BENCH-SCALE TECHNICAL REPORT

FRESHWATER VERIFICATION OF THE FASTBALLAST COMPLIANCE MONITORING DEVICE

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ABSTRACT

This technical report presents findings from bench-scale verification tests evaluating the performance of the FastBallast compliance monitoring device in freshwater. FastBallast was developed by Chelsea Technologies Ltd. of Surrey, UK.

The evaluation of the FastBallast compliance monitoring device 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. The FastBallast device employs Single Turnover Active Fluorometry (STAF) to rapidly quantify living organisms in ballast water samples in the ≥10 µm and <50 µm (nominally protists) regulated size class, providing an indication of compliance or exceedance of the International Maritime Organization (IMO) *International Convention for the Control and Management of Ships' Ballast Water and Sediments* Regulation D-2 Ballast Water Performance Standard (2004).

Verification testing composed of three phases in which results using the FastBallast device were compared to results using microscopic methods. Phase I testing was completed in two water types with laboratory-cultured organisms in the protist regulated size class, utilizing the single-celled protist *Haematococcus pluvialis* and colonial protist *Scenedesmus quadricauda*. Phase II testing was completed using naturally occurring Great Lakes organisms in the Duluth-Superior Harbor of Lake Superior. Phase III testing was completed using Duluth-Superior harbor water an ambient organism before and after treatment with a ballast water treatment (BWT) technology during three land-based trials. Data from all phases were analyzed for precision, accuracy, and reliability. Quantification/detection limits were calculated using data from Phase I testing.

Phase I testing showed that FastBallast was effective at quantifying single-celled protists but was less accurate at counting colonial protists. Increased turbidity and carbon content slightly impacted FastBallast results, however, both water types displayed strong correlations to microscopic counts. FastBallast results were lower than microscopic counts in all trials of Phase I. Phase II testing showed strong correlations between the FastBallast results and microscopic results of protists collected from the Duluth-Superior Harbor, however the counts reported by FastBallast were 4 to 10 times greater than the microscopic counts. Phase III testing showed FastBallast accurately measured uptake and treated discharge water from samples collected during a land-based BWT technology evaluation. FastBallast counts were more similar to the density of protist entities ≥10 µm in any dimension than they were to live density of individual protist cells comprising entities ≥10 µm in minimum dimension. The device was found to have minor operational issues but was found reliable for measuring organisms within the protist size class.

KEY WORDS

Compliance Monitoring Device, Ballast Water, Single Turnover Active Fluorometry

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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 FastBallast compliance monitoring device, hereafter, FastBallast developed by Chelsea Technologies Ltd., Surrey, UK.

FastBallast utilizes Single Turnover Active Fluorometry (STAF) to quantify living organisms in the ≥10 to <50 µm (nominally protist) regulated size class in marine, brackish, and freshwater. The purpose of the FastBallast analysis is to determine if ballast water discharge meets the IMO D-2 standard, which states that ships conducting ballast water management must discharge fewer than 10 viable organisms per mL that are in the protist size class.

The freshwater verification of FastBallast 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 the FastBallast device correlate to detailed microscopic analysis of freshwater laboratory-cultured organisms in the protist size class, both in single-celled and colonial protists?
2. Does water quality, specifically turbidity, transparency, and organic carbon content impact the results of FastBallast analysis compared to detailed microscopic analysis of freshwater laboratory-cultured organisms in the protist size class, both in single-celled and colonial protists?
3. Do results from sample analysis by the FastBallast correlate to detailed microscopic analysis of freshwater organisms in the protist µm size class collected from western Lake Superior?
4. Do results from sample analysis by the FastBallast correlate to detailed microscopic analysis of freshwater organisms in the protist size class 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, FastBallast was evaluated on the following verification factors (First et al., 2018 and IMO PPR 7/21, 2019):

- **Accuracy**: Measure of the overall agreement of a measured numerical value (device response) to a known value (accepted method of analysis as described in ETV Protocol (US EPA, 2010)). Additionally, accuracy will be further quantified by binary pass/fail value.
- **Precision**: Measure of mutual numeric agreement among individual measurements of the same property. Additionally, precision will be further quantified by binary pass/fail value.
- **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) *Chelsea Technologies Ltd. FastBallast Verification Plan* (LSRI, 2020a) and LSRI-GWRC 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 quality assurance and quality control (QA/QC) requirements. It was approved by both LSRI-GWRC and Chelsea Technologies Ltd. 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 FASTBALLAST DESCRIPTION

The FastBallast device evaluated by LSRI-GWRC is a portable, commercially available ballast water discharge compliance monitoring device. FastBallast is small in size (4.25” x 7.75” x 9.5”), lightweight (11 lb.) and enclosed in an IP67 rated hardcase, which is dustproof, waterproof, shockproof, and floats (Figure 1). FastBallast is operable at ambient temperatures from 0 to 40°C.

Figure 1. Photograph of the FastBallast device.

