BENCH-SCALE TECHNICAL REPORT

FRESHWATER VERIFICATION OF THE BALLAST EYE COMPLIANCE MONITORING DEVICE

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ABSTRACT

This technical report presents findings from freshwater verification tests evaluating the performance of the Satake Ballast Eye Viable Organism Analyzer VOA1000K compliance monitoring device, hereafter Ballast Eye. Ballast Eye was developed by Satake Corporation of Hiroshima, Japan.

The compliance monitoring device evaluation began in August 2020 and ended in December 2020 at the Lake Superior Research Institute (LSRI) of the University of Wisconsin-Superior (UWS) in Superior, Wisconsin, USA. Ballast Eye estimates the number of viable organisms and associated risk based on IMO D-2 ballast water discharge standards in the ≥10 and <50 µm (nominally protists) and ≥50 µm (nominally zooplankton) regulated size classes by measuring the fluorescence pulse number from fluorescein diacetate (FDA) stained organisms within a water sample.

The verification testing was composed of three phases. Phase I testing was completed in two water types with laboratory-cultured organisms in the two regulated size classes, utilizing the single-celled protist *Haematococcus pluvialis* and colonial protist *Scenedesmus quadricauda*, and the zooplankton *Daphnia magna* and *Eucyclops* spp. Phase II was completed using naturally occurring Great Lakes organisms in the Duluth-Superior Harbor of western Lake Superior in the two regulated size classes. Phase III testing was completed using Duluth-Superior harbor water and ambient organisms before and after treatment with a ballast water treatment technology (BWT) during three land-based trials. Data from all phases were analyzed for precision, accuracy, and reliability. Quantification/detection limits were calculated for Phase I data.

Phase I testing showed Ballast Eye was able to accurately estimate the number of zooplankton in high and low transparency water, while protist concentrations were not accurately determined. Phase II testing showed Ballast Eye was unable to accurately estimate the number or risk of ambient zooplankton or protists in Duluth-Superior harbor water. Phase III testing showed that Ballast Eye was able to accurately classify risk of ambient zooplankton or protists within uptake and treated discharge samples collected during land-based ballast water treatment technology testing at the Montreal Pier Facility located on the Duluth-Superior harbor.

KEY WORDS

Compliance Monitoring Device, Ballast Water, Fluorescein Diacetate (FDA)

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1 INTRODUCTION

A major focus area of the Lake Superior Research Institute's Great Waters Research Collaborative (LSRI-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. This report details the results of the LSRI-GWRC bench-scale evaluation of the Satake Ballast Eye viable organism analyzer VOA1000K, hereafter Ballast Eye. Developed by Satake Corporation of Hiroshima, Japan, the Ballast Eye method of analysis was added into the guidance on ballast water sampling and analysis of the Ballast Water Management convention as a new indicative analysis method in 2015. Ballast Eye participated in the 2019 Great Lakes Ballast Monitoring Practicums and learned that results obtained in the ≥50 µm size class correlated extremely well with microscopic analysis. Results of Ballast Eye correlated moderately well with microscopic analysis of samples in the ≥10 and <50 µm size class except at the lowest cell density where the device indicated a fail and microscopic analysis indicated a pass (Ram et al., 2019). In 2020, Ballast Eye was granted type approval by the American Bureau of Shipping.

Ballast Eye estimates the number of viable organisms in the ≥10 and <50 µm (nominally protists) and the ≥50 µm (nominally zooplankton) size classes within a sample by pulse counting fluorescence of fluorescein diacetate (FDA) stained organisms in a water sample.

The freshwater verification of Ballast Eye took place from August 2020 to December 2020 at the LSRI of University of Wisconsin-Superior (UWS) in Superior, WI, USA. The test objectives aimed to answer the following research and development questions:

1. Do results from sample analysis by Ballast Eye correlate to detailed microscopic analysis of freshwater laboratory-cultured organisms in the protist and zooplankton size classes?
    a. Does the presence of colonial protists in a sample impact the instrument's accuracy?
2. Does water quality, specifically turbidity, transparency and organic carbon content impact the results of Ballast Eye analysis compared to detailed microscopic analysis of freshwater laboratory-cultured organisms in the protist and zooplankton size classes, both in single-celled and colonial protists?
3. Do results from sample analysis by Ballast Eye correlate to detailed microscopic analysis of freshwater organisms in the protist and zooplankton size classes collected from western Lake Superior?
4. Do results from sample analysis by Ballast Eye correlate to detailed microscopic analysis of freshwater organisms in the protist and zooplankton size classes in uptake, control and treated discharge samples collected during land-based ballast treatment technology testing at Montreal Pier Facility (Superior, WI)?

To better answer these questions quantitatively, Ballast Eye was evaluated using the following verification factors (First et al., 2018 and IMO PPR 7/21, 2019):

- **Accuracy:** Measure of the overall agreement of a measured value (device response) to a known value (accepted method of analysis as described in ETV Protocol (US EPA, 2010)).
- **Precision:** Measure of mutual agreement among individual measurements of the same property.
- **Quantification limits:** Capability of an instrument to discriminate between measurement responses representing different levels of a variable of interest.
- **Reliability:** Ability to maintain integrity or stability of the device and data collection over time.

2 TEST METHODS

2.1 TEST PLAN AND SOPS

A Test/Quality Assurance Plan (TQAP) *Satake Ballast Eye Verification Plan* (LSRI, 2020a), and LSRI standard operating procedures (SOPs) were used to implement all test activities. The TQAP detailed sample and data collection and analysis, sample handling and preservation, data quality objectives, and the quality assurance and quality control (QA and QC) requirements. It was approved by both LSRI-GWRC and Satake Corporation prior to the start of the device verification activities. The SOPs followed throughout testing are described in the Methods section and listed in the References section of this report. These procedures facilitate consistent conformance to technical and quality system requirements and increase data quality.

2.2 BALLAST WATER COMPLIANCE MONITORING DEVICE DESCRIPTION

The Ballast Eye device evaluated by LSRI-GWRC is a portable, commercially available ballast water discharge compliance monitoring device. The Ballast Eye device weighs 8.8 lbs. and measures 9.5” x 12.5” x 6”. Ballast Eye was delivered in a compact backpack containing all equipment needed to quantify organisms in the protist (S-size) and zooplankton (L-size) size classes in a water sample (Figure 1). The device uses a pulse counting FDA method where fluorescence signals of stained organisms are detected and converted to corresponding organism numbers by a conversion formula. The components of Ballast Eye allow the analyst to stain, homogenize, and estimate the number of viable organisms and associated risk within the selected size-class. Results are reported as high risk (more viable organisms than specified in regulation IMO D-2 ballast water performance standard) or low risk (fewer or equal to the viable organisms than specified in regulation D-2 ballast water performance standard) and estimated organisms/mL (for the S-size class) or estimated organisms/m^3 (for the L-size class).

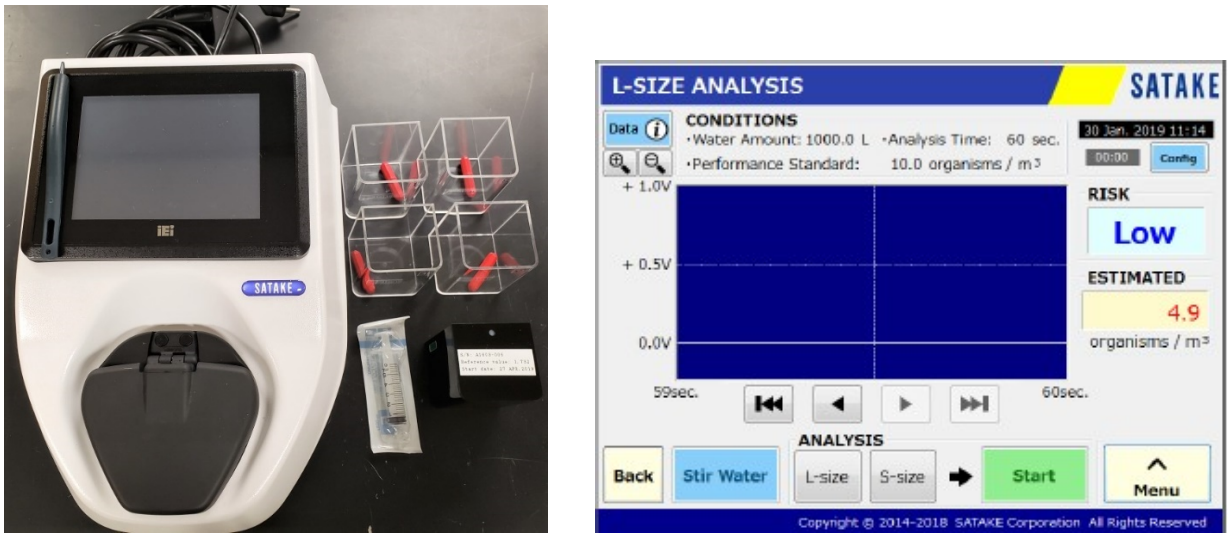

Figure 1. The image on the left is the Ballast Eye device, calibration cell, sample cells, stir bars, and a provided syringe. The image on the right depicts the Ballast Eye touch screen interface following analysis.

The Ballast Eye detection unit consists of LEDs, a stirrer, detector and band pass filters. Following staining, esterase enzymes located in living cells metabolize FDA to the fluorescent compound fluorescein. The LEDs provide blue light of a specific wavelength to excite FDA stained organisms causing them to emit green fluorescence. A photomultiplier tube is used to enhance detector sensitivity while optical filters decrease stray light to reduce noise. A detector measures a pulse signal as stained organisms pass through the monitoring area. Viable organisms produce strong pulse signals while dead organisms show weak/no pulse signal. By employing a suitable threshold, only pulse signals of viable organisms are calculated. The height of the fluorescence pulse produced allows the device to differentiate between organism counts in the two size classes (Ram et al., 2019).

Ballast Eye was operated in a dust-free environment to prevent the air filter from clogging, as recommended by the instruction manual. When the device was not in use it was stored in the provided backpack. During operation, a 4” space around the device was kept free to allow for operation and adequate exhaust. If the device or cell holder became wet with sample water it was wiped with a clean, wet cloth and then dried with a Kimwipe.

The provided reagents were kept frozen below 0^{o}C and thawed at room temperature (20^{o}C to 40^{o}C) prior to analysis. Reagents that were thawed were used within the day they were thawed. Sample water was within 20^{o}C to 30^{o}C range, as required by the user manual. GWRC staff measured the temperature of each sample collected and warmed samples to the acceptable range prior to analysis. When handling samples and reagents, GWRC staff wore protective gloves and safety glasses.

Before beginning analysis, the calibration cell was checked to ensure there were no scratches or dirt. The calibration cell was stored in the device cell chamber when not in use. All sample cells and the holder were cleaned of dirt and fingerprints prior to analysis. When handling the sample cell, GWRC staff held the cell above the scale line.

The instrument was turned on by pushing the power switch on the rear left of the analyzer to the ON position. The calibration cell was placed in the cell holder and the cap was closed. The Start screen appeared on the touch screen and a message indicating that the device was warming up appeared. The warm up took 10 minutes. Next, the calibration began, which took approximately 30 seconds. After calibration, the Main Menu icon on the touch screen was pressed. When the analysis screen appeared, the calibration cell was removed and the cap was closed.

S-sized samples (containing only organisms ≥10 µm and <50 µm) were analyzed by collecting 1 mL of a well-mixed sample with the supplied 5 mL syringe and dripping it into a thawed bottle of the provided Reagent S. Four mL of Milli-Q water was added with the supplied syringe. The bottle was inverted 2-3 times to mix. This sample volume is different than the 5 mL indicated in the device instruction manual, however, the developers indicated the 1 mL sample diluted with 4 mL Milli-Q water should be used and this was noted in the TQAP. While waiting the 15 minutes for the staining process to complete, the "Water Amount" on the Analysis screen was changed to reflect the volume of sample when necessary. Sample information was entered in the "Data" section and then "S-size" was selected at the bottom of the Analysis screen. At the end of 15 minutes, the bottle was inverted 2-3 times again, a stir bar was placed in the S-size sample cell and the stained sample was poured into the sample cell. Milli-Q water was poured into the sample cell to the 100 mL line to dilute the sample. The sample cell was placed in the cell holder and the cap was closed. The analysis was started by pressing "Start" on the touch screen. Results appeared in the Risk Window as "Low" or "High" along with an estimated concentration of organisms/mL.

L-size samples (containing only organisms ≥50 µm) were analyzed by pouring the mixed sample water into the appropriate cell to the 100 mL line, adding a stir bar, and adding one bottle of the provided Reagent L. The sample cell was then placed in the sample holder. The "Stir Water" button on the analysis screen was selected, which allowed the sample to mix for 10 seconds. The sample cell was removed from the analyzer and a 10-minute period was allowed for the staining process to be completed. During the 10 minutes, the "Water Amount" on the Analysis screen was changed to reflect the sample volume when necessary. Sample information was entered in the "Data" section and then "L-size" was selected at the bottom of the Analysis screen. Immediately after the 10-minute staining was complete, the sample cell was returned to the cell holder and the cap was closed. Analysis began after "Start" was selected on the touch screen and the output signal appeared on the Analysis screen for one minute. Results appeared in the Risk Window as "Low" or "High" risk along with an estimated concentration of organisms/m^3.

2.3 BALLAST WATER COMPLIANCE MONITORING DEVICE RECEIPT AND TRAINING

The Ballast Eye device was delivered via DHL and received on June 26th, 2020 by LSRI. A list of the contents shipped by Satake and received by LSRI is shown in Appendix 1. Sample reagents were considered expired due to a long hold time in customs during travel. Satake Corp. sent new reagents which were received before testing began. None of the expired reagents were used during testing. The *Ballast Eye Viable Organism Analyzer Instruction Manual* (Satake Corporation, 2019) was provided, as well as the basic instruction video.

2.4 EXPERIMENTAL DESIGN AND VERIFICATION METHODS

2.4.1 PHASE I

Phase I was conducted using known densities of laboratory-cultured freshwater organisms to compare the Ballast Eye analysis results to traditional laboratory/microscopic analysis. Freshwater organisms used represented two of the regulated size classes including a colonial alga, a single-celled alga (i.e., protists), and two types of zooplankton. Testing was done in two water types (see Section 2.4.1.1) to represent high transparency (laboratory water, LW) and low transparency (amended laboratory water, LW-TMH) conditions to determine whether increased turbidity and total suspended solids affect the ability of Ballast Eye to detect FDA in a water sample. Three replicates for each of the size classes and water types—at concentrations of organisms below, at, and above the D-2 ballast water discharge standard—were prepared and analyzed.

Ballast Eye samples were prepared and analyzed as described in Section 2.2. Before each trial, experimental blank samples of LW or LW-TMH were analyzed in the same manner as the samples containing organisms to ensure proper device operation.

