ± 0.5	10.0 ± 0.5	**11.0 ± 0.9**
	Treatment	9.2 ± 0.3	9.0 ± 0.6	8.3 ± 0.5	**8.8 ± 0.5**
Dissolved Oxygen (% Saturation)	Control	89 ± 0	89 ± 0	85 ± 1	**88 ± 2**
	Treatment	92 ± 1	95 ± 1	91 ± 1	**93 ± 2**
Dissolved Oxygen (mg/L)	Control	9.4 ± 0.0	9.2 ± 0.0	9.0 ± 0.1	**9.2 ± 0.2**
	Treatment	9.7 ± 0.1	9.9 ± 0.1	9.6 ± 0.0	**9.7 ± 0.1**

3.1.4. Water Quality in Sample Collection Tubs

Water quality measurements taken at the time of sample collection from the sample collection tubs during these trials are summarized in Table 9, and show very similar results for control and pre-treatment on intake. This result supports the evenness of the "Y" split of the control and treatment tracks. On intake, the post-treatment sample tub water quality was similar to the control/pre-treatment water quality for all parameters with the exception of specific conductivity and turbidity. The pH in the post-treatment tub was on average slightly lower than the control and pre-treatment intake tubs, however, the range of post-treatment pH values overlap the range of both control and pre-treatment intake pH values. The specific conductivity in the post-treatment sample collection tub (0.225 mS/cm) was on average lower than the control/pre-

treatment sample tubs (0.248 mS/cm and 0.249 mS/cm, respectively). In addition, the turbidity was on average higher in the post-treatment sample collection tub (14.6 NTU) as compared to the control/pre-treatment sample collection tubs (9.7 NTU and 9.5 NTU, respectively). On discharge, the control sample collection tub and treatment sample collection tubs had very similar water quality with the exception of chlorophyll. The chlorophyll concentration in the treatment discharge sample collection tubs (10.1 μg/L) was on average lower than in the control sample collection tub over all three trials (12.3 μg/L). This result agrees with the retention tank measurements as well as the biological data that shows a decreased density of phytoplankton in the treatment samples on discharge.

Table 9. Intake and Discharge Sample Collection Tub Water Quality (Average ± Std. Dev.) in PureBallast®, v.3 Ballast Water Treatment System Trials.

Parameter	Operation	Sample Type	Average (n=3)
Temperature (°C)	Intake	Control	**13.44 ± 1.89**
		Pre-Treatment	**13.50 ± 1.91**
		Post-Treatment	**13.59 ± 1.93**
	Discharge	Control	**14.48 ± 1.42**
		Treatment	**13.19 ± 1.62**
Specific Conductivity (mS/cm)	Intake	Control	**0.248 ± 0.003**
		Pre-Treatment	**0.249 ± 0.002**
		Post-Treatment	**0.225 ± 0.001**
	Discharge	Control	**0.245 ± 0.008**
		Treatment	**0.234 ± 0.010**
Salinity (ppt)	Intake	Control	**0.1 ± 0.0**
		Pre-Treatment	**0.1 ± 0.0**
		Post-Treatment	**0.1 ± 0.0**
	Discharge	Control	**0.1 ± 0.0**
		Treatment	**0.1 ± 0.0**
pH	Intake	Control	**7.7 ± 0.1**
		Pre-Treatment	**7.7 ± 0.1**
		Post-Treatment	**7.3 ± 0.4**
	Discharge	Control	**7.8 ± 0.1**
		Treatment	**7.7 ± 0.4**
Turbidity (NTU)	Intake	Control	**9.7 ± 2.5**
		Pre-Treatment	**9.5 ± 2.9**
		Post-Treatment	**14.6 ± 6.8**
	Discharge	Control	**11.2 ± 1.9**
		Treatment	**12.0 ± 2.4**
Chlorophyll (μg/L)	Intake	Control	**10.7 ± 0.7**
		Pre-Treatment	**11.1 ± 1.0**
		Post-Treatment	**10.0 ± 1.5**

	Discharge	Control	**12.3 ± 0.5**
		Treatment	**10.1 ± 0.8**
Dissolved Oxygen (% Saturation)	Intake	Control	**97 ± 2**
		Pre-Treatment	**96 ± 1**
		Post-Treatment	**98 ± 2**
	Discharge	Control	**96 ± 3**
		Treatment	**97 ± 2**
Dissolved Oxygen (mg/L)	Intake	Control	**10.1 ± 0.3**
		Pre-Treatment	**10.0 ± 0.3**
		Post-Treatment	**10.2 ± 0.3**
	Discharge	Control	**9.7 ± 0.2**
		Treatment	**10.1 ± 0.2**

3.1.5. Biological Challenge Conditions

The ≥ 50 μm size class of organisms in the intake water was the ambient assemblage of Duluth-Superior Harbor, and consisted largely of zooplankton. The live organism density on intake for the ≥ 50 μm size class ranged from 15,745/m^3 to 44,787/m^3 across trials with the maximum intake density achieved during Trial B (Table 10). The late season timing of the performance evaluation (i.e., late September to early October) resulted in these values being lower than the target density of > 100,000/m^3. However, the density of live organisms in the control discharge samples were nonetheless quite high, ranging from 19,893/m^3 to 75,071/m^3 with an average of 42,995/m^3 (Table 10), providing ample statistical power for a comparison between control and treatment. The live organism density in this larger size class increased over the two day retention time, which indicates a favorable holding environment in the control retention tank for organisms in the ≥ 50 μm size class.

The microzooplankton community at the test site was dominated by the rotifers Keratella (loricate) and Polyarthra and Synchaeta (illoricate or soft-bodied) which comprised 41 % to 72 % of total density. Bosminid cladocerans and cyclopoid and calanoid copepods were the dominant taxa in the macrozooplankton community. The density of live rotifers (hard- and soft-bodied) increased over the two day holding time in all of the trials.

The live organism density for the ≥ 10 and < 50 μm size class ranged from 221 cells/mL to 687 cells/mL, with an average of 433 cells/mL on intake (Table 10). The phytoplankton injection was terminated after 20 minutes during Trial C due to the BWTS filter clogging, which explains the low density of phytoplankton during this trial. A target density of > 1000 cells/mL was desired for the ≥ 10 and < 50 μm size class; however, this density was not met during any of the trials. This is again due to the seasonal timing of this performance evaluation and the low ambient phytoplankton densities in the Duluth-Superior Harbor during PureBallast®, v. 3 testing. The low ambient densities of phytoplankton were not conducive to collection of the large numbers of concentrated phytoplankton needed for injection on intake. As with the larger size class described above, the organism density in the ≥ 10 and < 50 μm size class increased over the

two day hold time during Trials B and C, resulting in control discharge densities with an average of 474 live cells/mL (Table 10); more than adequate to detect a treatment effect live cells/mL (Table 10).

The smaller regulated size class (≥ 10 and < 50 μm) was dominated by phytoplankton entities of diatoms, green algae, blue-green algae, chrysophytes and cryptophytes. Protozoans, including ciliates and flagellates, were also present, comprising up to 3 % of the assemblages in intake samples. Dominant taxa during these trials were *Aulacoseira* spp. (filamentous diatom), *Melosira* spp. (filamentous diatom), *Cyclotella* spp. (single-celled centric diatom), *Fragilaria* spp. (filamentous diatom), filamentous and sheet-forming cyanophytes (e.g., *Oscillatoria*, *Merismopedia*, *Lyngbya*), colonial (coccoid) green algae (e.g., *Gonium* and *Pandorina*), *Cryptomonas*/*Rhodomonas* spp. (single-celled cryptophytes), and other miscellaneous microflagellates.

Table 10. Live Plankton Concentrations (Average ± Standard Error of the Mean, SEM) in Intake and Control Discharge Water in Three Trials of the PureBallast®, v. 3 BWTS and in the Overall Test Cycle.

Live Organism Size Category	Target Density	Sample	Trial A	Trial B	Trial C	Summary (*n*=3)
≥ 50 μm (#/m^3)	>100,000	Intake	20,086	44,787	15,745	**26,872 ± 9044**
	>100	Control Discharge	34,020	75,071	19,893	**42,995 ± 16,549**
	--	Control Discharge (24 Hour Hold)	33,257	56,113	18,454	**35,941 ± 10,954**
≥ 10 and < 50 μm (#cells/mL)	>1000	Intake	399	687	221	**433 ± 136**
	>100	Control Discharge	393	721	308	**474 ± 126**

Microbial organism concentrations (i.e., < 10 μm size class), measured in most probable number (MPN) per volume, in the intake and control discharge samples during the PureBallast®, v.3 trials are provided in Table 11. Total coliform bacteria densities ranged from 246 MPN/100 mL to 305 MPN/100 mL on intake (Table 11). Of the total coliform bacteria on intake, approximately 26 % on average were *E. coli*, which ranged from 38 MPN/100 mL to 116 MPN/100 mL (Table 11). Enterococci ranged from 38 MPN/100 mL to 50 MPN/100 mL on intake (Table 11). Several of the intake samples collected and analyzed for total heterotrophic bacteria were below the limit of detection (i.e., < 200 MPN/mL), therefore, an overall average could not be determined but intake concentrations ranged from < 200 MPN/mL to 2933 MPN/mL (Table 11, Appendix 5). The density of total coliform bacteria, *E. coli*, and enterococci

decreased over the retention period (Table 11). This result is not surprising, as Duluth-Superior Harbor water is not a favorable environment for growth of these organisms. Total coliform bacteria density decreased to an average of 179 MPN/100 mL in the control discharge, which was a 36 % reduction in density compared to intake (Table 11). *E. coli* and enterococci densities decreased approximately 50 % on average, for a control discharge density average of 37 MPN/100 mL and 21 MPN/100 mL, respectively (Table 11). Heterotrophic bacteria ranged from < 200/mL to 467/mL in control discharge (Table 11, Appendix 5).

Table 11. *E. coli*, Enterococci, and Total Heterotrophic Bacteria Densities (Average ± SEM) in Intake and Control Discharge from Three Trials of the PureBallast®, v. 3 BWTS, and the Overall Test Cycle.

Microbial Organism	Sample	Trial A	Trial B	Trial C	Summary (*n*=3)
Total Coliform Bacteria (MPN/100 mL)	Intake	288 ± 13	305 ± 37	246 ± 24	280 ± 18
	Control Discharge	177 ± 22	207 ± 34	153 ± 13	179 ± 16
E. Coli (MPN/100 mL)	Intake	38 ± 4	65 ± 0.4	116 ± 16	73 ± 23
	Control Discharge	24 ± 2	33 ± 4	52 ± 2	37 ± 8
Enterococci (MPN/100 mL)	Intake	39 ± 4	50 ± 3	38 ± 9	42 ± 4
	Control Discharge	14 ± 4	23 ± 2	27 ± 7	21 ± 4
Total Heterotrophic Bacteria (MPN/mL)	Intake	2933 ± 2536	<200.00 to 600.00*****	<200.00 to 200.00*****	Could not Determine
	Control Discharge	<200.00 to 200.00*****	467 ± 176	116 ± 15	Could not Determine

*****Could not average replicates, as one or more are below the limit of detection (below 200 MPN/mL). See trimmed, raw data in Appendix 5.

3.2. Live Organisms in Treated Discharge

3.2.1. Regulated Plankton, ≥ 50 μm Size Class

The densities of live plankton in the post-treatment intake and treatment discharge from these trials are summarized in Table 12.

On intake, immediately post-treatment, the density of live organisms in the ≥ 50 μm size class ranged from 2,640/m^3 (Trial C) to 9,090/m^3 (Trial B) and averaged 6,232/m^3 (Table 12). This represented an immediate 77 % reduction in live organism density as compared to the intake density. Macrozooplankton observed live in post-treatment intake samples were Bosmina, copepods, and chironomid larvae; while Keratella, Polyarthra, Syncheata, and copepod nauplii were most commonly observed live from the microzooplankton group. Following a two day retention and second pass through the PureBallast®, v.3 BWTS on discharge, the density of live organisms in the ≥ 50 μm size class ranged from 445/m^3 (Trial A) to 1,871/m^3 (Trial B) and

averaged 947/m^3 (Table 12), which represents a 96 reduction from the intake live organism density. There was a statistically significant (p<0.05) reduction in live organism density between the post-treatment intake and the treatment discharge (Table 12). Copepods dominated the macrozooplankton observed live in the treatment discharge, but Bosmina and tardigrades were also observed (see Appendix 4 for listing of organisms found in treated discharge). In the microzooplankton group, Keratella were most frequently observed in the treatment discharge; Polyarthra, Syncheata, and other rotifers were also observed (Appendix 4). After a 24 hour hold time in one treatment sample collection tub, there was a further 2 % reduction in mean live organism density as compared to the treatment discharge, but this reduction was not statistically significant, compared to the treatment discharge sampled immediately. Post-discharge, 24 hour retention densities ranged from 288/m^3 (Trial C) to 686/m^3 (Trail B), with an average of 544/m^3 (Table 12). The results of the paired t-test comparing the control and treatment discharge are summarized in Table 13. There was a significant difference (p = 0.003) between the control and treatment discharge, indicating that for the ≥ 50 μm size class, the PureBallast® v.3 BWTS reduces the live organism density in the treated discharge when compared to the untreated discharge.

3.2.2. Regulated Plankton, ≥10 and <50 μm Size Class

Densities of live organisms in the ≥10 and <50 μm size class immediately post-treatment ranged from 62 cells/mL (Trial C, reduced augmentation) to 379 cells/mL (Trial B), with a test cycle average of 199 cells/mL (Table 12). Live organisms in the post-treatment intake had a similar diversity as that observed for untreated intake samples, although cyanophytes and colonial green algae were not observed. Following a two day retention time and second treatment using the PureBallast®, v.3 BWTS on discharge, the treatment discharge live density ranged from 36 cells/mL (Trial C, non-augmented) to 171 cells/mL (Trial B) and averaged 94 cells/mL (Table 12), for a 78 % reduction in live organism density as compared to the intake density. There was no significant difference (p<0.05) in live organism density between the post-treatment and the treatment discharge (Table 12). Again, diatoms dominated the live organisms found in the treatment discharge, while protozoans, cryptophytes, and dinoflagellates were occasionally observed. Cyanophytes were not observed in treated discharge and there was only one occurrence of a colonial green alga. The results of the paired t-test comparing the control and treatment discharge are summarized in Table 13. There was a significant difference (p=0.05) between the control and treatment discharge, indicating that for the ≥ 10 and < 50 μm size class, the PureBallast® v.3 BWTS reduces the live organism density in the treated discharge when compared to the control discharge.

Table 12. Live Plankton Densities (Average ± SEM) within Regulated Size Classes in Post-Treatment Intake and in Treatment Discharge During Three Trials of the PureBallast®, v.3 BWTS and the Overall Test Cycle. Note: Statistical comparisons were made within each regulated size class, not between size classes. Within each size class, treatment groups with densities having different superscript letters are significantly (p<0.05) different.

Live Organism Size Category	IMO G8 Guideline	Sample	Trial A	Trial B	Trial C	Summary (n=3)
≥ 50 μm (#/m^3)	NA	Post-Treatment Intake	6966	9090	2640	**6232 ± 1898[a]**
	<10/m^3	Treatment Discharge	445	1871	524	**947 ± 463[b]**
	NA	Treatment Discharge (24 Hour Hold)	657	686	288	**544 ± 128[b]**
≥10 and < 50 μm (#cells/mL)	NA	Post-Treatment Intake	155	379	62	**199 ± 94[a]**
	<10 /mL	Treatment Discharge	74	171	36	**94 ± 40[a]**

Table 13. Summary of Results from Paired t-tests Comparing Control Discharge Densities to Treatment Discharge Densities. Note: The hypothesis tested was that the PureBallast® v.3 BWTS significantly reduces the number of live organisms on discharge in comparison to the untreated, control discharge.