FastBallast was delivered in a padded backpack for easy transport. Included in the hardcase was a Panasonic ToughPad equipped with FaBtest software. The ToughPad connects to FastBallast via the supplied USB cable. The internal rechargeable battery in FastBallast has a battery life of 6 hours, while the battery life of the ToughPad will depend on factors such as brightness and wireless connection. FastBallast was supplied with reusable 20-mL sample cups, 30-mL syringes and 1-mL Pasteur pipettes. Additionally, a single Swinnex filtration unit, 41-µm and 30-µm nylon mesh filters and 8-µm membrane

filters were included in the delivery pack. The 41-µm filter was used for routine pre-filtration to remove cells >50 µm in size (Figure 2). The 30-µm filter was used only when prompted by FaBtest. FaBtest did not prompt the use of the 8-µm membrane filters, as the system was not used in flow through mode.

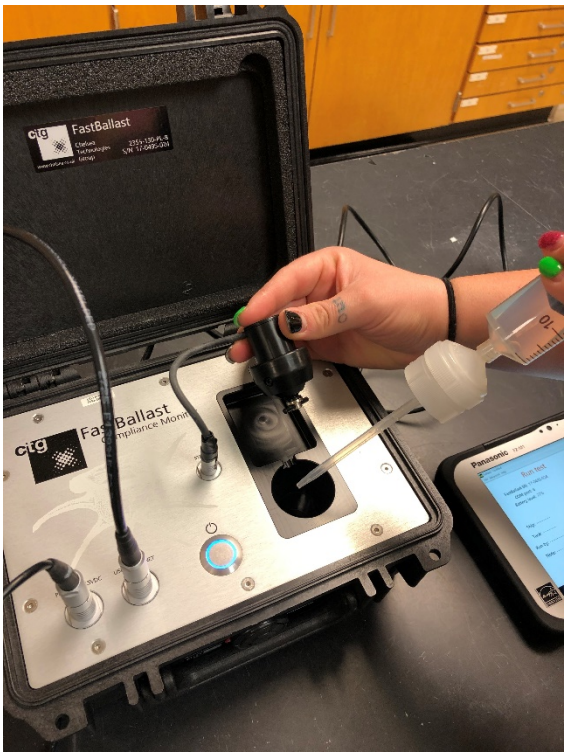

Figure 2. Photograph of sample injection into FastBallast chamber.

FastBallast utilizes STAF to provide an indicative analysis of the protist size class in ballast water samples to assess performance of ballast water treatment systems within ten minutes. Performance of a ballast water treatment (BWT) technology is evaluated with regards to the IMO D-2 Ballast Water Discharge Standard of <10 viable cells/mL. STAF uses higher intensity light for a shorter period of time than the Multiple Turnover Active Fluorometry widely used in other fluorometer models. This in turn leads to a shorter recovery period between excitations and thus faster analysis times and improved signal-to-noise ratios.

FastBallast utilizes patented technology based on Poisson theory to calculate variable fluorescence per cell. Four LED arrays excite chlorophyll and other light harvesting pigments within protist cells. The default settings use the royal blue (455 nm) and blue (470 nm) wavelengths. The fluorescence emission stimulated by light from the LEDs is detected within a waveband centered at 682 nm (deep red) (Chelsea Technologies, Ltd., 2020). The 682-nm waveband is closely matched to the variable fluorescence (Fv) emission from functional photosystem II (PSII) complexes within protists, providing indication of photochemical activity and cell vitality.

The STAF method incorporates a saturating pulse of light that is eight to ten times full sunlight and 100-400 µs in duration. The shorter pulse duration used in the ST method dramatically increases sampling

rate. The FastBallast sample chamber holds 20 mL of sample. Only 2.5% of the sample volume (0.5 mL) is being measured at any point in time during the test, but because the ST pulses are so short, measurements can be taken while the sample is stirred. Ten consecutive pulses are averaged to generate a data point every second and the stir speed ensures that the entire 20 mL volume is interrogated during the duration of the test (Chelsea Technologies, Ltd., 2020). The proprietary algorithm based on Poisson distribution is applied to the resulting array of Fv values to directly calculate cell density and Fv per cell. Fv per cell closely correlates with cell volume, thus providing an indication of cell size. This indication is particularly pertinent in the Great Lakes where cyanobacteria may be the dominant protist phylum where many are <10 µm in size. Additionally, since FastBallast only relies on the Fv component of the total fluorescence signal, background fluorescence such as Colored Dissolved Organic Matter (CDOM) or dead protist cells do not impact cell counts.

The FastBallast device is capable of performing level 1 and level 2 tests, both of which may result in a PASS or FAIL flag (in relation to the D-2 discharge standard) and a numeric value. Level 1 tests ran for approximately one minute while the total level 1 and level 2 test time was just over eight minutes. After 60 sample points, if the resulting numeric was no more than 0.08, the test ended as a level 1 PASS. If the numeric was greater than 0.08 at 60 points, FaBtest automatically progressed to a level 2 test. The default level 2 test was set to 480 points, which required just over eight minutes to complete. Following the level 2 test, samples with a numeric of no more than 1.0 were a PASS, while samples with a numeric of greater than 1.0 were a FAIL (Figure 3). This numeric describes how many times over or under the IMO D-2 discharge standard the sample was. For example, a numeric of 2.5 indicated that the sample was 2.5 times over the D-2 threshold, and thus contained 25 cells/mL.

