GREAT LAKES BALLAST WATER RESEARCH AND DEVELOPMENT PLAN: RESEARCH AREA 1, OBJECTIVE 2

LAND-BASED TECHNICAL REPORT

LAND-BASED EVALUATION OF THE EFFECTIVENESS OF THE OPTIMARIN BALLAST SYSTEM IN THE GREAT LAKES

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ABSTRACT

This technical report presents the land-based evaluation of the Optimarin Ballast System, Model 334/340FX2. This work was conducted to evaluate the potential of the system to be used as a flow-through water treatment method for the Laurentian Great Lakes, treating via filtration and UV exposure on uptake and UV exposure again on discharge.

The evaluation began September 2021 and ended October 2021. All analyses were conducted at either the Montreal Pier Facility or the Lake Superior Research Institute (LSRI) at the University of Wisconsin-Superior (UWS), both located in Superior, Wisconsin, USA.

Biological effectiveness was examined during a commissioning trial and five efficacy trials with overnight retention of harbor water at the Montreal Pier Facility that was amended to achieve *ETV Protocol* challenge conditions. Effectiveness was assessed in terms of remaining live organisms in three size classes per unit volume: organisms ≥50 µm in minimum dimension (nominally zooplankton), organism entities ≥10 µm in any dimension and with cell sizes <50 µm in minimum dimension (nominally protists), and organisms <10 µm in minimum dimension (e.g., total culturable heterotrophic bacteria, total coliform bacteria, *Escherichia coli*, *Enterococcus* spp., and toxigenic *Vibrio cholerae* O1 and O139). Samples were compared to the United States Coast Guard's (USCG) *Standards for Living Organisms in Ships' Ballast Water Discharged in U.S. Waters* (USCG, 2012) with a focus on the reduction in the number of propagules in treated water versus control water. The Optimarin system was found to be highly effective at reducing the densities of organisms in all three regulated size classes. In particular, the USCG ballast water discharge standard (BWDS) was met in the bacteria size class in all trials. The density of zooplankton in treated discharge was below the USCG BWDS in all but one of the test cycles. Protist densities were greater than USCG BWDS in all test cycles but densities of protists in treatment discharge were decreased by >95% compared to control discharge samples.

KEYWORDS

Ballast Water Treatment, UV, Great Lakes, Biological Effectiveness

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1 INTRODUCTION

A major focus area of the Lake Superior Research Institute's (LSRI) Great Waters Research Collaborative (GWRC) is providing unbiased, independent data in support of the accelerated development of technologies having the potential for preventing the introduction and/or controlling the spread of non-indigenous organisms within the Laurentian Great Lakes. GWRC undertakes research and development (R&D) testing of type-approved and prototype ballast water management systems (BWMS) at the Montreal Pier Facility located in the freshwater port of Duluth-Superior, Superior, WI. This report details the procedures for the land-based evaluation of the Optimarin Ballast System, Model 334/340FX2 (hereafter OBS) conducted during September and October 2021.

1.1 OBJECTIVES OF LAND-BASED EVALUATION

The GWRC land-based evaluation tested the OBS in the Great Lakes for R&D purposes (not for regulatory type approval). There is currently a lack of large-scale BWMS evaluation data specific to the Great Lakes system. The Great Lakes system has been identified as particularly challenging for BWMS treatment due to water quality conditions (e.g., low salinity, low temperature, high turbidity) and the unique operations of Laker vessels (e.g., high ballast flow rates, short voyage durations).

The focus of this testing is to generate primary data supporting the *GWRC's Great Lakes Ballast Water Research and Development Plan* (hereafter R&D Plan). This testing focused specifically on the biological efficacy (BE) of the OBS consistent with the *ETV Protocol* and examined the potential for the OBS to reduce the environmental risk (i.e., number of propagules) associated with ballast water discharge within the Great Lakes (NSF International, 2010). Ambient conditions were employed as the physical/chemical and biological challenge water conditions, except that total suspended solids (TSS), particulate organic matter (POM), and mineral matter (MM) were augmented during BE testing to meet the minimum requirements specified by the *ETV Protocol* (Table 3) and when needed to augment protist densities to meet the *ETV Protocol* (Table 3).

Following an initial commissioning trial, a total of five biological efficacy trials were completed. Biological efficacy is defined as the removal or death of organisms, and was measured in terms of live organisms in three size classes per unit volume: organisms ≥50 µm in minimum dimension (nominally zooplankton), organisms ≥10 and <50 µm in minimum dimension (nominally protists), and organisms <10 µm in minimum dimension (i.e., total heterotrophic bacteria, total coliform bacteria, *Escherichia coli*, *Enterococcus* spp., toxigenic *Vibrio cholerae* O1 and O139). To provide context, treatment discharge concentrations of live organisms were compared to the United States Coast Guard's (USCG) *Standards for Living Organisms in Ships' Ballast Water Discharged in U.S. Waters* (U.S. Coast Guard, 2012; Table 1). However, the endpoint of this evaluation was not pass/fail (qualitative) with respect to the USCG ballast water discharge standard (BWDS) and, instead, quantitative outcomes were reported.

Table 1. Maximum Treated Discharge Concentrations of Parameters for Verification of Biological Efficacy (BE) According to U.S. Coast Guard (USCG) Standards.

Verification Factor	Parameter	Maximum Concentration in Treated Discharge
Biological Efficacy	Organisms ≥50 µm in minimum dimension (nominally zooplankton)	<10 organisms per m^3
	Organisms ≥10 µm and <50 µm in minimum dimension (nominally protists)	<10 organisms per mL
	Organisms <10 µm in minimum dimension (nominally bacteria)	<250 CFU per 100 mL of *Escherichia coli*
		<100 CFU per 100 mL of *Enterococcus* spp.

2 DESCRIPTION OF GWRC AND THE MONTREAL PIER TESTING FACILITY

The tests described in this report took place at the GWRC Montreal Pier Facility, located in northwest Wisconsin, in the City of Superior. The Facility itself is situated within the St. Louis River Estuary within the Port of Duluth-Superior. This freshwater estuary forms where the St. Louis River meets the western end of Lake Superior (Figure 1, Figure 2, Figure 3). Specific details of the Montreal Pier Facility are provided in the GWRC Land-Based QAPP (Schaefer et al. 2021, draft).

Figure 1. Google Earth Image of Location of Montreal Pier Facility (star) within the Great Lakes - Saint Lawrence Seaway System.

Figure 2. Google Earth Image Showing Location of Montreal Pier Facility (star) within the St. Louis River Estuary and the Port of Duluth-Superior.

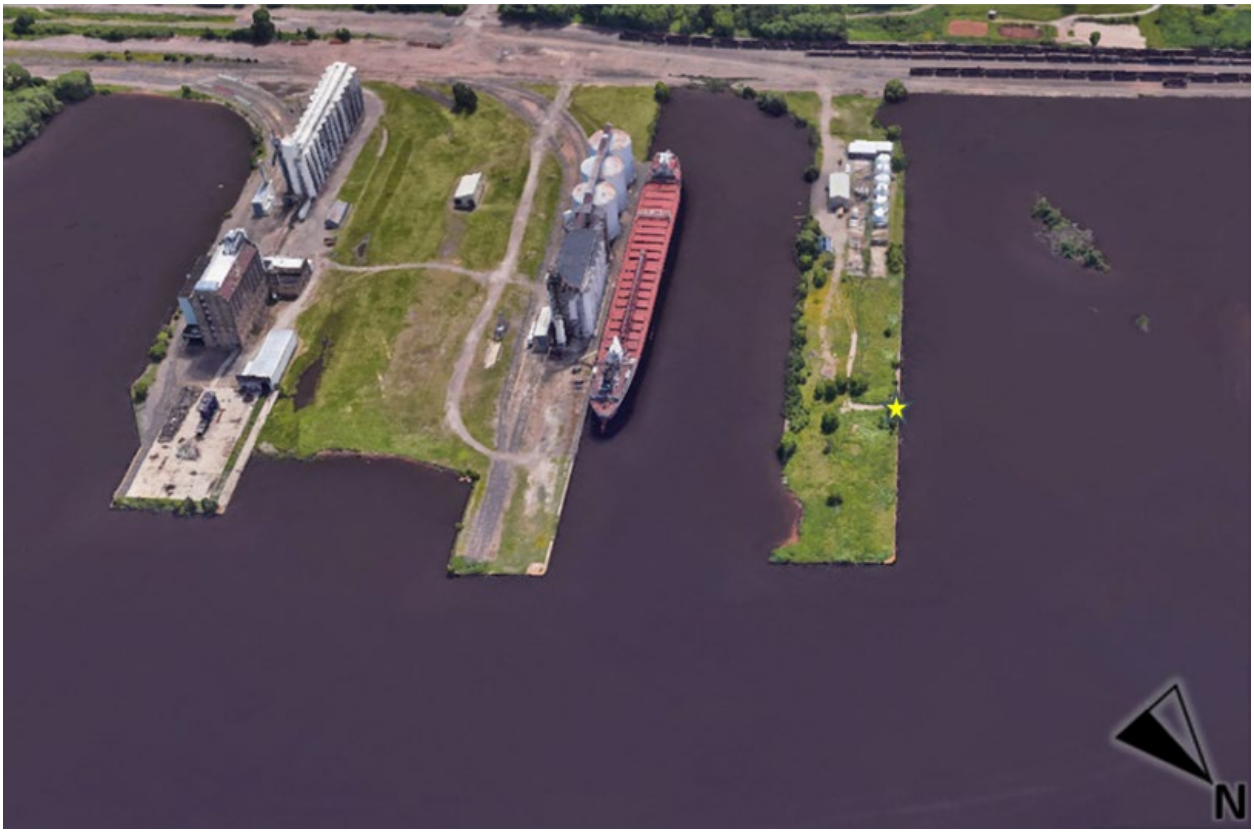

Figure 3. Close-Up Image of Montreal Pier (O-Dock). The Star Indicates the Location of the Facility's Bay Pump, Which Draws Water from the St. Louis River Estuary Through the Facility.

3 DESCRIPTION OF TECHNOLOGY

The OBS consists of six main components: UV system, filter, flow meter, flow pressure valve, UV power cabinets, and control system/PLC. The components can be adapted to various ballast water pump capacities. The OBS has received approval by both IMO (Certificate No.: TAP0000271 DNV GL) and USCG (Certificate No.: 162.060/1/3) for BWMS and has been tested in fresh, brackish, and sea water successfully. In order to comply with both regimes, the system is equipped with two different modes of operation. The differences between IMO and USCG modes are UV intensity range and UV intensity level when the lamps are dimmed. For the evaluation performed at Montreal Pier Testing Facility the OBS was operated in USCG mode. Instructions for this selection were provided in the Operations, Maintenance and Safety Manual (OMSM) for Optimarin Ballast System Section 4.4.4.1 (Optimarin AS, 2021).

The operational limits of the OBS, Model 334/340FX2, are presented in Table 2. The model number indicates that the system has a maximum UV capacity of 334 m^3/h when using both chambers, but due to power limitations at the facility, only one chamber can be powered limiting the flow to 167 m^3/h. The filtration capacity is either 340 m^3/h or 370 m^3/h, depending on the filter used. There are two filters that can be used in the OBS, a Filtrex ACB filter with 20 µm or a Boll filter with 25 µm basket. For purposes of this Great Lakes evaluation, the Filtrex ACB filter was used. The OBS shuts down if the flow exceeds 10% of the maximum for 120 seconds. The maximum allowable water flow to maintain necessary UV dose is 167 m^3/h, if the water quality of the ballast water causes the OBS to measure lower UV intensity in the chamber the flow may be regulated down to as low as 40 m^3/h to maintain the necessary UV dose. The absolute minimum flow through each UV chamber could not be less than 10 m^3/h to allow a safety margin that prevented the UV lamps from overheating. The holding time for USCG mode is 2 hours, but to accommodate sample analysis, an overnight hold time was employed. During its testing at the Montreal Pier Facility the system was not allowed to flow control in response to UV intensity due to water quality, specifically low transparency; the UV intensity was never high enough for the system to operate within its operational range in USCG mode no matter what flowrate was used. The Montreal Pier Facility hardware has not been validated to operate at the lower flow rates and it was uncertain if augmentation could be successfully accomplished at the decreased flow rate.

Table 2. OBS BWMS Operational Ranges and Tolerances Applicable to Land-based Tests Conducted at the Montreal Pier Facility.

Parameter	Montreal Pier Specific Operational Ranges
Flow range	Ballast: 40 – 167 m^3/h Deballast: 40 – 167 m^3/h
Filter capacity	45 – 340 m^3/h

Parameter	Montreal Pier Specific Operational Ranges
Back flush pump capacity	43 m^3/h @ 2.5 bar
Ballast water temperature range	-2 to +37°C
Ambient temperature	0 to +45°C
Relative humidity electronics	90%
UV intensity range	400 – 1100 W/m^2 in USCG mode The flow will be regulated between 40 – 167 m^3/h. UV chamber depending on measured UV intensity
OBS software version	2.0x

3.1 PRINCIPLE OF OPERATION

The OBS treated ballast water during uptake and discharge. During uptake, the ballast water was filtered through a 20 µm filter to remove large particles including zooplankton. Post filter, the water passed through the UV chamber where it was exposed to a high dose of UV light. The exposure is designed to kill/inactivate plankton, bacteria, and viruses. Water was held in the treatment tank for a minimum of 16 hours prior to discharge. During discharge the filter was bypassed but water passed through the UV chamber.

3.2 INSTALLATION AND COMMISSIONING

The OBS was received at the Montreal Pier Facility on August 13, 2021 (Figure 4). The developer provided GWRC staff with written instructions on operation and maintenance of the technology during testing and written human and environmental health and safety precautions associated with operation of the technology.