Size Class	Treatment Name	Mean Density (n=3)	Std. Dev.	SEM	t	p	Probability of Trial Resulting in No Difference
≥ 50 μm	Control Discharge	42,995[1] live/m^3	28,663	16,549	17.306	0.003	1 in 333
	Treatment Discharge	947[1] live/m^3	801	463			
≥10 and < 50 μm	Control Discharge	474 cells/mL	218	126	4.422	0.048	1 in 21
	Treatment Discharge	94 cells/mL	69	40			

[1]Data were not normally distributed, and were log-transformed to achieve normal distribution and equal variance prior to performing the paired t-test.

3.2.3. Regulated Organisms, <10 μm Size Class

Immediate post-treatment intake and treatment discharge microbial densities are summarized in Table 14. There was no significant difference between the post-treatment intake and treatment discharge densities for total coliform bacteria, *E. coli*, and enterococci (Table 14). There was an overall average of 5 MPN/100 mL total coliform bacteria in post-treatment intake, as compared to an average of <1 MPN/100 mL in treatment discharge (Table 14). Of the total coliform bacteria measured in post-treatment intake, 20 % were *E. coli* and averaged 1 MPN/100 mL. In

treatment discharge, the average *E. coli* density was <1 MPN/100 mL (Table 14). There was no significant difference between post-treatment intake and treatment discharge densities of enterococci, both averaged <1 MPN/100 mL (Table 14). Total heterotrophic bacteria results were variable, and ranged from 54 MPN/mL to 2233 MPN/mL in post-treatment intake samples (Table 14). In Trial A, there were less total heterotrophic bacteria in the treatment discharge (i.e., 246 MPN/mL) as compared to post-treatment intake (i.e., 2233 MPN/mL); however, the opposite result occurred in Trial C (Table 14). The densities appear to be similar in Trial B, although a direct comparison cannot be made because one of the treatment discharge samples was below the limit of detection (< 200 MPN/mL) and an overall average for the test could not be calculated (Table 14, Appendix 5).

For all the groups analyzed in the < 10 μm size class, there was a significant difference (p<0.05) between the control and treatment discharge densities, indicating that for the < 10 μm size class, the PureBallast® v.3 BWTS reduces the live organism density in the treated discharge when compared to the control discharge (Table 15).

Table 14. *E. coli*, Enterococci, and Total Heterotrophic Bacteria in Post-Treatment Intake and in Treatment Discharge during Three Trials of the PureBallast®, v.3 BWTS and the Test Cycle Average.

Microbial Organism	IMO G8 Guideline	Sample	Trial A	Trial B	Trial C	Summary (*n*=3)
Total Coliform Bacteria (MPN/100 mL)	N/A	Post-Treatment Intake	8 ± 2	4 ± 1	3 ± 0	**5 ± 2**
	N/A	Discharge	<1	<1	<1	**<1**
E. Coli (MPN/100 mL)	N/A	Post-Treatment Intake	2 ± 1	<1	2 ± 0	**1 ± 0**
	< 250 CFU/ 100 mL	Discharge	<1	<1	<1	**<1**
Enterococci (MPN/100 mL)	N/A	Post-Treatment Intake	<1	<1	<1	**<1**
	< 100 CFU/ 100 mL	Discharge	1 ± 0	<1	<1	**<1**
Total Heterotrophic Bacteria (MPN/mL)	N/A	Post-Treatment Intake	2233 ± 1027	171 ± 21	54 ± 8	**819 ± 708**
	N/A	Discharge	246 ± 27	<200.00 to 400.00*	232 ± 12	**Could not Determine**

*****Could not average replicates, as one or more are below the limit of detection (below 200 MPN/mL). See trimmed, raw data in Appendix 5.

Table 15. Summary of Results from Paired t-tests Comparing Control Discharge Densities (MPN/100 mL) to Treatment Discharge Densities (MPN/100 mL) of Live Organisms < 10 μm. Note: The hypothesis tested was the PureBallast® v.3 BWTS significantly reduces the number of live organisms on discharge in comparison to the untreated, control discharge.

Microbial Organism	Treatment Name	Mean Density (*n*=3)	Std. Dev.	SEM	*t*	*p*	Probability of Trial Resulting in No Difference[1]
Total Coliform Bacteria	Control Discharge	179	27	16	11.427	0.008	1 in 125
	Treatment Discharge	0.50	0.00	0.00			
E. Coli	Control Discharge	36.53	13.90	8.02	4.490	0.046	1 in 22
	Treatment Discharge	0.50	0	0			
Enterococci	Control Discharge	21.23	6.56	3.79	5.292	0.034	1 in 29
	Treatment Discharge	0.86	0.13	0.07			

3.3. Whole Effluent Toxicity (WET) Testing

GSI conducted WET tests as part of a separate trial that was conducted after the three valid trials in the test cycle were completed. Each test included a performance control using each species' medium or culture water. In all three WET tests, the performance control met the test acceptability criteria. This indicates that the organisms were healthy prior to test initiation, and that they were not damaged during the test due to handling. In addition, the untreated harbor water controls (0 % Effluent) met the test acceptability criteria for all three species tested. The average *S. capricornutum* density at test termination in the 0 % Effluent group (2,865,625 cells/mL) was slightly higher than the average density in the 100 % Effluent group (2,375,000 cells/mL); however, this difference was not statistically significant ($p<0.05$, Table 16). Therefore, there was no effect of the treatment discharge water on *S. capricornutum* growth in this trial. There was also no effect of the treatment discharge water on *P. promelas* survival or growth in this trial (Table 17). Finally, there was no effect of the treatment discharge water on *C. dubia* survival or number of young produced per female in this trial (Table 18).

Table 16. Average (±SEM) Final Density of *S. capricornutum* Exposed to PureBallast®, v.3 Treatment Discharge Whole Effluent.

Treatment Group	*S. capricornutum* Density (cells/mL)	Coefficient of Variation (CV %)
Algae Growth Media (Performance Control)	3,935,938 ± 232,407	11.8
0% Effluent (Untreated Harbor Water)	2,865,625 ± 81,070	5.7
100% Effluent	2,375,000 ± 204,825	17.2

Test acceptability criteria: Control flask must exceed $1 * 10^6$ cells/mL and not vary more than 20% among replicates.

Table 17. Average (±SEM) *P. promelas* Survival and Dry Weight per Surviving *P. promelas* Exposed to PureBallast®, v.3 Treatment Discharge Whole Effluent.

Treatment Group	Survival (%)	Dry Weight per Survivor (mg)
Laboratory Water (Performance Control)	100 ± 0	0.49 ± 0.01
0% Effluent (Untreated Harbor Water)	98.3 ± 1.8	0.51 ± 0.02
100% Effluent	100 ± 0	0.49 ± 0.01

Test acceptability criteria: 80% or greater survival in the controls; average dry weight per surviving organism in the controls equal to or exceed 0.25 mg.

Table 18. Average (±SEM) Survival and Total Reproduction of *C. dubia* Exposed to PureBallast®, v.3 Treatment Discharge Whole Effluent.

Treatment Group	Survival (%)	Reproduction (No. Young/Female)
C. dubia Culture Water (Performance Control)	100 ± 0	45.8 ± 2.0
0% Effluent (Untreated Harbor Water)	100 ± 0	46.5 ± 1.6
100% Effluent	100 ± 0	46.5 ± 3.9

Test acceptability criteria: 80% or greater survival and an average of 15 more young per female in the controls.

4.0. QUALITY MANAGEMENT

GSI uses a wide variety of quality management documents and records to implement its quality management system. These include quality system documentation (i.e., the GSI Quality Management Plan), project-specific documentation (i.e., Quality Assurance Project Plans), and routine procedures documentation (i.e., Standard Operating Procedures).

4.1 Quality Management Plan (QMP)

Detailed information on the structure and organization of GSI's quality system can be found in the GSI Quality Management Plan (GSI, 2010b). Electronic copies of this document are available upon request. The GSI QMP covers all aspects of GSI's commitment to quality including policies and procedures; criteria for and areas of application; roles, responsibilities, and authorities; assessment and response; and quality improvement. It is the framework for planning, implementing, documenting, and assessing the GSI's quality assurance and quality control (QAQC) activities.

4.2. Quality Assurance Project Plan (QAPP)

Additional information and details regarding the activities undertaken by GSI to assure the quality and credibility of its research at the Land-Based RDTE Facility can be found in GSI's Land-Based Quality Assurance Project Plan (GSI, 2010a). This document is available electronically upon request. The QAPP covers all aspects of quality assurance/quality control (QAQC), including data quality indicators, evaluation processes, performance measures and acceptance criteria; instrument certification and calibration; personnel training requirements; documents and records; data management; and QAQC assessments and response actions.

4.3. Standard Operating Procedures (SOPs)

SOPs are used to implement all GSI test activities. This facilitates consistent conformance to technical and quality system requirements and increases data quality. The SOPs include both programmatic and technical processes and procedures such as organism culturing; operation of the GSI Land-Based RDTE facility; sample collection, labeling, analysis and custody; and health and safety. Appendix 3 provides a list of GSI SOPs relevant to land-based test activities.

5.0. DISCUSSION OF RESULTS

The PureBallast® BWTS v.3 operated without interruption under the natural Duluth-Superior Harbor conditions for two out of the three research and development trials reported here. In the third trial, the BWTS filter clogged and the trial was briefly interrupted while the filter mechanism was reset. In all three trials, live organism densities in the two regulated size classes of plankton in treated discharge were significantly ($p<0.05$) lower than in control discharge, but well above IMO D-2 Standards. Densities of organisms $\geq$ 50 μm in minimum dimension in treated discharge exceeded the IMO standard of 10 live organisms per cubic meter by 2-3 orders

of magnitude (445 to 1871/m^3). Live densities in the ≥ 10 and < 50 μm size class exceeded the IMO limits of 10 live cells/mL by 1-2 orders of magnitude (36 cells/mL to 171 cells/mL). Holding the treated discharge for one day at ambient concentration did not result in significant additional die-off of organisms in the ≥ 50 μm size class. The treatment discharge densities of total coliform bacteria, *E. coli*, and enterococci were below the limit of detection (i.e., <1 MPN/mL) (though it should be noted that intake densities were already relatively low). For these three groups, the density of live organisms in the treated discharge was significantly (p<0.05) lower than the control discharge. Results from analysis of heterotrophic bacteria were variable, and differences between the treatment and control discharge could not be determined. The WET analysis detected no residual toxicity in the treated discharge.

These GSI testing outcomes relative to plankton are disappointing given the fact that tests performed on the same PureBallast® system components at the Norwegian Institute of Water Research (NIVA) yielded results consistent with the IMO standards. Part of the reason this BWTS discharge did not perform to IMO D-2 limits at GSI clearly had to do with the poor BWTS filter performance. The striking difference in filter function in tests conducted at GSI versus NIVA during the summer of 2010 must have arisen from filter performance sensitivity to something qualitative in the natural intake water conditions at GSI. That is, quantitatively GSI had lower concentrations of TSS, POC and organisms ≥ 10 and < 50 μm than required by IMO, and applied during tests at NIVA. However, NIVA's intake water from the Oslo fjord has naturally low concentrations of organisms in the ≥ 10 and < 50 μm size class. As a consequence NIVA supplements its sparse local ambient organism assemblage with dense concentrations of a single cell cultured organism (*Tetraselmis*). *Tetraselmis* is at the low side of the ≥ 10 and < 50 μm size range, and frequently below it depending on the chosen species, and likely did not present much of a challenge for the 40 μm filter of the PureBallast® BWTS v.2. Meanwhile, the diverse natural assemblage in the ≥ 10 and < 50 μm size range in Duluth-Superior Harbor was dominated by the common protist taxon *Aulacoseira* (previously known as *Melosira)*, a filamentous diatom. Filamentous diatoms are a known clogging issue for filters (Hess *et al*., 2002).

Had GSI amended its intake water to achieve IMO threshold levels, the problem would have been exacerbated. In contrast to the NIVA approach of using a single celled cultured organism, GSI concentrates natural algae and adds it to the intake stream to meet IMO-required thresholds for the ≥ 10 and < 50 μm size range. In addition, to meet IMO-required TSS levels, GSI uses Arizona Test Dust while NIVA uses Kaolinite-type clay mineral, and these additives have different particle size distributions.

At the same time, very low ambient UV transmittance of Duluth-Superior Harbor water during these tests likely impeded effectiveness of the secondary advanced oxidation treatment (AOT) stage in the BWTS. The PureBallast™ BWTS AOT™ component of the BWTS involves use of ultraviolet radiation, and was designed for water with significantly greater percent transmittance (%T) than was occurring naturally in Duluth-Superior Harbor during these tests. The %T levels at intake were 15.6 - 16.3 %T, extremely low even for Duluth-Superior Harbor, which ranged from 14.2 %T to 68.5 %T (34.1 average %T) during the 2009 and 2010. This latter condition resulted from high concentrations of dissolved organic material, however, and did not contribute to filter malfunction.

Thus two conditions likely account for the poor performance of the PureBallast™ BWTS at GSI and for the discrepancies between performance outcomes at GSI versus NIVA. The question then arises as to whether the GSI conditions under which the two BWTS components (filter and AOT) failed to perform effectively are always difficult for treatment systems and/or rare, i.e. not within the range of normal for harbors visited by ships. With respect to the level of challenge presented by GSI intake water to filters, it should be noted that other treatment system filters have performed effectively at GSI under both natural and IMO-consistent intake water conditions (see www.greatshipsinitiative.org). Thus, the sensitivity of the PureBallast™ BWTS filter to these conditions is not shared across filter types. With respect to the question of rarity of GSI challenge conditions for filtration, at least for the Great Lakes, GSI's intake concentrations of the dominant taxon, filamentous algae, are common in the ambient environment, even at augmented, IMO-consistent levels. For instance, algal monitoring data from near western Lake Erie ports (e.g., Toledo) have revealed cell densities of more than 100,000 cells/mL (Makarewicz, 1993). While much of that assemblage comprises small-celled blue-green algae such as *Anacystis*, more than 1500 cells/mL of that algal load was attributed solely to the taxon *Aulacoseira islandica*. Furthermore, recent monitoring data from Lake Erie indicate spring concentrations of *Aulacoseira islandica* as high as 2284 cells/mL (average = 828 cells/mL) (Reavie, 2009), much higher than that ever observed in even GSI's spiked intake samples. Total algal densities in Lake Erie are consistently higher than 15,000 cells/mL. In the GSI trials reported here using ambient levels of algae, live cells per mL were three orders of magnitude lower (ranging from 221 cells/mL in Trial C, to 399 cells/mL in Trial A).

With respect to the low UV Transmittance in the GSI challenge water during the summer of 2010, the story is quite different. High concentrations of dissolved organic carbon compounds result from run-off from cedar and other bogs containing tannin. The resulting brown coloration of the water often characterize river estuaries in the northern Great Lakes. But the %T levels confronted during the PureBallast™ BWTS tests were low even by Duluth-Superior Harbor standards, which averaged 34.1 %T in 2009-2010. Globally, the likelihood of such low UV transmittance conditions is not unique to Duluth-Superior Harbor, but it is relatively rare. As a practical matter, low %T at a given harbor can be anticipated in advance, such that the challenges to a UV based BWTS that they impose could be mitigated with management practices such as open ocean BWE in combination with treatment.