2.4.1.1 EXPERIMENTAL WATER PREPARATION

Two experimental water types were prepared as follows:

Laboratory Water (LW): The LW is municipal water from the City of Superior, Wisconsin (sourced from Lake Superior), that is accessed via hot and cold taps located in the LSRI testing lab which is passed through an activated carbon column in order to remove the majority of the chlorine. The remaining residual chlorine is removed through injection of sodium sulfite, and the resulting total residual chlorine concentration is below the limit of detection (i.e., <7.8 µg/L Cl_2). Typically, LW has a very low concentration of organic carbon and suspended solids, and a very high UV transmittance. Laboratory Water served as the experimental blank for Phase I testing with LW.

Amended Laboratory Water (LW-TMH): Prior to each test, LW-TMH was prepared by amending the necessary volume of LW with 12 mg/L pre-sterilized Fine Test Dust, 12 mg/L pre-sterilized Micromate™, and 20 mg/L humic acid according to LSRI SOP *AT/46 - Preparing Amended Laboratory Water Using Test Dust, Micromate, and Humic Acid Sodium Salt* (LSRI, 2020b). The amended water was mixed thoroughly until no visible clumps of Fine Test Dust or Micromate remained and a homogenous solution was achieved. Typically, LW-TMH is used to achieve challenge conditions similar to those stipulated in the U.S. Environmental Protection Agency (USEPA) Environmental Technology Verification (ETV) Program's *Generic Protocol for the Verification of Ballast Water Treatment Technology*, version 5.1 (USEPA, 2010). Amended Laboratory Water served as the experimental blank for Phase I testing with LW-TMH.

All acceptable water chemistry parameter ranges for LW and LW-TMH can be found in Table 1.

Table 1. Water chemistry parameter acceptable ranges for Phase I water types prepared for GWRC Bench-Scale evaluations.

Parameter	Units	Water Type	Acceptable Range for Initiating Bench-Scale Testing
Total Suspended Solids (TSS)	mg/L	LW	Less than reporting limit
		LW-TMH	11.9 - 30.3
Particulate Organic Matter (POM)	mg/L	LW	Less than reporting limit
		LW-TMH	4.1 - 12.1
Dissolved Organic Carbon (DOC)	mg/L	LW	Less than detection to 2
		LW-TMH	4.4 - 6.8
Non-Purgeable Organic Carbon (NPOC)	mg/L	LW	Less than detection to 2
		LW-TMH	5.1 - 13.1
Percent UV Transmittance at 254 nm (%T)	%	LW	93.0 - 100 (filtered and unfiltered)
		LW-TMH	25.5 - 35.5 (filtered and unfiltered)

2.4.1.2 PROTIST ENUMERATION

Experimental water was prepared as described in Section 2.4.1.1 and was spiked with stock mixtures of *Haematococcus pluvialis* or *Scenedesmus quadricauda* (approximately 10,000 cells/mL) to produce triplicate samples of protists with target live densities of 0, <10, 10-30, and 75-150 cells/mL. Standard laboratory microscopic methods of staining with FDA/CMFDA (5-chloromethylfluorescein diacetate) followed by examination with a compound microscope using epifluorescence, following LSRI SOP *GWRC/30 – Procedure for Protist Sample Analysis* (LSRI, 2020c), were used as a comparison to the rapid analysis results produced by Ballast Eye. Microscopic counts included cells "strictly" ≥10 and <50 µm in minimum dimension or total "allowable" microscopic count. Based on International Maritime Organization (IMO, 2004) and United States Environmental Protection Agency's Environmental Technology Verification (ETV) Program criteria (US EPA, 2010), "strictly" refers to organisms that range from ≥10 and <50 µm in minimum dimension, typically dominated by phytoplanktonic algae but often including some protozoans and suspended benthic algae. However, like many natural freshwater assemblages (Reavie & Cangelosi, 2020), most of the protist organisms (when taken as individual propagules) in the Duluth-Superior Harbor have a minimum cell dimension less than 10 µm, though most have at least one dimension greater than 10 µm. Therefore, total "allowable" microscopic counts included all cells in entities (i.e., single cells, colonies, filaments, etc.) that are ≥10 µm in any visible dimension. Multiple or single cell entities that were <10 µm in all visible dimensions were not counted. Large-celled *H. pluvialis* was enumerated using the "strictly" method while *S. quadricauda* was enumerated using the "allowable" method as the individual cells within each colony were <10 µm in minimum dimension. Each test concentration was verified to be within the target ranges by utilizing a microscopic blind count.

2.4.1.3 ZOOPLANKTON ENUMERATION

Experimental water was prepared as described in Section 2.4.1.1 and target numbers of *Daphnia magna* or *Eucyclops* spp. were added to the water to prepare triplicate samples at the following densities: 0, 5, 10, 15, and 50 organisms/m^3 for the zooplankton size class comparison. *Daphnia magna* were ≤48 hours old and collected the day of analysis. *Eucyclops* spp. were mixed age and collected the day prior to analysis. All organisms were counted by one analyst and verified by a second analyst before being transfer to the sample water for analysis.

2.4.2 PHASE II

Phase II testing was conducted using whole water collected at the Montreal Pier Facility located on the Duluth-Superior Harbor of western Lake Superior. The water was analyzed for live organisms in the protist and zooplankton size classes with Ballast Eye and by traditional microscopic techniques. Ballast Eye samples were prepared and analyzed as described in Section 2.2. Sample measurement values (as risk and estimated concentrations) obtained from Ballast Eye were recorded. Experimental blanks were prepared by filtering harbor water through a Whatman 934-AH filter (1.5-µm particle retention) to remove all plankton and the majority of suspended solids. The blank samples were processed and analyzed in the same manner as the samples containing organisms.

2.4.2.1 PROTIST ENUMERATION

For the assessment of the protist size class, two 20 L carboys of water were collected from the Duluth-Superior Harbor at the Montreal Pier Facility by filtering whole water samples through a 35-µm mesh to remove organisms ≥50 µm. An initial count of the organisms in the size class was determined following the method in LSRI SOP *GWRC/30* (LSRI, 2020c). Then, 10-15 L samples targeting the following live density ranges were prepared using harbor water filtered through a Whatman 934-AH filter (1.5-µm particle retention) to dilute the original protist sample: 0, 5-20, 30-50, and 51-150 live cells/mL "strictly" ≥10 µm and <50 µm as defined by the ETV protocol. Triplicate subsamples were prepared and analyzed with Ballast Eye as described in Section 2.2. Total live density was determined on the whole water samples following LSRI SOP *GWRC/30* (LSRI, 2020c). Protists were enumerated using the "strictly" and total "allowable" methods to determine the ability of Ballast Eye to count all biologically significant protists as described in Section 2.4.1.2. Each test concentration was verified to be within the designated range by a microscopic blind count. A detailed taxonomic analysis of the community composition of this size class was completed on preserved samples following LSRI SOP *GWRC/30* (LSRI, 2020c).

2.4.2.2 ZOOPLANKTON ENUMERATION

For the assessment of the zooplankton size class, Duluth-Superior Harbor water collected from the Montreal Pier Facility was concentrated through a 35-µm plankton net and collected into three 20-L carboys. All samples were diluted using harbor water filtered through a Whatman 934-AH filter (1.5-µm particle retention) to prepare four 10-15 L samples targeting the following live density ranges: 0, 5-20, 30-50, and 51-150 live organisms/m^3. The samples were mixed well prior to collecting a single subsample for standard microscopic analysis following LSRI SOP *GWRC/25-Procedure for Zooplankton*

Analysis (LSRI, 2020e) and triplicate subsamples for analysis with Ballast Eye following Section 2.2. Total live density and a general taxonomic categorization of the zooplankton community was determined (LSRI, 2020e).

2.4.3 PHASE III

Phase III testing was conducted at the Montreal Pier Test Facility during land-based evaluation of an ozone-based ballast water treatment technology (currently in development). The technology delivered ozone to ballast water through the production of ozone-impregnated nanobubbles. Samples were collected during three trials of the treatment technology evaluation and were analyzed using Ballast Eye and traditional microscopic counts. Each trial included analysis of untreated uptake water and treated discharge water. Samples were filtered through 35-µm filter to separate the organisms into their size classes.

2.4.3.1 PROTIST ENUMERATION

Protists were enumerated using the "strictly" and "total allowable" methods for the assessment of the protist size class, as described in section 2.4.2.1, following GWRC's standard operating procedures for microscopic analysis of organisms in the protist (LSRI, 2020c) and using Ballast Eye.

2.4.3.2 ZOOPLANKTON ENUMERATION

For the assessment of the zooplankton size class, uptake and treated discharge samples were analyzed for total live densities and general taxonomic categorization as described in Section 2.4.2.2, following GWRC's standard operating procedures for microscopic analysis of organisms in zooplankton (LSRI, 2020e) and using Ballast Eye.

2.4.4 STATISTICAL DATA ANALYSIS

Ballast Eye results and the data from microscopic counts were entered into Microsoft Excel for data analysis. The data was graphed using Excel by plotting microscopic counts on the x-axis versus the Ballast Eye results on the y-axis. Graphs were fitted with linear trendlines and R^2 values were calculated to measure closeness of fit to the data. The coefficient of variance (CV) was calculated for triplicate Ballast Eye results. The CV shows variability in a sample in relation to the sample mean. CV is a measure of precision and is calculated as the standard deviation of a data set divided by the mean and then multiplied by 100. A logistical regression analysis was performed on Phase I data to determine the probability Ballast Eye will detect an exceedance of the D-2 discharge standard based on sample concentration (First, 2018). The binary regression needed for the probability charts was performed using IBM SPSS Statistics, v.27 and plotted graphically using Excel.

The lower detection/quantification limits (LOD) of Ballast Eye were determined using the laboratory data generated during Phase I, as outlined in the proposed protocol submitted by IOC-UNESCO, ICES, and ISO (PPR 7/21, 07 October 2019). During Phase I, three replicate blank samples and three replicate samples below the discharge standard were analyzed. A signal to noise ratio was used to determine the effect of the noise on the relative error of a measurement.

Device reliability was determined for the combined dataset by calculating the percent completeness and the percentage of operation time. Percent completeness is calculated by comparing the number of datapoints that were planned during the evaluation to the number of datapoints that were recovered. The percentage of operation time is the total number of times that the device operated as designed without interruption (i.e., non-scheduled maintenance, non-scheduled calibration, or repair).

2.4.5 WATER QUALITY

Water quality measurements were made throughout the duration of Ballast Eye verification and involved determination of total suspended solids (TSS), percent transmittance at 254 nm (%T), particulate organic matter (POM), non-purgeable organic carbon (NPOC) and dissolved organic carbon (DOC), dissolved oxygen (DO), temperature, specific conductivity, and pH.

TSS analysis was conducted according to LSRI SOP *SA/66 – Analyzing Total Suspended Solids (TSS), Particulate Organic Matter (POM), and Mineral Matter (MM)* (LSRI, 2017b). Briefly, accurately measured sample volumes (± 1%) were vacuum filtered through pre-ashed, pre-washed, dried, and pre-weighed glass fiber filters (i.e., Whatman 934-AH). After the sample volume was filtered, the filter 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 on the filter 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). Mineral matter concentrations are determined by subtracting the POM concentration from the TSS concentration.

Analysis of %T was conducted according to LSRI SOP *SA/69 – Determining Percent Transmittance (%T) of Light in Water at 254 nm* (LSRI, 2018). For analysis of the filtered aliquot, an appropriate volume of sample was filtered through a glass fiber filter (i.e., Whatman 934-AH). A Perkin Elmer Lambda 35 UV-Vis spectrophotometer was used to measure %T of the unfiltered (%TU) and filtered (%TF) 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 measured in a pre-rinsed sample cuvette with a 1 cm path length.

Analysis of NPOC/DOC was conducted according to LSRI SOP *SA/47 – Measuring Organic Carbon in Aqueous Samples* (LSRI, 2006) on a Shimadzu Model TOC-L Total Organic Carbon Analyzer. Before analysis, the samples were acidified to a pH <2 with concentrated hydrochloric acid (HCl; ~0.2% v/v). Samples were then purged with high purity air to remove the inorganic carbon and purgeable organic carbon and injected into the analyzer. Samples amended with Micromate (i.e., LW-TMH) were sonicated for a minimum of 30 minutes with a stir bar and stirred continuously on a stir plate while being manually injected into the instrument. An organic carbon stock solution which had a concentration of 1,000 mg/L carbon was used to prepare a working standard of 50 mg/L C which was also acidified to a pH <2 with concentrated HCl. The standard was used to generate a calibration curve which was then used to determine the concentration of organic carbon in the samples.

During Phase I, measurement of DO was conducted using a YSI ProSolo Dissolved Oxygen meter and dissolved oxygen/temperature probe, which was calibrated daily following LSRI SOP *GLM/34-Calibrating, Maintaining, and Using the YSI ProSolo Handheld Meter and Optical Dissolved Oxygen/Temperature Probe* (LSRI, 2019). Temperature was measured using a Fisher digital thermometer that was calibrated quarterly following LSRI SOP *GLM/17 – Procedure for Thermometer Verification and Calibration* (LSRI, 1995). Specific conductivity was measured using an Oakton Model CON 110 or an Oakton Model CON 150 Conductivity/TDS/Temperature Meter that is calibrated on a monthly basis following LSRI SOP *GLM/26 - Procedures for Calibrating and Using the Oakton CON 110 Conductivity/TDS/Temperature Meter* (LSRI, 2011) or *GLM/28 - Procedures for Calibrating and Using the Oakton CON 150 Conductivity/TDS/Temperature Meter* (LSRI, 2021a), respectively. Accuracy was also verified daily prior to sample analysis using a Daily Check Standard (0.0100M potassium chloride).

Measurement of pH was conducted using an Orion 3 Star meter and Orion 8157BNUMD pH probe. Both instruments were calibrated daily following LSRI SOP *GLM/05 – Procedure for Calibration and Operation of pH Meters Utilizing Automatic Temperature Compensation (ATC)* (LSRI, 1992). A check buffer of pH 8.00 was also measured after calibration to verify the accuracy of the calibration. During Phase II and II testing, DO, temperature, pH and conductivity, and turbidity were measured using a YSI EXO2 sonde LSRI SOP FS/41–*Deployment and Storage of YSI EXO2 Multiparameter Sondes* (LSRI, 2021b) which was calibrated prior to each test cycle following LSRI SOP FS/39 – *Calibration of YSI EXO2 Mulitparameter Water Quality Sondes* (LSRI, 2017c).

2.5 DEVIATIONS

During the course of conducting testing with Ballast Eye, there were deviations that occurred from the TQAP. Those deviations are listed in Table 2 along with corrective actions that were taken as a response to the deviation and perceived impact of the deviation on the test results**.**

Table 2. Deviations from the Test/Quality Assurance Project Plan (TQAP) encountered during Ballast Eye device verification.