GWRC managed the installation and commissioning of the OBS at the Montreal Pier Facility. Plumbing installation was completed the first week of September and pressure testing was conducted the second week. Electrical installation was delayed by the need for a second smaller power supply to power the OBS's flow control valve. Once installed, GWRC engineers identified that the option to operate only one UV chamber was not available. Optimarin was able to remote control the OBS and enable the single chamber option.

The commissioning trial began on September 14th. The system functioned well during uptake. During the cleaning operation that followed, a socket weld on the temporary 8" PVC pipe that connects the OBS to

the facility came apart. This was not the fault of the OBS. The repair delayed the discharge of the commissioning trial, creating a hold time of approximately 46 hours. GWRC invalidated the commissioning trial due to the extended hold time and completed five more trials at a hold time of approximately 16 hours.

The Optimarin AS contact during GWRC land-based testing at the Montreal Pier Facility was:

Dan Åge Langeland & Kim Stian Haugland
Phone number: +47 51 11 45 33
After hours phone number: +47 469 49 485
Email: support@optimarin.com

Figure 4. OBS System Installation in Pole Building at Montreal Pier Facility.

4 TEST METHODS

4.1 TEST PLAN AND SOPS

A TQAP (Test Quality Assurance Plan); Land-Based Evaluation of the Effectiveness of the Optimarin Ballast System in the Great Lakes (Schaefer et al., 2021) and standard operating procedures (SOPs) were used to implement all testing activities. These procedures facilitated consistent conformance to technical and quality system requirements and increased data quality. The TQAP detailed sample and data collection and analysis, sample handling and preservation, data quality objectives, and the QAQC requirements. It was signed by LSRI-GWRC and the technology developer on August 23, 2021, prior to the start of land-based test activities. The SOPs followed throughout testing are described in the methods section and listed in the references section of this report.

4.2 AUGMENTATION OF WATER

GWRC followed the procedures detailed in *LSRI/SOP/GWRC/23 – Water Augmentation for the Montreal Pier Facility* when augmentation was required during testing. The *ETV Protocol's* challenge conditions matrix requires POM levels greater than 4 mg/L and MM concentrations greater than 20 mg/L, which means a TSS (TSS = POM + MM) concentration greater than 24 mg/L. To meet these target concentrations, GWRC measured the harbor water MM and POM levels ~24 hours prior to uptake operations to determine the amount of augmentation needed for each parameter. To increase the MM, ISO 12103-1, A3 Medium Test Dust (Powder Technology, Inc.; Arden Hills, Minnesota) was added. To increase the POM, Micromate (micronized humate product for liquid suspension, Mesa Verde Resources, Placitas, NM) was added. Approximately 64% of Micromate added to the augmented uptake water contributes to the POM concentration. Micromate is micronized humates plus clay suspending agent. It contains humic and fluvic acids (greater than 24%) plus an insoluble humic fraction. The Medium Test Dust contributes 88.3% of its mass to the measurable MM concentration due to 11.7% filter loss during analysis. GWRC determined the mass of solids needed to increase the challenge water conditions to levels at or above those listed in Table 3. The solids were added to a Solids Injection System (SIS) tank located prior to the Y-split in the facility piping, mixed thoroughly, and injected into the uptake stream at a constant rate using a peristaltic pump (Figure 5).

When protist augmentation was required to achieve challenge conditions, collection and concentration of organisms in this size class from the Duluth-Superior Harbor was completed following *LSRI/SOP/GWRC/23*. Briefly, Duluth-Superior Harbor water is pumped through a pair of rotary drum filters utilizing two sizes of mesh (21 and 40 µm). After collection and concentration, the organisms are stored outdoors in a holding tub equipped with aeration to maintain the population of organisms prior to injection during uptake. The collected organisms are not held longer than 48 hours. Prior to injection, the protist concentrate is mixed, sampled, and analyzed for live cell density. In addition, ambient levels of live protists in the Duluth-Superior Harbor water were determined to calculate the volume of protist concentrate that is needed to meet uptake challenge condition densities. The Organism Diaphragm Injection System (ODIS) injects the calculated amount of protist concentrate needed to the uptake water at a constant rate via an electric, double diaphragm pump for the entire duration of the uptake procedure (Figure 5).

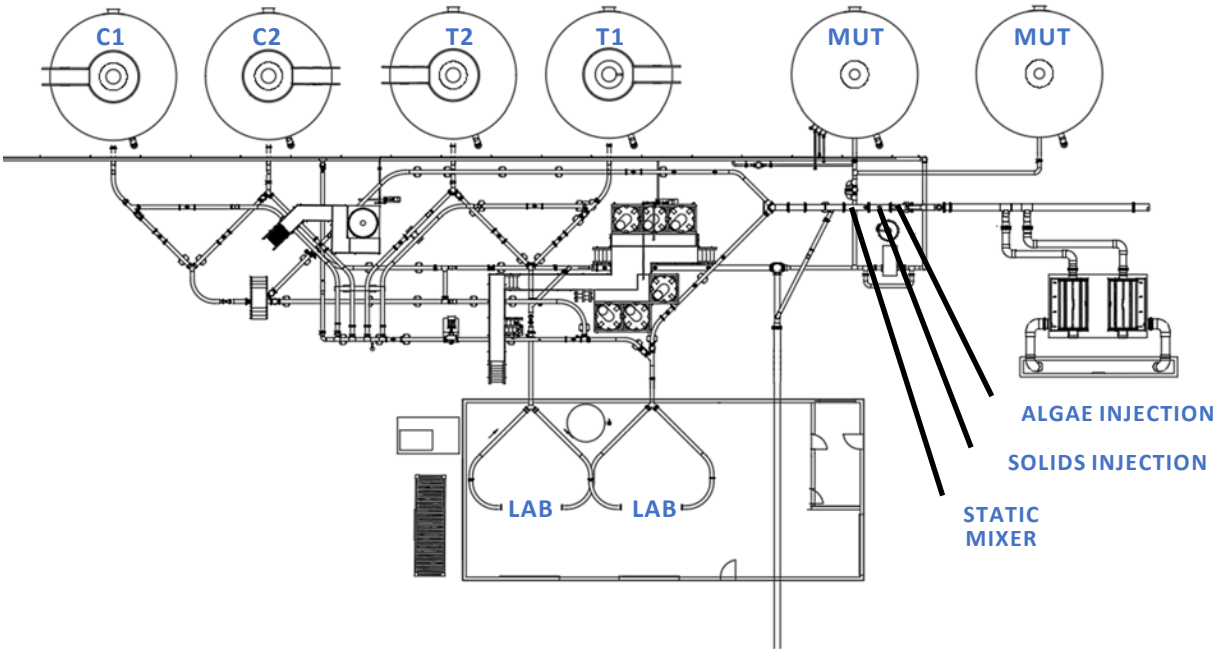

Figure 5. Montreal Pier Facility Showing Augmentation Sites.

The flow from the SIS was isolated from the ODIS to reduce the risk of organism mortality. Once injected into the main line, an inline static mixer mixed the material before the line splits into control and treatment tracks (Figure 5). This arrangement ensures the injected material was uniformly mixed before the flows are split into the control and treatment tracks.

4.3 BIOLOGICAL EFFICACY TRIALS

Each biological efficacy trial consisted of an uptake operation (ballasting simulation) with filtration and UV treatment, retention of control water and treated water, and a discharge operation (deballasting simulation) with further UV treatment. During uptake, ambient water was pumped from the Duluth-Superior Harbor of Lake Superior through the Montreal Pier Facility at a flow rate of approximately 330 m^3/h (2x 165 m^3/h); half of the water was directed through the control track and into control retention tank C1, and the other half of the water through the treatment track and into treatment retention tank T1 (Figure 6). The fill continued until the target retention volume (225 m^3) was reached in both tanks. Mechanical mixers in C1 and T1 were set to 10% capacity creating a very gentle mixing to keep non-neutrally buoyant organisms suspended in the water column. Treatment system operational data was recorded automatically by facility sensors or by the Facility engineering staff in a laboratory notebook.

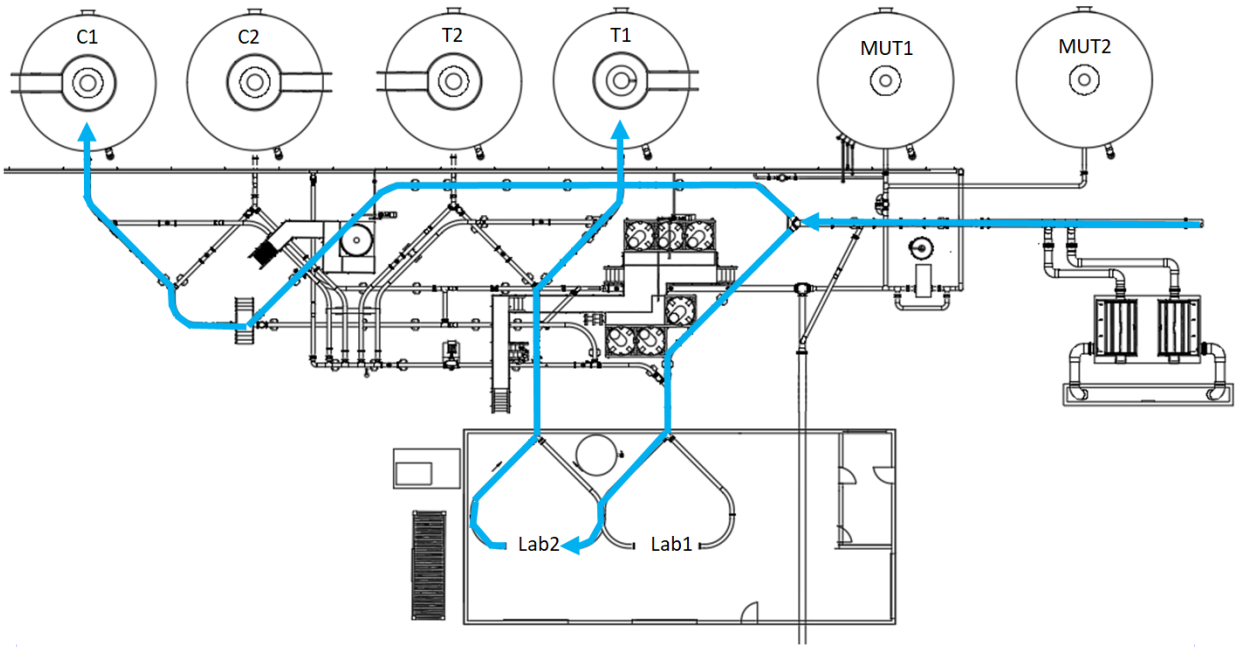

Figure 6. Flow Path for Filling Treatment Retention Tank 1 through Lab 2 and Filling Control Retention Tank 1.

To minimize live organism contamination of the treated discharge water, prior to discharge, the facility lines were flushed with municipal water and then steamed at 205°C for a minimum of ten minutes as detailed in *LSRI/ SOP/GWRC/21 – Cleaning and Performing Cleaning Validation of the Montreal Pier Facility*, 2021. The sample collection tubs on the treatment track were also cleaned following *LSRI/SOP/GWRC/24 – Cleaning the Sample Collection Tubs and Zooplankton Collection Equipment*, 2021. The lines and tubs were verified to contain <1 live zooplankton/m^3 and <1 live protist cell/mL prior to performing the discharge operation during the commissioning trial. The cleaning validation was only conducted during the commissioning trial because the OBS treats both during uptake and discharge. As such, water in the retention tank that has passed one dose of UV was passed through the steam cleaned lines prior to receiving a second dose of UV and discharged. Untreated water was not permitted into the treatment lines after the cleaning process.

Following retention and Facility line cleaning, the retention tanks were discharged sequentially. The treatment retention tank was always discharged prior to the control retention tank. The retention tanks were discharged through the facility lines and to the Duluth Superior Harbor (Figure 7).

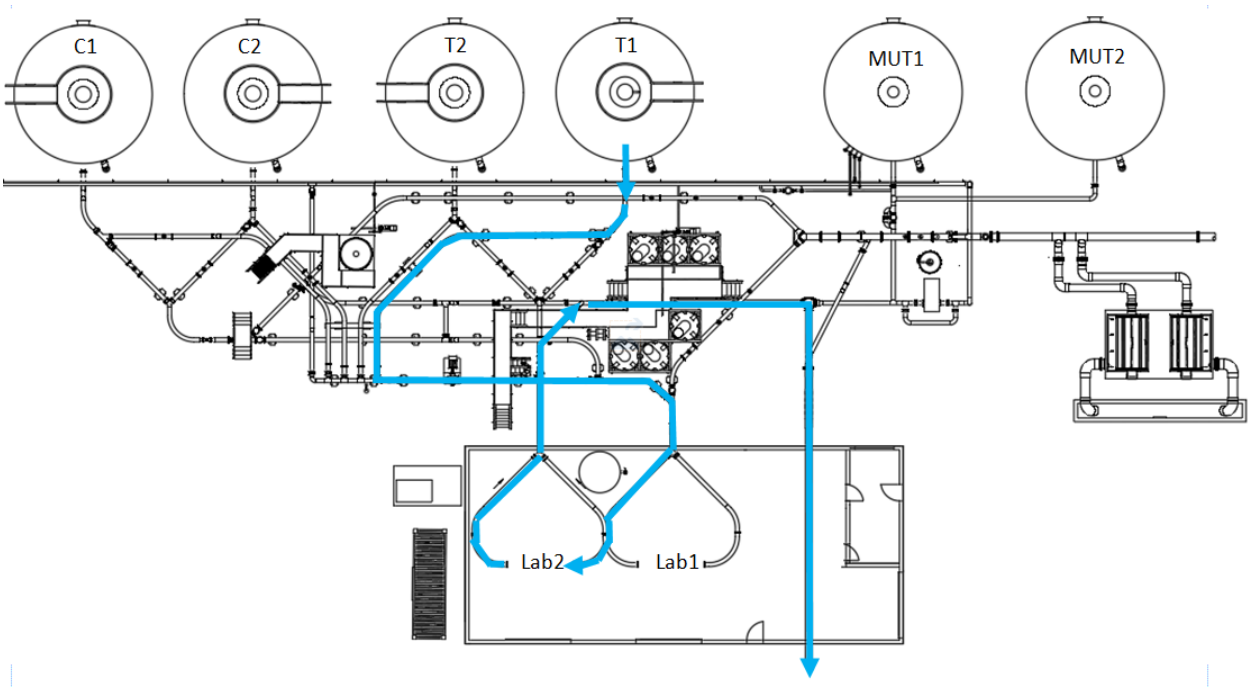

Figure 7. Discharge of Treatment Retention 1 through Lab 2 to Harbor.