6.0. CONCLUSIONS

The version of the PureBallast® ballast water treatment system (BWTS) v.3 tested at the Great Ships Initiative (GSI) land-based testing facility in September and October 2010 combined a 40 μm filtration system with an Advanced Oxidation Technology (AOT™). The PureBallast® BWTS v.3 operated without interruption under the natural Duluth-Superior Harbor conditions for two out of the three trials; during the third trial, the BWTS filter clogged and the trial was briefly interrupted while the filter mechanism was reset. In all three trials, live organism densities in the two regulated size classes of plankton in treated discharge were significantly (p<0.05) lower than in control discharge, but well above IMO D-2 standards. Densities of organisms $\geq$ 50 μm in minimum dimension in treated discharge exceeded the IMO standard of 10 live organisms per cubic meter by 2-3 orders of magnitude (445 to 1871/cubic meter). Live densities in the $\geq$ 10

and < 50 μm size class exceeded the IMO limits of 10 live cells/L by 1-2 orders of magnitude (36 cells/mL to 171 cells/mL). The density of live total coliform bacteria, *E. coli*, and enterococci in the treated discharge was significantly (p<0.05) lower than the control discharge. Results from analysis of heterotrophic bacteria were variable, and differences between the treatment and control discharge could not be determined. The Whole Effluent Toxicity (WET) Analysis detected no residual toxicity in the treated discharge. These results differed from findings generated by the Norwegian Institute of Water Research (NIVA) on the same or similar system components. The difference between GSI and NIVA test outcomes can be explained in part by more challenging conditions for filtration at the GSI site, which are not unique to Duluth-Superior Harbor, and which have not led to malfunction of other filters tested at GSI. The difference was also a result of the extraordinarily low UV transmittance of the source water, which posed a greater challenge to the UV-based AOT within the PureBallast® BWTS. GSI UV Transmittance conditions are natural and not unique to Duluth-Superior Harbor, but relatively rare. Low UV transmittance of source water in Duluth-Superior Harbor resulted from high concentrations of dissolved organic material, and did not contribute to filter malfunction.

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APPENDICES

1. **GSI Land-Based Test Plan for the AlfaWall PureBallast® Ballast Water Management System**

2. **Performance Evaluation Summary for Type-Approved and Modified PureBallast® BWTS, and Research and Development Testing of the PureBallast® BWTS.**

3. **List of GSI SOPs Relevant to the Commissioning of PureBallast® v.1 and v.2 and Performance Evaluation of PureBallast® v.3.**

4. **Average Density (per m^3) of Live Zooplankton in Treatment Discharge during the Trials of the PureBallast®, v.3 Ballast Water Treatment System. Organisms are Grouped by Taxa in the ≥50 μm Size Class, Additional Live Organisms <50 μm, and Excluded Live Organisms.**

5. **Average Density (MPN per volume) of Organisms in the ≤10-μm Size Class Intake (Pre- and Post-Treatment) and Discharge (Control and Treatment) during the Trials of the PureBallast®, v.3 Ballast Water Treatment System.**

APPENDIX 1 - GSI Land-Based Test Plan for the AlfaWall PureBallast® Ballast Water Management System.

GSI Land-Based Freshwater Test Plan for the AlfaWall PureBallast® Ballast Water Management System

July 15, 2010

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EXECUTIVE SUMMARY

The Great Ships Initiative (GSI) provides independent no-cost performance verification testing services to developers of ballast treatment systems and processes at a purpose-built, land-based ballast treatment test facility located in the Duluth-Superior Harbor of Lake Superior. GSI test protocols are consistent with the requirements of the International Convention for the Control and Management of Ships Ballast Water and Sediments (International Maritime Organization, 2004). GSI procedures, methods, materials and findings are publicly accessible on the GSI website (www.greatshipsinitiative.org).

In August through October 2010, the GSI will conduct land-based tests on two versions of the PureBallast® System. During a series of five consecutive valid trials, a new version of the PureBallast® System (hereafter referred to as "AW 2") will be evaluated for its ability to: (a) successfully treat ballast water without interruption, (b) meet IMO D-2 discharge standards after a five-day holding time, and (c) discharge water after the five day retention period that is environmentally benign (i.e., no residual toxicity) pursuant to United States Environmental Protection Agency water quality criteria. Subsequently, and as time permits, additional trials up to five valid trials will be conducted on the original PureBallast® System, (hereafter referred to as "AW 1") which already has IMO final approval.

GSI land-based fresh water ballast treatment testing draws ambient water from Duluth-Superior Harbor, and amends it for these tests with solids and concentrated harbor phytoplankton to achieve IMO-consistent challenge conditions. Residual toxic effects of whole treated effluent (WET tests) will be evaluated on an array of test species in at least two trials of the AW 2.

1.0. INTRODUCTION

1.2. The Great Ships Initiative

The Great Ships Initiative (GSI)[5] 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 established superlative freshwater ballast treatment evaluation capabilities at three scales—bench, land-based, and on board ship.

The GSI awards its independent status-testing services to developers of ballast treatment systems and processes determined to be promising. GSI status-testing is performed at the scale appropriate to the state of development of the target treatment system, with the goal of facilitating the rapid progression of meritorious ballast treatment systems through the research and development and approval processes to a market-ready condition.

GSI has no involvement, intellectual or financial, in the mechanics, design or market success of the actual treatment systems it tests. To ensure that GSI tests are uncompromised by any real or perceived individual or team bias relative to test outcomes, GSI test activities are subject to rigorous quality assurance, quality control (QA/QC) procedures and documentation. This QA/QC attention also assures high quality and credible evaluation findings.

GSI has worked to standardize and intercalibrate its protocols to evaluate the performance of ballast water treatment systems with IMO guidelines, United States Environmental Protection Agency ETV draft protocols, and other test facilities. GSI test protocols are as consistent with the requirements of the International Maritime Organization's (IMO) Convention for the Control and Management of Ships Ballast Water and Sediments (IMO, 2004) and federal requirements as practicable. In particular, GSI testing directly supports IMO G8 and G9 evaluations. GSI procedures, methods, materials and findings are also not proprietary, and are publicly accessible on the GSI website (www.greatshipsinitiave.org).

Ms. Allegra Cangelosi of the Northeast-Midwest Institute is the Principal Investigator and Manager of the GSI. Researchers from the University of Wisconsin-Superior's Lake Superior Research Institute (LSRI), and the University of Minnesota-Duluth's Natural Resources Research Institute (NRRI), among others, provide critical scientific and technical expertise and implementation services to GSI's biological research activities, and the GSI generally. Dr. Mary Balcer is the project's lead zooplankton ecologist. Dr. Euan Reavie leads all phytoplankton analysis. Mr. Matthew TenEyck leads the bench-testing and Whole Effluent Toxicity (WET) tests. AMI Consulting Engineers provide engineering expertise in support of GSI testing activities. A GSI Advisory Committee comprising top-level officials of key stakeholder groups helps steer the GSI providing crucial assistance in making GSI award decisions and fund-raising. The GSI Advisory Committee includes elected leadership, environmental organizations, port directors and federal officials from the United States and Canada, and industry representatives. The American Great Lakes Ports Association advises the project, assuring that the GSI is

[5] www.greatshipsinitiative.org

meeting the needs of the maritime industry; and coordinating maritime industry and supply chain outreach.

To date, all GSI tests are supported by general project funds which derive from federal, state and port grants and contributions, and in-kind contributions by industry, local government and universities. Over time, GSI will begin to charge treatment developers for a portion of the testing costs associated with type approval testing for United States regulatory purposes. The largest contributor of GSI operating funds is the United States Department of Transportation, including its Maritime Administration, and the Saint Lawrence Seaway Development Organization. GSI also receives significant funds and in-kind contributions from the National Oceanic and Atmospheric Administration, the Canadian St. Lawrence Seaway Management Corporation, the City of Superior, Wisconsin, and approximately ten U.S. and Canadian ports in the Great Lakes.

In August, September and October 2010, the GSI will conduct land-based tests on two versions of the PureBallast® System. During the series of five consecutive valid trials, PureBallast® System will be evaluated for its ability to: (a) successfully treat ballast water without interruption, (b) meet IMO D-2 discharge standards after a five-day holding time, and (c) discharge water after the five day retention period that is environmentally benign (i.e., no residual toxicity) pursuant to United States Environmental Protection Agency water quality criteria.

1.3. The GSI Land-Based RDTE Test Facility

GSI tests evaluate the biological efficacy of a ballast water treatment system at its purpose-built, land-based ballast treatment test facility located in the Duluth-Superior Harbor of Lake Superior (Figures 1-3). The facility draws raw intake water from Duluth-Superior Harbor at 400 m^3/hr to 680 m^3/hr. This main flow of intake water can be amended with TSS and endemic Harbor algae just prior to being split into control and treatment tracks (see injection points A and B; Figure 4).

Figure 1. Location of the GSI's Land-Based RDTE Facility in Superior, Wisconsin.

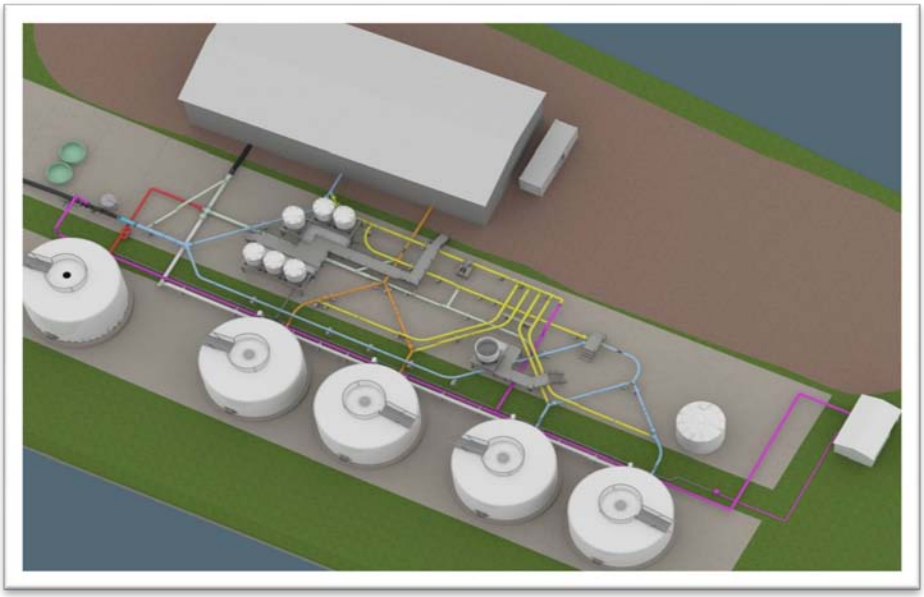

Figure 2. Computer-Generated Rendering of the GSI Land-Based RDTE Facility.

Figure 3. Photo of the GSI Land-Based RDTE Facility.

A Y-split in the intake piping at the facility simultaneously channels one half of the flow (200 m^3/hr to 340 m^3/hr) to a treatment track and the other half (also 200 m^3/hr to 340 m^3/hr) to a matched control track (Figure 4). The treatment track directs water through the experimental treatment system and into a 200 m^3 cylindrical retention tank. The control track by-passes the treatment system and channels water directly into a matched control retention tank (Figure 4). After a retention period, water is discharged sequentially from the treatment and control retention tanks at 340 m^3/hr. The water is directed either back to the harbor, to a 260 m^3 wastewater storage tank for subsequent discharge to the sewer, or recirculated to a second set of facility retention tanks (Figure 5). Information on the facility's validation is available on request.

Water is sampled continuously throughout ballasting functions (intake or discharge) through in-line sample points. Each sample point is made up of one to three identical sample ports with a center-located elbow-shaped pitot tube (90 ^{o}) bent towards the direction of water flow used to sample the water. This pitot design is based on a design developed and validated by the U.S. Naval Research Laboratory in Key West, Florida, and empirically at GSI. Intake sampling uses sample ports at paired intake sample points (SP#2 and SP#3) on the control and treatment tracks (Figure 2). Discharge sampling occurs through sample ports at sampling points SP#9 or SP#10 (Figure 2). All four SPs are made up of three sample ports.

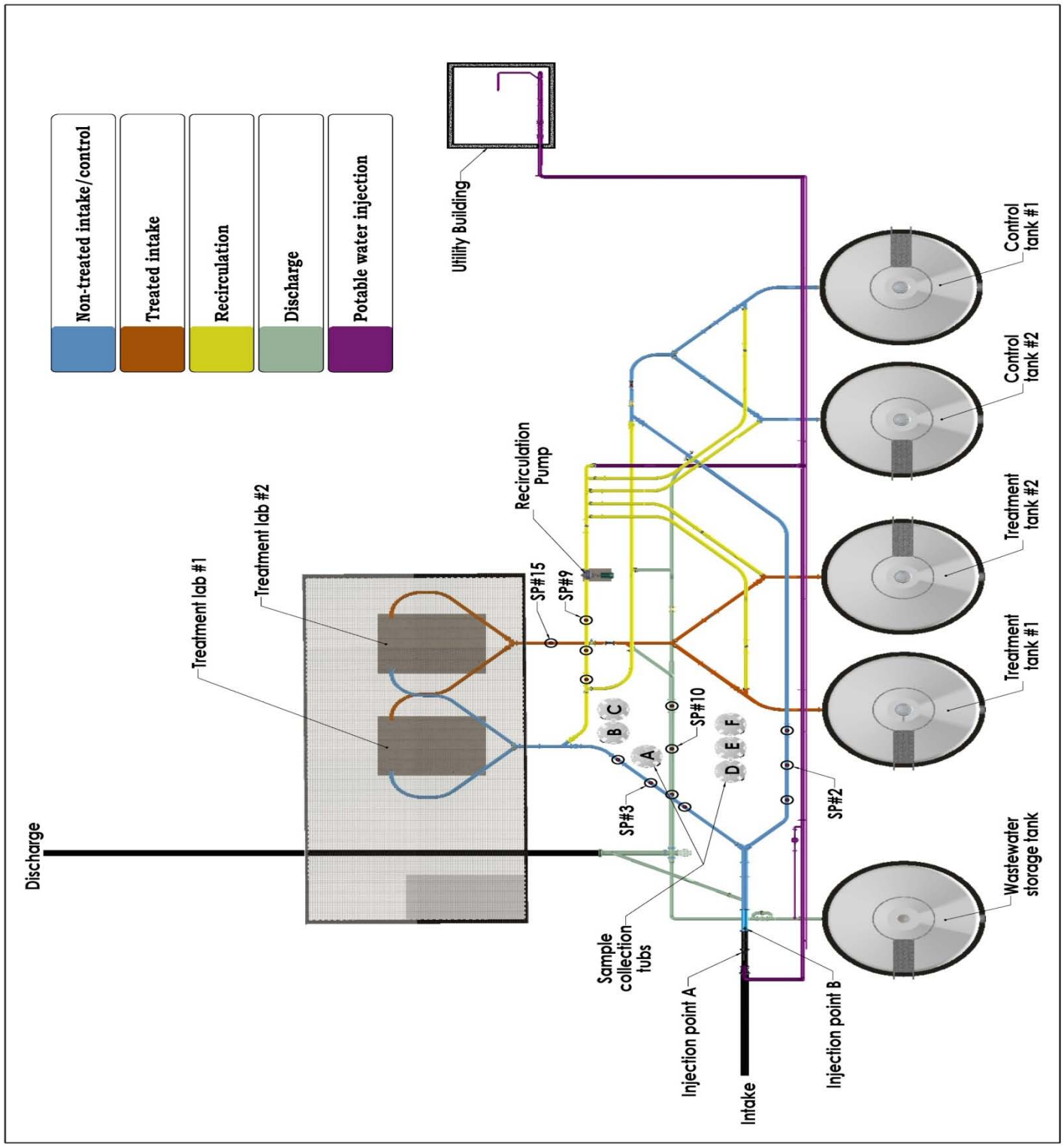

Figure 4. Simplified Schematic of the GSI Land-Based RDTE Facility Showing Location of Sample Points, Injection Points, Retention Tanks, and Treatment and Controls Tracks.