Test	Description and Root Cause of Deviation or Quality Control Failure	Description of Corrective Action(s)	Description of Impact on the Project/Test	Data Qualified? (Y/N)
Phase II Zooplankton 31 August 2020	The Test Plan called for NPOC and DOC to be analyzed on the stock water, however, only DOC was analyzed. **Root cause**: Parameters were overlooked in the Test Plan.	Better review of Test Plan. Summarize data as soon as possible so it's more apparent if parameters have been overlooked.	Minimal, all other water quality parameters were measured.	N
Phase II Zooplankton 1 September 2020	The Test Plan called for NPOC and DOC to be analyzed on the stock water, however, only DOC was analyzed. **Root cause**: Parameters were overlooked in the Test Plan.	Better review of Test Plan. Summarize data ASAP so it's more apparent if parameters have been overlooked.	Minimal, all other water quality parameters were measured.	N

Test	Description and Root Cause of Deviation or Quality Control Failure	Description of Corrective Action(s)	Description of Impact on the Project/Test	Data Qualified? (Y/N)
Phase II Zooplankton 1 September 2020	The microscopically determined concentrations of zooplankton were 29, 60, and 170 live organism/m^3 in the 5-20, 30-50, and 51-150 live organism/m^3 size ranges, respectively. **Root Cause:** When working with natural assemblages of organisms, there is inherent variability.	No corrective action taken. The microscopically determined concentration of zooplankton was close to the target range and when working with samples from the environment, there will be some variability when making dilutions.	Minimal effect, the concentrations were slightly out of range and each concentration was distinct from the other. With the Ballast Eye system, the maximal organism concentration that is able to be enumerated by the device is 150 live organism/m^3.	Y
Phase I LW *D. magna* 2 September 2020	The Test Plan called for NPOC and DOC to be analyzed on the stock water, however, only DOC was analyzed. %T was not analyzed within 24 hours of collection. **Root cause**: Parameter was overlooked in the Test Plan.	Better review of Test Plan. Summarize data as soon as possible so it's more apparent if parameters have been overlooked.	Minimal as LW was the stock water. As seen historically with LW at LSRI, it is expected that the organic carbon concentration in the NPOC sample would've been very similar to that in the DOC sample.	N
Phase I LW-TMH *D. magna* 10 September 2020	%T was not analyzed within 24 hours of collection. **Root cause**: Parameter was overlooked in the Test Plan.	Better review of Test Plan and Standard Operating Procedures.	Minimal, Filtered and Unfiltered %T values were within the acceptable range for LW-TMH.	N
Phase II Protist 2 October 2020	%T was not analyzed within 24 hours of collection. **Root cause**: Parameter was overlooked in the Test Plan.	Better review of Test Plan and Standard Operating Procedures.	Minimal, Filtered and Unfiltered %T values were very similar to those of Harbor Water that was collected and analyzed on the same day.	N
Phase III Zooplankton and Protist 8 October 2020, 9 October 2020, 15 October 2020, 22 October 2020	For the Phase III testing, only uptake samples and treatment discharge samples were analyzed with all three compliance monitoring devices. The Test Plan stated that control discharge water would also be analyzed. **Root cause:** The treatment system being used had a short treatment time and no retention time, so the time between the uptake sample and the control discharge sample would have likely led to no difference in the uptake and control discharge samples counts.	None needed as the decision to eliminate the control discharge samples does not impact the analysis of the other samples. The goal of the test was to look at samples that would be above and below the discharge standard and this was accomplished with the uptake and treatment discharge samples.	Minimal, the goal of the Test Plan was achieved.	N

Test	Description and Root Cause of Deviation or Quality Control Failure	Description of Corrective Action(s)	Description of Impact on the Project/Test	Data Qualified? (Y/N)
Phase I LW-TMH HP 19 November 2020	The Unfiltered %T sample from the LW-TMH stock solution produced a result of 23.6 %T which is outside the acceptable range for unfiltered LWTMH (25.5-35.5%). **Root cause:** Using a new method to prepare LW-TMH without updating the acceptable ranges for parameters using the data we've accrued since the new method was implemented.	The method for preparing LW-TMH has changed in the last year and SOP AT/46 was created. However, we have not re-evaluated our data since adopting the new LW-TMH preparation method. New data should be added to historical data to update the acceptable range for parameters measured.	Minimal, all other water quality parameters were within the target range for test initiation.	N
Phase I LW-TMH SQ 11 December 2020	The Unfiltered T% sample from the LW-TMH stock solution produced a result of 25.1 %T which is outside the acceptable range for unfiltered LWTMH (25.5–35.5%). **Root cause:** Using a new method to prepare LW-TMH without updating the acceptable ranges for parameters using the data we've accrued since the new method was implemented.	The method for preparing LW-TMH has changed in the last year and SOP AT/46 was created. However, we have not re-evaluated our data since adopting the new LW-TMH preparation method. New data should be added to historical data to update the acceptable range for parameters measured. Data is in the process of being re-evaluated.	Minimal, all other water quality parameters were within the target range for test initiation.	N

3 BALLAST EYE OPERATIONAL PERFORMANCE

During the testing period, no operational performance issues occurred that affected testing. The seam on one sample cell separated while cleaning and drying the sample cell and was no longer used for sample analysis. Four sample cells were provided, two were dedicated S-size cells and two were dedicated L-size cells. Stir bars were not used exclusively between organism sizes, however when one stir bar was used there was a distinct sound while stirring. To verify the stirrer was working properly, GWRC staff followed the Maintenance and Inspection section of the *Ballast Eye Viable Organism Analyzer Instruction Manual,* the stirrer was checked and found to be operational.

4 RESULTS

Findings from the Ballast Eye Phase I, Phase II, and Phase III tests are presented in the following subsections. In the results tables with estimated organism concentration values reported, the values have been highlighted and labeled to align with what Ballast Eye analysis indicates regarding compliance with IMO's *International Convention for the Control and Management of Ships' Ballast Water and*

Sediments Regulation D-2 Ballast Water Performance Standard (2004). In all results tables that follow, green highlighting and L superscript indicates low risk (within D-2 regulations) and red highlighting and H superscript indicates high risk (above D-2 regulations). Regulation D-2 specifies that ships conducting ballast water management shall discharge:

- <10 viable organisms/mL ≥10 µm and <50 µm in minimum dimension
- <10 viable organisms/m^3 ≥50 µm in minimum dimension

4.1 PHASE I

4.1.1 HAEMATOCOCCUS PLUVIALIS

A subsample of *H. pluvialis* (Figure 2) was measured and cells were found to have an average size of 20.95 µm (17.8-22.4 µm cell size range). Results from Ballast Eye and microscopic counts (LSRI, 2020c) from LW and LW-TMH samples containing *H. pluvialis* are shown in Table 3. Target concentrations of the *H. pluvialis* in both water types were 0 (experimental blank), <10, 10-30, and 75-150 cells/mL. Samples were measured in triplicate to acquire an average live cell density and the coefficient of variation (CV) for each sample concentration. In LW, the final microscopic cell count averages for each range were 0, 5.6, 23.4, and 103 cells/mL and the average estimated concentration as detected by Ballast Eye were 3.4, 9.0, 26.2, and 213.1 cells/mL. In LW-TMH samples, the final microscopic cell count averages were 0, 3.9, 19.9, and 89.0 cells/mL and the average estimated concentration as detected by Ballast Eye were 7.4, 8.3, 24.9, and 153.9 cells/mL. In both tests, Ballast Eye estimated greater than 0 cells/mL in the blank samples (containing no organisms). Correction for the measurements made in the blank samples would make the sample measurements more accurate. Samples with organism concentrations just above the D-2 regulation were the most accurate. The high organism concentration sample range was determined with regard to Ballast Eye's upper limit of detection, 150 live cells/mL. Although the device accurately assessed the samples as high risk, the numerical result was not an accurate assessment of the organism concentration. The CV in LW samples ranged from 23.6 to 56.8 and generally increased with higher concentrations of cells, with the exception being the highest CV was in the blank sample. The CV in the LW-TMH samples ranged from 4.4 to 74.9.

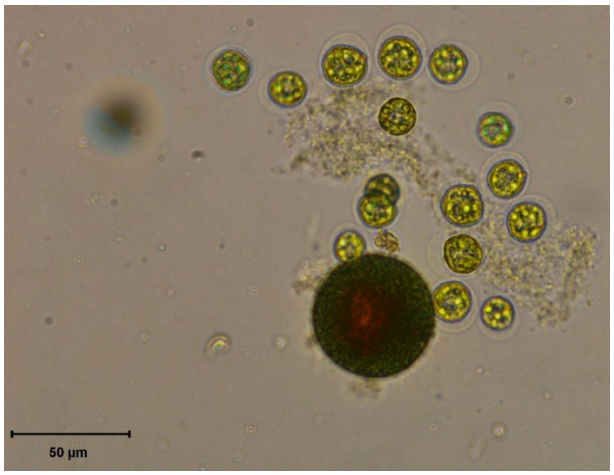

Figure 2. *H. pluvialis* cells.

Table 3. Phase I "strictly" microscopic cell counts and Ballast Eye estimated cell count results for *H. pluvialis* in LW and LW-TMH.

Sample Description	LW Samples			LW-TMH Samples		
	Ballast Eye Estimated (cells/mL)	Mean (CV)	Cells "Strictly" ≥10 and <50 µm (cells/mL)	Ballast Eye Estimated (cells/mL)	Mean (CV)	Cells "Strictly" ≥10 and <50 µm (cells/mL)
0 cells/mL (Blank)	5.1^{L}	3.4 (56.8)	0.0	10.6^{H}	7.4 (74.9)	0.0
	3.8^{L}			10.6^{H}		
	1.3^{L}			1.0^{L}		
<10 cells/mL	10.9^{H}	9.0 (23.6)	5.6	11.2^{H}	8.3 (34.9)	3.9
	6.7^{L}			5.4^{L}		
	9.3^{L}			8.3^{L}		
10-30 cells/mL	24.0^{H}	26.2 (30.9)	23.4	23.7^{H}	24.9 (4.4)	19.9
	35.2^{H}			25.9^{H}		
	19.5^{H}			25.0^{H}		
75-150 cells/mL	121.0^{H}*	213.1 (38.1)	103	207.4^{H}	153.9 (32.9)	89.0
	274.6^{H}			147.8^{H}		
	243.8^{H}*			106.6^{H}		

*Samples were analyzed in duplicate due to unexpected result, duplicate result is reported.

Figure 3 shows the "strictly" cells/mL count (LSRI, 2020c) versus the Ballast Eye estimated live cell density for each water type. A linear regression was performed and the corresponding equation and R^2 value for each water type is shown. The R^2 value for both LW and LW-TMH was >0.86, indicating a relatively high level of accuracy for the device. However, at the lowest and highest cell counts, accuracy was lower in both water types than it was at the two intermediate concentrations.

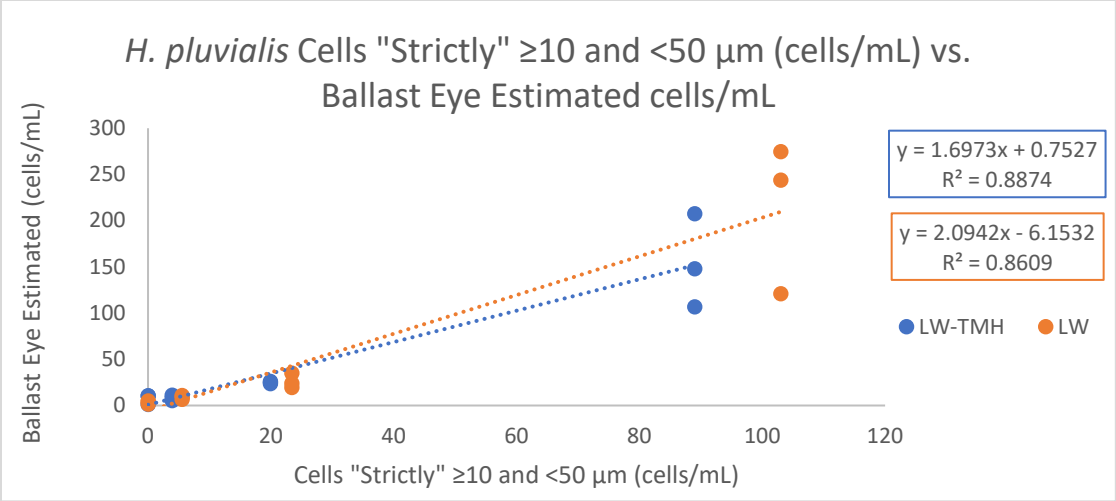

Figure 3. Phase I *H. pluvialis* "strictly" microscopic cell counts vs. Ballast Eye estimated cell counts in LW and LW-TMH.

4.1.2 *SCENEDESMUS QUADRICAUDA*

The cells that comprise the colonial *S. quadricauda* are outside of the ≥10 and <50 µm size class, therefore, this species is not a measurement target of Ballast Eye. Section 4.1.2 is for reference only and addresses Great Lakes-specific objectives of this project (see §1, Objective 1.a.).

S. quadricauda (Figure 4) were used during Phase One of this project because this protist species is representative of Great Lakes biology. Within the Great Lakes, and freshwater generally, many protist taxa are colonial with the entity (i.e., colonial form) being larger than 10 µm in minimum dimension but comprised of cells that are less than 10 µm in minimum dimension (Kim et al., 2016; Reavie & Cangelosi, 2020). A subsample of *S. quadricauda* was assessed (Figure 4) and measured (Table 4) by GWRC staff. Colonies had an average length of 22.7 µm (14-32 µm), and length including the spines was an average of 40.1 µm (23-51 µm; Figure 4). Cells were found to have an average width of 7.7 µm (6-9 µm cell range) and average length of 18.0 µm (14-27 µm cell range). The average colony size is 2-4 cells, although colonies observed during the evaluation of the device ranged from 1-8 cells.

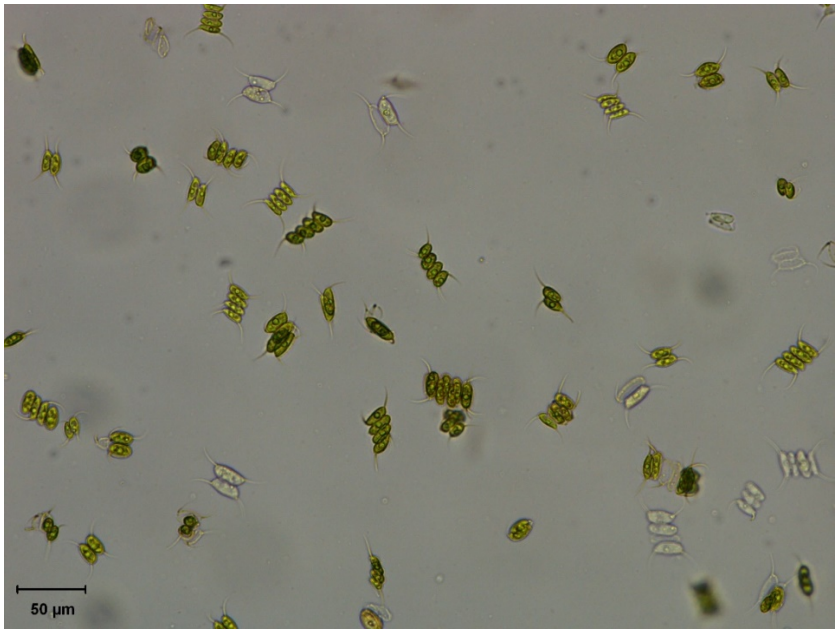

Figure 4. *S. quadricauda* cells and colonies.