4.4 SAMPLE AND DATA COLLECTION

The samples collected and measurements that were made during the biological efficacy trials were described in the TQAP. Sample collection tubs and ports indicated in the test plan are illustrated in Figure 8. Tubs 1–6 are fed by the identified sample ports and allow for time-integrated sub-samples of water going into/out of the control and treatment retention tanks during ballasting/deballasting operations. Each trial was considered an independent replicate. The following parameters were measured in the control and treatment lines: temperature, salinity, TSS, POM, MM, ultraviolet transmittance (UV-T), dissolved organic matter (DOC), non-purgeable organic matter (NPOC), specific conductivity, turbidity, chlorophyll *a*, dissolved oxygen, pH, ballast main flow rate, and ballast main pressure. Sample tub flow rate, density of live zooplankton, density of live protists, and density of bacteria were determined on time-integrated samples collected from sample tubs.

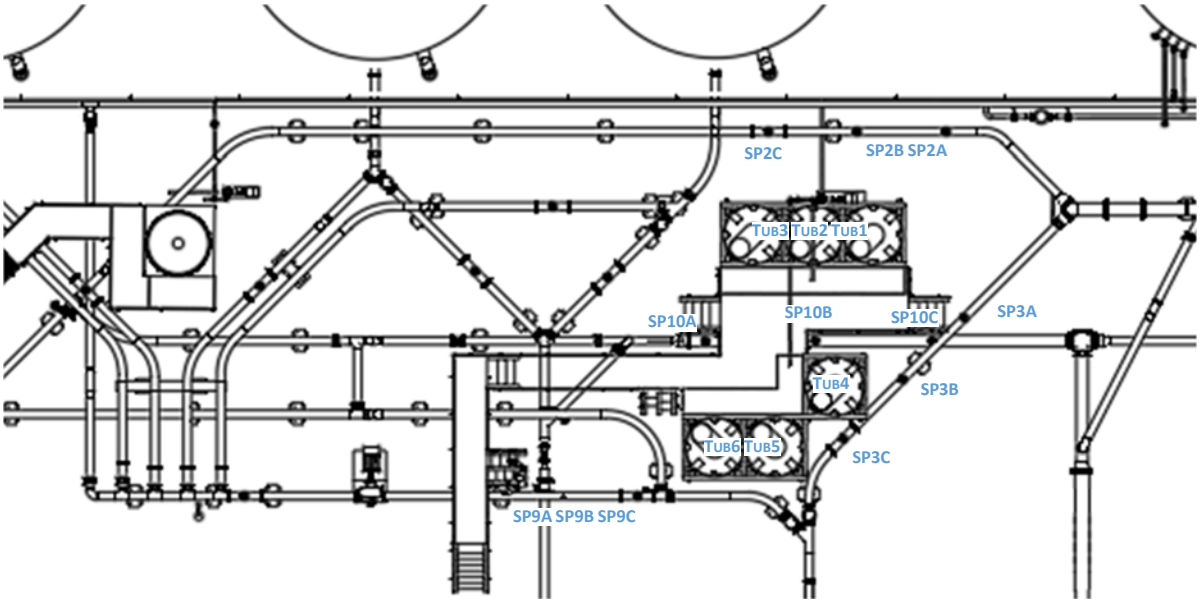

Figure 8. Schematic of Sample Ports and Sample Tubs.

4.4.1 UPTAKE ACCEPTANCE CRITERIA

The evaluation of the OBS was conducted with augmented source water from the Duluth-Superior Harbor meeting the *ETV Protocol* challenge conditions (Table 3) during the commissioning and five biological efficacy trials. The challenge condition's non-biological requirements physically challenge the OBS equipment. In order to ensure testing was sufficiently robust, the acceptance criteria outlined in Table 3 were targeted for all biological efficacy trials.

Table 3. Uptake Acceptance Criteria for Water Chemistry and Biological Parameters.

Parameter (units)	Uptake Acceptance Criteria
Total Pumped Volume (m^3)	250
Temperature (°C)	4 – 35
Total Suspended Solids (mg/L)	Min. 24
Particulate Organic Matter (mg/L)	Min. 4
Mineral Matter (mg/L) = TSS – POM	Min. 20
Non-Purgeable Organic Carbon/Dissolved Organic Matter, as Carbon (mg/L)	Min. 6
Zooplankton	Minimum of 100,000 live organisms/m^3 with at least 5 species present across 3 phyla.

Parameter (units)	Uptake Acceptance Criteria
Protists	Minimum of 1,000 live organisms/mL with at least 5 species present across 3 phyla.
Bacteria	Minimum concentration of 1,000/mL as culturable aerobic heterotrophic bacteria.

4.4.2 CONTROL DISCHARGE ACCEPTANCE CRITERIA

To ensure that each biological efficacy trial was robust and that the majority of organism mortality measured was the result of the treatment system process and not of unhealthy organisms (or other non-treatment processes), the control discharge must meet the minimum density criteria outlined in Table 4.

Table 4. Biological Acceptance Criteria for Control Discharge.

Parameter	Sample Approach	Control Discharge Acceptance Criteria
Zooplankton	Time-Integrated, Concentrated	≥100 live organisms per m^3
Protists	Time-Integrated	≥100 live cells per mL
Bacteria	Time-Integrated	≥500 per mL as culturable aerobic heterotrophic bacteria

4.4.3 TOTAL SUSPENDED SOLIDS (TSS), PARTICULATE ORGANIC MATTER (POM), MINERAL MATTER (MM), AND PERCENT UV TRANSMITTANCE (UV-T)

Analysis of TSS, POM, and determination of MM were conducted according to *LSRI/SOP/SA/66 – Analyzing Total Suspended Solids, Particulate Organic Matter, and Mineral Matter*. In this procedure, accurately measured sample volumes (± 1%) were vacuum filtered through pre-ashed, washed, dried, and pre-weighed glass fiber filters (i.e., Whatman 934-AH). After each sample was filtered, it was dried in an oven and brought to constant weight. TSS values were determined based on the weight of particulates collected on the filter and the volume of water filtered. The residue from the TSS analysis was ignited to a constant weight at 550°C in a muffle furnace. The concentration of POM was determined by the difference of the dry weight of the particulates on the filter before and after ignition (the mass lost to combustion). MM concentrations were calculated following analysis of TSS and the determination of POM. The reporting limit for TSS/POM/MM was dependent on the volume filtered. Typically, an 800 mL sample was analyzed which had a reporting limit of 1.25 mg/L TSS/POM/MM.

Two aliquots of approximately 10 mL from each TSS sample were used to measure percent transmittance (%T). Sample analysis was conducted according to *LSRI/SOP/SA/69 – Determining Percent Transmittance of Light in Water at 254 nm*. Water that was filtered through a Whatman 934-AH filter for TSS determination constituted the filtered aliquot. A UV-Vis spectrophotometer was used to measure %T of the unfiltered and filtered sample aliquots. Milli-Q water was used as a reference to adjust the spectrophotometer to 100% T and then each unfiltered and filtered sample aliquot was analyzed in a pre-rinsed sample cuvette with a 1 cm path length.

4.4.4 NON-PURGEABLE ORGANIC CARBON (NPOC) AND DISSOLVED ORGANIC CARBON (DOC)

Sample analysis for NPOC and DOC were conducted according to *LSRI/SOP/SA/47 – Measuring Organic Carbon in Aqueous Samples*. Upon arrival at LSRI, an aliquot of each sample was filtered through a Whatman GF/F filter and acidified with hydrochloric acid (HCl) for analysis of DOC. An aliquot of each sample was acidified with hydrochloric acid (HCl) for analysis of NPOC. A Shimadzu Total Organic Carbon Analyzer (Model TOC-L) was used for analysis of NPOC and DOC. Concentrations of NPOC and DOC were determined based on a calibration curve developed on the analyzer using organic carbon standards prepared from a 1000 mg/L Total Organic Carbon Standard (e.g., Aqua Solutions, Inc.). The Limit of Detection (LOD) for NPOC/DOC was 0.77 mg/L and the Limit of Quantitation (LOQ) was 2.6 mg/L.

4.4.5 ZOOPLANKTON

Analysis of zooplankton samples was conducted according to *LSRI/SOP/GWRC/25 – Procedure for Zooplankton Analysis* and took place immediately after collecting and concentrating the individual samples. Due to the wide variation in sizes of organisms in this size class, separate analysis procedures performed by separate analysts were implemented for each of two sub-size classes: “microzooplankton” (e.g., rotifers, copepod nauplii, and dreissenid veligers) and “macrozooplankton” (e.g., copepods, cladocerans, and other macroinvertebrates). Microzooplankton, though usually larger than 50 µm, are generally less than 100 µm in minimum dimension, and were examined in a Sedgwick-Rafter counting chamber with a compound microscope at a magnification of 40–100X. Macrozooplankton are always greater than 50 µm, and generally greater than 200 µm in minimum dimension, and were analyzed in a Ward’s counting wheel at a magnification of 20–30X using a dissecting microscope.

Analysis of live organisms in samples with high densities of zooplankton (i.e., ambient challenge water, control uptake, and control discharge) were enumerated using the dead/total method described in *LSRI/SOP/GWRC/25* by an indirect count of a portion of the original concentrated sample. First, all the dead organisms present in a subsample were counted, then dilute acetic acid was added to effectively kill the organisms. A second count followed, which included the total number of organisms in the subsample. The number of dead organisms was subtracted from the number of total organisms resulting in the number of live organisms in the subsample. Analysis for both micro and macrozooplankton included the analysis of a least 600 organisms collectively when possible or four subsamples each.

The treatment discharge samples had lower live organism densities, thereby allowing for analysis of a

greater proportion of the sample and in some cases the entire sample. The sample was analyzed for a period of up to six hours following concentration and collection. During this analysis, only live organisms were enumerated using standard movement and response to stimuli techniques. Both micro and macrozooplankton will be counted together by taxonomists on a Ward's wheel.

4.4.6 PROTISTS

Live analysis occurred within six hours of sample collection, with samples stored in a laboratory refrigerator during the interim. Sample analysis was conducted according to *LSRI/SOP/GWRC/30 – Procedure for Protist Sample Analysis.* Briefly, a 1.5 mL subsample of the concentrated sample was transferred to a 5 mL sample container, with 6 µL of fluorescein diacetate (FDA) viability stain stock solution and 15 µL of chloromethylfluorescein diacetate (CMFDA) added. The subsample was allowed to incubate in the dark for 10 minutes. The incubated sample was mixed and immediately transferred to a Sedgwick-Rafter cell (approximately 1.0 mL), covered, and placed on the stage of a compound microscope set for simultaneous observation using brightfield and epifluorescence. Prepared samples were counted for 20 minutes maximum and then counts were repeated two more times, preparing a new slide each time, until three replicates were analyzed, 6 mL of water was counted for treatment or cleaning samples, or the sample holding time was reached, whichever came first.

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 (not counted); and
- Ambiguous = cells whose level of fluorescence was difficult to discern.

Records were kept of transect lengths and widths using the Sedgewick-Rafter grid so that the total counted area and volume analyzed could be calculated. Cells between 10 and 50 µm in minimum dimension were counted and totaled. The remaining concentrated sample in the 25 mL bottle was archived using Lugol's solution for long-term storage.

4.4.7 BACTERIA

For analysis of bacteria samples, multiple procedures based on standard methods listed in the ETV protocol, were employed to enumerate total culturable heterotrophic bacteria, *Escherichia coli*, *Enterococcus* spp., and toxigenic *V. cholerae O1 and O139*.

For culturable, aerobic, heterotrophic bacteria, analysis followed *LSRI/SOP/GWRC/32 – Procedure for Enumerating Culturable Heterotrophic Bacteria.* Briefly, 1 mL samples were diluted in a ten-fold dilution series using sterile ambient harbor water. A volume of 100 µL of the appropriate dilutions were spread on Heterotrophic Plate Count Agar (HPCA) in triplicate. HPCA Plates were incubated at 20°C for 7 days after which colonies were counted and reported in CFU/mL. A second analysis was conducted for heterotrophic bacteria using IDEXX SimPlates® for Heterotrophic Plate Counts (HPC). Briefly, a volume of 100 µL to 1 mL of sample was pipetted onto the center of the SimPlate and media was added to a final

volume of 10 mL. HPC SimPlates were incubated at 35°C for 48-72 hours after which colonies were counted under a UV light and reported in MPN/mL.

The density of total coliform bacteria and *E. coli* was determined using *LSRI/SOP/SA/56 – Detection and Enumeration of Total Coliforms and E. coli Using Colilert®*, which was based on IDEXX's patented Defined Substrate Technology (DST®). Briefly, 100 mL of sample was mixed with Colilert media, poured into the Quanti-Tray/2000, sealed, and incubated at 35°C for 24–28 hours. Positive wells were counted, and results were reported in MPN/100 mL, which correlates with CFU/100 mL.