Sample water at a given sampling point (i.e., intake line of the control track, intake line of the treatment track, or the discharge line for the control and treatment tracks) is transferred simultaneously and continuously throughout ballasting operations (intake or discharge) from replicate sample ports to replicate 3.8 m^3 sample collection tubs via clean 3.8 cm ID flexible hoses and automated flow-controlled pneumatic diaphragm valves. Flow control valves and system logic assure that sample flow rates are equivalent and proportional to intake and discharge flow rates throughout each operation. Flow rates are recorded every 5 seconds during the test trials from three locations at automated valves on the control track, treatment track, and on the discharge line. Pressure readings are also recorded every 5 seconds throughout the facility.

An on-site mobile field laboratory (Figure 3a) and stationary structure (Figure 3b) provide bench-scale facilities to support time sensitive assays associated with the GSI land-based tests, including live analysis of phytoplankton and zooplankton. The laboratories are climate-controlled, and have enough bench space to allow for simultaneous microscopic and analytical analysis of samples by multiple analysts.

Figure 3a. The GSI Mobile Field Laboratory.

Figure 3b. The GSI Stationary Laboratory.

1.4. Treatment Performance Requirements in Regulation D-2

The International Convention for the Control and Management of Ships Ballast Water and Sediments was adopted by consensus at a Diplomatic Conference at IMO in London on Friday 13 February, 2004. Annex D-2 of the Convention relates to ballast water performance standards for ships conducting ballast water management, including use of a ballast water treatment system to effectively treat the ballast water. The regulation states that ships conducting ballast water management shall discharge:

- Less than 10 viable organisms per m^3 greater than or equal to 50 μm in minimum dimension;
- Less than 10 viable organisms per mL less than 50 μm in minimum dimension and greater than or equal to 10 μm in minimum dimension; and
- Discharge of the indicator microbes shall not exceed the specified concentrations. The indicator microbes, as a human health standard, include, but are not be limited to:
  - Toxicogenic *Vibrio cholerae* (O1 and O139) with less than 1 colony forming unit (cfu) per 100 mL or less than 1 cfu per 1 gram (wet weight) zooplankton samples;

- *Escherichia coli* - less than 250 cfu per 100 mL;
- Intestinal *Enterococci* - less than 100 cfu per 100 mL.

1.5. GSI Testing to G8 and G9 Requirements in IMO Convention

All current protocols, guidelines and requirements are open to interpretation especially in these early stages of implementation, and few if any facilities meet all requirements in the strictest sense. Accordingly, it is ultimately up to an Administration to decide if the testing conducted by GSI and the system meets their requirements for Type Approval Certification.

The fundamental approach of GSI is to conduct independent, scientifically-sound, rigorous, and quality assured evaluations of ballast water treatment systems. At the same time, GSI seeks immediate relevance of its freshwater land-based testing to regulatory processes such as those outlined in the IMO Convention and those under development domestically in the United States and Canada. To that end, GSI protocols are rooted in the essential features of the IMO G8 guidelines for testing, and the draft ETV protocols under development by the United States Coast Guard and United States Environmental Protection Agency. All aspects of the testing infrastructure (e.g. flow rate, retention tank size, sample size, sample collection and analysis equipment and data logging) are directly consistent with these requirements. It formally partners with the Maryland-based Maritime Environmental Resource Center (MERC) to assure that GSI freshwater land-based testing can be directly complemented by comparable brackish/salt water testing.

With respect to physical/chemical and biological characteristics of the intake stream, GSI is fortunate in that its feed water source naturally meets many of the IMO G8 requirements for intake organism densities and physical/chemical conditions during the testing season (Table 1). However, ambient conditions do fluctuate in all natural systems. Therefore, for these tests, GSI will augment intake water to better assure that initial challenge water conditions meet requirements in the IMO G8 guidelines throughout the trial series. During initial filling of control and test retention tanks, fine grade Arizona Test Dust, particulate organic matter, and concentrated algae harvested from the Duluth-Superior Harbor will be metered into the intake stream before the flow split to the control and treatment tracks. Details on these processes are provided below. Target intake levels of these parameters appear in Table 1.

Table 1. Ranges of Various Physical/Chemical and Biological Parameters in Ambient Water from Duluth-Superior Harbor (June – September) in Comparison to Draft U.S. EPA/ETV and IMO G8 Recommended Challenge Conditions.

Parameter	DRAFT U.S. EPA ETV[6]	Recommended IMO G8[7]	Historic Ranges Duluth/Superior Harbor	Target Values for Augmented Duluth-Superior Water
Temperature (^{0}C)	4 – 35	–	5 – 25	5 – 25
Salinity (psu)	< 1	Two salinities, >10 psu difference	0 – 1	0 - 1
Total Suspended Solids (TSS, mg/L)	Min. 24	> 50	2 – 21	50
Particulate Organic Carbon (POC, mg/L)	Min. 4	> 5	0.5 – 2.1	≥5
Dissolved Organic Carbon (DOC, mg/L)	Min. 6	> 5	3 – 30	3 - 30
Mineral Matter (MM, mg/L)	Min. 20	--	--	Min. 20
Zooplankton (> 50 μm/m^3)	Min. 75,000	> 100,000	100,000 - 3,000,000	100,000 – 3,000,000
Phytoplankton (10 - 50 μm/mL)	Min. 750	> 1,000	25 – 1,200	> 1,000
Heterotrophic Bacteria (CFU/mL)	Min. 750	> 10,000	> 1,000 MPN/mL	1,000 MPN/mL

6 U.S. Environmental Protection Agency, Environmental Technology Verification Program. DRAFT Generic Protocol for the Verification of Ballast Water Treatment Technologies. March, 2010.
7 IMO MEPC 57, Annex 3: Revised Guidelines for Approval of Ballast Water Management Systems (G8). April 4, 2008.

2.0. METHODS

The GSI land-based evaluation of the PureBallast® System will be carried out in keeping with the methods summarized in this Test Plan and detailed in GSI Standard Operating Procedures. Some refinements may be necessitated by circumstance or opportunity, but these will be carefully noted. The following sections describe how each parameter and variable will be sampled and analyzed during the trials at GSI. Additional details can be found at www.nemw.org/GSI/SOPS.htm. All SOPs relevant to the tests, as amended, also are presented Appendix 2. Any deviations from these SOPs during the performance of the tests will be minor and will not affect data quality.

2.1. Experimental Goals and Design

The PureBallast® System performance evaluation will involve physical and biological characterization of water upon ballasting (uptake/intake of water), and enumeration, sizing, and live/dead analysis of organisms in control and treated discharge water after a five-day in-tank holding time. GSI biological characterizations support direct comparison with the IMO D-2 organism categories and standards. During a series of five consecutive valid trials, the treatment system will be tested for its ability to: (a) successfully treat ballast water without interruption, (b) meet IMO D-2 discharge standards after a five-day holding time, and (c) discharge water after the five day retention period that is environmentally benign (i.e., no residual toxicity) pursuant to United States Environmental Protection Agency water quality criteria. A valid trial will be considered one in which intake challenge conditions and control discharge densities of live organisms meet the IMO G8 guidelines, and in which the facility operated properly.

2.1.1 Treatment System and Test Facility Calibration Trials

GSI will conduct two calibration test runs of the PureBallast® System. The calibration runs are undertaken to assure the facility and the treatment system are operating properly. During these calibration trials, adjustments to the system will be documented only for internal reference by the treatment developer. If there are no such adjustments, and the trials are valid, they will be subsumed into the set of five test trials.

2.1.2. Valid Trials

Once the two calibration trials are complete, if there are adjustments to either the treatment system or the facility, the PureBallast® System and the facility will be set for type approval testing by the treatment developer, and the GSI Facility Manager, respectively, and five valid trials of the PureBallast® System will immediately follow the calibration runs. If no such changes took place, no changes will be made to system or facility settings, and three valid trials of the PureBallast® System will immediately follow the calibration runs. Any further adjustments to either component of the testing (the treatment system or the facility) will be carefully noted and subject to QA/QC documentation.

GSI runs concurrent, but staggered, tests using two sets of matched 200 cubic meter tanks. Treatment and control intake operations for a given trial are always simultaneous, and treatment and control discharge operations are always sequential.

2.1.3. Preventing Cross-Contamination

The GSI facility lines are flushed with potable water using a self-propelled spiral-action water jet mechanism. The operation is undertaken between each facility intake or discharge operation. The thoroughness of the cleansing process is checked by partially filling catchment tubs with potable water and then draining that water through a plankton net and examining the filtrate for evidence of residual organisms. The facility is deemed clean only if the rinse water is completely free of organisms visible with a compound microscope. Nets and other sample collection equipment are likewise validated for cleanliness prior to each sampling operation.

2.1.4. Challenge Conditions

Ambient conditions will be employed as the physical/chemical challenge conditions, except that Fine Test Dust and artificial POM will be added to the facility intake to assure levels are in keeping with IMO G8 guidelines. The solids injection procedure is detailed in GSI/SOP/LB/G/O/5 – *Procedure for Injecting Organisms and Solids into the GSI Land-Based RDTE Facility*. Using TSS as an example, Fine Test Dust (ISO 12103-1, A2; nominal 0-80 μm particle size; Powder Technology Incorporated; Burnsville, MN) is pre-weighed at LSRI, and sterilized by baking in an oven at 190 °C for one hour. One day prior to the test trial, ambient TSS is measured in the Duluth-Superior Harbor. On the day of the test trial, the volume of harbor water to be used in the Solids Injection System (SIS) tank is determined in order to augment the intake water to 60 mg/L TSS, and the SIS tank is filled. The prepared Fine Test Dust is poured into the SIS tank slowly to prevent clumping, and the dust is mixed for a minimum of 20 minutes prior to the start of the trial. The test dust is injected into the intake water for the entire duration of the fill at a constant rate using a peristaltic pump located at Injection Point A (Figure 4).

Biological challenge conditions are largely ambient as well except that organism densities in the smaller of the two plankton size classes (i.e., 10 - 50 μm) are enhanced to assure consistency with IMO G8 required thresholds. The solids and phytoplankton injection systems are kept separate to reduce the risk of interference. The phytoplankton injection procedure is detailed in GSI/SOP/LB/G/O/5 – *Procedure for Injecting Organisms and Solids into the GSI Land-Based RDTE Facility*. One to two days prior to the test trial, phytoplankton entities from the Duluth-Superior Harbor are collected and concentrated using 20 - 50 μm plankton nets. The concentrated phytoplankton entities are stored at the GSI Land-Based RDTE Facility in holding ponds. Prior to injection, the water containing concentrated phytoplankton is mixed, sampled, and analyzed for viable cell density. In addition, a sample of Duluth-Superior Harbor water is collected to determine the ambient viable cell density. Based on the density of cells in the phytoplankton concentrate and ambient intake water, the volume of spiked concentrate that would be needed to achieve a concentration of 1500 cells/mL in intake water is calculated. This volume is added to an Organism Pressure Injection System (OPIS) vessel. The OPIS vessel is pressurized to 25 psi greater than the system pressure. The phytoplankton concentrate is added at

a constant rate to the intake water via the pressure differential for the entire duration of the intake procedure via Injection Point B (Figure 4). A static mixer, installed in the main intake line just after the two metering systems (SIS and OPIS) and prior to the main system "Y split" (Figure 4), assures that the concentrations of these additives is equivalent in the control and treatment tracks of the facility. Gentle agitators installed in the tanks assure that that live organisms, especially spiked algal particles that may settle to the bottom of the tank during the retention period are accounted for to the greatest extent possible in the discharge water analysis (SOP to be developed prior to AlfaWall tests.

2.2. Water Quality Analysis

2.2.1. Analysis of Total Suspended Solids (TSS)

Samples for TSS analysis are collected during intake only as follows:

- Three 1 L whole water samples are collected from the pre-treatment line (SP #3, Figure 4) at approximately 10, 30, and 50 minutes after the start of the intake procedure, and
- Three 1 L whole water samples are collected from the post-treatment line at approximately 10, 30, and 50 minutes after the start of the intake procedure (SP #16, Figure 4).

Samples are collected in-line rather than from the sample collection tubs to avoid settling of suspended solids. This approach assured a more accurate measurement of solids and organic carbon in the intake water.

For analysis, the samples are vacuum filtered through pre-washed, dried, and pre-weighed Whatman 934-AH glass fiber filters. After the sample is filtered it is dried in an oven and brought to constant weight. TSS values are determined based on the weight of particulates on the filter and the volume of water filtered.

Quality control sample analysis consists of analyzing approximately ten percent of the samples in duplicate. A TSS reference standard (QCI, 711, ULTRA Scientific) is analyzed on multiple occasions along with TSS samples to confirm the accuracy of the data being generated.

2.2.2. Analysis of Non-Purgeable Organic Carbon (NPOC) and Dissolved Organic Carbon (DOC), and Determination of Particulate Organic Carbon (POC) Concentrations

In these tests, NPOC is measured as a surrogate for total organic carbon (TOC), though it may be a slight underestimate of TOC. The analytical instrument used to measure NPOC purges the sample with air to remove inorganic carbon before measuring organic carbon levels in the sample. Thus, the NPOC analysis does not incorporate any volatile organic carbon which may be present in the sample.

Aliquots of the same samples that are analyzed for TSS are also analyzed for NPOC and DOC. Before the TSS analysis is conducted, aliquots of approximately 50 mL of the sample are transferred to glass bottles and acidified with hydrochloric acid for NPOC analysis. An aliquot of the filtrate from the TSS analysis is transferred to a glass bottle and acidified for analysis of DOC. A Shimadzu Total Organic Carbon Analyzer (Model TOC-5050A) is employed for analysis of both NPOC and DOC. Concentrations of NPOC and DOC are determined based on a calibration curve developed on the instrument using organic carbon standards prepared from potassium hydrogen phthalate. Reported particulate organic carbon concentrations (POC) are determined as the difference between the NPOC and DOC values for a sample.

Quality control sample analysis consisted of analyzing approximately 10 % of the samples in duplicate. A reference standard (#516 Demand, Environmental Resource Associates) is analyzed daily to confirm the accuracy of the data being generated.

2.3. Viable Organism Analysis

Sample water for analysis of viable organisms is simultaneously collected from replicate sample ports into identical 3.8 m^3 collection tubs during each intake or discharge operation. Volumes retained varied with the operation (intake versus discharge) and treatment (control versus treatment), depending upon anticipated organism concentrations, but are always greater than IMO guideline volumes. The water in each collection tub constitutes an independent time integrated replicate sample of the 200 m^3 experimental water mass.

2.3.1. Organisms Greater than 50 µm in Minimum Dimension

2.3.1.1. Sample Collection

During the intake operation, i.e. the filling of the treatment and control 200 m^3 retention tanks, the following time-integrated sample volumes are collected by continuous flow from the intake lines simultaneously:

- 2 - 4 m^3 from the pre-treatment intake line,
- 2 - 4 m^3 from the control intake line, and
- 2 - 4 m^3 from the immediate post-treatment intake line.