When initial microscopic counts were being performed, the vital stain FDA/CMFDA did not fluoresce green as expected, rather the cells fluoresced red—as they naturally do when exposed to fluorescence — due to the response of chlorophyll under blue wavelengths. This indicated that the usual green signal from FDA/CMFDA was being overwhelmed or replaced by a strong chlorophyll signal and/or stains were not functioning for *S. quadricauda.* A heat-kill procedure was performed with the *S. quadricauda* to ensure no false negatives were being reported. A sample of *S.quadricauda* was placed in a beaker of freshly boiled water for 10 minutes, the sample was allowed to cool and stained using the FDA/CMFDA protocol described in GWRC/30. No glowing cells were observed in the heat-killed sample, indicating no biasing of the results with false negatives. Given that the results from the heat-killed sample verified red fluorescence provided an alternative vital signal, and literature-based evidence supported the use of red fluorescence of the chlorophyll signal as a measure of vitality in protists (Pouneva, 1997), the testing proceeded. For *S. quadricauda*, the FDA/CMFDA staining procedure in GWRC/30 was not effective, which was an unanticipated outcome. However, the deviation from GWRC/30 that allowed for use of chlorophyll fluorescence was a justified alternative for this species and was supported by internal experiments and literature.

Microscopic (LSRI, 2020c) and Ballast Eye results from LW and LW-TMH samples containing *S. quadricauda* are shown in Table 5. Target concentrations of the *S. quadricauda* in both water types were 0 (experimental blank), <10, 10-30, and 75-150 cells/mL. Triplicate samples were analyzed to acquire an average live cell density and coefficient of variation for each sample concentration. All blank samples analyzed resulted in microscopic counts of 0 cells/mL in both LW and LW-TMH. In LW, the final microscopic cell count averages for each range were 0, 6.8, 14.2, and 89.4 cells/mL and the average estimated counts as detected by Ballast Eye were 1.9, 2.7, 4.0, and 1.9 cells/mL. In the LW-TMH, the final microscopic cell count averages were 0, 2.6, 16.2, and 91.2 cells/mL and the average estimated

counts as detected by Ballast Eye were 3.3, 1.4, 2.7, and 3.5 cells/mL. The CV in LW samples ranged from 30.1 to 68.4 and generally did not vary depending on concentrations of cells. The CV in the LW-TMH samples ranged from 44.7 to 109.4. Ballast Eye grossly underestimated the densities of *S. quadricauda* in all concentrations and both water types. It is not known whether this effect is the result of the device not being able to distinguish individual cells within colonies, or due to the FDA stain not indicating vitality in the organisms, similar to the FDA/CMFDA.

Table 4. Measurements of *S. quadricauda* cells and colonies with and without the spines.

Cell Length (µm)	Cell Width (µm)	Colony Length without Spines (µm)	Colony Length with Spines (µm)	Colony Width without Spines (µm)	Colony Width with Spines (µm)	Number of Cells in the Colony
17	6	25	47	17	34	4
27	9	32	50	27	37	4 (8, cells possibly dividing)
14	7	27	49	14	26	4
18	7	28	51	18	35	4
16	7	14	23	16	34	2
17	9	16	32	17	36	2
17	9	17	29	17	33	2

Table 5. Phase I total "allowable" microscopic cell counts and Ballast Eye estimated cell count results for *S. quadricauda* in LW and LW-TMH.

	LW Samples			LW-TMH Samples		
Sample Description	Ballast Eye Estimated (cells/mL)	Mean (CV)	Total "Allowable" Microscopic Count** (cells/mL)	Ballast Eye Estimated (cells/mL)	Mean (CV)	Total "Allowable" Microscopic Count**(cells/mL)
0 cells/mL (Blank)	2.6^{L}	1.9 (43.1)	0.0	7.4^{L}*	3.3 (109.4)	0.0
	2.2^{L}*			1.9^{L}		
	1.0^{L}			0.6^{L}		
<10 cells/mL	2.6^{L}	2.7 (30.1)	6.8	1.3^{L}	1.4 (103.8)	2.6
	1.9^{L}			0.0^{L}		
	3.5^{L}			2.9^{L}		
10-30 cells/mL	5.4^{L}	4.0 (40.9)	14.2	1.3^{L}	2.7 (44.7)	16.2
	4.5^{L}			3.5^{L}		
	2.2^{L}			3.2^{L}		
75-150 cells/mL	1.9^{L}	1.9 (68.4)	89.4	5.1^{L}	3.5 (56.2)	91.2
	0.6^{L}			4.2^{L}		
	3.2^{L}			1.3^{L}		

*Samples were analyzed in duplicate due to unexpected results, duplicate result is reported.

** The "strictly" microscopic count would have been 0 cells/mL since all of the individual cells measured were outside of the "strictly" size class.

Figure 5 shows the microscopic cells/mL count versus the Ballast Eye estimated cells/mL for each water type. A linear regression was performed and the corresponding equation and R^2 value for each water type is shown. The R^2 value for both LW and LW-TMH were <0.06 indicating a low level of accuracy for the device regardless of cell concentrations within the sample. This may be due to the fact that the cells that comprise the colonial *S. quadricauda* are outside the ≥10 and <50 µm size class, therefore this taxa is not a measurement target of Ballast Eye. In addition, Ballast Eye does not detect chlorophyll a fluorescence, only fluorescein fluorescence (from FDA) which was not effective for this species.

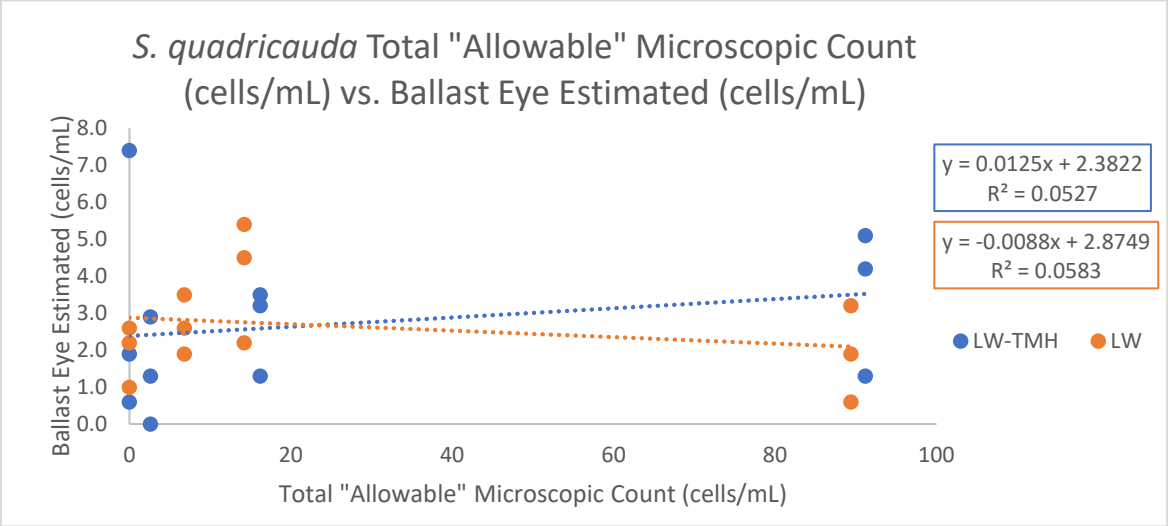

Figure 5. Phase I microscopic *S. quadricauda* total "allowable" cell counts vs. Ballast Eye estimated cell counts in LW and LW-TMH.

4.1.3 DAPHNIA MAGNA

Results from Ballast Eye analysis of LW and LW-TMH samples containing *D. magna* are shown in Table 6. The size of *D. magna* on average was 1045 µm ± 109 µm. No CV values are provided for the organism counts of the zooplankton samples because all of the samples were counted by one GWRC staff member and the count was verified by a second GWRC staff member, which resulted in all samples having the same density (i.e., the target density) of organisms. The number of organisms added to each sample were 0 (experimental blank), 5, 10, 15 and 50 organisms/m^3. Triplicate samples were prepared and analyzed to determine an average organisms/m^3 and CV for each sample concentration. In LW samples, the average estimated concentrations as measured by Ballast Eye were 1.4, 6.6, 14.4, 19.5, and 50.3 organisms/m^3. In LW-TMH samples, the average estimated concentrations as measured by Ballast Eye were 0.1, 4.5, 9.7, 17.1, and 47.6 organisms/m^3. The estimated risk determined by Ballast Eye correlated with the D-2 ballast water discharge standard in all samples except the organism concentration 10 organisms/m^3 (equal to the D-2 regulation). The CV in LW samples ranged from 1.5 to 120.8 and decreased with higher organism concentrations. The CV in the LW-TMH samples ranged from 4.1 to 86.6

and generally increased with lower organism concentrations, with the exception of the high concentration samples having the same CV as the mid-range concentration.

Table 6. Phase I visual organism counts and Ballast Eye estimated count results using *D. magna* in LW and LW-TMH.

Sample Description	LW Samples			LW-TMH Samples		
	Ballast Eye Estimated (organisms/m³)	Mean (CV)	Visual Organism Count (organism/m³)	Ballast Eye Estimated (organisms/m³)	Mean (CV)	Visual Organism Count (organism/m³)
0 organisms/m³ (Blank)	0.0[L]	1.4 (120.8)	0	0.2[L]	0.1 (86.6)	0
	3.2[L]			0.0[L]		
	0.9[L]			0.2[L]		
5 organisms/m³	8.3[L]	6.6 (23.0)	5	3.2[L]	4.5 (38.4)	5
	5.3[L]			6.5[L]		
	6.3[L]			3.9[L]		
10 organisms/m³	11.6[H]	14.4 (17.5)	10	9.9[L]	9.7 (8.9)	10
	15.1[H]			8.8[L]		
	16.5[H]			10.5[H]		
15 organisms/m³	18.5[H]	19.5 (6.5)	15	16.7[H]	17.1 (4.1)	15
	19.0[H]			17.9[H]		
	20.9[H]			16.7[H]		
50 organisms/m³	51.2[H]	50.3 (1.5)	50	51.0[H]	47.6 (8.7)	50
	50.0[H]			43.0[H]		
	49.8[H]			48.8[H]		

Figure 6 shows the organism count versus the Ballast Eye estimated organisms/m³ for each water type. A linear regression was performed and the corresponding equation and R^2 value for each water type is shown. The R^2 value for both LW and LW-TMH was 0.9857, indicating a high level of accuracy for the device. However, at the lowest organism concentration the accuracy was lower in both water types.

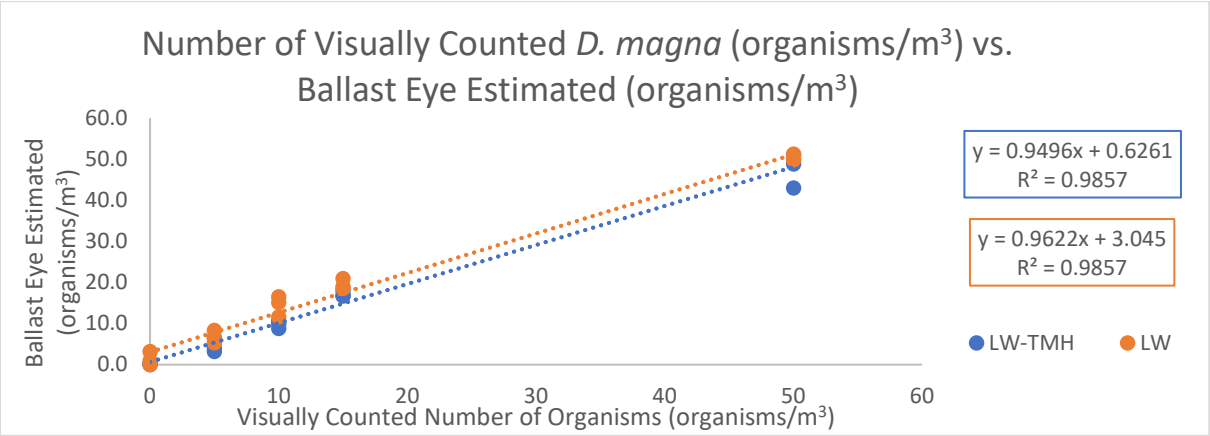

Figure 6. Phase I visual *D. magna* counts vs. Ballast Eye estimated organism counts in LW and LW-TMH.

4.1.4 *EUCYCLOPS* SPP.

Results from Ballast Eye analysis of LW and LW-TMH samples containing mixed age *Eucyclops* spp. are shown in Table 7. The length of *Eucyclops* ranged from 0.75-1.02 mm with a mean of 0.93 mm; and the width ranged from 0.24-0.34 mm with a mean of 0.30 mm. No CV values are provided for visual organism counts of *Eucyclops* spp. because all samples were counted by one GWRC staff member and the count was verified by a second GWRC staff member, which resulted in all samples having the same density (i.e., the target density) of organisms. The number of organisms added to the samples were 0, 5, 10, 15, and 50 organisms/m^3. Triplicate samples were analyzed to acquire an average organisms/m^3 and CV for each sample concentration. In LW samples, the average estimated counts as measured by Ballast Eye were 0.0, 2.7, 7.0, 12.8, and 38.4 organisms/m^3. In LW-TMH samples, the average estimated counts as measured by Ballast Eye were 0.0, 2.8, 6.2, 13.7, and 32.2 organisms/m^3. The estimated risk determined by Ballast Eye correlated with the D-2 ballast water discharge standard in all samples except the organism concentration 10 organisms/m^3 (equal to the D-2 regulation). The CV in LW samples ranged from 5.8 to 31.2 and generally decreased with higher organism concentrations. The CV in the LW-TMH samples ranged from 2.6 to 73.2 and generally decreased with higher organism concentrations.

Table 7. Phase I visual organism counts and Ballast Eye estimated count results using *Eucyclops* spp. in LW and LW-TMH.