The density of Enterococci was determined using *LSRI/SOP/SA/62 – Detection and Enumeration of Enterococcus Using IDEXX Enterolert®*, which is also based on IDEXX's patented Defined Substrate Technology (DST®). Briefly, 100 mL sample and media were mixed, poured into the Quanti-Tray/2000, sealed, and incubated at 41°C for 24–28 hours. Results were reported in MPN/100 mL, which correlates with CFU/100 mL.

The determination of the presence of toxigenic *V. cholerae* in water samples followed *LSRI/SOP/GWRC/33 – Procedure for the Detection and Enumeration of Culturable Toxigenic Vibrio cholerae O1 and O139*. Briefly, a portion of the sample (e.g., 100 mL of treated sample and a volume of control sample expected to yield 20–200 colonies) was concentrated on a filter and incubated overnight on thiosulfate citrate bile salts sucrose (TCBS) agar, a selective media used to isolate and differentiate *Vibrio* species. Yellow colonies (presumptive *V. cholerae*; serotype undetermined) were counted (CFU/100 mL) and recorded as CFU/100 mL. For samples that exhibited growth on TCBS agar, RNA and DNA colony lift filters would have been prepped and fixed at LSRI. Filters associate with positive DFA samples would have been shipped to a qualified lab (e.g., Maryland Pathogen Research Institute; College Park, MD) to complete the RNA and DNA Colony Blot Hybridizations that detect the *ctxA* gene (Huq et al., 2012) to determine the presence of *V. cholerae* serotype O1 and O139.

In addition to the Colony Blot Hybridization Procedure, an additional 100 mL volume of sample was filtered and incubated in a solution of 2.5% yeast extract and nalidixic acid to enlarge and elongate viable substrate responsive cells. Viable *V. cholerae* O1 and O139 cells were enumerated (per 100 mL) using direct fluorescent antibody (DFA) test kits (New Horizons Diagnostics; Columbia, MD). The Cholera DFA (serotype O1) and Bengal DFA (serotype O139) tests consisted of a monoclonal antibody, specific for the surface antigen of either O1 or O139 lipopolysaccharide in the outer membrane of *V. cholerae*, which were directly labeled with fluorescein isothiocyanate (FITC). Cells were counted under the microscope and reported as cells/100 mL.

5 BIOLOGICAL EFFICACY TEST RESULTS

Data for biology, water quality and chemistry, Montreal Pier Facility operations, and OBS operations summarizing the commissioning trial and five successful BE test cycles are presented below. The dates of uptake and discharge for the commissioning and test cycles, as well as duration of treatment and irregularities encountered when conducting a trial are displayed in Table 5. As described in Section 3.2,

the commissioning trial was planned to be approximately 21–22 hours of retention but a pipe repair was required delaying the discharge for 24 hours, for a total hold time of approximately 46 hours.

Table 5. Dates and Duration of Test Cycles during Biological Efficacy Evaluation. NA= Not Applicable.

Test Cycle	Uptake Date	Discharge Date	Hold Time	Solids Augment-ation	Organism Augment-ation	Notes
COM	14 Sept 2021	16 Sept 2021	~ 46 hours	Yes	No	8" PVC pipe came apart during cleaning procedure, repair and curing required delay in discharge
1	21 Sept 2021	22 Sept 2021	~22 hours	Yes	No	NA
2	28 Sept 2021	29 Sept 2021	~ 22 hours	Yes	No	NA
3	30 Sept 2021	1 Oct 2021	~ 21 hours	Yes	No	On uptake: System shut down after sampling completed due to a low-pressure alarm. The two back flushes that normally occur did not happen. The uptake was not affected. The system was restarted so it could shut down properly. On discharge: system was incorrectly started in ballast mode. The system was quickly shutdown and restarted in deballast mode.
4	6 Oct 2021	7 Oct 2021	~ 21 hours	Yes	No	NA
5	13 Oct 2021	14 Oct 2021	~ 21 hours	Yes	Yes	Uptake volumes slightly reduced in order to achieve protist uptake required densities

5.1 BIOLOGICAL, WATER QUALITY, AND FACILITY OPERATIONAL CONDITIONS ON UPTAKE

This section summarizes biological and water quality challenge conditions during uptake sampling of commissioning and test cycles. Table 6 displays operational parameters measured during the uptake of the commissioning trial and five test cycles. Uptake duration ranged from 84.2 minutes to 92.3 minutes. The shortest uptake occurred during test cycle 5 where the duration was intentionally decreased to accommodate low protist densities in the uptake water and available augmentation water. The shorter duration and uptake volume allowed uptake protist densities to be optimized although they still fell short of *ETV Protocol* requirements (Table 7). Treatment retention tank volume ranged from 231 m^3 to

252 m^3. The lowest treatment volume occurred during test cycle 5. With the exclusion of test cycle 5, the lowest treatment volume was 248 m^3. The treatment flow rate was 164 m^3/h for all trials, which is within the range specified in Table 2.

The UV intensity of the OBS on treatment uptake ranged from 221 W/m^2 to 347 W/m^2 (Table 6). This intensity is less than the minimum UV intensity indicated in the OMSM for the Optimarin Ballast System (2021) to operate in USCG mode. This intensity is impacted by water quality parameters such as total suspended solids and dissolved organic carbon and is not an indication that the OBS system is not operating correctly.

The uptake concentration of organisms in the three regulated size classes (zooplankton, protist and bacteria) are presented in Table 7. In all trials, the density of zooplankton met ETV minimum challenge conditions. The requirement to have a minimum of 5 zooplankton species present across 3 phyla was met in all trials (Figure 9). The protist densities met the ETV uptake challenge conditions during commissioning and in Trials 1 – 3. The protist uptake density for Trial 4 and Trial 5 did not meet the ETV minimum at 999 live cells/mL and 554 live cells/mL, respectively. The final two trials were conducted in mid-October when ambient temperatures were declining, which contributed to the low protist densities. The requirement to have a minimum of 5 protist species across 3 phyla was met in the uptake for all test cycles. As is typical of conditions in the harbor, the phyla represented in all trials included mainly cyanophytes, green algae, cryptophytes, and diatoms. In addition, some trials also had chrysophytes, protozoans, and dinoflagellates. Total culturable heterotrophic bacteria met the ETV minimum challenge conditions in all trials. Total coliform bacteria were enumerated as part of the *E.* coli procedure; however, results were not reported in the tables as there are currently no ballast water discharge standards for total coliform bacteria. Total coliform bacteria densities on uptake ranged from an average of 382.4 – 1841.9 MPN/100mL (SEM of 55 – 144 MPN/100mL) for the commissioning and all five biological efficacy trials.

Table 6. Operational Parameters Measured on Uptake during Commissioning and Three Biological Efficacy Trials.

Test Cycle	Uptake Duration (min)	Control Flow Rate (m^3/h) (Std Dev)	Control Retention Tank Volume (m^3)	Treatment Flow Rate (m^3/h) (Std Dev)	Treatment Retention Tank Volume (m^3)	Main Y Pressure (bar)	Pre-Treatment Pressure (bar)	Post-Treatment Pressure (bar)	UV Intensity (W/m^2)	Sample Flows (m^3/h) (Std Dev)	Sample Tub Volumes (m^3)
COM	91.0	159 (18)	242	164 (4)	248	2.65 (0.13)	2.48 (0.15)	0.35 (0.16)	347 (17)	2.06 (0.33), 2.05 (0.34), 2.07 (0.32)	3.12, 3.12, 3.13
1	91.5	163 (10)	249	164 (5)	250	2.63 (0.10)	2.47 (0.12)	0.35 (0.16)	330 (2)	2.10 (0.14), 2.09 (0.17), 2.11 (0.14)	3.20, 3.20, 3.21
2	91.3	166 (17)	252	164 (5)	248	2.62 (0.10)	2.45 (0.13)	0.35 (0.15)	323 (20)	2.07 (0.27), 2.07 (0.28), 2.08 (0.26)	3.16, 3.16, 3.18
3	91.8	167 (9)	256	164 (5)	251	2.61 (0.10)	2.45 (0.12)	0.35 (0.16)	299*	2.09 (0.21), 2.08 (0.23), 2.09 (0.21)	3.20, 3.19, 3.21
4	92.3	165 (20)	255	164 (4)	252	2.61 (0.10)	2.46 (0.13)	0.35 (0.16)	301 (12)	2.10 (0.35), 2.08 (0.23), 2.10 (0.19)	3.22, 3.22, 3.23
5	84.2	168 (10)	236	164 (4)	231	2.62 (0.09)	2.47 (0.12)	0.33 (0.14)	221 (32)	2.27 (0.25), 2.26 (0.25), 2.27 (0.21)	3.19, 3.18, 3.20

* Only one light intensity measured during ballasting operation

Table 7. Concentration of Organisms in the Three Regulated Size Classes (Standard Error of the Mean) in Uptake Water Samples Collected during Commissioning and Three Biological Efficacy Test Cycles.

Test Cycle	≥ 50 µm live/m^3 (Zoo-plankton)	Mean ≥ 10 µm and < 50 µm live cells/mL (SEM) (Protists)	Mean < 10 µm (Bacteria)			
			Total heterotrophic bacteria Spreadplates CFU/mL (SEM)	Total heterotrophic Bacteria Simplates MPN/mL (SEM)	*E. coli* MPN/100 mL (SEM)	Enterococci MPN/100 mL (SEM)
ETV minimum	10^5	1000	1000	NA	NA	NA
COM	4.8E+05	1,810 (382)	19,000 (2,000)	2,300 (367)	32.7 (1.4)	24.2 (2.8)
1	4.3E+05	1,768 (313)	7,000 (684)	733 (133)	114.9 (16.2)	41.2 (3.4)
2	1.7E+05	3,009 (82)	5,100 (433)	466.7 (67)	42.9 (0.3)	15.5 (1.7)
3	1.3E+0.5	2,457 (275)	5,800^ (1,060)	273.3 (82)	154.2 (9.5)	38.2 (5.7)
4	2.2E+05	999* (24)	4,900^^ (698)	157.0 (36.7)	52.5 (3.2)	30.6 (6.8)
5	8.0E+05	554* (42)	11,000 (333)	887.0 (57)	59.3 (2.9)	55.0 (12.3)

* Indicates the value is less than ETV uptake requirement

^ Value reported are colony counts not associated with the contaminant. May reflect a count lower than actual sample value.

^^ Value reported is for sample analysis that occurred just over the 24-hour hold time limit. Reported sample may be lower than actual sample value.

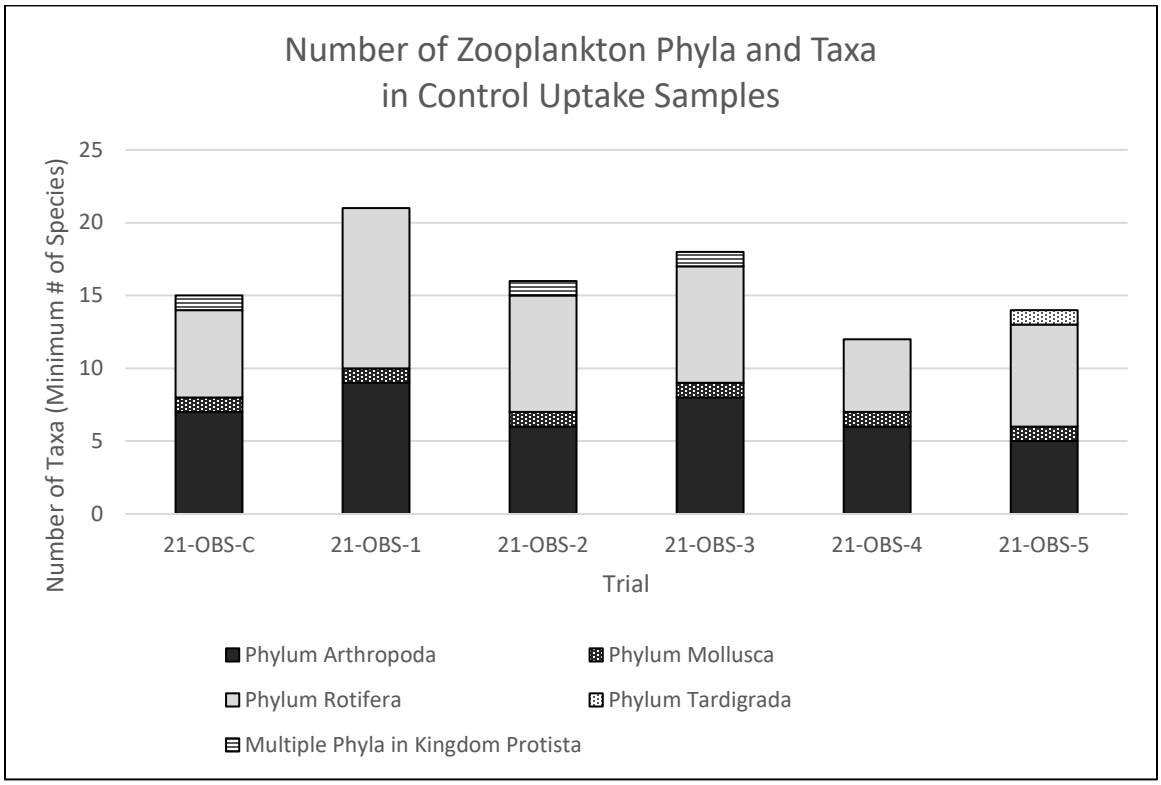

Figure 9. Zooplankton Control Uptake Phyla and Taxa.