During discharge:

- One 2 - 4 m^3 time-integrated sample is continuously collected from the control discharge, and
- Two to three replicate time-integrated samples of 2 - 4 m^3 each (total volume 4 to 9 m^3) are continuously collected from the treatment discharge.

Flow control valves and system logic assured that sample flow rates are equivalent and proportional to intake and discharge flow rates throughout each operation. Immediately after filling, the entire sample volumes are drained from the sample collection tubs and concentrated

through 35 μm (50 μm diagonal dimension) plankton nets into 1 L cod-ends for microscopic examination. See *GSI/SOP/LB/RA/SC/6 - Procedure for Zooplankton Sample Collection*.

2.3.1.2. Live/Dead and Size Analysis

Live/dead analysis takes place within two hours of collecting and concentrating the individual samples. Microzooplankton (e.g., rotifers, copepod nauplii, veligers, etc.) and macrozooplankton (e.g., crustaceans), all generally greater than 50 μm in minimum dimension (with the exception noted below) are analyzed simultaneously by separate taxonomists. Microzooplankton subsamples are analyzed in a Sedgewick-Rafter counting chamber by examination under a compound microscope at a magnification of 40X to 100X. Macrozooplankton are analyzed in a Ward's Counting Wheel at a magnification of 20 to 30X using a dissecting microscope. Due to high densities, quantification of zooplankton in intake and control discharge samples requires analysis of sub-samples and extrapolation to the entire sample volume. For these samples, a subsample is removed for analysis using a Henson-Stempel pipette. The treatment discharge samples has lower densities allowing analysis of a greater sample volume. Treatment discharge samples are split in half using a Folsom Plankton Splitter. Half of the sample is analyzed for macrozooplankton and the other half is examined for microzooplankton. The proportion and total concentration of live versus dead organisms is determined using standard movement and response to stimuli techniques.

Quality assurance measures include live/dead analysis of at least 10 % of treatment discharge samples, and 10 % of control intake and discharge samples by two separate taxonomists. The average percent similarity of taxonomic identification and the average relative percent difference of the number of live organisms counted are calculated for all second analyses. These data quality measurements are compared against the data quality objectives outlined in the *GSI Quality Assurance Project Plan (QAPP) for Land-Based Tests* (GSI, 2010), and the percentage of data quality measurements meeting the data quality objectives is determined for microzooplankton and macrozooplankton separately.

Because freshwater zooplankton are in general smaller than their salt and brackish water counterparts, the larger regulated size category (greater than 50 μm in minimum dimension) does not incorporate all live zooplankton that may be present in a freshwater assemblage. This freshwater phenomenon raises special issues with respect to assessing zooplankton densities for the purpose of comparison with the IMO D-2 standard. If individuals of these smaller species occur in discharge samples during these tests, they will be counted, sized and reported, but the data will be kept distinct from tallies directly relevant to regulated size classes. See *GSI/SOP/LB/RA/SA/2 - Procedure for Zooplankton Sample Analysis*.

2.3.2. Organisms 10 – 50 µm in Minimum Dimension

2.3.2.1. Sample Collection

For live analysis of organisms 10 – 50 μm in minimum dimension, one sample of 1 L is collected immediately after filling from the pre-treatment sample collection tub and one sample of 1 L is collected from the immediate post-treatment sample collection tub. During discharge, one

sample of 1 L is collected from the control tank via sample collection tub, and three samples of 1 L each are collected from the replicate treatment sample collection tubs. Analysis occurred on-site within 1.5 hours of sample collection, with samples stored in coolers during the interim. Prior to analysis, samples are concentrated through a 10 μm plankton net and stored in a 25 mL sample container. See *GSI/SOP/LB/RA/SC/3 - Procedure for Algae/Small Protozoa Sample Collection*.

2.3.2.2. Sample Analysis

For analysis, a 1.5 mL subsample of the concentrated sample is transferred to a 2 mL sample container, with 4 μL of Fluorescein Diacetate (FDA) stock solution added. The subsample is then allowed to incubate in the dark for 5 minutes. The 1.5 mL incubated algae sample is mixed and 1.1 mL is immediately transferred to a Sedgwick-Rafter cell, covered and placed on the stage of a microscope that is set for simultaneous observation using brightfield and epifluorescence. At least two horizontal transects are counted (an area known to reflect greater than 1 mL of original sample water). If time permits, additional transects are counted to increase statistical power. This results in greater than 100 live cells counted from the pre-treatment intake and control discharge samples, and often fewer than 10 live cells counted in two transects for post-treatment intake and treatment discharge samples. Single cell entities and cells comprising colonial and filamentous entities are characterized as follows: alive = cells showing obvious green fluorescence from cell contents; dead = cells showing no or very little evidence of green fluorescence from cell contents; and ambiguous = cells or entities that cannot be clearly identified as alive or dead (should be uncommon). Records are kept of transect lengths and widths so that the total counted area and volume analyzed can be calculated later.

Entities less than 10 μm in all visible dimensions or greater than 50 μm in minimum dimension are not counted. Counting and measurement of all other entities followed standard procedures for individuals (length and width), colonies (e.g., number of cells, cell length and width) and filaments (e.g., number of cells, cell length and width or total filament length if cells cannot be discerned). The remaining concentrated sample in the 25 mL bottle is archived using a preservative (formalin or Lugol's) for long-term storage.

Quality assurance measures include analysis of at least at least two treatment discharge samples and at least one control intake/discharge sample by two separate taxonomists using a dual-headed microscope (i.e., both taxonomists analyze the same sample at the same time). The average percent similarity of taxonomic identification and the average relative percent difference of the number of live organisms counted are calculated for all second analyses. These data quality measurements are compared against the data quality objectives outlined in the *GSI Quality Assurance Project Plan (QAPP) for Land-Based Tests* (GSI, 2010), and the percentage of data quality measurements meeting the data quality objectives is determined. See *GSI/SOP/LB/RA/SA/1 - Procedure for Algae/Small Protozoan Sample Analysis*.

2.3.3. Bacteria

Control and treatment samples are collected and analyzed for heterotrophic bacteria, two specific indicator pathogens: *E. coli* and enterococci, and viable toxigenic *Vibrio cholerae*.

2.3.3.1. Sample Collection

One liter whole water samples are collected as follows:

- On intake, three are collected immediately after filling from the pre-treatment sample collection tubs, and three are collected from the post-treatment sample collection tubs.
- On discharge, three are collected from a control sample collection tub and three are collected from a treatment sample collection tub.

All samples are collected according to *GSI/SOP/LB/RA/SC/4 – Procedure for Microbial Sample Collection*, and are immediately transported in an insulated cooler to the LSRI and analyzed as individual replicates.

2.3.3.2. Sample Analysis

Viable heterotrophic bacteria are enumerated according to *GSI/SOP/BS/RA/MA/1 – Procedure for Quantifying Heterotrophic Plate Counts (HPCs) using IDEXX's SimPlate® for HPC Method*. This method utilizes the IDEXX SimPlate® for HPC Method (IDEXX Laboratories, Inc.; Westbrook, Maine), which is based on IDEXX Laboratories' patented multiple enzyme technology.

The presence and abundance of *E. coli* (*GSI/SOP/BS/RA/MA/4 - Procedure for the Detection and Enumeration of Total Coliforms and E. coli Using IDEXX's Colilert*®) and enterococci (*GSI/SOP/BS/RA/MA/3 - Procedure for the Detection and Enumeration of Enterococcus using Enterolert*™) are determined using Colilert® and Enterolert™, respectively, which are both based on IDEXX's patented Defined Substrate Technology (DST®).

RNA and DNA colony blots are prepared at the LSRI following *GSI/SOP/LB/RA/MA/6 - Procedure for the Colony Blot Preparation for Enumeration of Culturable Vibrio chloreae*, a procedure in which the RNA or DNA of potential *Vibrio Cholerae*, and a limited number of additional species which may grow on the selective media, is fixed to a filter. Filters which exhibit colony growth are then shipped to the Maryland Pathogen Research Institute at the University of Maryland (College Park, MD) for analysis of potential viable toxigenic *V. cholerae*. Viable toxigenic *V. cholerae* is assayed with a commercial DFA kit specific for serogroup O1 (New Horizons Diagnostics) using monoclonal antibodies tagged with fluorescein isothiocyanate (FITC) (Hasan *et al*., 1994).

Quality control samples include a media blank and a positive control for *E. coli*/total coliforms and *Enterococcus spp*.; a media and peptone-saline diluent blank for heterotrophic bacteria; and a thiosulfate citrate bile salts sucrose (TCBS) agar blank, and DNA, and RNA blanks for *Vibrio spp.* Quality assurance measures include analysis of at least 10 % of the samples in duplicate. The average relative percent difference of all duplicates analyzed during the test trials is calculated separately for *E. coli*, *Enterococcus spp*., heterotrophic bacteria, and *Vibrio spp*. In addition, at least 10 % of the samples are counted by two separate analysts and the average relative percent difference for all second counts is determined. These data quality measurements

are compared against the data quality objectives outlined in the *GSI Quality Assurance Project Plan (QAPP) for Land-Based Tests* (GSI, 2010), and the percentage of data quality measurements meeting the data quality objectives is determined.

2.4. Ambient Physical/Chemical Water Conditions Analysis

Temperature, salinity, dissolved oxygen, chlorophyll fluorescence, turbidity and pH are measured every 15 minutes during the test trials by two identical multi-parameter probes (calibrated according to manufactures specifications) placed, one each, into the control and test tanks. A calibrated, hand-held instrument is used to measure temperature, salinity, and dissolved oxygen from the control sample collection tub, the pre-treatment sample collection tub, and post-treatment sample collection tub during intake. In addition, temperature, salinity, and dissolved oxygen are measured during discharge from one control sample collection tub and two or three treatment sample collection tubs. See *GSI/SOP/LB/RA/SC/8 - Procedure for Collecting Physical/Chemical Data and Samples at the GSI Land-Based RDTE Facility*.

2.5. Whole Effluent Toxicity Analysis

GSI's whole effluent toxicity testing involves tests for chronic toxicity involving three freshwater species as arrayed in Table 3. Toxicity tests are conducted on treated water from all five test trials.

Table 3. Standard Operating Procedures Relative to Whole Effluent Toxicity Testing.

GSI SOP Code	Test Type	Test Species	Test Endpoint
GSI/SOP/BS/RA/WET/1	Chronic	Cladoceran (*Ceriodaphnia dubia)*	Survival and Reproduction
GSI/SOP/BS/RA/WET/2	Chronic	Fathead Minnow (*Pimephales promelas)*	Survival and Growth
GSI/SOP/BS/RA/WET/3	Chronic	Green Alga (*Selenastrum capricornutum)*	Growth

2.5.1. Standard Whole Effluent Toxicity Tests

One set of tests—Standard Whole Effluent Toxicity Tests (Standard WET)—measures toxicity following five days storage in the land-based facility's 200 m^3 retention tanks. For these tests, samples are collected for analysis of residual toxicity at discharge. Sample water, stored in large HDPE containers, is immediately transported to the LSRI and is used immediately upon arrival to set up the Standard WET tests. Following initial set up of the tests, the remaining sample water is held at 4 °C in the dark to retain as much of the initial toxicity as possible, and portions of the discharge sample water is warmed to 25 °C each day to serve as renewal water for the bioassay. A dilution series, using Duluth-Superior Harbor water, is run for each species. All tests are conducted in temperature-controlled incubators, water baths, or at ambient room temperature following the SOPs listed in Table 3.

2.5.2. Cold Whole Effluent Toxicity Tests

A second set of trials—Cold Whole Effluent Toxicity (Cold WET) tests—is conducted to estimate the TRC, TRO and toxicity effects on organisms under cold water conditions. Treated water is collected continuously from a sample port just downstream of the treatment system (SP #15) and diverted into a sample collection tub during the filling of the treatment retention tank. A 50 L whole water subsample is extracted and placed in a dark, refrigerator set at 4 ^{o}C for five days, thus simulating cold temperature tank retention. A portion of the sample water is warmed to 25 °C prior to initial set up of the Cold WET assay, and is warmed prior to daily renewal as described above for the Standard WET assay. There is no dilution series for the Cold WET assay; test organisms (*Selenastrum capricornutum, Ceriodaphnia dubia, and Pimephales promelas*) are exposed to 100 % sample water. The Cold WET assay is conducted concurrently with the Standard WET assay following the SOPs listed in Table 3.

2.5.3. Statistical Analysis for WET Assay

Data are analyzed using the Comprehensive Environmental Toxicity Information Systems program (version 1.7, Tidepool Scientific Software, McKinleyville, CA). Data analyses includes normality, homogeneity of variance, one-way analysis of variance (ANOVA), and a suite of tests for comparison between treatment means. Non-normal survival data are transformed using the natural log (EPA, 2002) to normalize the data. The endpoints of the chronic toxicity tests are:

- Lowest Observed Effect Concentration (LOEC), i.e., the lowest concentration in a test with a statistically significant difference in response from the control response.
- No Observed Effect Concentration (NOEC), i.e., the highest concentration in a test for which there is no statistically significant difference in response from that of the control.
- Median Lethal Concentration (LC_{50}), i.e., the concentration resulting in death of 50 % of exposed individuals by a predetermined time.
- Effective Concentration (EC_{25}), i.e., the concentration resulting in inhibiting a biological function (e.g. growth, reproduction) of 25 % of exposed individuals by a predetermined time.

These measures are extrapolations of statistical results to the experimental endpoints. Mean percent survival, mean dry weight values, mean cell density, and mean number of young per female for the laboratory controls and treatments are analyzed with a statistical significance level of 0.05.

2.5.5. Determination of Quality of Test Organisms for WET Assay

Whole Effluent Toxicity tests are initiated with healthy, vigorous organisms. To determine the overall health of the test organisms, reference toxicant tests are performed with *Ceriodaphnia dubia* and *Pimephales promelas* prior to the start of each definitive test or at least once per month. To determine the validity of the Standard and Cold WET tests, percent survival, dry

weights of survivors, mean cell density, and mean number of young per female in the controls are compared to the test acceptability criteria published in the U.S. EPA's Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms (4^{th} edition, 2002). Class I standardized weights are used as a check for the organism drying process and the performance of the balance. Daily and weekly calibration of test meters ensures optimal performance.

2.6. Data Recording

Biological and chemical data is recorded by hand (using indelible ink) on pre-printed data collection forms and/or in bound laboratory notebooks that are uniquely-identified and are specific to the treatment technology being tested. The types of biological and chemical data collected include: sample collection data (e.g., date, time, and location of collected samples), water quality and chemistry analysis data (e.g., TSS, TOC, and active substance concentration), microbial analysis data (e.g., sample preparation, incubation, and direct counts), phytoplankton analysis data (e.g., number of live and number of dead entities), zooplankton analysis data (e.g., sample concentration; number of dead, total, and live organisms), and whole effluent toxicity test data (e.g., test set up, direct counts, and test take down).

The data that are recorded on pre-printed data collection forms are secured in uniquely-identified three ring binders, specific to the type of data and to the treatment technology. Biological and chemical data that are recorded by hand are entered into either a MS Access Database that was designed, developed, and is maintained by the GSI Database Manager, or the data are entered into a MS Excel Spreadsheet. The electronic data files are stored on the LSRI's secured Local Area Network (LAN) that can be accessed only by relevant GSI personnel. The GSI Database Manager is the single point of control for access to the LSRI LAN. The LSRI LAN is automatically backed up every 24 hours. The electronic data files are also stored on the GSI's internal SharePoint website, which acts as a secondary data backup/storage mechanism. All original raw data from verification testing of each treatment technology are stored in a climate-controlled, secure archive room at the LSRI for five years after the final report is finalized.