Sample Description	LW Samples			LW-TMH Samples		
	Ballast Eye Estimated (organisms/m^3)	Mean (CV)	Visual Organism Count (organisms/m^3)	Ballast Eye Estimated (organisms/m^3)	Mean (CV)	Visual Organism Count (organisms/m^3)
0 organisms/m^3 (Blank)	0.0^L	0.0 (NC)	0	0.0^L	0.0 (NC)	0
	0.0^L			0.0^L		
	0.0^L			0.0^L		
5 organisms/m^3	3.6^L	2.7 (31.2)	5	4.4^L	2.8 (73.2)	5
	2.0^L			0.5^L		
	2.4^L			3.4^L		
10 organisms/m^3	7.1^L	7.0 (22.2)	10	5.6^L	6.2 (11.4)	10
	8.5^L			6.1^L		
	5.4^L			7.0^L		
15 organisms/m^3	12.6^H	12.8 (9.9)	15	14.1^H	13.7 (2.6)	15
	11.6^H			13.6^H		
	14.1^H			13.4^H		
50 organisms/m^3	35.9^H	38.4 (5.8)	50	30.4^H	32.2 (5.3)	50
	39.4^H			32.5^H		
	40.0^H			33.8^H		

NC = Not Calculable

Figure 7 shows the organism count verses the Ballast Eye estimated organisms/m^3 for each water type. A linear regression was performed and the corresponding equation and R^2 value for each water type is shown. The R^2 value for both LW and LW-TMH was >0.97, indicating a high level of accuracy for the device. However, it consistently underestimated densities in both water types.

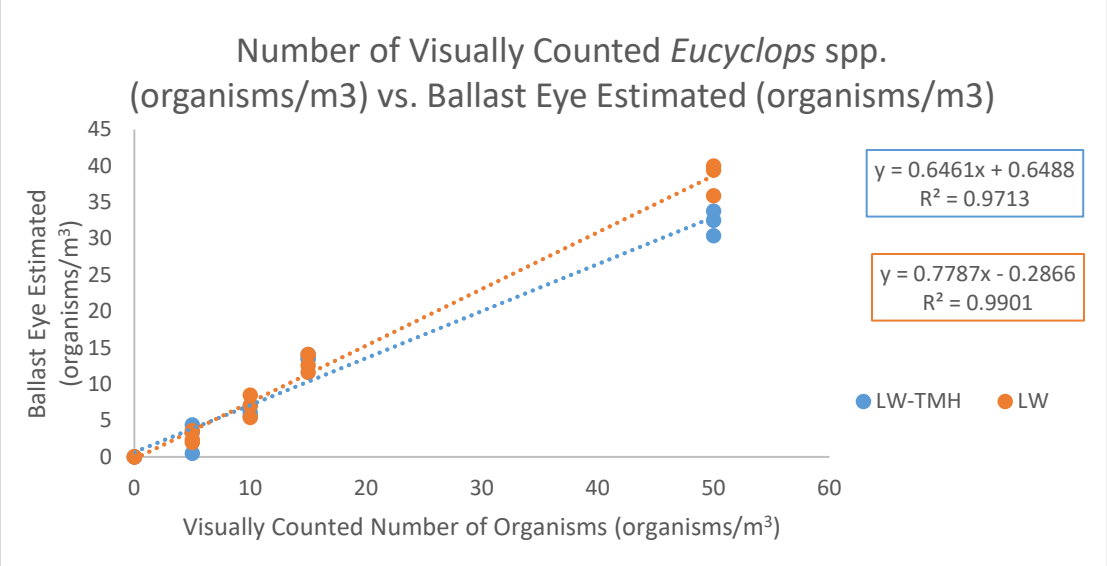

Figure 7. Phase I visual *Eucyclops* spp. counts vs. Ballast Eye estimated organism counts in LW and LW-TMH.

4.1.5 PHASE I WATER QUALITY AND CHEMISTRY

Water quality measurements taken during Phase I testing with Ballast Eye are shown in Table 8. Samples of stock water solution were collected prior to addition of organisms; the measurements were within LSRI historical ranges for each of the experimental water types.

Table 8. Water quality measurements made in LW and LW-TMH collected during Phase I testing with Ballast Eye.

Organism(s)	Water Type	Temperature (°C)	pH	Dissolved Oxygen (mg/L)	Conductivity (µS/cm)
H. pluvialis	LW	23.2	7.11	5.0	137.3
H. pluvialis	LW-TMH	24.8	7.04	4.7	141.8
S. quadricauda	LW	25.6	7.00	4.1	140.7
S. quadricauda	LW-TMH	24.8	7.03	5.0	165.8
D. magna	LW	24.8	7.57	7.3	150.5
D. magna	LW-TMH	23.8	7.84	8.1	160.6
Eucyclops spp.	LW	23.9	7.25	7.3	152.1
Eucyclops spp.	LW-TMH	22.9	7.79	8.7	158.1

Water chemistry measurements taken during Phase I testing with Ballast Eye are shown in Table 9. Samples of stock water solution were collected prior to addition of organisms. All LW and LW-TMH samples were within acceptable ranges (Table 1) for all established parameters. However, some LW-TMH samples were outside the acceptable ranges for % Transmittance Unfiltered, which was due to a change in LW-TMH preparation method (see Deviations from November 19, 2020, December 4, 2020, and December 12, 2020 in Table 2).

Table 9. Water chemistry parameter measurements made in LW and LW-TMH collected during Phase I Ballast Eye testing.

Organism(s)	Water Type	TSS (mg/L)	%T Filtered	%T Unfiltered	NPOC (mg/L)	DOC (mg/L)	POM (mg/L)	MM (mg/L)
H. pluvialis	LW	<1.25	98.4	98.3	0.9[J]	1.2[J]	<1.25	<1.25
H. pluvialis	LW-TMH	20.3	25.8	23.6*	9.6	6.7	8.2	12.1
S. quadricauda	LW	<2.50	98.5	98.9	1.0[J]	1.0[J]	<2.50	<2.50
S. quadricauda	LW-TMH	21.5	27.7	25.1*	9.3	6.4	8.5	13.0
D. magna	LW	<1.25	98.1	97.7	NM	0.93[J]	<1.25	<1.25
D. magna	LW-TMH	22.0	29.8	27.2	8.8	5.8	9.2	12.8
Eucyclops spp.	LW	<1.25	98.4	98.4	1.1[J]	1.0[J]	<1.25	<1.25
Eucyclops spp.	LW-TMH	21.5	29.5	27.5	9.3	6.2	8.8	12.7

*Values are outside of the acceptable range.
[J] Indicates that the data point is between the Limit of Detection (LOD) and Limit of Quantification (LOQ).
NM= Not Measured

4.2 PHASE II

Results from Phase II testing of the protist and zooplankton size classes in Duluth-Superior harbor water using Ballast Eye compared to traditional microscopic enumeration methods are discussed below.

4.2.1 PROTISTS

Phase II testing for protists occurred on two separate occasions. The first trial was repeated due to variable cell counts caused by a high number of filamentous protist forms and the failure to achieve target densities in the samples (Appendix 2). The trial was later repeated successfully, and the results of the total live density analysis of organisms in the protist size class and the results of Ballast Eye analysis of the Duluth-Superior Harbor water samples are shown in Table 10. The ambient harbor density of protists on the day of the verification test was 524.0 cells/mL. Appendix 3 shows the detailed taxonomic assessment and community composition counts for the Duluth-Superior Harbor water used for the protist sample dilutions. The target density ranges were 0 (experimental blank), 5-20, 30-50, and 51-150 cells/mL. The "strictly" ≥10 µm and <50 µm microscopic counts were within the acceptable range with concentrations of 0.0, 17.8, 31.2, and 90.6 cells/mL. The total "allowable" live cell density resulting from microscopic counts were: 0.0, 25.4, 62.6, and 174.8 cells/mL; approximately 50-70% of the protist community did not fit the strict definition of the size class according to the ETV Protocol. The experimental blanks (and dilution water) were verified through microscopic analysis using FDA/CMFDA to have no live organism density (0 cells/mL). Samples were measured in triplicate to acquire an average (cells/mL) and CV for each sample concentration. The average estimated counts as detected by Ballast Eye were 8.9, 13.3, 4.7, and 10.3 cells/mL. The estimated risk detected by Ballast Eye did not correlate with the D-2 ballast water discharge standard for any sample concentration. The CV ranged from 31.7 to 95.9.

Table 10. Phase II ambient protist microscopic counts and Ballast Eye estimated cell count in Duluth-Superior Harbor water.

Sample Description	Ballast Eye Estimated (cells/mL)	Mean (CV)	Microscopic Counts	
			Cells "Strictly" ≥10 and <50 µm (cells/mL)	Total "Allowable" Microscopic Count (cells/mL)
0 cells/mL (Blank)	18.6[H]	8.9 (95.9)	0.0	0.0
	5.1[L]			
	2.9[L]			
5-20 cells/mL	17.3[H]	13.3 (47.1)	17.8	25.4
	6.1[L]			
	16.6[H]			
30-50 cells/mL	8.6[L]	4.7 (93.1)	31.2	62.6
	0.0[L]			
	5.4[L]			
51-150 cells/mL	7.4[L]	10.3 (31.7)	90.6	174.8
	13.8[H]			
	9.6[L]			

Figure 8 shows the results of the "strictly" microscopic cells/mL versus the Ballast Eye estimated cells/mL. A linear regression was performed and the corresponding equation and R^2 value are shown. The R^2 value is <0.001 indicating a low level of accuracy for the device.

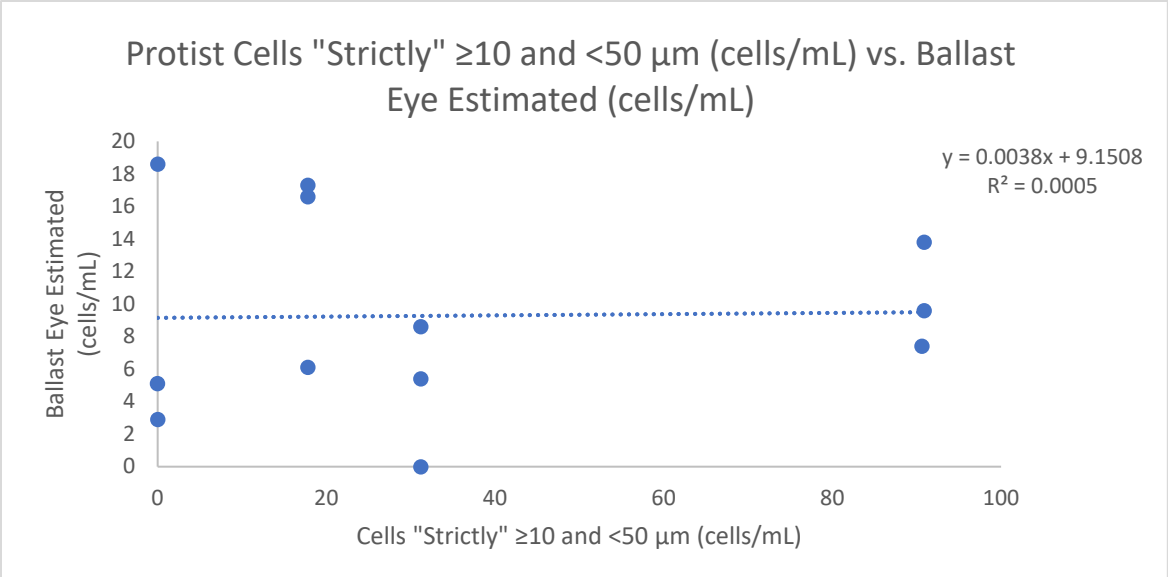

Figure 8. Phase II "strictly" microscopic counts of ambient protists vs. Ballast Eye estimated counts in Duluth-Superior Harbor water.

4.2.2 ZOOPLANKTON

The results of the live density analysis of organisms in the zooplankton size class and the results of Ballast Eye analysis of the Duluth-Superior Harbor water samples are shown in Table 11. The ambient

harbor density of live zooplankton on the day of the verification test was 2.0E+04 organisms/m^3. Appendix 4 shows the detailed taxonomic assessment of the organisms in this size class. The target concentrations were 0 (experimental blank), 5-20, 30-50, and 51-150 organisms/m^3. Sample concentrations were 0.0, 29.0, 60.0, and 170.0 organisms/m^3. Sample concentrations did not meet the target concentrations due to the inherent variability when working with natural harbor assemblages (see Deviation from Phase II Zooplankton, 1 September 2020 in Table 2), however the sample concentrations were still distinctly different from one another. The experimental blanks (and dilution water) were verified through microscopic analysis to have no live organism density (0 organisms/m^3). Samples were measured in triplicate to acquire an average (organisms/m^3) and CV for each sample concentration. The estimated risk generally did not correlate with the D-2 regulations. Ballast Eye did not detect any high-risk samples during the test set. The CV ranged from 52.0 to 81.7, however, Ballast Eye did not detect any organisms in two low concentration samples and a CV was not calculable. The data from Phase I testing, with *D.magna* (i.e. cladoceran) and *Eucyclops* spp. (i.e. copepod), demonstrates the ability of FDA to stain these types of organisms. It is unclear whether the FDA stain is as effective when used on a natural assemblage of zooplankton where over 80% of the total population were rotifers.

Table 11. Phase II visual ambient zooplankton counts and Ballast Eye estimated counts in Duluth-Superior Harbor water.

Sample Description	Ballast Eye Estimated (organisms/m^3)	Mean (CV)	Visual Organism Count (organisms/m^3)
0 organisms/m^3 (Blank)	0.0[L]	0.0 (NC)	0.0
	0.0[L]		
	0.0[L]		
5-20 organisms/m^3	0.0[L]	0.0 (NC)	29.0
	0.0[L]		
	0.0[L]		
30-50 organisms/m^3	3.1[L]	1.6 (81.7)	60.0
	0.7[L]		
	1.0[L]		
51-150 organisms/m^3	2.7[L]	4.5 (52.0)	170.0
	3.6[L]		
	7.1[L]		

NC = Not Calculable

Figure 9 shows the microscopic organisms/m^3 versus the Ballast Eye estimated organisms/m^3. A linear regression was performed and the corresponding equation and R^2 value are shown. The R^2 value was >0.70, indicating a low level of accuracy for the device. However, the device did not indicate any high-risk samples.