The water quality parameters measured on uptake are recorded in Table 8. The ETV minimum challenge conditions for TSS, POM, and MM were met for all trials. The challenge condition for DOC was not met on uptake for any of the test cycles. Historically, DOC concentrations in the Duluth-Superior Harbor at the Montreal Pier Facility are at or above 6 mg/L (the ETV minimum challenge condition for DOC), with a yearly average being 14.1 mg/L. DOC concentrations were much lower this year than in the past as seen in Table 8. Augmentation with Micromate minimally contributes to the DOC concentration, but not enough to increase the low concentrations present in the harbor this year to within the ETV challenge conditions. Although there is not an ETV requirement for %UVT filtered, it is reported in Table 8 because it could impact the effectiveness of the OBS treatment.

Table 8. Water Quality Parameter Averages (Standard Deviation) Measured from Three Discrete Grab Samples Collected during Control Uptake.

Test Cycle	TSS (mg/L) (Std Dev)	POM (mg/L) (Std Dev)	MM (mg/L) (Std Dev)	% UVT filtered (Std Dev)	NPOC (mg/L) (Std Dev)	DOC (mg/L) (Std Dev)
ETV minimum	>24	>4	>20	-	-	> 6
COM	29.0 (1.5)	6.4 (0.2)	22.6 (1.3)	71.2 (0.2)	5.8 (0.1)	4.2 (0.1)
1	35.7	7.1	28.5	72.8	5.6	3.9

	(1.0)	(0.2)	(0.9)	(0.1)	(0.2)	(0.1)
2	30.4 (2.0)	6.1 (0.4)	24.3 (1.6)	70.6 (0.1)	5.3 (0.5)	4.2 (0.0)
3	25.3 (1.1)	5.1 (0.2)	20.2 (1.0)	70.8 (0.1)	4.8 (0.3)	4.1 (0.1)
4	28.4 (1.6)	5.7 (0.4)	22.7 (1.3)	69.9 (0.5)	4.6 (0.2)	3.9 (0.1)
5	31.5 (1.7)	6.6 (0.2)	24.9 (1.5)	65.5 (0.2)	5.2 (0.5)	4.2 (0.0)

The water chemistry values measured via a flow cell and multiparameter sonde during uptake operations are shown in Table 9. Temperature average values ranged from 16.23 – 18.79 °C on uptake during the commissioning trial through Trial 5. The temperatures in the later trials were cooler due to them being conducted later in the fall. Salinity values were stable at 0.08 – 0.09 psu during all uptake cycles. Dissolved oxygen values on uptake ranged from 8.39 – 10.12 mg/L O_2. Specific conductivity average values varied from a minimum of 169.7 – 198.1 µS/cm. Average pH values measured during uptake were 7.50 – 7.91. Turbidity and chlorophyll *a* values had more variability than the other parameters that were measured. It has been observed that the turbidity values during uptake can fluctuate widely based on variable sediment load or bubbles present in the flow cell. The minimum turbidity values ranged from 15.82 – 23.62 FNU. The minimum values are more indicative of uptake conditions than the averages for turbidity due to the variability with that parameter. Chlorophyll *a* average values ranged from 2.28 – 7.54 µg/L.

Water chemistry measurements taken in the control and treatment retention tanks are displayed in Table 10. Water chemistry values in the treatment retention tanks showed minimal different than the control tanks for salinity, conductivity and pH. Water temperature in the treatment tank was approximately 0.2 °C warmer than in the control retention tanks during all test cycles. Average dissolved oxygen values in the treatment tank were generally 0.3 mg/L higher than in the control tank during all test cycles. There was no clear trend in the difference in turbidity measurements between the control and treatment tanks. In the commissioning trial, turbidity was higher in the treatment tank. In test cycle 1, there was no difference in turbidity between control and treatment tanks, and in test cycles 2 – 5 the turbidity was lower in the treatment tank than in the control tank. In all trials, the chlorophyll *a* measurement was lower in the treatment retention tank than in the control tank. This is an indication that the treatment was effective at causing a decrease in protist densities.

Table 9. Water Chemistry Parameters Measured in a Flow Cell During Control Uptake.

Test Cycle	Temperature (°C)			Salinity (PSU)			Dissolved Oxygen (mg/L)			Specific Conductivity (µS/cm)			pH			Turbidity (FNU)			Chlorophyll *a* (µg/L)		
	Avg	Min	Max	Avg	Min	Max	Avg	Min	Max	Avg	Min	Max	Avg	Min	Max	Avg	Min	Max	Avg	Min	Max
COM	18.79	18.77	18.81	0.08	0.08	0.08	8.76	8.39	9.63	171.6	171.3	171.9	7.85	7.76	7.90	109.6	16.38	3713	3.99	2.98	4.72
1	17.86	17.77	17.97	0.08	0.08	0.08	9.26	8.67	10.23	171.5	169.4	173.1	7.72	7.65	7.87	22.05	16.53	129.2	6.26	3.92	10.38
2	16.23	16.19	16.32	0.08	0.08	0.08	9.58	9.29	10.21	169.7	169.4	170.3	7.85	7.80	7.87	807.0	15.63	27369	7.54	5.38	9.13
3	16.44	16.43	16.49	0.08	0.08	0.08	10.12	9.79	10.52	176.3	175.9	176.9	7.91	7.90	7.94	105.9	15.82	1064	4.62	2.59	7.31
4	16.75	16.72	16.80	0.09	0.09	0.09	8.48	7.32	9.38	181.4	180.3	182.4	7.66	7.56	7.75	3440	19.19	19910	3.69	2.15	8.78
5	16.47	16.46	16.53	0.09	0.09	0.09	8.39	8.15	9.25	198.1	197.6	198.4	7.50	7.37	7.64	298.1	23.62	1312	2.28	1.68	4.61

Table 10. Water Chemistry Parameters Measured in Control (C) and Treatment (T) Retention Tanks During Control and Treatment.

Test Cycle		Temperature (°C)			Salinity (PSU)			Dissolved Oxygen(mg/L)			Specific Conductivity (µS/cm)			pH			Turbidity (FNU)			Chlorophyll *a* (µg/L)		
		Avg	Min	Max	Avg	Min	Max	Avg	Min	Max	Avg	Min	Max	Avg	Min	Max	Avg	Min	Max	Avg	Min	Max
COM	C	18.62	18.18	18.87	0.08	0.08	0.08	8.13	7.81	8.47	171.6	171.3	171.9	7.80	7.75	7.85	11.94	9.37	16.29	3.09	1.84	4.24
	T	18.70	18.26	19.00	0.08	0.08	0.08	8.58	8.39	8.68	171.7	171.4	171.9	7.86	7.80	7.89	12.44	10.50	14.53	2.01	1.26	3.25
1	C	17.94	17.57	18.10	0.08	0.08	0.08	8.89	8.81	8.98	171.4	171.3	171.5	7.72	7.70	7.74	13.55	11.89	15.49	5.30	4.21	6.37
	T	18.07	17.64	18.23	0.08	0.08	0.08	9.23	9.20	9.25	166.8	166.6	166.9	7.68	7.44	7.75	13.58	11.80	15.20	2.68	2.15	3.56
2	C	16.41	16.26	16.52	0.08	0.08	0.08	9.19	9.09	9.31	169.6	169.4	169.6	7.86	7.83	7.90	12.89	11.49	15.26	7.27	5.18	12.66
	T	16.54	16.41	16.64	0.08	0.08	0.08	9.45	9.42	9.46	168.9	168.8	169.1	7.84	7.66	7.91	11.97	10.95	13.39	3.36	2.78	4.34
3	C	16.49	16.41	16.56	0.08	0.08	0.08	9.29	9.17	9.41	176.5	176.4	176.6	7.94	7.92	7.96	10.72	9.65	12.22	4.81	3.37	6.51
	T	16.62	16.57	16.68	0.08	0.08	0.08	9.58	9.56	9.61	177.1	177.0	177.1	7.80	7.72	7.87	10.16	9.42	11.08	3.07	2.30	4.10
4	C	16.67	16.50	16.75	0.09	0.09	0.09	8.26	8.19	8.37	181.4	181.3	181.4	7.64	7.62	7.65	12.87	11.68	14.86	2.42	1.92	3.09
	T	16.83	16.65	16.91	0.09	0.09	0.09	8.50	8.49	8.58	181.7	181.6	181.8	7.57	7.48	7.65	12.33	11.49	14.50	1.72	1.36	2.22
5	C	16.37	16.18	16.46	0.09	0.09	0.09	7.95	7.84	8.09	198.0	197.9	198.0	7.53	7.51	7.55	16.28	15.00	18.28	1.92	1.54	2.30
	T	16.51	16.30	16.62	0.09	0.09	0.09	8.32	8.31	8.36	198.3	198.3	198.4	7.56	7.51	7.59	15.48	14.09	16.46	1.51	1.20	1.89

5.2 BIOLOGICAL, WATER QUALITY, AND FACILITY OPERATIONAL CONDITIONS ON DISCHARGE

The operational parameters measured during the treatment discharge and control discharge are shown in Table 11 and Table 12, respectively. The control discharge always occurred after the treatment discharge to prevent contamination of treated lines with live organisms. There were no significant differences in the flow rates, line pressures, or sample volumes between the control and treatment discharges. The post treatment pressure was higher in the commissioning trial than in any of the other test cycles. Conversely, the control discharge pressure was lower during the commissioning trial than it was in test cycles 1 – 5. The shorter discharge duration for test cycle 5 treatment was due to the volume being lower to accommodate low ambient protist densities, as described in Section 5.1. UV intensity on treatment discharge ranged from 207 to 356 W/m^2. This intensity is less than the minimum UV intensity indicated in the OMSM for the Optimarin Ballast System (2021) to operate in USCG mode. This intensity is impacted by water quality parameters such as total suspended solids and dissolved organic carbon and is not an indication that the OBS system is not operating correctly.

Table 11. Operational Parameters Measured during Treatment Discharge Procedures. Standard Deviation values are indicated in parenthesis following the parameter.

Test Cycle	Treatment Discharge Duration (min)	Treatment Flow Rate (m^3/h) (Std Dev)	Volume Treated on Discharge (m^3)	Pre-Treatment Pressure (bar)	Post-Treatment Pressure (bar)	UV Intensity (W/m^2)	Sampling Flow Rates (m^3/h)	Sample Volumes (m^3)
COM	82.7	162 (3)	243	1.16 (0.05)	0.87 (0.03)	342 (2)	2.47 (0.25), 2.46 (0.26), 2.47 (0.28)	3.41, 3.41, 3.41
1	81.3	161 (2)	242	1.17 (0.02)	0.48 (0.01)	356 (1)	2.43 (0.23), 2.45 (0.23), 2.44 (0.26)	3.31, 3.34, 3.33
2	81.3	163 (2)	245	1.21 (0.02)	0.48 (0.01)	314 (1)	2.43 (0.25), 2.44 (0.25), 2.44 (0.26)	3.31, 3.32, 3.32

Test Cycle	Treatment Discharge Duration (min)	Treatment Flow Rate (m^3/h) (Std Dev)	Volume Treated on Discharge (m^3)	Pre-Treatment Pressure (bar)	Post-Treatment Pressure (bar)	UV Intensity (W/m^2)	Sampling Flow Rates (m^3/h)	Sample Volumes (m^3)
3	80.3	162 (1)	236	1.21 (0.03)	0.48 (0.03)	340 (6)	2.53 (0.33), 2.55 (0.33), 2.54 (0.34)	3.41, 3.43, 3.42
4	80.7	162 (3)	242	1.23 (0.08)	0.48 (0.04)	302 (3)	2.54 (0.29), 2.56 (0.29), 2.55 (0.30)	3.45, 3.46, 3.45
5	75.0	164 (8)	227	1.23 (0.08)	0.49 (0.04)	207 (0)	2.53 (0.31), 2.54 (0.31), 2.54 (0.31)	3.17, 3.19, 3.18

Table 12. Operational Parameters Measured during Control Discharge Procedures. Standard Deviation values are indicated in parenthesis following the parameter.

Test Cycle	Control Discharge Duration (min)	Control Flow Rate (m^3/h) (Std Dev)	Control Volume (m^3)	Control Discharge Pressure (bar)	Sampling Flow Rates (m^3/h)	Sample Volumes (m^3)
COM	71.3	163 (9)	232	0.92 (0.09)	2.49 (0.18), 2.48 (0.18), 2.49 (0.21)	3.15, 3.14, 3.16
1	76.0	162 (6)	233	1.25 (0.09)	2.47 (0.24), 2.47 (0.25), 2.50 (0.29)	3.14, 3.13, 3.15
2	82.3	163 (2)	240	1.21 (0.07)	2.47 (0.24), 2.47 (0.26), 1.28 (1.32)	3.41, 3.40, 1.77
3	81.0	165 (2)	244	1.12 (0.08)	2.59 (0.21), 2.58 (0.22), 2.61 (0.22)	3.50, 3.49, 3.51
4	80.0	165 (5)	242	1.16 (0.08)	2.59 (0.24), 2.59 (0.24), 2.60 (0.26)	3.52, 3.51, 3.53

Test Cycle	Control Discharge Duration (min)	Control Flow Rate (m^3/h) (Std Dev)	Control Volume (m^3)	Control Discharge Pressure (bar)	Sampling Flow Rates (m^3/h)	Sample Volumes (m^3)
5	76.0	165 (4)	223	1.21 (0.08)	2.58 (0.26), 2.57 (0.30), 2.59 (0.25)	3.27, 3.27, 3.29

The densities of the organisms in the regulated size classes in the control and treatment discharge are displayed in Table 13. ETV control discharge minimum densities were met for all regulated size classes, indicating that retention times did not negatively impact organism densities. The density of zooplankton in treated discharge was below the USCG BWDS limit for all test cycles except test cycle 5. Table 14 summarizes the taxonomy of the zooplankton in the discharge samples, differentiating between microzooplankton and macrozooplankton. Protist densities were greater than the USCG BWDS in all test cycles. During the commissioning trial and test cycles 4 and 5, the density was closer to the acceptable discharge limit, but in test cycles 4 and 5, the ETV minimum uptake density for protists was not met. Total coliform bacteria are currently not included in the USCG BWDS, therefore, they are not reported in Table 13. However, average total coliform densities ranged from 227.4 – 1362.2 MPN/100mL (SEM of 6 – 128 MPN/100 mL) in the control discharge samples, and were <1 MPN/100 mL in all treatment discharge samples indicating 100% mortality. Although the densities of *E. coli* and *Enterococci* were below the discharge standard in control uptake samples, the treated samples were all < 1 MPN/100 mL, indicating complete mortality. Total heterotrophic bacteria densities measured by spread plates and SimPlates were both lower by at least an order of magnitude in the treated discharge samples than in the control discharge samples. *Vibrio* counts are not shown due to all results being <1 CFU or cell/100 mL.