In-tank water quality data (e.g., temperature, pH, dissolved oxygen, salinity, turbidity, and chlorophyll-a) is measured every fifteen minutes during each retention period and automatically recorded in a Microsoft (MS) Excel spreadsheet. Facility data (e.g., flow rates and pressure measurements) are electronically recorded every five seconds during intake and discharge. This data is exported to MS Excel for subsequent analysis, and stored by AMI Engineers on a secure network, as well as on GSI SharePoint for addition storage and archiving.

A percentage of data that is recorded by hand and entered into MS Access or Excel is verified against the original raw data, this also includes verification of formulas/calculations (i.e., hand-calculation of data) done using MS Access or Excel. The percentage of verified raw data depends on the amount of raw data that was generated, and ranges from 10 % to 100 % of the original raw data. Data validation is detailed in Section 7 of the *GSI Quality Assurance Project Plan (QAPP) for Land-Based Tests* (GSI, 2010). This section also details the acceptable values, where appropriate, for the following quality objectives: accuracy, precision, completeness, comparability, representativeness, and sensitivity.

3.0. QUALITY MANAGEMENT

3.1. Documents and Records

GSI uses a wide variety of quality management documents and records to implement its quality system. These include quality system documentation (i.e., the GSI Quality Management Plan), project-specific documentation (i.e., Quality Assurance Project Plans), and routine procedures documentation (i.e., Standard Operating Procedures).

3.1.1. Quality Management Plan (QMP)

The GSI QMP details the structure of the GSI's quality system from an organizational perspective. It covers all aspects of GSI's commitment to quality including policies and procedures; criteria for and areas of application; roles, responsibilities, and authorities; and assessment and response. It is the framework for planning, implementing, documenting, and assessing the GSI's quality assurance and quality control (QAQC) activities.

The GSI Senior Quality Systems Officer is responsible for preparing the QMP, with the document based on the U.S. EPA's "*EPA Requirements for Quality Management Plans*" to the greatest extent possible. The QMP is distributed to the GSI PI for review in draft form. Once a draft is finalized, the document is approved and forwarded to GSI senior research personnel and QAQC officers. Draft and final copies of the document are posted to the GSI SharePoint intranet website. The GSI's QMP is valid for a maximum period of five years, with an annual review and revision (as needed) occurring at the end of each calendar year. Copies of this document are available on request.

3.1.2. Quality Assurance Project Plans (QAPP)

The GSI's Land-Based Quality Assurance Project Plan (GSI, 2010) describes the activities undertaken by GSI to assure the quality and credibility of its research at the land-based facility. The QAPP covers all aspects of quality assurance/quality control (QAQC), including data quality indicators, evaluation processes, performance measures and acceptance criteria; instrument certification and calibration; personnel training requirements; documents and records; data management; and QAQC assessments and response actions.

The GSI Senior Quality Systems Officer, in conjunction with the GSI Senior QAQC Officer, is responsible for developing the QAPP. The plans follow the format of the U.S. Environmental Protection Agency's (EPA's) "*EPA Guidance for Quality Assurance Plans*" to the greatest extent possible. Draft QAPPs are distributed to relevant GSI senior research personnel for review and comment. Once a draft is finalized, the documents are then passed on to the GSI PI for review and approval. Draft and final copies of QAPPs are posted to the GSI SharePoint intranet website; the final versions may also be posted to the GSI public website. Once approved, the QAPP is valid for a period of five years, though they are reviewed annually and revised as needed. Copies of this document are available on request.

3.1.3. Standard Operating Procedures (SOPs)

SOPs are used to implement all GSI test activities. This facilitates consistent conformance to technical and quality system requirements and increases data quality. The SOPs include both programmatic and technical processes and procedures such as organism culturing; operation of the GSI Land-Based RDTE facility; sample collection, labeling, analysis and custody; and safety. Appendix 1 provides a list of GSI SOPs relevant to land-based test activities.

GSI SOPs are developed by the relevant GSI senior research personnel in conjunction with the GSI Senior Quality Systems Officer and GSI Senior QAQC Officer. The GSI Senior Quality Systems Officer is responsible for distributing finalized SOPs to the GSI PI for approval. Draft and final copies of all SOPs are posted to the GSI SharePoint website; the final versions are also posted to the GSI public website (www.greatshipsinitiative.org). All GSI SOPs are updated on an as-needed basis.

To date approximately 50 SOPs have been finalized, with many more in draft form or planned. The SOPs follow a common format and include specific QAQC procedures and metrics. GSI SOPs are grounded in published standard methods. They are also consistent with international and domestic guidelines where they exist. All GSI SOPs are subject to periodic review and revision to assure that the most up to date approaches are employed.

3.1.4. Notebooks, Forms and Records

Bound field and laboratory notebooks, each having a unique identification code, are used to record observations, sampling details, and laboratory and field measurements. Notebooks are also used to record instrument and equipment calibration and maintenance information. GSI personnel are responsible for maintaining the notebooks on site, creating electronic copies, and posting to the GSI SharePoint website for storage and archiving.

Specific forms are used to record sample collection and analysis data. All relevant GSI senior research personnel are responsible for ensuring that the forms are correctly filled out. They are also responsible for maintaining the forms on file, creating electronic copies, and posting to the GSI SharePoint website for storage and archiving. In general, hard copies of all forms are stored in three-ring binders, each with a unique identification code.

Specific forms are also used to record sample custody, handling and storage information. Chain of custody forms are employed only when an outside laboratory is contracted to conduct sample analyses. All relevant GSI senior research personnel are responsible for ensuring that the forms are correctly filled out at the time of changes to sample custody, and sample handling and storage. They are also responsible for maintaining the forms on file, creating electronic copies, and posting to the GSI SharePoint website for storage.

In addition, specific forms are used to record operation, maintenance and safety information. The GSI Land-Based RDTE Facility Operations Manager is responsible for ensuring that all forms associated with safety (i.e., confined space entry permit forms, daily safety checklist) and operation and maintenance of the land-based test facility are correctly filled out. It is the

responsibility of the GSI Land-Based RDTE Facility Operations Manager to ensure that equipment maintenance and instrument calibration is properly documented, and that forms are maintained on file, and also posted to the GSI SharePoint website for storage.

3.2. Assessment

GSI assesses its quality system on a project by project (or test by test) basis using a variety of tools. The purpose, procedural details, and implementation frequency of each of these assessment tools are outlined below.

3.2.1. Project-Specific QAPP Audits

GSI QAQC Officers assess the implementation of project-specific QAPPs (i.e., the GSI Land-Based QAPP) during each test of a ballast treatment system. At the end of the test duration, the officers provide a report to the GSI Senior Quality Systems Officer and GSI PI. The report includes a Table listing deviations to the specific QAPP associated with the testing. The following Table headings are to be used:

- QAPP Section
- QAPP Page No.
- Description
- Deviation/Inconsistency
- Date
- GSI Personnel
- Reconciliation/Corrective Act

The report also includes an assessment of personnel training requirements and certification, as well as procedures for storing and archiving documents and records; sample labeling, handling and custody requirements; and instrument and equipment maintenance. GSI QAQC Officers post final copies of the QAPP audit reports to the GSI SharePoint website for archiving and storage.

3.2.2. Project-Specific SOP Audits

GSI QAQC Officers assess the implementation of project-specific SOPs during each test of a ballast treatment system. At the end of the test duration, the officers provide a report to the GSI Senior Quality Systems Officer and GSI PI. The report includes a Table listing deviations to the specific SOPs that were used during the testing. The following Table headings are to be used:

- SOP Code
- SOP Title
- Description
- Deviation/Inconsistency
- Date
- GSI Personnel

- Reconciliation/Corrective Act

GSI QAQC Officers post final copies of the SOP audit reports to the GSI SharePoint website for archiving and storage.

3.2.3. Project-Specific Data Recording and Archiving Audits

Following completion of test activities associated with a specific ballast treatment test, GSI QAQC Officers verify data recording and archiving procedures by randomly evaluating data recording forms and field notebooks for completion, compliance and correct storage procedures. This includes the GSI Land-Based RDTE Facility Daily Safety Check List, zooplankton enumeration datasheets, phytoplankton enumeration datasheets, sampling station logs, chain of custody forms, etc. GSI QAQC Officers also undertake regular random data verification checks by comparing electronic records (i.e., in database or Excel format) with raw datasheets (i.e., paper forms). This is a manual inspection process and though rather time consuming, is an essential procedure for discovering errors. Findings are summarized in a report provided to the GSI Senior Quality Systems Officer and GSI PI. Final reports are saved to GSI SharePoint for storage and archiving.

3.2.4. Project-Specific Data Quality Assessments

Following completion and verification of a data set associated with a specific ballast treatment test, GSI QAQC Officers determine if the data quality objectives outlined in the relevant GSI QAPP have been successfully met. Findings are summarized in a series of Tables detailing the data quality indicators by type of analysis, e.g., zooplankton, phytoplankton, microbes, etc. Reports are provided to the GSI Senior Quality Systems Officer and GSI PI; final copies are stored on GSI SharePoint.

3.2.5. Project-Specific Performance Criteria Assessments

Following completion and verification of a data set associated with a specific ballast treatment test, GSI QAQC Officers also determine if the performance criteria outlined in the relevant GSI QAPP have been successfully met. Findings are summarized in a Table detailing the performance criteria and test results. The Table is provided in a report to the GSI Senior Quality Systems Officer and GSI PI. Final copies of the report are saved to GSI SharePoint for storage and archiving.

3.3. Response

GSI quality management personnel convene to discuss quality system audits and assessment outcomes following completion of a specific ballast treatment test. Personnel use the results of audits and assessments to develop recommendations and directives for actions to correct work or data that do not conform to GSI quality standards. They then compile a report listing the recommendations and directives. This report is provided to the GSI PI, relevant GSI senior research team personnel and to those individuals involved in the follow-up to ensure visibility and timeliness. Reports are also posted to the GSI SharePoint website for storage and archiving.

APPENDIX 1. GSI SOPs Relevant to Land-Based Testing.
Note: SOPs are subject to revision and available for download from:
http://www.nemw.org/GSI/protocols.htm

Document Type	Document Code	Title	Scale	Category	Subcategory
SOP	GSI/SOP/G/A/RK/1	Procedure for Record Keeping	General	Administration	Record Keeping
SOP	GSI/SOP/G/RA/DM/1	Procedure for Data Entry, Data Quality Control and Database Management	General	Research Activities	Data Management
SOP	GSI/SOP/G/RA/SC/1	Procedure for Custody of GSI Samples	General	Research Activities	Sample Custody
SOP	GSI/SOP/G/RA/SC/3	Procedure for Labeling Samples Collected at the GSI Land-Based RDTE Facility	General	Research Activities	Sample Custody
SOP	GSI/SOP/BS/RA/WET/1	Procedure for Assessing Chronic Residual Toxicity of a Ballast Treatment System to *Ceriodaphia dubia*	Bench-Scale	Research Activities	Residual Toxicity
SOP	GSI/SOP/BS/RA/WET/2	Procedure for Assessing Chronic Residual Toxicity of a Ballast Treatment System to the Fathead Minnow (*Pimephales promelas*)	Bench-Scale	Research Activities	Residual Toxicity
SOP	GSI/SOP/BS/RA/WET/3	Procedure for Assessing Chronic Residual Toxicity of a Ballast Treatment System to the Green Alga (*Selenastrum capricornutum*)	Bench-Scale	Research Activities	Residual Toxicity
SOP	GSI/SOP/BS/RA/MA/1	Procedure For Quantifying Heterotrophic Plate Counts (HPCs) Using IDEXX's SimPlate® for HPC Method	Bench-Scale and Land-Based	Research Activities	Microbial Analysis
SOP	GSI/SOP/BS/RA/MA/2	Procedure for Assessing Antimicrobial Activity Using Time-Kill Method	Bench-Scale	Research Activities	Microbial Analysis
SOP	GSI/SOP/BS/RA/MA/3	Procedure for the Detection and Enumeration of Enterococcus using Enterolert™	Bench-Scale and Land-Based	Research Activities	Microbial Analysis
SOP	GSI/SOP/BS/RA/MA/4	Procedure for the Detection and Enumeration of Total Coliforms and E. coli Using IDEXX's Colilert®	Bench-Scale and Land-Based	Research Activities	Microbial Analysis
SOP	GSI/SOP/BS/RA/MA/5	Procedure for the Detection and Enumeration of Male-Specific (F+) Coliphage Using Double Agar Layer Technique (DAL)	Bench-Scale	Research Activities	Microbial Analysis
SOP	GSI/SOP/BS/RA/MA/6	Procedure For Colony Blot Preparation for the Enumeration of Culturable Vibrio cholerae and Presence of ctxA Gene	Bench-Scale	Research Activities	Microbial Analysis
SOP	GSI/SOP/BS/RA/MP/1	General Microbiology Preparation Procedures	Bench-Scale	Research Activities	Microbial Analysis
SOP	GSI/SOP/BS/RA/C/1	Procedure for Analyzing the Concentration of Ozone in Water	Bench-Scale and Land-Based	Research Activities	Chemistry

SOP	GSI/SOP/BS/RA/C/2	Procedure for Determining Total Residual Oxidants (TRO) in Water	Bench-Scale and Land-Based	Research Activities	Chemistry
SOP	GSI/SOP/BS/RA/C/3	Procedures for Measuring Organic Carbon in Aqueous Samples	Bench-Scale and Land-Based	Research Activities	Chemistry
SOP	GSI/SOP/BS/RA/C/4	Procedure for Determining Percent Transmittance (%T) of Light in Water at 254 nm	Bench-Scale and Land-Based	Research Activities	Chemistry
SOP	GSI/SOP/BS/RA/C/5	Procedure for Measuring Organic Compounds using High Performance Liquid Chromatography (HPLC)	Bench-Scale and Land-Based	Research Activities	Chemistry
SOP	GSI/SOP/BS/RA/C/6	Procedure for Analyzing Total Residual Chlorine Concentrations in Water	Bench-Scale and Land-Based	Research Activities	Chemistry
SOP	GSI/SOP/BS/RA/C/7	Procedure for Analyzing Hydrogen Peroxide Concentrations in Water	Bench-Scale and Land-Based	Research Activities	Chemistry
SOP	GSI/SOP/BS/RA/C/8	Procedure for Analyzing Total Suspended Solids (TSS)	Bench-Scale and Land-Based	Research Activities	Chemistry
SOP	GSI/SOP/BS/RA/C/9	Procedure for pH Meter Calibration and pH Measurement for Ballast Treatment Systems Utilizing pH as the Active Substance	Bench-Scale and Land-Based	Research Activities	Chemistry
SOP	GSI/SOP/BS/RA/L/1	Procedure for Conducting a Scientific Search of Peer-Reviewed Literature, Including Use of Quantitative Structure Activity Relationships (QSAR)	Bench-Scale	Research Activities	Literature
SOP	GSI/SOP/LB/G/O/1	Procedure for Operating the GSI Land-Based RDTE Facility	Land-Based	General	Operation
SOP	GSI/SOP/LB/G/O/2	Procedure for Sampling and Testing Water Prior to Waste Water Treatment Facility Reception	Land-Based	General	Operation
SOP	GSI/SOP/LB/G/O/3	Procedure for Cleaning the Retention Tanks and Other Equipment at the GSI Land-Based RDTE Facility	Land-Based	General	Operation
SOP	GSI/SOP/LB/G/O/5	Procedure for Injecting Organisms and Solids into the GSI Land-Based RDTE Facility	Land-Based	General	Operation
SOP	GSI/SOP/LB/G/S/1	Procedure for Ensuring Worker Health and Safety at the GSI Land-Based RDTE Facility	Land-Based	General	Safety
SOP	GSI/SOP/LB/RA/SC/1	Procedure for Collecting Biological Sample Water Via In-Line Sample Ports	Land-Based	Research Activities	Sample Collection
SOP	GSI/SOP/LB/RA/SC/2	Procedure for Collecting Biological Samples From Within The Retention Tanks Using A Submersible Pump	Land-Based	Research Activities	Sample Collection