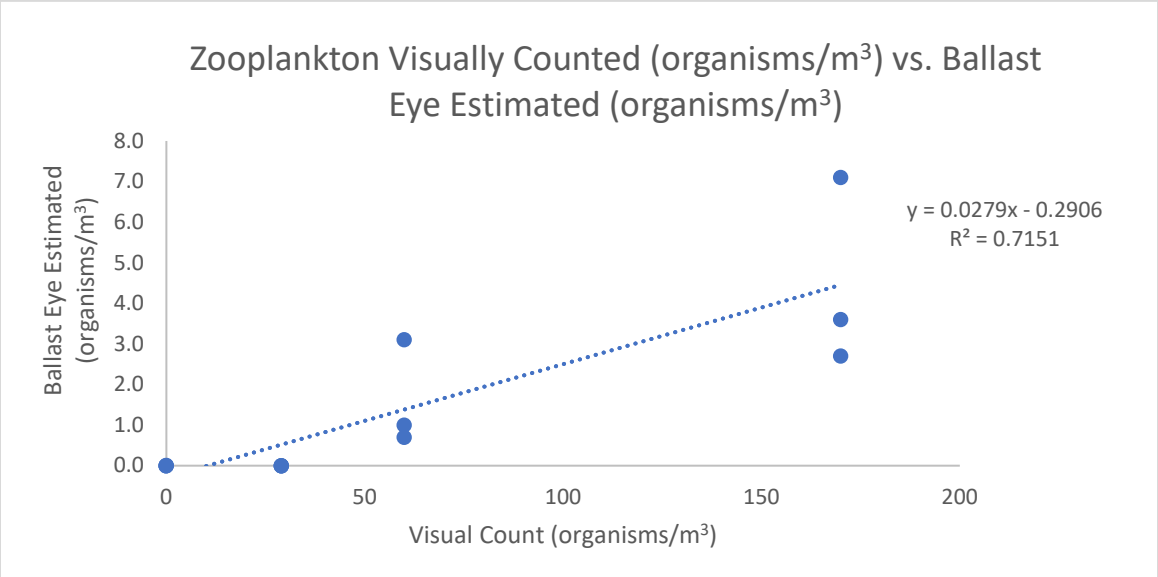

Figure 9. Phase II visual counts of ambient zooplankton vs. Ballast Eye estimated counts in Duluth-Superior Harbor water.

4.2.3 WATER CHEMISTRY AND QUALITY

Water chemistry and water quality analysis was conducted during the Phase II testing on Duluth-Superior Harbor water in order to provide the developer with data to show how naturally occurring turbidity and total suspended solids may impact Ballast Eye test results. The water quality values obtained during the Phase II testing are shown in Table 12 and water chemistry values are shown in Table 13 and are within historical ranges measured in the Duluth-Superior Harbor.

Table 12. Water quality values measured during Phase II of Ballast Eye testing.

Sample Description	Temperature (°C)	pH	Dissolved Oxygen (mg/L)	Conductivity (µS/cm)	Turbidity (FNU)
Protist Dilution Water	13.2	7.72	9.9	177.5	10.6
Protist Source Water	14.5	7.93	9.6	177.8	10.0
Zooplankton Dilution Water	20.1	7.61	8.1	193.3	15.0
Zooplankton Source Water	19.3	7.67	8.2	177.8	12.1

Table 13. Water chemistry parameter measurements made during Phase II testing with Ballast Eye.

Organism(s)	Date of Analysis	Water Type	TSS (mg/L)	%T Filtered	%T Unfiltered	NPOC (mg/L)	DOC (mg/L)	POM (mg/L)	MM (mg/L)
Protist	10/2/20	Harbor Water Prior to Filtration (Dilution Water)	7.5	48.7	44.9	7.8	7.3	1.4	6.1
Protist	10/5/20	Harbor Water (Source Water)	4.8	47.1	42.8	8.3	7.6	1.0	3.8
Zooplankton	8/31/20	Harbor Water Prior to Filtration (Dilution Water)	9.9	36.0	31.0	NM	8.9	1.6	8.3
Zooplankton	9/1/20	Harbor Water (Source Water)	8.1	37.1	32.5	NM	8.6	1.5	6.6

NM= Not Measured

4.3 PHASE III

Phase III testing occurred on three individual testing events. The BWT technology utilized produced ozone impregnated nanobubbles which are highly oxidative and eliminate microscopic organisms in the treated water. Ozone was analyzed during Phase III testing as well as after treatment was completed to ensure ozone levels returned to non-detectable concentrations before beginning either analysis method. Analysis was conducted on uptake and treatment discharge samples. The BWT technology had a short treatment time, therefore control discharge sample analysis was omitted from the Ballast Eye assessment (see Deviations from Phase III Zooplankton and Protist in Table 2). Results from Phase III testing of the protist and zooplankton size classes in Duluth-Superior harbor water using Ballast Eye compared to traditional microscopic analysis are discussed below.

4.3.1 PROTISTS

Results of the live density analysis of organisms in the protist size class and the results of Ballast Eye analysis of the Duluth-Superior Harbor uptake and treated discharge samples are shown in Table 14. The estimated risk, on average, did correlate with the D-2 ballast water discharge standard. The uptake organism concentration was outside of the Ballast Eye's upper limit of detection, 150 live cells/mL. Although the device accurately assessed the samples as high risk, the numerical result is not a good

indication of how the device is analyzing the sample. A linear regression analysis was not performed due to the small data set.

Table 14. Phase III microscopic counts of ambient protist and Ballast Eye estimated cell counts for untreated uptake and treated discharge samples.

Sample Description	Ballast Eye Estimated (cells/mL)	Microscopic Counts	
		Cells "Strictly" ≥10 and <50 µm (cells/mL)	Total "Allowable" Microscopic Count (cells/mL)
Phase III-1 Uptake	17.0[H]	300.4	665.7
Phase III-2 Uptake	22.1[H]	252.6	660.8
Phase III-3 Uptake	26.9[H]	152.9	354.9
Phase III-1 Treatment	0.0[L]	0.4	34.4
Phase III-2 Treatment	2.2[L]	0.0	0.0
Phase III-3 Treatment	0.3[L]	0.0	0.0

4.3.2 ZOOPLANKTON

Results of the live density analysis of organisms in the zooplankton size class and the results of Ballast Eye analysis of the Duluth-Superior Harbor uptake and treated discharge samples are shown in Table 15. Appendix 5 and Appendix 6 shows the taxonomic characterization of the organisms in the zooplankton size class during Phase III testing. The estimated risk, on average, did not correlate with the D-2 ballast water discharge standard. The uptake organism concentration was far outside of the Ballast Eye's upper limit of detection, 150 live organisms/m^3. Although the device accurately assessed the samples as high risk, the numerical result is not a good indication of how the device is analyzing the sample. The Ballast Eye developer has recommended that uptake water samples should not be concentrated as the concentrated organism densities will likely be above the range of Ballast Eye. This description should be clarified in the Ballast Eye User Manual. A linear regression analysis was not performed due to the small data set.

Table 15. Phase III visual counts of ambient zooplankton and Ballast Eye estimated counts for untreated uptake and treated discharge samples.

Sample Description	Ballast Eye Estimated (organisms/m^3)	Microscopic Counts (live organisms/m^3)
Phase III-1 Uptake	463.4[H]	1.50E+05
Phase III-2 Uptake	405.6[H]	1.2E+05
Phase III-3 Uptake	310.3[H]	4.4E+04
Phase III-1 Treatment	1.7[L]	13.2
Phase III-2 Treatment	0.7[L]	17.3
Phase III-3 Treatment	6.5[L]	3.9

4.3.3 WATER CHEMISTRY AND QUALITY

Water quality (Table 16) and water chemistry (Table 17) analysis was conducted during the Phase III testing prior to running the BWT technology. Three replicate uptake samples of Duluth-Superior harbor water were analyzed in conjunction with each test.

Table 16. Water quality measurements from uptake samples during Phase III testing.

Sample Description	Temperature (°C)	pH	Dissolved Oxygen (mg/L)	Conductivity (µS/cm)	Turbidity (FNU)
Phase III-1	11.5	6.89	10.3	167.4	42.9
Phase III-2	10.1	7.53	10.3	189.8	49.5
Phase III-3	6.3	7.02	11.7	206.5	16.2

Table 17. Water chemistry parameter measurements from uptake samples during Phase III testing.

Sample Description	TSS (mg/L)	%T Filtered	%T Unfiltered	NPOC (mg/L)	DOC (mg/L)	POM (mg/L)	MM (mg/L)
Phase III-1	7.9	51.1	47.2	7.1	6.8	1.4	6.8
Phase III-2	11.0	48.0	41.1	7.6	7.3	1.7	9.2
Phase III-3	4.5	39.2	35.5	9.0	8.5	NC	NC

NC=Not Calculable

5 STATISTICAL ANALYSIS

The data collected during Phase I was analyzed to determine the probability of Ballast Eye detecting exceedances of the D-2 ballast water discharge standard based upon cell concentration within a water sample (First et al., 2018). Logistical regression analysis (IBM SPSS Statistics, v.27) was used to determine the probability of correctly predicting an exceedance along a range of concentrations. A logistics regression was not performed on data from Phase II or III. The device accuracy during Phase II testing did not allow for a logistics regression to be done on either data set. Phase III results were not within the range of the Ballast Eye limit of detection, therefore the generated probability graph would be misleading.

Figure 10 shows the probability of Ballast Eye detecting an exceedance based on a sample's concentration using results from Phase I. Each organism was analyzed separately to determine its probability. Probability is expressed on a scale of 0 to 1; 0 means the device will not detect an exceedance, 1 means the device will detect an exceedance (i.e., at 0.5 the device has a 50% chance of detecting an exceedance). When analyzing samples containing *D. magna*, Ballast Eye has a 66.7% chance of correctly detecting an exceedance when there are 10 organisms/m^3. When analyzing samples containing *Eucyclops* spp., Ballast Eye has a 97.5% chance of correctly detecting an exceedance when there are 13 organisms/m^3, but only a 3.4% chance of detecting an exceedance when there are 12 organisms/m^3. When analyzing samples containing *H. pluvialis*, Ballast Eye has a 72.7% chance of correctly detecting an exceedance when there are 10 cells/mL, but a 23.5% chance of detecting an

exceedance when there are 0 cells/mL. Although not included in Figure 10, when analyzing samples containing *S. quadricauda*, Ballast Eye will not reliably detect an exceedance at any sample concentration (cells/mL). This colonial protist was included to meet Great Lakes-specific objectives of this project, and is not a target of Ballast Eye.

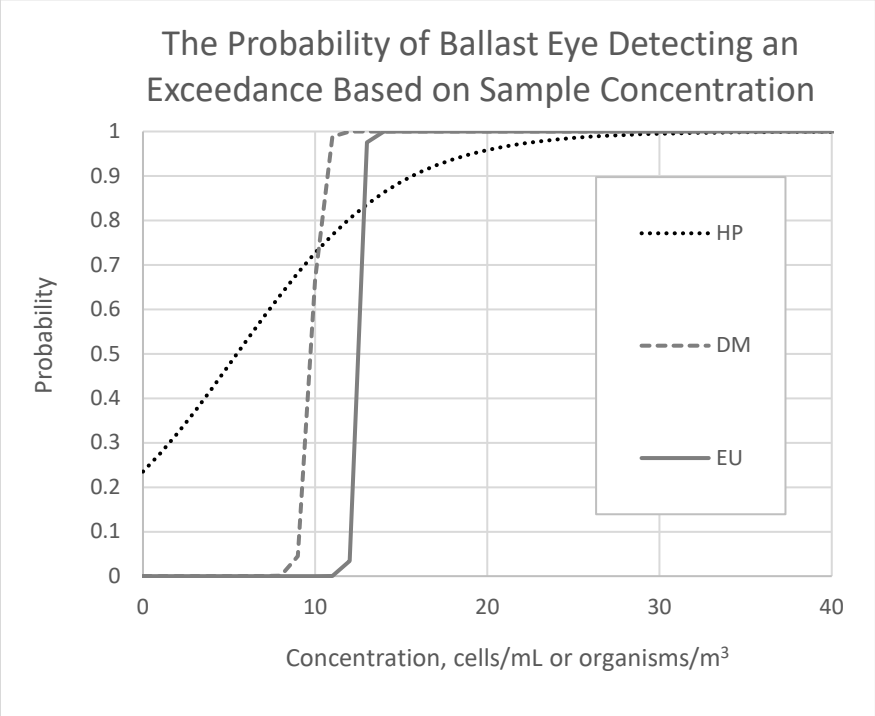

Figure 10. Probability of Ballast Eye detecting exceedances based on the cell/organism concentration of a sample using Phase I data. The legend indicates which line corresponds to the individual cell/organism type: *H. pluvialis* (HP), *D. magna* (DM), and *Eucyclops* spp. (EU).

A lower limit of detection (LOD) was calculated for Ballast Eye using data from Phase I with respect to organism size. The LOD was calculated by multiplying the standard deviation of all replicates and corresponding Student t-value, as stated in *Analytical Detection Limit Guidance & Laboratory Guide for Determining Method Detection Limits* (Wisconsin Department of Natural Resources Laboratory Certification Program, 1996). A signal to noise ratio (S/N) was used to determine if random error effected the significance of the calculated LOD, this was calculated by dividing the mean by the standard deviation of the combined data set. The S/N can range from 1 to 10 but is typically within 3 to 5. A S/N below 2.5 indicates that the random error of the measurements is too high. Zooplankton organism concentrations used for analysis were: 0 (blank), 5, and 10 organisms/m^3. There were three replicates of four samples for each concentration. The blank and five concentration data sets had a S/N of <2.5 and the LOD was greater than the concentration of the samples. Therefore, the LOD needed to be calculated at a higher concentration. The 10 organism/m^3 data set had a S/N of 2.6 and a LOD of 9.8 organisms/m^3 for L-size organisms. Protist data did not yield a LOD for S-size organisms. Analysis was performed on the combined and individual organism data sets, but the S/N was either too low or the LOD was too high.

During all three phases of testing, device reliability was determined by calculating the percent completeness and the percentage of operation time of the combined dataset. Any duplicated results—denoted with an (*) in the report tables—are considered unplanned data points. There were 144 planned data points for the device validation testing. There were 148 data points recovered. Therefore, the percent completeness for the combined dataset is 103%. There were no non-scheduled device maintenance/calibration or repairs performed that halted testing. Therefore, the percentage of operation time is 100%.

6 DEVICE USABILITY AND COMPATIBILITY WITH GREAT LAKES CONDITIONS

Ballast Eye must be operated in a dust-free environment to prevent the air filter from clogging, as recommended by the instruction manual. In practice, this may be difficult for analyses conducted onboard a Great Lakes bulk cargo carrier. During cargo loading and ballast discharge, the cargo itself can create a lot of particulates in the area around the dock. Future shipboard testing onboard a Great Lakes vessel could be very useful to determine how these environmental factors impact analysis and operation.

One concern noted by analysts was the requirement that samples be between 20 and 30˚C when stained and analyzed. For much of the Great Lakes shipping season (i.e., typically March through December), average surface water temperature is less than 10°C and may only reach an average of ≥20°C during a few months (Figure 11). These seasonal variations in Great Lakes water temperature would require that samples be warmed after collection to meet this requirement. Rapidly warming the samples could potentially cause mortality in protists and zooplankton leading to the device producing artificially low organism concentrations.