Table 13. Concentration of Organisms in the Three Regulated Size Classes in Treated Discharge Water Samples as Compared to Control Discharge Water Samples during Commissioning and Three Biological Efficacy Test Cycles.

Test Cycle	Treated Discharge						Control Discharge					
	≥ 50 µm live/m³ (Zoo-Plankton)	≥ 10 µm and < 50 µm live cells/mL (Protists)	< 10 µm (Bacteria)				≥ 50 µm live/m³ (Zoo-Plankton)	≥ 10 µm and < 50 µm live cells/mL (SEM) (Protists)	< 10 µm (Bacteria)			
			Total heterotrophic bacteria Spreadplates (CFU/mL) (SEM)	Total heterotrophic bacteria Simplates (MPN/mL) (SEM)	*E. coli* (MPN/100 mL) (SEM)	Enterococci (MPN/100 mL) (SEM)			Total heterotrophic bacteria Spreadplates (CFU/mL) (SEM)	Total heterotrophic bacteria Simplates (MPN/mL) (SEM)	*E. coli* (MPN/100 mL) (SEM)	Enterococci (MPN/100 mL) (SEM)
USCG BWDS	10	10	NA	NA	250	100	100	100	500	NA	NA	NA
COM	2.9	12	490 (23)	75 (7.5)	<1 (NA)	<1 (NA)	1.4E+06	974 (94)	11,000 (577)	720 (87)	17.4 (2.7)	9.7 (1.4)
1	7.8	52	128 (32)	37 (5.7)	<1 (NA)	<1 (NA)	4.4E+05	2,109 (146)	11,000 (666)	867 (133)	54.2 (2.9)	20.9 (3.1)
2	2.1	51	230 (35)	58.3 (8)	<1 (NA)	<1 (NA)	2.4E+05	2,188 (123)	9,900^ (666)	203.3 (18)	24.5 (5.2)	7.4 (0.6)
3	4.1	41	140 (10)	12.3 (2.3)	<1 (NA)	<1 (NA)	3.9E+05	1,848 (74)	5,000 (376)	154.3 (3)	53.2 (5.3)	20.8 (3.4)
4	6.4	21	310 (52)	50.6 (3.2)	<1 (NA)	<1 (NA)	3.8E+05	977 (136)	6,200 (1,184)	230.0 (50.3)	22.4 (1.2)	8.6 (1.7)
5	100.6	24	170 (50)	16.0 (7.0)	<1 (NA)	<1 (NA)	9.3E+05	350 (38)	51,000 (2,963)	3,140 (333)	40.9 (4.8)	44.7 (6.8)

* One or more values used to calculate the average were below the detection limit. When this occurs, half of the detection limit is used and the SEM is not able to be calculated.

^ Value reported is count not associated with the contaminant. May reflect a count lower than actual sample value.

Table 14. Summary of Zooplankton Taxa Present in Discharge Samples.

Phyla		Live Zooplankton Density (#/m³)														
		Macrozooplankton				Microzooplankton										
		Arthropoda			Nematoda	Rotifera									Unknown	Multiple Phyla in Kingdom Protista
Trial	Taxa*	Cladocera	Copepoda	Insecta												
		Bosminidae	Cyclopoida	Caddisfly Larva	Nematode ≥50 µm	Bdelloidea	Conochilus	Dicranophoridae	Kellicottia	Keratella	Ploesoma	Polyarthra	Synchaeta	Tricocerca	Egg ≥50 µm	Motile Protists
21-OBS-C				0.3		1.8		0.6			0.3					
21-OBS-1		1.9	0.4			0.4		0.4		3.4		0.4	0.4			0.7
21-OBS-2		0.9	0.6		0.3					0.3						
21-OBS-3		1.8						0.3		0.6		0.3	0.9		0.3	
21-OBS-4		3.1	0.3			0.3				2.0			0.6			
21-OBS-5		60.1	1.6				1.6	0.8	1.6	31.2	0.8	0.8	0.8	1.6		
Minimum Dimension (µm)		Generally >200 µm			74*	54†	57†	55†	67†	55†	91†	53†	78†	47†	62*	>50*

*Minimum dimension measurements are from eyepiece micrometer measurements during analysis.

†Minimum dimension measurements are averages from historical data from the Duluth-Superior Harbor at Montreal Pier Facility.

Water quality parameters measured on water collected from control and treatment discharge are presented in Table 15. There was minimal difference between the control and treatment tanks in the values measured for these water quality parameters. TSS, POM and MM values on discharge were much lower than on uptake in the same test cycle. This is likely due to settling in the tanks during retention. The measurements of %UVT filtered, NPOC and DOC were unaffected by the OBS treatment and only slightly differed from the values measured on uptake (Table 8).

Table 15. Average (Standard Deviation) Water Quality Parameters from Discrete Grab Samples Collected During Discharge Operations.

Test Cycle	Sample Type	TSS (mg/L) (Std Dev)	POM (mg/L) (Std Dev)	MM (mg/L) (Std Dev)	% UVT filtered (Std Dev)	NPOC (mg/L) (Std Dev)	DOC (mg/L) (Std Dev)
COM	Control	6.6 (0.6)	2.0 (0.3)	4.6 (0.4)	71.7 (0.3)	5.1 (0.5)	4.1 (0.1)
	Treatment	8.1 (0.5)	2.2 (0.5)	5.9 (0.8)	72.0 (0.4)	5.0 (0.2)	4.0 (0.1)
1	Control	13.6 (1.0)	3.0 (0.1)	10.6 (0.9)	73.6 (0.2)	5.2 (0.1)	3.8 (0.1)
	Treatment	14.0 (0.6)	2.9 (0.3)	11.1 (0.4)	73.7 (0.2)	5.3 (0.2)	4.0 (0.2)
2	Control	12.7 (0.7)	2.7 (0.2)	9.9 (0.5)	70.4 (0.2)	4.5 (0.4)	4.1 (0.2)
	Treatment	12.3 (0.8)	2.3 (0.1)	10.0 (0.7)	71.7 (0.4)	5.1 (0.1)	4.0 (0.2)
3	Control	10.3 (0.7)	2.4 (0.3)	7.8 (0.4)	71.9 (0.1)	4.2 (0.0)	3.7 (0.1)
	Treatment	9.7 (0.5)	2.0 (0.2)	9.4 (2.6)	72.4 (0.1)	5.1 (0.0)	4.0 (0.1)
4	Control	10.9 (0.7)	2.5 (0.3)	8.4 (0.4)	70.2 (0.1)	4.3 (0.2)	3.8 (0.1)
	Treatment	10.9 (0.7)	2.4 (0.3)	8.5 (0.5)	70.8 (0.1)	4.7 (0.3)	3.7 (0.1)
5	Control	11.4 (0.8)	2.6 (0.2)	8.7 (0.7)	63.9 (0.1)	5.1 (0.3)	4.5 (0.4)
	Treatment	11.2 (0.7)	2.2 (0.1)	9.0 (0.7)	65.8 (0.4)	5.4 (0.3)	4.5 (0.2)

The water chemistry parameters as measured with a sonde deployed in a flow cell during the control and treatment discharge are presented in Table 16. As was noted in the retention tanks (Table 10) salinity, specific conductivity, and pH values did not vary between control and treatment discharge samples. Values measured for temperature and dissolved oxygen were slightly higher in the treatment discharge samples than the control discharge samples. Turbidity was not consistently different between average control and treatment discharge values. In test cycles commissioning, 1 and 4 the turbidity was higher in the treatment discharge while in test cycles 3 and 5 the average turbidity was lower in treatment discharge. Chlorophyll *a* values were lower in the treated discharge water than in the control discharge water, which was indicative of the treatment causing a decline in protist densities. Microscopic analysis concurred with this indication (Table 13).

Table 16. Water Chemistry Parameters Measured in a Flow Cell with a Sonde During Control and Treatment Discharge. NM= Not Measured.

Test Cycle		Temperature (°C)			Salinity (PSU)			Dissolved Oxygen(mg/L)			Specific Conductivity (µS/cm)			pH			Turbidity (FNU)			Chlorophyll *a* (µg/L)		
		Avg	Min	Max	Avg	Min	Max	Avg	Min	Max	Avg	Min	Max	Avg	Min	Max	Avg	Min	Max	Avg	Min	Max
C	C	18.83	18.75	18.95	0.08	0.08	0.08	7.95	7.77	8.11	172.2	172.1	172.2	7.73	7.73	7.76	10.86	10.27	11.28	2.23	2.12	2.38
	T	18.89	18.81	18.89	0.08	0.08	0.08	8.38	8.33	8.75	171.9	170.8	172.0	7.82	7.81	7.88	12.23	11.31	18.41	1.07	1.01	1.14
1	C	17.44	16.54	17.52	0.08	0.07	0.08	8.99	8.82	10.38	166.3	145.7	176.3	7.73	7.72	7.79	13.88	13.42	17.84	4.96	3.80	5.42
	T	17.57	16.16	17.64	0.08	0.08	0.08	9.37	9.20	10.47	166.4	162.3	169.5	7.77	7.76	7.88	14.67	14.25	15.08	1.85	1.75	1.93
2	C	16.33	16.16	16.35	0.08	0.08	0.08	9.12	9.00	9.29	169.1	168.8	169.4	7.82	7.81	7.88	13.12	12.76	13.82	6.25	5.57	7.01
	T	16.57	16.50	16.59	0.08	0.08	0.08	9.51	9.47	99.57	169.0	168.7	169.4	7.90	7.90	7.93	13.07	12.60	13.47	2.22	2.08	2.34
3	C	16.45	16.44	16.48	0.08	0.08	0.08	9.30	9.14	9.41	177.3	177.2	177.3	7.85	7.85	7.87	11.03	10.68	11.37	6.52	5.61	8.19
	T	16.75	16.74	16.75	0.08	0.08	0.08	9.56	9.53	9.61	177.4	177.2	177.5	7.93	7.93	7.94	10.91	10.49	11.31	1.81	1.67	1.92
4	C	16.41	15.19	16.42	0.09	0.09	0.09	8.24	8.20	8.45	181.7	181.6	181.8	7.67	7.66	7.71	12.97	12.66	13.39	2.56	1.53	2.71
	T	16.78	16.74	16.79	0.09	0.09	0.09	8.60	8.24	8.82	181.8	181.4	182.0	7.70	7.69	7.75	13.29	12.83	13.71	1.16	1.06	1.24
5	C	15.90	15.80	15.95	0.09	0.09	0.09	7.86	7.82	8.14	198.4	198.3	198.4	7.56	7.56	7.58	17.15	16.68	17.83	1.94	1.84	2.08
	T	16.30	16.25	16.34	0.09	0.09	0.09	8.39	8.32	8.59	198.5	198.4	198.6	7.60	7.60	7.62	16.56	16.03	17.05	1.14	1.08	1.21

6 DEVIATIONS

During testing with OBS, there were several deviations that occurred from the test plan which are shown in Table 17. None of the deviations had significant impact on the outcome of the testing.

Table 17. Corrective Action Log for OBS.

Test ID Code	1. Deviation or Quality Control Failure 2. Root Cause of Deviation	Description of Corrective Action(s)	Description of Impact on the Project/Test
21-OBS-C through 21-OBS-5	**1.** Augmented uptake had a DOC (~4mg/L) lower than the *ETV Protocol* (min. 6 mg/L). This occurred for all test cycles **2.** The ambient harbor water is lower than historic range. Our augmentation process does not consider DOC concentration.	N/A	A DOC concentration lower than *ETV Protocol* lead to increased percent transmittance of the water and may have presented less of a challenge to the OBS system.
21-OBS-C-U	**1.** Total coliform precision criteria in duplicate failed. PC calculated at 0.275, current PC is 0.236. Duplicate out of 95% confidence interval per IDEXX calculations. **2.** No root cause established.	N/A	Minimal; Total coliform data not required
21-OBS-C-U	**1.** *Vibrio* +cont on TCBS plate contained no colonies, positive control had been filtered only with stock culture #4. **2.** Positive control tube #4 used on analysis date did not exhibit colonies on a spread plate made 9/15/21. Spread plate made with positive control #5 on 9/16/21 and plate was positive.	Positive control tube #4 was discarded, positive control plates will be made by spread plate or filtering with a diluent.	Minimal due to no colony lifts performed on 9/15/21 and samples used as preemptive counts for discharge analysis. Control sample filtered did exhibit *Vibrio* colonies.
21-OBS-C-D	**1.** HPC for portion of samples Control M-2 and Control M-3 used media lot LR450 which expired 5/5/21. **2.** Analyst error.	Old lot removed and media discarded. Sterility check on media lot was negative, and HPC analysis on samples contained positive wells.	Minimal as control samples 2 and 3 were similar in counts to control sample 1 for all dilutions.