SOP	GSI/SOP/LB/RA/SC/3	Procedure for Algae/Small Protozoa Sample Collection	Land-Based	Research Activities	Sample Collection
SOP	GSI/SOP/LB/RA/SC/4	Procedure for Microbial Sample Collection	Land-Based	Research Activities	Sample Collection
SOP	GSI/SOP/LB/RA/SC/5	Procedure for MS-2 Bacteriophage Sample Collection	Land-Based	Research Activities	Sample Collection
SOP	GSI/SOP/LB/RA/SC/6	Procedure for Zooplankton Sample Collection	Land-Based	Research Activities	Sample Collection
SOP	GSI/SOP/LB/RA/SC/7	Procedure for Preparing Lugol's Solution	Land-Based	Research Activities	Sample Collection
SOP	GSI/SOP/LB/RA/SC/8	Procedure for Collecting Physical/Chemical Data and Samples at the GSI Land-Based RDTE Facility	Land-Based	Research Activities	Sample Collection
SOP	GSI/SOP/LB/RA/SA/1	Procedure for Algae/Small Protozoan Sample Analysis	Land-Based	Research Activities	Sample Analysis
SOP	GSI/SOP/LB/RA/SA/2	Procedure for Zooplankton Sample Analysis	Land-Based	Research Activities	Sample Analysis

Exhibit B
List of Additional Insured

Northeast-Midwest Institute
50 F St. NW
Washington, DC 20001

Lake Superior Research Institute
University of Wisconsin-Superior
P.O. Box 2000
Superior, WI 54880

AMI Consulting Engineers PA
1 East 1st Street, Suite 403
Duluth, MN 55802

Benson Electric Company
1102 North 3rd Street
Superior, WI 54880

Rockwell Automation
4411 Venture Avenue
Duluth MN 55811

JR Jensen Construction Co
814 21st Avenue East
Superior, WI 54880

J C Custom Welding
489 Amos Way Northwest
Bemidji, MN 56601

APPENDIX 2 - Performance Evaluation Summary for Type-Approved and Modified PureBallast® BWTS, and Research and Development Testing of the PureBallast® BWTS.

Prior to performance evaluation of the PureBallast®, v.3 BWTS, GSI conducted commissioning trials on the type-approved PureBallast® BWTS and a modified version of the PureBallast® BWTS, PureBallast® v.2 (testing period was 26 August 2010 to 03 September 2010). In addition, GSI conducted research and development testing (R&D testing) on the filter component of the PureBallast® BWTS. This R&D testing was initiated during the performance evaluation period on 26 August 2010 and 31 August 2010, while the majority of the R&D testing took place from 08 September 2010 to 23 September 2010. One successful commissioning trial was completed on the PureBallast BWTS v.2; the methods, results, and discussion from this test are presented below.

METHODS, RESULTS, AND DISCUSSION

The commissioning trials were conducted according to GSI's SOPs, which can be found at www.greatshipsinitiative.info. All SOPs relevant to the PureBallast® performance evaluation and R&D tests (type-approved PureBallast®, PureBallast® v.2, and PureBallast® v.3), as amended, also are listed by analysis category in Appendix 3.

Experimental Objectives

The objectives of this commissioning trial of the PureBallast®, v.2 BWTS were to characterize the physical, chemical, and biological aspects of the challenge water on intake, as well as, to analyze and quantify live organisms from the regulated size classes (i.e., < 10 μm, ≥ 10 and < 50 μm, and ≥ 50 μm) in the control and treatment discharge water after a specified retention time.

Operational Parameters and Challenge Conditions

Operational parameters, i.e., flow rate, pressure, retention tank volume, and volume sampled were measured continuously every five seconds during intake using in situ sensors and are summarized in Table 16 below. In total, four commissioning trials were attempted and one trial was successfully completed (trial ID code 10-A2-2). The completed commissioning trial was initiated with augmented TSS (to achieve 55 mg/L TSS on intake) and phytoplankton, but ambient POC was used as the challenge conditions. The filter clogged approximately five minutes after the start of injection. As a result, the PureBallast® BWTS was stopped, the filter was refreshed, and the trial was continued with only the phytoplankton injection (there was no solids injection after restart). The sample collection and analysis methods that were used during the completed commissioning trial (10-A2-2) are as previously described for the PureBallast®, v.3 BWTS except that the retention time for this trial was five days and whole effluent toxicity of the treatment discharge was not assessed.

Table 19. Operational log of attempted PureBallast® BWTS (type-approved and modified version) performance evaluation trials and research and development testing. One successful performance evaluation trial (10-A2-2) was completed out of four trials attempted in the test cycle. All trials without an identification code were conducted as part of the research and development testing.

Trial ID	Operation Dates	Filter	Backwash Cycle Duration	Flow Duration (min)	Flow Rate (m^3/hr)	Pressure (bar)	Target TSS (mg/L)	Target POC (mg/L)	Target PP (cells/mL)	Engineering Comments
10-A2-1	26 AUG 10 - Trial Aborted	40 μm	Continuous	25	250	2.2	55	5	1500	After injection started, there was approximately 3 min. of operation before the filter clogged. Backwashing was ineffective.
NA[1]	26 AUG 10	50 μm	Continuous	12	250	2.2	55	Ambient	Ambient	Increased filter mesh size, and reduced POC and PP concentration had little impact. Filter clogged and backwashing was not effective.
10-A2-2	27 AUG 10 – 01 SEP 10	40 μm	100 sec	60	250	2.2	55	Ambient	1500	Filter clogged and backwashing was not effective. TSS injection was stopped, the filter refreshed, and the trial continued.
NA[1]	31 AUG 10	40 μm	Not Recorded	15	250-200	2.2-3.5	25	Ambient	Ambient	Filter was tested at different speeds and different injection amounts. Lower flow rate, increased pressure, and reduced loadings appear to help.
10-A2-3	02 SEP 10 – Trial Aborted	40 μm	Continuous	60	200	3.2	25	5	1500	Lower TSS, higher pressure, and lower flow could not overcome problems associated with PP injection. Two filter candles blew 35 minutes into trial. Pressure differential across treatment dropped from 2.6 bar to 0.9 bar.
10-A2-4	03 SEP 10 – Trial Aborted	50 μm wedge wire	Not Recorded	25	200	3.2	25	5	1500	Lower TSS, higher pressure, and lower flow rate could not overcome problems associated with PP injection. Trial aborted due to filter clogging 3 min. into trial. Filter backwashing had little effect and differential pressure increased rapidly once injection started.
NA[1]	08 SEP 10	15 pcs 50 μm + 5 plugged	Not Recorded	75	200-160	3.2-3.8	Ambient	Ambient	Ambient	Filter operated over 75 min. using ambient harbor water, lower flow rate, and higher pressure. Over time, flow rate dropped and the pressure increased. Backwashes were not effective and pressure differential grew.
NA[1]	08 SEP 10	40 μm	Not Recorded	86	160-110	3.5-3.8	Ambient	Ambient	Ambient	Ambient harbor water, lower flow rate, and higher pressure led to longer duration of operation. Outlet valve on filter is partially closed manually during a backwash, which is effective at reducing the pressure differential over the treatment system.

Trial ID	Operation Dates	Filter	Backwash Cycle Duration	Flow Duration (min)	Flow Rate (m^3/hr)	Pressure (bar)	Target TSS (mg/L)	Target POC (mg/L)	Target PP (cells/mL)	Engineering Comments
NA[1]	21 SEP 10	40 μm	Not Recorded	49	250	3.2-3.6	Ambient	Ambient	Ambient	At 250 m^3/hr, higher pressure, and ambient harbor water[2] the filter performance degraded over time. Backwashes are effective when the effluent valve partially closed manually during backwash.
NA[1]	22 SEP 10	40 μm	40 min	160	250- 230	3.3-3.5	Ambient	Ambient	Ambient	System performed without issue in ambient harbor water[2]. Four successful backwashes at 40 minute intervals.
NA[1]	23 SEP 10	40 μm	40 sec	50	250	3.2-3.5	25	Ambient	Ambient	With 1/2 IMO required TSS in otherwise ambient harbor water[2], system performed without issue. Filter backwashes at 40 sec. cycles.
NA[1]	23 SEP 10	40 μm	40 sec	33	250	3.2-3.5	25	Ambient	Ambient	With 1/2 IMO required TSS in otherwise ambient harbor water[2], system performed without issue. Filter backwashes at 40 sec. cycles.

[1] Not Applicable: This trial was a research and development trial and was not conducted for the purposes of collecting water chemistry/quality or biological data. Therefore, this trial did not receive an identification code.
[2] Well below IMO guidelines for TSS, POC, and phytoplankton density in challenge water

The completed commissioning trial (10-A2-2) was not conducted according to IMO guidelines for challenge water; in addition, the solids injection was terminated after the filter became clogged. The TSS on intake was ambient Duluth-Superior Harbor water averaging 3.2 mg/L (Table 20). The overwhelming majority of the 16.5 mg/L NPOC consisted of DOC (16.4 mg/L), and POC was 0.2 mg/L (Table 20). The water quality parameters measured from the sample collection tubs immediately after phytoplankton and microbial sample collection on intake are similar between all three sample tubs measured (Table 21). The biological challenge conditions are described in Table 22 for all three regulated size classes. There were 239,321 live organisms/m^3 in the ≥ 50 μm size class on intake, which met the target density of >100,000/m^3. The live organism density increased during the five-day retention time to 293,975/m^3 in the control discharge, indicating favorable holding conditions in the control retention tank. In the $\geq$10 and < 50 m size class, there were 827 live cells/mL on intake, less than the target density of 1500 cells/mL but close to the IMO guidelines for challenge conditions. The live organism density in the control discharge decreased over the five-day retention time to 349 live cells/mL, but met the goal of >100 cells/mL.

The treatment tank water quality was measured automatically every 15 minutes during the five-day retention period. The average temperature and salinity was similar in the control and treatment tank during retention (Table 23). The average specific conductivity was lower in the treatment tank (0.172 mS/cm) than in the control tank (0.201 mS/cm), while the pH was slightly higher in the treatment tank (7.58) as compared to the control tank at an average of 7.48. The average turbidity in the treatment tank, 4.5 NTU, was slightly lower than the control tank (5.7); this result is likely due to the PureBallast® filter and the removal of a portion of the ambient solids from the treatment track on intake. The average total chlorophyll in the treatment tank, 9.5 µg/L, was lower as compared to the control tank at 11.3 µg/L. The biological data supports this reduction, as a reduction in live organisms from the $\geq$ 10 and < 50 μm size class (consisting mainly of phytoplankton) was seen in the treatment discharge as compared to the control discharge. The reduction of plankton also likely explains the increase in dissolved oxygen in the treatment tank (87.6% and 7.77 mg/L) as compared to the control tank (77.2% and 6.87 mg/L). A similar comparison between treatment discharge and control discharge can be seen in the water quality in the sample collection tubs, which was measured on discharge immediately after the whole-water samples were taken (Table 21).

Table 20. Average (*n*=3, ±std. dev.) total suspended solids (TSS), non-purgeable organic carbon (NPOC), dissolved organic carbon (DOC), particulate organic carbon (POC), and mineral matter (MM) measured during intake. The trial was initiated with TSS augmented to achieve 55 mg/L on intake; however, the solids injection was terminated after the filter became clogged.

Parameter	Target Concentration	10-A2-2
TSS (mg/L)	Ambient	3.2 ± 0.2
NPOC (mg/L)	Ambient	16.5 ± 0.3
DOC (mg/L)	Ambient	16.4 ± 0.3
POC (mg/L)	Ambient	0.2 ± 0.2
MM (mg/L)	Ambient	3.0 ± 0.2

Table 21. Water quality measurements taken from the sample collection tubs immediately after phytoplankton and microbial whole-water samples were collected during intake and discharge. The treatment discharge values are the average (±std. dev.) of the three treatment discharge sample collection tubs.

Parameter	Operation	Sample Type	Value
Temperature (°C)	Intake	Control	**20.06**
		Pre-Treatment	**19.50**
		Post-Treatment	**19.11**
	Discharge	Control	**23.47**
		Treatment	**21.52 ± 0.60**
Specific Conductivity (mS/cm)	Intake	Control	**0.171**
		Pre-Treatment	**0.171**
		Post-Treatment	**0.171**
	Discharge	Control	**0.192**
		Treatment	**0.175 ± 0.003**
Salinity (ppt)	Intake	Control	**0.08**
		Pre-Treatment	**0.08**
		Post-Treatment	**0.08**
	Discharge	Control	**0.09**
		Treatment	**0.08 ± 0.00**
pH	Intake	Control	**7.73**
		Pre-Treatment	**7.79**
		Post-Treatment	**7.72**
	Discharge	Control	**7.57**
		Treatment	**7.41 ± 0.06**
Turbidity (NTU)	Intake	Control	**Not Measured**
		Pre-Treatment	**Not Measured**

		Post-Treatment	**Not Measured**
	Discharge	Control	**10.2**
		Treatment	**6.3 ± 2.7**
Chlorophyll (μg/L)	Intake	Control	**Not Measured**
		Pre-Treatment	**Not Measured**
		Post-Treatment	**Not Measured**
	Discharge	Control	**11.5**
		Treatment	**8.2 ± 0.1**
Dissolved Oxygen (% Saturation)	Intake	Control	**93.4**
		Pre-Treatment	**93.1**
		Post-Treatment	**92.3**
	Discharge	Control	**85.5**
		Treatment	**87.1 ± 1.2**
Dissolved Oxygen (mg/L)	Intake	Control	**8.49**
		Pre-Treatment	**8.55**
		Post-Treatment	**8.55**
	Discharge	Control	**7.27**
		Treatment	**7.68 ± 0.19**

Table 22. Biological challenge conditions on intake and live organism densities in the control discharge in the three regulated size classes. Values reported for the <10 μm size class are the average (±SEM) of triplicate samples collected from the pre-treatment tub on intake and the control tub on discharge.

Live Organism Size Class		Target Density	Sample	10-A2-2
≥ 50 μm (#/m^3)		>100,000 (Ambient)	Intake	**239,321**
		>100	Control Discharge	**293,975**
≥ 10 and < 50 μm (#cells/mL)		>1500 (Augmented)	Intake	**826.79**
		>100	Control Discharge	**349.35**
< 10 μm	***E. coli* (MPN/100 mL)**	Ambient	Intake	**657±97**
	Total Coliforms (MPN/100 mL)			**1458±255**
	***Enterococcus spp.* (MPN/100 mL)**			**>1254**
	Total Heterotrophic (MPN/mL)			**3400±551**
	***E. coli* (MPN/100 mL)**	Ambient	Control Discharge	**25 ± 4**
	Total Coliforms (MPN/100 mL)			**115 ± 10**
	***Enterococcus spp.* (MPN/100 mL)**			**30 ± 7**
	Total Heterotrophic (MPN/mL)			**1700 ± 115**

Table 23. Average (±std. dev.) water quality measured from the control and retention tanks during the five-day holding time. Measurements were taken automatically every 15 minutes.