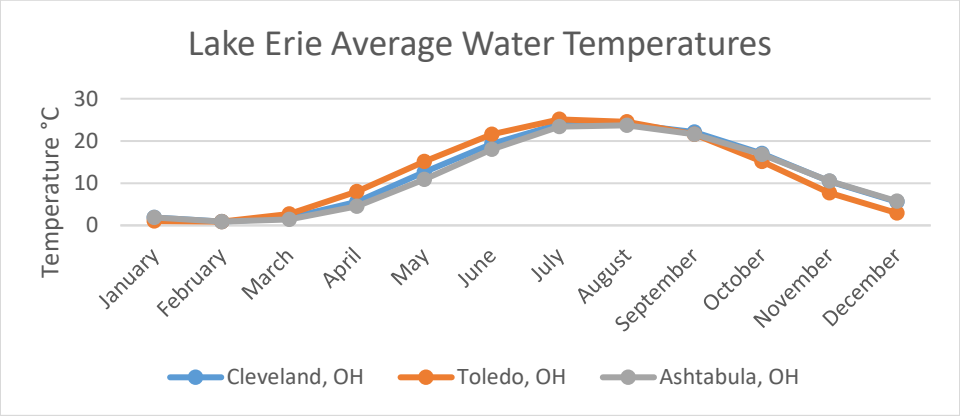

Figure 11. Ten-year average water temperatures measured at Lake Erie ports.

7 QUALITY ASSURANCE/QUALITY CONTROL – DATA QUALITY OBJECTIVES

7.1 PROTIST TESTING

No quality control (QC) counts were conducted during the *H. pluvialis* or *S. quadricauda* testing due to COVID-19 restrictions.

7.2 ZOOPLANKTON TESTING

During testing with *D. magna* and *Eucyclops* spp., data quality was ensured by having a second analyst conduct counts on a minimum of 10% of the samples. This minimum was exceeded in both of the tests, with 100% of the samples having QC counts conducted (Table 18). The Relative Percent Difference was 0% for all samples counted in duplicate.

Table 18. Relative Percent Difference of samples counted for *D. magna* and *Eucyclops* spp. samples during Ballast Eye Phase I testing.

Test Species - Water Type	Percent of Samples with QC Counts	Data Quality Objective	Relative Percent Difference Between Counts
D. magna - LW	100%	RPD ≤10%	0%
D. magna - LW-TMH	100%		0%
Eucyclops spp. - LW	100%		0%
Eucyclops spp. - LW-TMH	100%		0%

7.3 WATER CHEMISTRY AND WATER QUALITY

The data quality objectives (DQO) for water chemistry analyses conducted during the evaluation of Ballast Eye are summarized in Table 19. Data quality objectives were met for all measures of precision, bias, and accuracy. The percent completeness exceeded the required percentage for all parameters except NPOC.

Table 19. Data Quality Objectives, criteria, and performance measurement results from water chemistry analyses conducted during Ballast Eye evaluation.

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: 13.0%	%TF: 0.2 ± 0.2%
			%TU: 13.0%	%TU: 0.0 ± 0.0%
			NPOC: 16.7%	%NPOC: 8.0 ± 7.3%
			DOC: 13.0%	%DOC: 8.6 ± 5.8%
			POM: 13.0%	POM: 0.0 ± 0.0%
			TSS: 13.0%	TSS: 0.0 ± 0.0%
Bias, Filter Blanks	%T method blanks were prepared by filtering Milli-Q water samples (one per analysis date).	>98% average %T	Number of %T Method Blanks Analyzed: 16	Method Blanks (%T): 99.8 ± 0.6%
	TSS/POM method blanks were prepared by	<1.25 mg/L average TSS/POM	Number of TSS Method Blanks Analyzed: 16	Method Blanks (TSS): <1.25 ± 0

Data Quality Indicator	Evaluation Process/Performance Measurement	Data Quality Objective	Performance Measurement Result	
	filtering Milli-Q water samples from a 1-L sample bottle (one per analysis date) and then drying, weighing, washing and weighing the filter.			
			Number of POM Method Blanks Analyzed: 16	Method Blanks (POM): <1.25 ± 0
Bias, Filter Blanks	NPOC blanks were prepared by acidifying a volume of Milli-Q water to 0.2% with concentrated hydrochloric acid.	<0.48 mg/L average NPOC	Number of NPOC Blanks Analyzed: 34	Blanks (NPOC): <0.48 ± 0
	DOC method blanks were prepared by filtering Milli-Q water samples (one per analysis date).	<1.6 mg/L average DOC	Number of DOC Method Blanks Analyzed: 16	Method Blanks (DOC): <1.6 ± 0
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: 24.4%	NPOC/DOC Spike Recovery: 98.7 ± 3.0
	Performance was measured by average percent difference (%D) between all measured and nominal reference standard values.	One per analysis day <20% average %D	Percentage of Analysis Days Containing a Reference Standard	Reference Standard Percent Difference
			TSS: 100%	TSS: 1.8 ± 0.9%
			POM: 100%	POM: 2.5 ± 1.2%
			NPOC: 100%	NPOC: 9.1 ± 1.3%
		A least one per 10 samples <10% average %D	Percentage (vs. total samples) Check Standards	NPOC 10 mg/L Standard % Difference
			NPOC/DOC: 78%	3.6 ± 2.3%
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		>90%C	TSS: 100%	

Data Quality Indicator	Evaluation Process/Performance Measurement	Data Quality Objective	Performance Measurement Result
	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).		%T Filtered: 100%
			%T Unfiltered: 100%
			**NPOC: 78%
			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 – Qualitative.	TSS/POM RL: 1.25 mg/L based on filtering 800 mL of sample
			NPOC/DOC LOD: 0.48 mg/L
			NPOC/DOC LOQ: 1.6 mg/L
			Determined 2/7/2020

**Completeness NPOC: NPOC samples were not collected for Phase I testing in LW with *D. magna* or Phase II testing with zooplankton (Harbor water monitoring days) due to overlap of sample collection and parameter was overlooked in the Test Plan.

8 CONCLUSIONS AND DISCUSSION

The LSRI-GWRC freshwater verification of Ballast Eye met the stated objectives as outlined in the TQAP (LSRI, 2020a) and described below. The reported deviations that occurred during testing do not impact LSRI-GWRC's ability to draw conclusions on Ballast Eye performance during this verification. Ballast Eye was operated in accordance with the developer's instructions and operated reliably during all reported tests.

Objective 1 and 1a: Do results from sample analysis by Ballast Eye correlate to detailed microscopic analysis of freshwater laboratory-cultured organisms in the protist and the zooplankton size classes? Does the presence of colonial protists in a sample impact the instrument's accuracy?

The sample results from Ballast Eye during Phase I testing with *H. pluvialis* and *S. quadricauda* in both LW and LW-TMH (Table 3 and Table 5) indicated the presence of cells in the protist size class in the experimental blank samples. Therefore, Ballast Eye did not accurately estimate low concentrations of S-sized organisms within the water samples (in either high transparency or low transparency water). If Ballast Eye were able to correct for these estimated readings, it could more accurately estimate live cell densities in sample concentrations near the D-2 ballast water discharge standard.

Ballast Eye did correctly classify risk in LW and LW-TMH testing with *H. pluvialis* samples containing concentrations of this single-celled protist above the D-2 ballast water discharge standard. The results of Phase I testing using *S. quadricauda* appear to indicate that Ballast Eye is unable to accurately detect moderate to high concentrations of colonial S-sized organisms within a sample, if the cells that comprise those colonies are <10 µm in minimum dimension. This is indicated by a low R^2 value of <0.06 in samples of both water types, and the low risk classification assigned to all *S. quadricauda* samples by the device. This species was selected for Phase I of this project because it represents many colonial protists within the Great Lakes, wherein, the entity (i.e., colony) is >10 µm but the cells that comprise the entity are <10 µm. Given that the cells of *S. quadricauda* are <10 µm, this species is not a target of Ballast Eye, and was included in this project to meet Great Lakes-specific objectives. It should be noted that these results could also be attributed to the failure of FDA to effectively stain or overcome the strong chlorophyll fluorescence of live *S. quadricauda* cells. Microscopic counts were conducted using chlorophyll fluorescence because the usual green signal from FDA/CMFDA was being overwhelmed or replaced by a strong chlorophyll signal and/or stains were not functioning for *S. quadricauda.* It is possible this is an uncommon problem associated with this species, but tests on additional species are recommended to confirm performance of the device with respect to colonial forms of protists.

The analysis of the S-sized organisms was conducted differently than recommended in the *Ballast Eye Viable Organism Analyzer Instruction Manual* (Satake Corporation, 2019). However, it was conducted according to the direction of Satake Corporation, and as indicated in the TQAP (2020a). The difference is, the manual states a 5 mL sample is stained, but the TQAP reflected the request of the Satake Corporation that a 1 mL sample be diluted with 4 mL of Milli-Q water and then stained. It would be beneficial to assess whether analyzing samples as indicated in the manual results in more accurate and precise results.

When analyzing samples containing *D. magna* in both LW and LW-TMH, Ballast Eye indicated low density presence of organisms in two-thirds of the experimental blank samples. If Ballast Eye were able to correct for these estimated readings in the blank sample, it would more accurately be able to estimate organism densities in samples containing organism concentrations below the D-2 ballast water discharge standard. The sample results from Ballast Eye during Phase I testing using *D. magna* and *Eucyclops* spp. (Table 6 and Table 7) in both LW and LW-TMH highly correlates to the detailed microscopic analysis of freshwater laboratory-cultured organism in the zooplankton size class. This is indicated by the R^2 values of >0.9 in all LW and LW-TMH samples containing L-sized organisms. The small CV for each sample concentration also suggests that the device can accurately estimate L-sized organism concentrations and risk category in high transparency and low transparency water.

Objective 2: Does water quality, specifically turbidity, transparency and organic carbon content impact the results of Ballast Eye analysis compared to detailed microscopic analysis of freshwater laboratory-

cultured organisms in the protist and the zooplankton size classes, both in single-celled and colonial protists?

Water quality, specifically turbidity, transparency, and organic carbon content appears to have minimal impact on the results of Ballast Eye analysis compared to detailed microscopic analysis of freshwater laboratory-cultured organisms in the protist and the zooplankton size classes. LW-TMH contains higher DOC, NPOC and TSS concentrations. Increased TSS in turn increases turbidity and decreases percent transmittance. The data from Ballast Eye indicate very similar results, R^2 values within ±0.03, between LW and LW-TMH for all individual organisms analyzed.

Objective 3: Do results from sample analysis by Ballast Eye correlate to detailed microscopic analysis of freshwater organisms in the protist and the zooplankton size classes collected from western Lake Superior?

Results from sample analysis by Ballast Eye do not correlate to detailed microscopic analysis of freshwater organisms in the protist size class collected from western Lake Superior. This outcome is indicated overall by a very low R^2 value (0.0005) and by the large CV for each concentration (Table 10). More specifically, Ballast Eye estimated an average concentration of 8.9 cells/mL in the experimental blank sample where no organisms were present, an average concentration of 4.7 cells/mL in the 30-50 cells/mL concentration, and an average concentration of 10.3 cells/mL in the 51-150 cells/mL concentration. These issues suggest that the device is unable to accurately determine the concentration of ambient S-sized organisms in water collected from the Duluth-Superior harbor. As noted previously, it would be beneficial to determine whether the analysis would be more accurate and precise if a 5 mL sample were stained rather than 1 mL as GWRC was instructed to do by the Satake Corporation for these tests.

Results from sample analysis by Ballast Eye do not correlate to detailed microscopic analysis of freshwater organisms in the zooplankton size class collected from western Lake Superior. This outcome is indicated overall by a low R^2 value (0.7151) and the large CV for higher concentrations (Table 11 and Figure 9). More specifically, the Ballast Eye device estimated an average concentration of 0.0 organisms/m^3 in the 5-20 organisms/m^3 concentration, an average concentration of 1.6 organisms/m^3 in the 30-50 organisms/m^3 concentration, and an average concentration of 4.5 organisms/m^3 in the 51-150 organisms/m^3 concentration. These issues suggest that the device is unable to accurately determine the concentration or risk of ambient L-sized organisms in water collected from the Duluth-Superior harbor.

Objective 4: Do results from sample analysis by Ballast Eye correlate to detailed microscopic analysis of freshwater organisms in the protist and the zooplankton size classes in uptake and discharge samples collected during land-based ballast treatment technology testing at Montreal Pier Facility (Superior, WI)?

Although Ballast Eye appears to be able to distinguish between uptake and treated water by correctly estimating the risk for the protist size class, the estimated live densities vary greatly

from the microscopic counts (Table 14). Uptake sample microscopic "strictly" counts of the protist size class ranged from 152.9-300.4 cells/mL while the Ballast Eye device estimated an average of 22.0 cells/mL. The uptake organism concentration was outside of the Ballast Eye's upper limit of detection, 150 live cells/mL. Although Ballast Eye accurately assessed the samples as high risk, the numerical result is not a good indication of how well the device is analyzing the sample. Treated sample microscopic "strictly" counts ranged from 0.0-0.4 cells/mL and the Ballast Eye device estimated an average of 0.8 cells/mL, so these values correlate well in comparison, as did the classification of these samples as low risk.

Results from sample analysis by Ballast Eye do not correlate to detailed microscopic analysis of freshwater organisms in the zooplankton size class in uptake and treated water (Table 15). Although Ballast Eye accurately assessed the uptake samples as high risk, the estimated live densities vary greatly from the microscopic counts. Uptake sample microscopic counts ranged from 4.4E+04 to 1.50E+05 organisms/m^3 while the Ballast Eye device estimated an average of 393.1 organisms/m^3. The uptake organism concentration was far outside of the Ballast Eye's upper limit of detection, 150 live organisms/m^3. Although Ballast Eye accurately assessed the samples as high risk, the numerical result is not a good indication of how well the device is analyzing the sample. After seeing the results of this testing, the developer indicated that the uptake water should not be concentrated as this may cause estimated concentrations to exceed the upper detection limit of Ballast Eye. Treated sample microscopic counts ranged from 3.9-17.3 organisms/m^3 and Ballast Eye estimated an average of 3.0 organisms/m^3. Ballast Eye did not correctly assign all treated samples to the low risk category. The ballast water treatment system used in this test is in development and is not approved by the IMO or USCG.

Results from this verification indicate that Ballast Eye has potential for use as a compliance monitoring device in the Great Lakes for organisms in the zooplankton size class. However, it was less effective in natural water samples with native assemblages of zooplankton than it was with laboratory cultured organisms in laboratory water or amended laboratory water. Ballast Eye was not as successful at analysis of the protist size class with laboratory-cultured organisms in either water type or natural assemblages.

9 REFERENCES

First, M.R., et al. (2018). A test of the framework designed to evaluate compliance monitoring devices for ballast water exchange. Management of Biological Invasion, Vol. 9, Issue 4:505-513.

International Maritime Organization. (2019). Proposed protocol for the verification of ballast water compliance monitoring devices, Submitted by IOC-UNESCO, ICES, and ISO at PPR 7/21 on 07 October 2019.