Test ID Code	1. Deviation or Quality Control Failure 2. Root Cause of Deviation	Description of Corrective Action(s)	Description of Impact on the Project/Test
21-OBS-1-D	**1.** Quantitative positive control for E. coli failed; MPN= 344.8 MPN/100mL. Acceptable range is 49-313 MPN/100mL. **2.** Possible cause is prolonged rehydration of organisms allowing growth of E. coli prior to sealing in Q-trays, or pH of water being out of range used for the dilutions.	Same lot of IDEXX QC kit, media, Quanti-Trays, and STDI used for dilutions was used for 21-BWT-C trial on 9-24-21 and passed	None
21-OBS-2	**1.** Flow control valve for sample tub 3 failed and couldn't control flow. **2.** Positioner failed and couldn't control the valve.	Sample tub was not used.	None
21-OBS-3-D	**1.** OBS was started in ballasting mode during the discharge rather than deballasting mode. **2.** Operator Error	The system was shut down once the error was recognized and the valves were manually closed. About 7% of the test volume was lost. A similar volume was discharged without sampling on the control side to make the two discharges similar.	Minimal- Volumes discharged were reduced but still above requirements for an ETV test.
21-OBS-2	**1.** Post deployment check was not conducted on sondes**.** **2.** Analyst error	N/A - sondes were calibrated for	Minimal - as subsequent sonde calibration did not have any issues
21-OBS-3-U	**1.** HPC media blank displayed 1 positive well. Final concentration of media blank is calculated at <2 MPN/1 mL, which is still below the LOD. **2.** Possible contamination from STDI.	Bottle of media was disposed of and was used for 21-OBS-3-D HPC and media blank was negative associated with batch.	Minimal - media blank for 21-OBS-3-D was negative, all other media blanks from same batch of media were blank, and diluent blank was negative for 21-OBS-3-U

Test ID Code	1. Deviation or Quality Control Failure 2. Root Cause of Deviation	Description of Corrective Action(s)	Description of Impact on the Project/Test
21-OBS-2-D	**1.** HPCA method blank was positive indicating diluent contamination (FSW080921DNN-3). **2.** FSW likely contained a slow growing organism that was not removed during filter sterilization and grew during storage (batch was filter sterilized but not autoclaved).	Remaining FSW and diluent tubes used for this date were discarded. All colonies that appeared on BLK plate were similar (yellow), therefore only non-yellow colonies, that did not appear to be from contamination, were counted.	Treatment HPCA samples are unaffected as reported values are from un-diluted samples. Values reported for control samples are the count of colonies unlike the contaminant and reflect a count lower than the actual value (i.e., contaminant colonies compete, overgrow sample colonies). Reported data has been flagged.
21-OBS-3-U	**1.** HPCA method blank was positive indicating diluent contamination (FSW080921DNN-3). Final concentration of method blank is calculated at <1 CFU/mL. **2.** FSW likely contained a slow growing organism that was not removed during filter sterilization and grew during storage (batch was filter sterilized but not autoclaved).	Remaining FSW and diluent tubes used for this date were discarded. All colonies that appeared on BLK plate were similar (yellow), therefore only non -yellow colonies, that did not appear to be from contamination, were counted.	Values reported for control samples are the count of colonies unlike the contaminant and reflect a count lower than the actual value (i.e., contaminant colonies compete, overgrow sample colonies). Reported data has been flagged.
21-OBS-3	**1.** Uptake requirements were not met for challenge water augmentation. The Mineral Matter was less than 20.0 mg/L (ETV required limit). **2.** The ambient harbor water mineral matter decreased 4.4 mg/L overnight. Augmentation calculations are based on the previous days harbor water chemistry.	N/A	Minimal. The Mineral Matter concentration was 19.2 mg/L, which is below the ETV required limit. POM and TSS concentrations were within the ETV required limit.

Test ID Code	1. Deviation or Quality Control Failure 2. Root Cause of Deviation	Description of Corrective Action(s)	Description of Impact on the Project/Test
21-OBS-1-D	**1.** Positive control for *Vibrio* on TCBS plates did not exhibit any colonies. **2.** Due to positive control being filtered with STDI and colonies did not survive. Previous spread plate of positive control was positive.	Future filtration of positive control will be done using 1X PBS.	None
21-OBS-3-D	**1.** Negative control for *Vibrio* DFA kit was positive. **2.** Could be due to bleeding within wells from positive control on analysis slide.	Moving position of positive controls within slides to prevent possible contamination.	None
21-OBS-4-U	**1.** HPC and HPCA blank from diluent tube was positive. **2.** FSW likely contained a slow growing organism that was not removed during filter sterilization and grew during storage (batch was filter sterilized but not autoclaved).	All remaining media bottles (even those that had not been opened/used) from same FSW date and diluent tubes were discarded. Samples were re-analyzed (22 minutes over the 24 hour hold time).	No impact on HPC due to undiluted samples being reported. HPCA was re-analyzed on 10-7-21 just over the 24 hour hold time. Reported values could be lower.
21-OBS-4-U	**1.** HPCA media blank contained 2 mold colonies. Final concentration calculated at <1 CFU/mL. **2.** Unknown	N/A Minimal to no mold colonies present on other plates. Mold colonies not counted or reported.	Minimal since not counted or reported.
21-OBS-5-U	**1.** HPCA media blank contained 4 mold colonies. Final concentration calculated at <1 CFU/mL. Mold colonies not exhibited in samples and method blank associated with batch was negative. **2.** Unknown	N/A Minimal to no mold colonies present on other plates. Mold colonies not counted or reported.	Minimal since not counted or reported.

Test ID Code	1. Deviation or Quality Control Failure 2. Root Cause of Deviation	Description of Corrective Action(s)	Description of Impact on the Project/Test
21-OBS-C-Discharge through 21-OBS-5-Discharge	**1.** The water quality samples collected on discharge came from a different sample port than the one identified in the test plan. The reason is occurred is that the wrong sample port was listed in the test plan. On control discharge events, all discrete water quality samples are collected from SP9a and all treatment discharge discrete samples are collected from SP15. **2.** Error in test plan.	Collected samples from the appropriate sample port for this, and the remaining test cycles	None
21-OBS-C through 21-OBS-5	1. 10% of protist samples were not counted twice by the same analyst to estimate precision. 2. Analyst error	No corrective action taken	No impact. This does not affect the primary data and protist analysts are experienced and historically able to pass QC counts without trouble.

7 QUALITY ASSURANCE/QUALITY CONTROL

7.1 BACTERIA TESTING

The data quality objectives (DQO) for bacteria analyses conducted during the evaluation of OBS are summarized in Table 18. Data quality objectives for precision, bias, accuracy, and completeness (i.e., method blanks, duplicate agreements, and quantitative positive and negative controls) were all within acceptable limits with the exception of the frequency at which QA counts were completed for heterotrophic bacteria spread plates. Due to time constraints and staffing limitations, only 5.5% of heterotrophic bacteria spread plates were recounted by a second analyst. All analysts passed an internal

proficiency assessment prior to conducting independent analysis, and QA counts completed prior to and coincident with OBS were within acceptable ranges.

Table 18. Data Quality Objective Table for OBS Bacteria Analysis.

Data Quality Indicator	Evaluation Process/ Performance Measurement	Data Quality Objective	Performance Measurement Result
Precision	Samples (10%) are analyzed in duplicate – with performance measured R_{log} not greater than precision criterion (PC)	R_{log} not greater than 0.667 for *E. coli*	***E. coli:*** 12 of 54 (22%) reported samples were analyzed in duplicate; R_{log} for 2 were not calculable as both samples were < 1 MPN/100mL, remaining 10 average R_{log} = 0.084
		R_{log} not greater than 0.236 for Total Coliforms	**Total Coliforms*:*** 12 of 54 (22%) reported samples were analyzed in duplicate; R_{log} for 2 were not calculable as both samples were < 1 MPN/100mL, remaining 10 average R_{log} = 0.103
		R_{log} not greater than 0.349 for *Enterococcus* spp.	***E. faecium:*** 12 of 54 (22%) reported samples were analyzed in duplicate; R_{log} for 2 were not calculable as both samples were < 1 MPN/100mL, remaining 10 average R_{log} = 0.123
		R_{log} not greater than 0.450 for heterotrophic bacteria simplates	**Heterotrophic Bacteria SimPlates:** 12 of 54 (22%) reported samples were analyzed in duplicate; R_{log} for 6 were not calculable as at least 1 sample was <2 MPN/1mL, remaining 6 average R_{log} = 0.119
		R_{log} not greater than 0.296 for heterotrophic bacteria spreadplates	**Heterotrophic Bacteria Spread plates:** 10 of 54 (19%) reported samples were analyzed in duplicate; R_{log} for 4 were not calculable as counts were >300, remaining 6 average R_{log} = 0.056
Bias, Operator	Samples (10%) are counted by two separate analysts with performance measured by average relative percent difference	<20% average RPD	***E. coli*/Total Coliforms:** 12 of 54 (22%) reported samples were recounted by second analyst; Average RPD = 1.56% for *E. coli* and 0.57% for total coliforms. 3 of 54 (5.5%) samples analyzed were recounted by same

Data Quality Indicator	Evaluation Process/ Performance Measurement	Data Quality Objective	Performance Measurement Result
	(RPD) of all second counts.		analyst; Average RPD = 2.24% for *E. coli* and 0% for total coliforms.
			E. faecium: 12 of 54 (22%) reported samples were recounted by second analyst; Average RPD = 5.04%. 3 of 54 (5.5%) samples analyzed were recounted by same analyst; Average RPD = 0.81%.
		<20% average RPD	**Heterotrophic Bacteria SimPlates:** 9 of 54 (17%) reported samples were recounted by second analyst; Average RPD = 2.80%. 9 of 54 (17%) samples analyzed were recounted by same analyst; Average RPD = 0.11%.
		<20% average RPD	**Heterotrophic Bacteria Spread plates:** 3 of 54 (5.5%) reported samples were recounted by second analyst; Average RPD = 6.38%. No samples analyzed were recounted by same analyst.
Bias, Positive Control	Qualitative positive control samples (American Type Culture Collection) are analyzed on each analysis date or IDEXX-QC samples are analyzed as a quantitative positive control at least once per ballast water treatment system test.	Results must be greater than the limit of detection.	***E. coli*/Total Coliforms:** Qualitative Positive controls >1 MPN/100 mL n=9; Quantitative positive controls 49-313 MPN/100mL n=3
			E. faecium: Qualitative Positive controls >1 MPN/100 mL n=9; Quantitative positive controls 52-150 MPN/100mL n=3
			Heterotrophic Bacteria SimPlates: Qualitative Positive controls >1 MPN/1 mL n=11; Quantitative positive controls 188-580 MPN/1 mL n=1
			Heterotrophic Bacteria Spread plates: Qualitative Positive controls >1 CFU/1 mL n=12; Quantitative positive controls 188-580 MPN/100mL n=1.

Data Quality Indicator	Evaluation Process/ Performance Measurement	Data Quality Objective	Performance Measurement Result
Bias, Negative Control	Qualitative negative control samples (American Type Culture Collection) are analyzed on each analysis date or IDEXX-QC samples are analyzed as a negative control at least once per ballast water treatment system test.	Results must be less than the limit of detection.	***E. coli*/Total Coliforms:** Qualitative Positive controls <1 MPN/100 mL n=9; Quantitative positive controls <1 MPN/100mL n=3
			E. faecium: Qualitative Positive controls <1 MPN/100 mL n=9; Quantitative positive controls <1 MPN/100mL n=3
Bias, Method Blank	One per analysis day; Sterilized water or diluent (similar matrix sample) analyzed using same method as samples on each analysis date.	Results must be less than the limit of detection.	***E. coli*/Total Coliforms:** All method blanks <1 MPN/100 mL, n=12
			E. faecium: All method blanks <1 MPN/100 mL, n=12
			Heterotrophic Bacteria SimPlates: 10 of 11 method blanks <2 MPN/1 mL, n=11. One method blank positive due to contaminated diluent. Reported undiluted samples.
			Heterotrophic Bacteria Spread plates: 10 of 12 method blanks <1 CFU/mL, n=12. Three method blanks positive due to contaminated diluent. Control samples flagged.
Bias, Media Blank	One per analysis day, media only; incubated coincident with samples	Results must be less than the limit of detection.	**Heterotrophic Bacteria Simplates:** 12 of 12 media blanks incubated with samples <2 MPN/1 mL; All media sterility checks done prior to analysis were negative. One media blank contained 1 positive well, but method blank was negative.
			Heterotrophic Bacteria Spread plates: 13 of 13 media blanks incubated with samples <1 CFU/mL; All sterility checks done prior to analysis were negative. Two media blanks were positive, but

Data Quality Indicator	Evaluation Process/ Performance Measurement	Data Quality Objective	Performance Measurement Result
			only contained 2-4 mold colonies which were not counted in associated samples
Accuracy	IDEXX-QC samples are analyzed as a quantitative positive control at least once per ballast water treatment system test.	***E. coli:*** 49-313 MPN/100 mL	***E. coli:*** 2 of 3 quantitative analyses within IDEXX acceptance ranges (n=3), one was out of range due to prolonged rehydration of organisms prior to sealing in Quanti-Tray.
			16 Sep. 2021; 290.9 MPN/100 mL 22 Sep. 2021; 344.8 MPN/100 mL 29 Sep. 2021; 142.1 MPN/100 mL
		Total Coliforms: 29-99 MPN/100 mL	**Total Coliforms:** All quantitative analyses within IDEXX acceptance ranges (n=3)
			16 Sep. 2021; 60.9 MPN/100 mL 22 Sep. 2021; 68.9 MPN/100 mL 29 Sep. 2021; 46.4 MPN/100 mL
		E. faecalis: 52-150 MPN/100 mL	***E. faecalis:*** All quantitative analyses within IDEXX acceptance ranges (n=3)
			16 Sep. 2021; 105.0 MPN/100 mL 22 Sep. 2021; 137.6 MPN/100 mL 29 Sep. 2021; 113.0 MPN/100mL
		***E. faecalis* as Heterotrophic Bacteria:** 188 -580 MPN/1 mL with IDEXX Simplate for HPC analysis, results may vary for other methods	**Heterotrophic Bacteria:** All quantitative analyses within IDEXX acceptance range (n=1)
			22 Sep. 2021; 276 MPN/ mL for IDEXX SimPlates 22 Sep. 2021; 360 MPN/ mL for Spread plates