Parameter	Retention Tank	10-A2-2
Temperature (°C)	Control	21.07 ± 1.05
	Treatment	21.24 ± 1.06
Specific Conductivity (mS/cm)	Control	0.201 ± 0.001
	Treatment	0.172 ± 0.001
Salinity (ppt)	Control	0.1 ± 0.0
	Treatment	0.1 ± 0.0
pH	Control	7.48 ± 0.02
	Treatment	7.58 ± 0.02
Turbidity (NTU)	Control	5.7 ± 0.7
	Treatment	4.5 ± 0.6
Chlorophyll (μg/L)	Control	11.3 ± 1.2
	Treatment	9.5 ± 0.6
Dissolved Oxygen (% Saturation)	Control	77.2 ± 1.0
	Treatment	87.6 ± 1.0
Dissolved Oxygen (mg/L)	Control	6.87 ± 0.22
	Treatment	7.77 ± 0.24

Viable Organisms in Treated Discharge

The live organism densities in the three regulated size classes can be seen in Table 24. The ≥ 50 μm size class had 7580 live organisms/m^3 in the treatment discharge as compared to 293,975/m^3 in the control discharge. Although the target density (i.e., IMO guideline) of < 10/m^3 was not met, the treatment discharge density represents a reduction from the pre-treatment intake density of over 96 %. The treatment discharge had 62 live cells/mL from the ≥ 10 and <50 μm size class, as compared to the control discharge density of 349 cells/mL. The treatment discharge density for this size class also did not meet the target density of < 10 cells/mL; however, the density was reduced by 93 % from the pre-treatment density on intake.

Table 24. Live organism densities on intake, immediately post-treatment, and in the treatment discharge in the three regulated size classes. Values reported for the < 10 μm size class are the average (±SEM) of triplicate samples collected from the post-treatment tub on intake and the three treatment discharge tubs.

Live Organism Size Category		Target Density	Sample	10-A2-2
≥ 50 μm (#/m^3)		Ambient	Intake Post-Treatment	**44,974**
		<10	Treatment Discharge	**7,580**
≥ 10 and < 50 μm (#cells/mL)		Ambient	Intake Post-Treatment	**477**
		<10	Treatment Discharge	**62**
< 10 μm	***E. coli* (MPN/100 mL)**	Ambient	Intake Post-Treatment	**11 ± 3**
	Total Coliforms (MPN/100 mL)			**28 ± 5**
	***Enterococcus spp.* (MPN/100 mL)**			**9 ± 1**
	Total Heterotrophic (MPN/mL)			**>738**
	***E. coli* (MPN/100 mL)**	<250	Treatment Discharge	**<1**
	Total Coliforms (MPN/100 mL)	Ambient		**<1**
	***Enterococcus spp.* (MPN/100 mL)**	<100		**36 ± 2**
	Total Heterotrophic (MPN/mL)	Ambient		**549±44**

APPENDIX 3 - List of GSI SOPs Relevant to the Commissioning of PureBallast® v.1 and v.2 and Performance Evaluation of PureBallast® v.3.

SOP CODE	SOP TITLE	CATEGORY	SUBCATEGORY
GSI/SOP/G/A/RK/1	Procedure for Record Keeping	Administration	Record Keeping
GSI/SOP/G/RA/DM/1	Procedure for Data Entry, Data Quality Control and Database Management	Research Activities	Data Management
GSI/SOP/G/RA/SC/2	Procedure for Labeling Samples Collected at the GSI Land-Based RDTE Facility	Research Activities	Sample Custody
GSI/SOP/BS/RA/GL/1	Procedure for Verification of Laboratory Balances	Research Activities	General Laboratory
GSI/SOP/BS/RA/WET/1	Procedure for Assessing Chronic Residual Toxicity of a Ballast Treatment System to *Ceriodaphia dubia*	Research Activities	Residual Toxicity
GSI/SOP/BS/RA/WET/2	Procedure for Assessing Chronic Residual Toxicity of a Ballast Treatment System to the Fathead Minnow (*Pimephales promelas*)	Research Activities	Residual Toxicity
GSI/SOP/BS/RA/WET/3	Procedure for Assessing Chronic Residual Toxicity of a Ballast Treatment System to the Green Alga (*Selenastrum capricornutum*)	Research Activities	Residual Toxicity
GSI/SOP/BS/RA/MA/1	Procedure For Quantifying Heterotrophic Plate Counts (HPCs) Using IDEXX's SimPlate® for HPC Method	Research Activities	Microbial Analysis
GSI/SOP/BS/RA/MA/3	Procedure for the Detection and Enumeration of Enterococcus using Enterolert™	Research Activities	Microbial Analysis
GSI/SOP/BS/RA/MA/4	Procedure for the Detection and Enumeration of Total Coliforms and *E. coli* Using IDEXX's Colilert®	Research Activities	Microbial Analysis
GSI/SOP/BS/RA/MP/1	General Microbiology Preparation Procedures	Research Activities	Microbial Procedures
GSI/SOP/BS/RA/C/3	Procedures for Measuring Organic Carbon in Aqueous Samples	Research Activities	Chemistry
GSI/SOP/BS/RA/C/4	Procedure for Determining Percent Transmittance (%T) of Light in Water at 254 nm	Research Activities	Chemistry
GSI/SOP/BS/RA/C/6	Procedure for Analyzing Total Residual Chlorine Concentrations in Water	Research Activities	Chemistry
GSI/SOP/BS/RA/C/8	Procedure for Analyzing Total Suspended Solids (TSS)	Research Activities	Chemistry
GSI/SOP/BS/RA/C/9	Procedure for pH Meter Calibration and pH Measurement for Ballast Treatment Systems Utilizing pH as the Active Substance	Research Activities	Chemistry

GSI/SOP/LB/G/O/1	Procedure for Operating the GSI Land-Based RDTE Facility	General	Operation
GSI/SOP/LB/G/O/2	Procedure for Sampling and Analyzing Treated Water in the GSI Land-Based RDTE Facility's Retention Tanks Prior to Discharge	General	Operation
GSI/SOP/LB/G/O/3	Procedure for Cleaning and Verifying Cleanliness of the Retention Tanks and Piping at the GSI Land-Based RDTE Facility	General	Operation
GSI/SOP/LB/G/O/4	Procedure for Cleaning Sampling Equipment at the GSI Land-Based RDTE Facility	General	Operation
GSI/SOP/LB/G/O/5	Procedure for Injecting Organisms and Solids into the GSI Land-Based RDTE Facility	General	Operation
GSI/SOP/LB/G/O/7	Procedure for Maintaining Solids Suspension in the GSI Land-Based RDTE Facility's Retention Tanks	General	Operation
GSI/SOP/LB/G/C/4	Procedure for Calibration, Deployment, and Storage of YSI Multiparameter Water Quality Sondes	General	Calibration
GSI/SOP/LB/G/S/1	Procedure for Ensuring Worker Health and Safety at the GSI Land-Based RDTE Facility	General	Safety
GSI/SOP/LB/RA/SC/1	Procedure for Collecting Biological Sample Water via In-Line Sample Ports	Research Activities	Sample Collection
GSI/SOP/LB/RA/SC/3	Procedure for Algae/Small Protozoa Sample Collection	Research Activities	Sample Collection
GSI/SOP/LB/RA/SC/4	Procedure for Microbial Sample Collection	Research Activities	Sample Collection
GSI/SOP/LB/RA/SC/6	Procedure for Zooplankton Sample Collection	Research Activities	Sample Collection
GSI/SOP/LB/RA/SC/8	Procedure for Collecting Physical/Chemical Data and Samples at the GSI Land-Based RDTE Facility	Research Activities	Sample Collection
GSI/SOP/LB/RA/SA/1	Procedure for Algae/Small Protozoan Sample Analysis	Research Activities	Sample Analysis
GSI/SOP/LB/RA/SA/2	Procedure for Zooplankton Sample Analysis	Research Activities	Sample Analysis

APPENDIX 4 - Average Density (per m³) of Live Zooplankton in Treatment Discharge during the Trials of the PureBallast®, v.3 Ballast Water Treatment System. Organisms are Grouped by Taxa in the ≥ 50 μm Size Class, Additional Live Organisms < 50 μm, and Excluded Live Organisms.

Test Trials:	Trial A	Trial B	Trial C
Total Vol. Analyzed for MacroZooplankton, m^3:	**2.19**	**2.03**	**2.11**
Total Vol. Analyzed for MicroZooplankton, m^3:	**0.17**	**0.09**	**0.20**
Live Organisms ≥ 50 μm in minimum dimension			
Taxa Group	**Avg. Density (per m^3)**	**Avg. Density (per m^3)**	**Avg. Density (per m^3)**
Calanoid and Cyclopoid Copepods	1.5	1.5	1.5
Bosmina	2.5	2.0	1.5
Chydoridae	1.0		0.5
Chironomid	0.5		
Other MacroZP (Not Specified)		1.0	
Copepod Nauplii	5.5	17.5	
Rotifera	433.5	1785.0	511
Other MicroZP (Not Specified)		66.0	10.5
> 50 μm Total:	**444.5**	**1873.0**	**525.0**
Additional Live Organisms < 50 μm in minimum dimension			
Taxa Group	**Avg. Density (per m^3)**	**Avg. Density (per m^3)**	**Avg. Density (per m^3)**
Chironomid	1.5	0.0	0.0
Trichocerca Rotifer	5.5	0.0	5.0
< 50 μmTotal:	**7.0**	**0.0**	**5.0**
Live Organisms that were Excluded – All Sizes			
Taxa Group	**Avg. Density (per m^3)**	**Avg. Density (per m^3)**	**Avg. Density (per m^3)**
Nematode	3.5	2.5	1.5
Bdelloid	42.5	34.5	25.0
Monostyla/Lecane	79.0	226.5	42.0
Excluded Total:	**125.0**	**263.5**	**68.5**
Additional Organisms from ≥ 10 and < 50 μm – Not Quantified			
Taxa Group	**Observations/ Comments**	**Observations/ Comments**	**Observations/ Comments**
Protozoa (i.e., Vorticella, Codonella, and Other)	Present	Present	Present
Phytoplankton (i.e., Gonium and Other)	Not Observed	Present	Not Observed
Bacteria	Not Observed	Present	Not Observed

APPENDIX 5 - Average Density (MPN per volume) of Organisms in the < 10 μm Size Class Intake (Pre- and Post-Treatment) and Discharge (Control and Treatment) during the Trials of the PureBallast®, v.3 Ballast Water Treatment System.

				Total Coliform Density	*E. coli* Density	*Enterococcus spp*. Density
TRIAL	Sample Location	Sample Tub	Rep.	MPN/100 mL	MPN/100 mL	MPN/100 mL
A	Pre-Treatment	4	1	313.0	44.3	35.5
	Pre-Treatment	4	2	275.5	30.1	46.5
	Pre-Treatment	4	3	275.5	39.9	35.5
	Post-Treatment	6	1 DUP	8.6	< 1.0	1.0
	Post-Treatment	6	1	8.6	4.1	1.0
	Post-Treatment	6	2	5.2	< 1.0	1.0
	Post-Treatment	6	3	11.0	2.0	< 1.0
	Control Discharge	1	1	214.3	27.5	5.2
	Control Discharge	1	2	139.6	24.6	17.3
	Control Discharge	1	3	160.7	19.9	18.7
	Control Discharge	1	3 DUP	193.5	22.8	19.7
	Treatment Discharge	4	1	< 1.0	< 1.0	1.0
	Treatment Discharge	4	1 DUP	< 1.0	< 1.0	1.0
	Treatment Discharge	5	1	< 1.0	< 1.0	1.0
	Treatment Discharge	6	1	< 1.0	< 1.0	1.0
B	Pre-Treatment	4	1	365.4	65.7	51.2
	Pre-Treatment	4	2	235.9	66.3	56.1
	Pre-Treatment	4	2 DUP	387.3	63.1	34.1
	Pre-Treatment	4	3	238.2	64.4	54.6
	Post-Treatment	6	1	3.1	< 1.0	1.0
	Post-Treatment	6	2	5.2	1.0	1.0
	Post-Treatment	6	3	5.1	1.0	< 1.0
	Control Discharge	1	1	275.5	39.9	19.9
	Control Discharge	1	2	172.3	32.3	26.5
	Control Discharge	1	3	172.2	27.9	23.3
	Treatment Discharge	4	1 DUP	< 1.0	< 1.0	1.0
	Treatment Discharge	4	1	< 1.0	< 1.0	< 1.0
	Treatment Discharge	5	1	< 1.0	< 1.0	1.0
	Treatment Discharge	6	1	< 1.0	< 1.0	< 1.0
C	Pre-Treatment	4	1	209.8	111.2	53.8
	Pre-Treatment	4	2	290.9	146.7	37.3
	Pre-Treatment	4	3	235.9	90.9	22.6
	Post-Treatment	6	1	3.1	2.0	< 1.0
	Post-Treatment	6	2	2.0	2.0	< 1.0

TRIAL	Sample Location	Sample Tub	Rep.	Total Coliform Density MPN/100 mL	*E. coli* Density MPN/100 mL	*Enterococcus spp*. Density MPN/100 mL
	Post-Treatment	6	3	1.0	< 1.0	< 1.0
	Post-Treatment	6	3 DUP	5.2	3.1	< 1.0
	Control Discharge	1	1	178.9	55.6	16.9
	Control Discharge	1	2 DUP	150.0	51.2	26.2
	Control Discharge	1	2	133.3	53.8	19.9
	Control Discharge	1	3	137.4	47.1	39.7
	Treatment Discharge	4	1	< 1.0	< 1.0	1.0
	Treatment Discharge	5	1	< 1.0	< 1.0	< 1.0
	Treatment Discharge	6	1	< 1.0	< 1.0	1.0

TRIAL	Sample Location	Sample Tub	Rep.	Total Heterotrophic Bacteria Density MPN/mL
A	Pre-Treatment	4	1	8000
	Pre-Treatment	4	2	600
	Pre-Treatment	4	3	200
	Post-Treatment	6	1	200
	Post-Treatment	6	2	3000
	Post-Treatment	6	3	3500
	Control Discharge	1	1	< 200
	Control Discharge	1	2	200
	Control Discharge	1	3	< 200
	Control Discharge	1	3 DUP	200
	Treatment Discharge	4	1	299
	Treatment Discharge	5	1	231
	Treatment Discharge	6	1	209
B	Pre-Treatment	4	1	< 200
	Pre-Treatment	4	2	200
	Pre-Treatment	4	2 DUP	< 200
	Pre-Treatment	4	3	600
	Post-Treatment	6	1	166
	Post-Treatment	6	2	137
	Post-Treatment	6	3	209
	Control Discharge	1	1	400
	Control Discharge	1	2	800
	Control Discharge	1	3	200
	Treatment Discharge	4	1	200
	Treatment Discharge	4	1 DUP	400
	Treatment Discharge	5	1	200
	Treatment Discharge	6	1	< 200
C	Pre-Treatment	4	1	< 200

				Total Heterotrophic Bacteria Density
TRIAL	**Sample Location**	**Sample Tub**	**Rep.**	**MPN/mL**
	Pre-Treatment	4	2	200
	Pre-Treatment	4	3	< 200
	Post-Treatment	6	1	40
	Post-Treatment	6	2	68
	Post-Treatment	6	3	53
	Post-Treatment	6	3 DUP	56
	Control Discharge	1	1	124
	Control Discharge	1	2	137
	Control Discharge	1	3	86
	Treatment Discharge	4	1	248
	Treatment Discharge	5	1	239
	Treatment Discharge	6	1	209