Kim, Y.-H., Jang, P.-G., Jung, S. W., & Choi, K.-H. (2016). Performance testing of ballast water management systems: Revisiting the interpretation of organisms ≥10 µm and ≤50 µm in minimum dimension. Aquatic Ecosystem Health & Management, 19(4), 355–359. https://doi.org/10.1080/14634988.2016.1257899

Lake Superior Research Institute. (2020a). Great Lakes Verification of Ballast Water Compliance Monitoring Devices: Satake Corporation Ballast Eye Verification Plan.

Lake Superior Research Institute. (2020b). SOP/AT/46-Preparing Amended Laboratory Water using Test Dust, Micromate, and Humic Acid Sodium Salt.

Lake Superior Research Institute. (2020e, Draft). SOP/GWRC/25 - Procedure for Zooplankton Analysis.

Lake Superior Research Institute. (2020c). SOP/GWRC/30 - Procedure for Protist Analysis.

Lake Superior Research Institute. (1992). SOP/GLM/05 v.6 - Procedure for Calibration and Operation of pH Meters Utilizing Automatic Temperature Compensation (ATC).

Lake Superior Research Institute. (1995). SOP/GLM/17 v.3 - Procedure for Thermometer Verification and Calibration.

Lake Superior Research Institute. (2011). SOP/GLM/26 v.2 - Procedures for Calibrating and Using the Oakton CON 110 Conductivity/TDS/Temperature Meter.

Lake Superior Research Institute. (2021a). SOP/GLM/28 - Procedures for Calibrating and Using the Oakton CON 150 Conductivity/TDS/Temperature Meter.

Lake Superior Research Institute. (2019). SOP/GLM/34 - Calibrating, Maintaining, and Using the YSI ProSolo Handheld Meter and Optical Dissolved Oxygen/Temperature Probe

Lake Superior Research Institute. (2006). SOP/SA/47 v.3 - Procedures for Measuring Organic Carbon in Aqueous Samples.

Lake Superior Research Institute. (2017b). SOP/SA/66 v.2 - Analyzing Total Suspended Solids, Particulate Organic Matter, and Mineral Matter.

Lake Superior Research Institute. (2018). SOP/SA/69 v.1 - Laboratory Determination of Percent Transmittance of Light in Water at 254 nm.

Lake Superior Research Institute. (2017c). SOP/FS/39 v. 2 - Procedure for Calibration of YSI EXO 2 Multiparameter Water Quality Sondes.

Lake Superior Research Institute. (2021b). SOP FS/41 - Deployment and Storage of YSI EXO2 Multiparameter Sondes.

Pouneva, I. (1997). Evaluation of Algal Culture Viability and Physiological State by Fluorescent Microscopic Methods. *Bulg. J. Plant Physiol*., 23(1-2), 67-76.

Ram, J.L., et al. (2019). Ballast Management in the Great Lakes: Priorities and Technologies for Indicative Testing. https://ballastwater.org/wp-content/uploads/2020/02/Priorities-and-Technologies-for-Indicative-Testing_Ballast_Water_Dr_Ram.pdf

Reavie, E.D. and Cangelosi, A.A., (2020). Ballast water management system certification testing requirements for protists are a poor fit for the Great Lakes. *Journal of Great Lakes Research*, 46(1), pp.237-242

Satake Corporation. (2019). Ballast Eye Viable Organism Analyzer Instruction Manual.

Wisconsin Department of Natural Resources Laboratory Certification Program. (1996). Analytical Detection Limit Guidance & Laboratory Guide for Determining Method Detection Limits.

United States Environmental Protection Agency. (2010). Generic Protocol for the Verification of Ballast Water Treatment Technology. U.S. Environmental Protection Agency, Washington, DC, EPA/600/R-10/146.

Appendix 1. Checklist of items received from Satake Corporation, June 26, 2020. In the calibration cell row, LSRI staff noted "in VOA" which means the calibration cell was transported within the device cell chamber.

RNC Checklist of contents

		SATAKE → LSRI	LSRI → SATAKE
Viable organism analyzer	1pc.	✓ ☑	☐
Instruction manual	1pc.	✓ ☑	☐
Safety data sheet	1pc.	✓ ☑	☐
Yellow card	1pc.	✓ ☑	☐
Information paper (Additional Information)	1pc.	✓ ☑	☐
Rental Agreement	1pc.	✓ ☑	☐
Cloth	1pc.	✓ ☑	☐
Stylus	1pc.	✓ ☑	☐
Introduction video (DVD)	1pc.	✓ ☑	☐
Calibration cell	1pc.	✓ in VOA ☑	☐
Sample cell for L-size	2pcs.	✓ ☑	☐
Sample cell for S-size	2pcs.	✓ ☑	☐
Stirring bar	4pcs.	✓ ☑	☐
AC adapter	1pc.	✓ ☑	☐
Power code (type A)	1pc.	✓ ☑	☐
Back pack	1pc.	✓ ☑	☐
Reagent L (For L-size)	11pcs.	✓ ☑	No need to return
Reagent S (For S-size)	11pcs.	✓ ☑	No need to return
		Confirmer SATAKE Shinya Fushida (Sign) Shinya Fushida (Name)	Confirmer LSRI (Sign) _____ (Name)

Appendix 2. Phase II ambient protist counts and Ballast Eye analysis in Duluth-Superior Harbor water. Microscopic counts were outside the acceptable range.

Sample Description	Ballast Eye Estimated (cells/mL)	Mean (CV)	Microscopic Counts	
			Cells Strictly ≥10 and <50 µm (cells/mL)	Total "Allowable" Cells (cells/mL)
0 cells/mL	0.3[L]	4.2 (98.4)	0.0	0.0
	3.8[L]			
	8.6[L]			
5-20 cells/mL	4.5[L]	3.2 (36.8)	2	4.9
	2.9[L]			
	2.2[L]			
30-50 cells/mL	8.6[L]	6.3 (38.3)	5.5	23.5
	6.4[L]			
	3.8[L]			
51-150 cells/mL	7.0[L]	6.5 (23.3)	12.2	48.9
	4.8[L]			
	7.7[L]			

Appendix 3. Detailed taxonomic assessment of organisms in Phase II protist testing.

Taxonomy	min. dimension < 10 µm cells/mL	min. dimension > 10 µm cells/mL
Blue Greens		
***Microcystis* - like coccoid**	9	NA
Other filamentous cells	91	-
Filamentous-no cells (length)	21	-
Greens		
***Scenedesmus* - type desmid**	11.5	-
Coccoid	58	-
Single spindle	0.5	NA
Euglenoid	-	0.5
Cryptophytes (and other small flagellates)		
***Cryptomonas/ Chroomonas* - types**	0.5	1
Round microflagellates	-	3
Diatoms		
Chain *(Aulacoseira, Melosira, S. binderanus*)	186	33.5
Asterionella	2.5	-
Centric nonchain *(Cyclotella, Stephanodiscus)*	3	145.5
Synedra – like (includes nitzschioid)	1	-
Naviculoid (or other single pennate)	-	2
Chrysophytes		
Mallomonas	0.5	-
Protozoans and Animals		
Ciliate	NA	0.5

Totals	<10 total cells/mL	>10 total cells/mL
Blue Greens	121	0
Greens	70	0.5
Cryptophytes	0.5	4
Diatoms	192.5	181
Chrysophytes	0.5	0
Protozoans and Animals	0	0.5
All Taxa	384.5	186
Total Cells Counted:	**570.5**	

Appendix 4. Detailed taxonomic characterization of organisms in Phase II zooplankton testing.

Ballast Eye Phase II ≥50 µm Size Class Taxonomy								
	Starting Density Sample		51–150 Live Organisms/m³ Sample		30–50 Live Organisms/m³ Sample		5–20 Live Organisms/m³ Sample	
Taxonomy	**Total Organisms /m³**	**Live Organisms /m³**	**Total Organisms /m³**	**Live Organisms /m³**	**Total Organisms /m³**	**Live Organisms /m³**	**Total Organisms /m³**	**Live Organisms /m³**
Cladocerans								
Bosmina	566.4	549.9	7	7	2	0	5	4
Daphnia	192.5	181.5	3	1	1	1	1	1
Ceriodaphnia	5.5	5.5	-	-	-	-	-	-
Sidid	115.5	44.0	-	-	-	-	-	-
Copepods								
Calanoids	384.9	296.9	5	5	1	1	1	1
Cyclopoids	154.0	148.5	7	7	-	-	1	0
Nauplii	1,899.7	1,122.6	20	16	5	5	4	3
Mollusca								
Dreissenid	647.6	647.6	2	1	3	3	1	1
Other Organisms								
Egg	86.4	86.4	2	2	-	-	-	-
Ostracod	5.5	5.5	1	1	-	-	-	-
Planaria	-	-	1	1	-	-	-	-
Protista >50 µm	518.1	518.1	7	7	2	2	1	1
Rotifers								
Asplanchna	86.4	43.2	-	-	-	-	-	-
Dicranophoridae	86.4	86.4	-	-	-	-	-	-
Collotheca	43.2	43.2	-	-	-	-	-	-
Conochilus	906.7	906.7	10	8	4	4	-	-
Kellicottia	86.4	43.2	1	1	-	-	-	-
Keratella	4,878.8	4,576.6	47	43	21	16	11	10
Ploesoma	259.1	259.1	4	4	1	1	-	-
Polyarthra	12,866.2	9,412.2	91	62	49	22	18	8
Synchaeta	863.5	820.3	7	3	5	4	-	-
Tricocerca	474.9	259.1	1	1	3	1	-	-
Total	**$2.5x10^4$**	**$2.0x10^4$**	**216**	**170**	**97**	**60**	**43**	**29**
Percent Live	**80%**		**79%**		**62%**		**67%**	

Appendix 5. Phase III detailed taxonomic characterization of organisms in uptake samples during zooplankton testing.

Ballast Eye Phase III Untreated Uptake Sample ≥50 µm Size Class Taxonomy						
	Phase III-1		Phase III-2		Phase III-3	
Taxonomy	Total Organisms /m³	Live Organisms /m³	Total Organisms /m³	Live Organisms /m³	Total Organisms /m³	Live Organisms /m³
Cladocerans						
Bosmina	$9.3x10^3$	$9.1x10^3$	$7.8x10^3$	$7.5x10^3$	$4.9x10^3$	$4.6x10^3$
Ceriodaphnia	-	-	-	-	35	35
Chydoridae	$1.1x10^2$	$1.1x10^2$	$1.1x10^2$	$1.1x10^2$	-	-
Daphnia	$1.5x10^3$	$1.4x10^3$	$3.4x10^2$	$2.6x10^2$	$2.4x10^2$	$2.1x10^2$
Holopedium	$1.1x10^2$	$1.1x10^2$	-	-	35	0
Sidids	57	57	$1.5x10^2$	75	-	-
Copepods						
Calanoids	$2.2x10^3$	$1.9x10^3$	$6.4x10^2$	$3.0x10^2$	$1.3x10^3$	$1.2x10^3$
Cyclopoids	$4.2x10^3$	$3.9x10^3$	$2.2x10^3$	$1.6x10^3$	$3.5x10^3$	$3.1x10^3$
Harpacticoid	-	-	-	-	35	35
Nauplii	$7.0x10^3$	$4.9x10^3$	$3.3x10^3$	$2.4x10^3$	$2.3x10^3$	$1.2x10^3$
Mollusks						
Dreissenid	$8.2x10^2$	$8.2x10^2$	$3.0x10^2$	$3.0x10^2$	-	-
Other Organisms						
Oligochaetes	57	57	-	-	-	-
Planaria	$2.3x10^2$	$2.3x10^2$	75	75	35	35
Protista >50	$8.2x10^2$	$8.2x10^2$	$4.2x10^3$	$4.2x10^3$	$4.8x10^2$	$4.8x10^2$
Rotifers						
Asplanchna	$4.1x10^2$	$4.1x10^2$	$3.0x10^2$	$3.0x10^2$	$1.2x10^2$	$1.2x10^2$
Bdelloid	$8.2x10^2$	$8.2x10^2$	$6.0x10^2$	$6.0x10^2$	$3.6x10^2$	$3.6x10^2$
Collotheca	$4.1x10^2$	$4.1x10^2$	$6.0x10^2$	$6.0x10^2$	$1.2x10^2$	$1.2x10^2$
Conochilus	$4.1x10^3$	$4.1x10^3$	$6.0x10^2$	$6.0x10^2$	$4.8x10^2$	$3.6x10^2$
Dicranophoridae	-	-	$1.2x10^3$	$1.2x10^3$	$1.2x10^2$	$1.2x10^2$
Euchlanis	-	-	$3.0x10^2$	$3.0x10^2$	-	-
Gastropus	$4.1x10^2$	$4.1x10^2$	-	-	$2.4x10^2$	$1.2x10^2$
Kellicottia	$1.2x10^3$	$1.2x10^3$	$6.0x10^2$	$6.0x10^2$	$3.6x10^2$	$2.4x10^2$
Keratella	$1.8x10^4$	$1.8x10^4$	$2.0x10^4$	$1.8x10^4$	$7.4x10^3$	$7.1x10^3$
Monostyla	-	-	$6.0x10^2$	$6.0x10^2$	-	-
Notholca	-	-	$3.0x10^2$	$3.0x10^2$	-	-
Polyarthra	$5.5x10^4$	$4.5x10^4$	$3.0x10^4$	$2.5x10^4$	$1.1x10^4$	$7.3x10^3$
Pompholyx	-	-	$3.0x10^2$	$3.0x10^2$	-	-
Synchaeta	$5.8x10^4$	$5.1x10^4$	$5.6x10^4$	$4.9x10^4$	$1.9x10^4$	$1.8x10^4$
Trichotria	$4.1x10^2$	$4.1x10^2$	-	-	-	-
Tricocerca	$2.9x10^3$	$2.9x10^3$	$1.5x10^3$	$1.5x10^3$	$6.0x10^2$	0
Total	**$1.7x10^5$**	**$1.5x10^5$**	**$1.3x10^5$**	**$1.2x10^5$**	**$5.3x10^4$**	**$4.4x10^4$**
Percent Live	**88%**		**87%**		**83%**	

Appendix 6. Phase III detailed taxonomic characterization of organisms in discharge samples during zooplankton testing.

Ballast Eye Phase III Treated Discharge Sample ≥50 µm Size Class Taxonomy			
	Phase III-1	**Phase III-2**	**Phase III-3**
Taxonomy	**Live Organisms /m³**	**Live Organisms /m³**	**Live Organisms /m³**
Cladocerans			
Bosmina	-	-	2.6
Copepods			
Nauplii	1.3	-	-
Other Organisms			
Tardigrade	1.3	2.7	-
Rotifers			
Bdelloid	10.6	13.3	1.3
Keratella	-	1.3	-
Total	**13.2**	**17.3**	**3.9**