Data Quality Indicator	Evaluation Process/ Performance Measurement	Data Quality Objective	Performance Measurement Result
Representativeness	All samples are collected, handled, and analyzed in the same manner.	Not Applicable – Qualitative.	All bacterial samples were collected, handled, and analyzed in the same manner (using the appropriate LSRI/GWRC SOPs).
Comparability	Routine procedures are conducted according to appropriate SOPs to ensure consistency between tests.	Not Applicable – Qualitative.	The LSRI/GWRC SOPs listed in Appendix A were used for all bacterial analyses conducted.
Completeness	Percentage of valid (i.e., collected, handled, analyzed correctly and meet DQOs) bacterial samples measured out of the total number of bacterial samples collected. Performance is measured by percent completeness (%C).	>90% C.	***E. coli* /Total Coliforms:** 54 of 54 samples = 100% Completeness
			E. faecium: 54 of 54 samples = 100% Completeness
			Heterotrophic Bacteria SimPlates: 54 of 54 samples = 100% Completeness
			Heterotrophic Bacteria Spread plates: 54 of 54 samples = 100% Completeness
Sensitivity	The limit of detection (LOD) for the analytical method used is reported.	Dependent upon the analytical technique used. Adjusted for volume used.	***E. coli*/Total Coliforms: LOD:** 1 MPN/100 mL
			***E. faecium:* LOD:** 1 MPN/100 mL
			Heterotrophic Bacteria SimPlates: LOD: 2 MPN/1 mL

7.2 PROTIST

As stated in Table 16, no precision calculation was done for protist analysis during the OBS evaluation. Bias was evaluated on an uptake sample and treatment discharge sample from test cycle 21-OBS-4 for a total of 11.1% of samples being analyzed for bias during the OBS evaluation period. The benchmark for bias in protist samples is bias <20% and this was achieved with an average bias of 10.7% in the two samples that were analyzed.

7.3 ZOOPLANKTON

The DQO for zooplankton analysis conducted during the evaluation of OBS are summarized in Table 19. Data quality objectives were met for all measures of precision and bias.

Table 19. Data Quality Objectives for Zooplankton Sample Analysis.

Data Quality Indicator	Evaluation Process/ Performance Measurement	Data Quality Objective	Zooplankton Sample	Performance Measurement Result	
				Micro-ZP	Macro-ZP
Precision	Analyze at least two slides (i.e., microzooplankton subsamples) or two counting wheels (i.e., macrozooplankton) from all samples analyzed via the "dead/total" counting method.	Coefficient of variation among subsamples (%CV) ≤30%	21-OBS-C Control Uptake	5.9%	10.3%
			21-OBS-C Control Discharge	1.8%	13.8%
			21-OBS-1 Control Uptake	9.8%	12.8%
			21-OBS-1 Control Discharge	9.3%	5.5%
			21-OBS-2 Control Uptake	14.3%	7.0%
			21-OBS-2 Control Discharge	4.9%	4.4%
			21-OBS-3 Control Uptake	13.7%	18.7%
			21-OBS-3 Control Discharge	14.3%	5.4%
			21-OBS-4 Control Uptake	4.3%	4.8%
			21-OBS-4 Control Discharge	0.9%	1.0%
			21-OBS-5 Control Uptake	7.9%	10.0%
			21-OBS-5 Control Discharge	5.0%	2.6%
Bias	Ensure a second, qualified analyst analyzes a minimum of 10% of control samples collected per set of BWMS trials.	Percent Taxonomic Similarity (PSC) ≥80%	21-OBS-3 Control Uptake	93.9%	83.3%
		Relative Percent Difference (RPD) ≤20%		1.7%	14.9%

7.4 WATER QUALITY AND CHEMISTRY

The DQO for water quality and water chemistry analysis conducted during the evaluation of OBS are summarized in Table 20. Data quality objectives were met for all measures of precision, bias, and accuracy. The percent completeness exceeded the required percentage for all parameters.

Table 20. Data Quality Objective for Water Quality Sample Analysis.

Data Quality Indicator	Evaluation Process/Performance Measurement	Data Quality Objective	Performance Measurement Result	
Precision	Samples (10%) were collected and analyzed in duplicate with performance measured by average relative percent difference (RPD).	< 20% average RPD	Percentage of Samples Collected and Analyzed in Duplicate:	Duplicate Relative Percent Difference
			%TF: 11.1%	%TF: 0.2 ± 0.2%
			%TU: 11.1%	%TU: 0.4 ± 0.3%
			NPOC: 11.1%	%NPOC: 2.9 ± 1.4%
			DOC: 11.1%	%DOC: 4.5 ± 3.2%
			POM: 11.1%	POM: 4.8 ± 5.6%
			TSS: 11.1%	TSS: 2.3 ± 1.1%
Bias, Filter Blanks	%T method blanks were prepared by filtering deionized water samples (one per analysis date).	> 98% average %T	Number of %T Method Blanks Analyzed: 10	Method Blanks (%T): 100.0 ± 0.4%
	TSS/POM method blanks were prepared by filtering deionized water samples from a 1L sample bottle (one per analysis date) and then drying, weighing, ashing and weighing the filter.	< 1.25 mg/L average TSS/POM	Number of TSS Method Blanks Analyzed: 10	Method Blanks (TSS)= 0.0 ± 0.0
			Number of POM Method Blanks Analyzed: 10	Method Blanks (POM)= 0.1 ± 0.1
	NPOC blanks were prepared by acidifying a volume of deionized water to 0.2% with concentrated hydrochloric acid.	< 0.77 mg/L average NPOC	Number of NPOC Blanks Analyzed: 25	Blanks (NPOC)= 0.3 ± 0.1
	DOC method blanks were prepared by filtering deionized water samples (one per analysis date).	< 2.6 mg/L average DOC	Number of DOC Method Blanks Analyzed: 10	Method Blanks (DOC)= 0.4 ± 0.1
Accuracy	Samples (10%) were spiked with a total organic carbon spiking solution with performance measured by average spike-recovery (SPR).	75% - 125% average SPR	Percentage of NPOC/DOC Samples Spiked: 12.2%	NPOC/DOC Spike Recovery= 98.5 ± 4.9%
	Performance was measured by average percent difference	One per analysis	Percentage of Analysis Days Containing a Reference Standard:	Reference Standard Percent Difference

Data Quality Indicator	Evaluation Process/Performance Measurement	Data Quality Objective	Performance Measurement Result	
	(%D) between all measured and nominal reference standard values.	day < 20% average D	TSS: 100%	TSS: 3.0 ± 1.8%
			POM: 100%	POM: 4.8 ± 1.9%
			NPOC: 100%	NPOC: 8.0 ± 2.8%
		A least one per 10 samples < 10% average D	Percentage (vs total samples) Check Standards:	NPOC 10 mg/L Standard % Difference
			NPOC/DOC: 28%	5.8 ± 2.6%
Represent-ativeness	All samples were collected, handled, and analyzed in the same manner.	Not Applicable – Qualitative.	All water chemistry/quality samples were collected, handled, transported, and analyzed in the same manner using the appropriate SOPs.	
Comparability	Routine procedures were conducted according to appropriate SOPs to ensure consistency between tests.	Not Applicable – Qualitative.	The SOPs listed in the methods and references section were used for all water chemistry and water quality analyses.	
Completeness	Percentage of valid (i.e., collected, handled, analyzed correctly and meeting DQOs) water chemistry samples measured out of the total number of water chemistry samples collected. Performance is measured by percent completeness (%C).	> 90% C	TSS: 100%	
			%T Filtered: 100%	
			%T Unfiltered: 100%	
			NPOC: 100%	
			DOC: 100%	
Sensitivity	The limit of detection (LOD) and limit of quantification (LOQ) for each analyte and analytical method utilized was determined annually unless a reporting limit was used based on the amount filtered as was the case with TSS/POM.	Not Applicable	TSS/POM RL: 1.25 mg/L based on filtering 800 mL of sample	
			NPOC/DOC LOD: 0.77mg/L	
			NPOC/DOC LOQ: 2.6 mg/L	
			Determined 14 December 2020	

8 CONCLUSIONS AND DISCUSSION

The LSRI-GWRC land-based evaluation of the OBS BWMS met the stated objectives as outlined in the Test Plan (Schaefer et al., 2021). The reported deviations and quality control failures do not impact LSRI-GWRC's ability to draw conclusions on the performance of OBS during testing. The system was operational during all reported tests and was operated in accordance with the developer's instructions. However, UV intensity range of operation of the OBS BWMS was never within the range specified. It should be noted, UV transmittance (UV-T) of the uptake water, impacted the intensity delivered by the OBS system, UV-T for the test water (Superior Harbor water) is historically lower than 70% transmittance, which makes the test water challenging for UV BWMS, generally.

The overall conclusion from the biological effectiveness testing with OBS is that the system is highly effective at controlling concentrations of bacteria, protists, and zooplankton in water at or near ETV challenge conditions for both water quality and organism density. Bacteria met discharge standard in all test cycles, and zooplankton met discharge standards in all test cycles with the exception of one. Uptake densities during that test cycle were elevated due to augmentation (Table 7) and 100.6 live zooplankton/m^3 were present upon discharge (Table 13). Protist densities did not meet the discharge standard for any of the trials, but the densities were reduced by about 95% in all test cycles. Additionally, the treatment system caused minimal impact on water chemistry and water quality parameters.

9 APPENDIX A

Appendix A. LSRI and GWRC Standard Operating Procedures Associated with OBS Biological Efficacy Testing.

CATEGORY	TITLE	CODE
Field Studies	Procedure for Calibration of YSI EXO2 Series Multiparameter Water Quality Sondes	LSRI/SOP/FS/39
Field Studies	Procedure for Operation and Storage of YSI EXO2 Multiparameter Water Quality Sondes	LSRI/SOP/FS/41
General Lab Maintenance	Procedure for Calibration and Operation of pH Meters Utilizing Automatic Temperature Compensation (ATC)	LSRI/SOP/GLM/05
General Lab Maintenance	Procedure for Verification of Laboratory Balances	LSRI/SOP/GLM/12
General Lab Maintenance	Procedure for Thermometer Verification and Calibration	LSRI/SOP/GLM/17
General Lab Maintenance	Procedure for Verifying the Accuracy of Laboratory Pipettes	LSRI/SOP/GLM/27
General Lab Maintenance	Calibrating, Maintaining, and Using the HQ30d and HQ40d Meter and LDO101 Optical Electrode to Measure Dissolved Oxygen in Water Samples	LSRI/SOP/GLM/30
General Lab Maintenance	Autoclave Use and Maintenance	LSRI/SOP/GLM/33
Great Waters Research Collaborative	Operating the Montreal Pier Facility	LSRI/SOP/GWRC/19
Great Waters Research Collaborative	Cleaning and Performing Cleaning Validation of the Montreal Pier Facility	LSRI/SOP/GWRC/21
Great Waters Research Collaborative	Procedure for Calibrating the Pressure Sensors and Flow Meters at the Montreal Pier Facility	LSRI/SOP/GWRC/22

CATEGORY	TITLE	CODE
Great Waters Research Collaborative	Water Augmentation for the Montreal Pier Facility	LSRI/SOP/GWRC/23
Great Waters Research Collaborative	Procedure for Cleaning the Sample Collection Tubs and Zooplankton Sample Collection Equipment	LSRI/SOP/GWRC/24
Great Waters Research Collaborative	Procedure for Zooplankton Analysis	LSRI/SOP/GWRC/25
Great Waters Research Collaborative	Collecting Water Chemistry Samples and Data at the Montreal Pier Facility	LSRI/SOP/GWRC/27
Great Waters Research Collaborative	Collecting Protist and Microbial Samples at the Montreal Pier Facility	LSRI/SOP/GWRC/28
Great Waters Research Collaborative	Zooplankton Sample Collection at the Montreal Pier Facility	LSRI/SOP/GWRC/29
Great Waters Research Collaborative	Procedure for Protist Analysis	LSRI/SOP/GWRC/30
Great Waters Research Collaborative	Labeling Samples Collected at the Montreal Pier Facility	LSRI/SOP/GWRC/31
Great Waters Research Collaborative	Procedure for Enumerating Culturable Heterotrophic Bacteria	LSRI/SOP/GWRC/32
Great Waters Research Collaborative	Procedure for the Detection and Enumeration of Culturable Toxigenic *Vibrio chloerae* O1 and O139	LSRI/SOP/GWRC/33
Recordkeeping	Sample Custody and Tracking	LSRI/SOP/REC/15
Sample Analysis	Measuring Organic Carbon in Aqueous Samples	LSRI/SOP/SA/47
Sample Analysis	Detection and Enumeration of Total Coliforms and *E. coli* Using IDEXX Colilert®	LSRI/SOP/SA/56

CATEGORY	TITLE	CODE
Sample Analysis	Detection and Enumeration of *Enterococcus* Using IDEXX Enterolert®	LSRI/SOP/SA/62
Sample Analysis	Analyzing Total Suspended Solids, Particulate Organic Matter, and Mineral Matter	LSRI/SOP/SA/66
Sample Analysis	Laboratory Determination of Percent Transmittance of Light in Water at 254 nm	LSRI/SOP/SA/69

10 REFERENCES

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