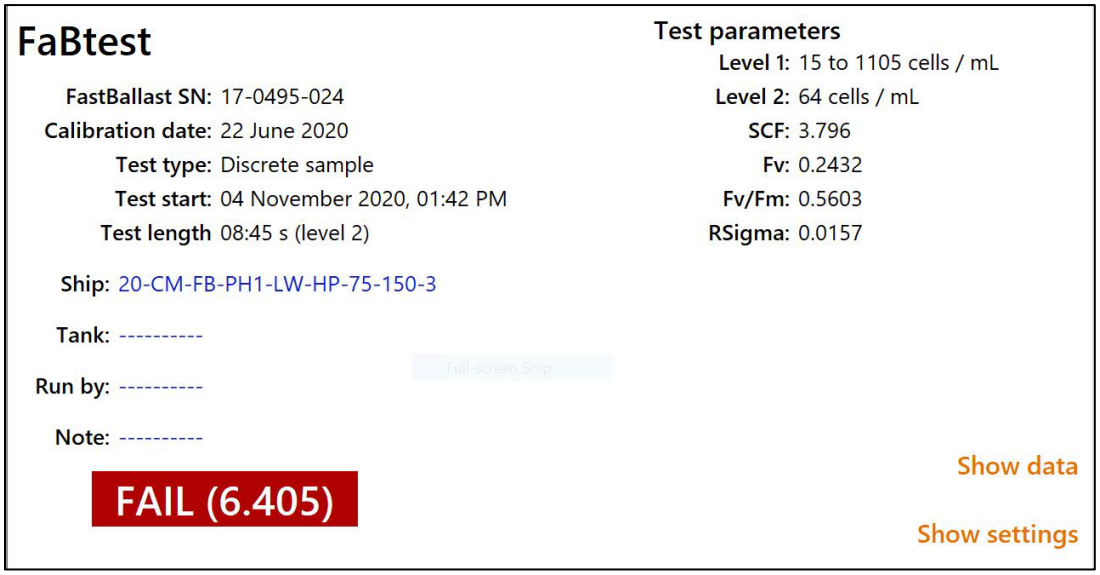

Figure 3. FaBtest screenshot at the end of a level 2 test.

Samples were processed prior to analysis using the 41-µm filters supplied with the system to remove cells larger than 50 µm which may have obscured cells in the protist size class from being analyzed by FastBallast. A 20-mL sample was drawn up in the supplied 30-mL syringe that was fitted with the tip of a

1-mL Pasteur pipette (Figure 4, left). The syringe was turned upside down and the entire sample was drawn up into the syringe. The Pasteur pipette tip was removed and replaced with a Swinnex filtration unit containing a 41-µm filter (Figure 4, right). The sample was then passed through the filtration unit and into the sample chamber and the stir unit was inserted into the chamber.

Figure 4. Photograph of Syringe fitted with tip of Pasteur pipette (left) and with Swinnex filtration unit (right).

The Run button on the ToughPad (Figure 1), was pressed to initiate testing. The screen passed through a series of displays beginning with the Test Setting title which was replaced by the Optimizing Setup title, indicating start up for measurements, and ended with the Test Running title, indicating FastBallast had begun taking measurements. The desired value for the signal "Range" was between 30-100%. Values outside of the desired range were flagged with yellow (<30%) or red (>100%) and FaBtest adjusted the signal gain automatically to bring the range within desired values. Once the number of "Points" was greater than 6, the Show Data button appeared in the lower right corner of the screen (Figure 3). Clicking on the Show Data button switched the testing screen to the data screen. The top panel of the data screen showed accumulating activity values while the lower panel showed the most recent acquisition as a black line and the mean of all traces as a purple line. At the end of the level 1 test, if the activity numeric was ≤0.08, the test ended with a PASS, and if the numeric was >0.08 the test continued to a level 2 reading. Data files were archived within timestamped files on the ToughPad at the end of each sample/test.

2.3 FASTBALLAST DEVICE TRAINING

The FastBallast device was delivered via FedEx to LSRI in on July 6, 2020. Upon arrival, the system was inspected to ensure no damage was sustained during shipping. Chelsea Technologies Ltd. was notified of the receipt of the FastBallast. No formal training was necessary, however all relevant LSRI-GWRC staff reviewed all training materials and handbooks supplied by Chelsea Technologies, Ltd. prior to initiation of testing. Any questions concerning operation were directed to Mary Burkitt-Gray or Kevin Oxborough of Chelsea Technologies, Ltd.

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 FastBallast analysis results to traditional microscopic analysis. Freshwater organisms used represented the regulated protist size class and included a single-celled alga and a colonial alga. Testing was done in two water types to represent high transparency (LW) and low transparency (LW-TMH)

conditions to determine whether increased turbidity and carbon content affect the ability of STAF utilized by the FastBallast device to detect organisms in the protist size class in a sample. The goal with sample preparation was to have samples that were below, near, and above the IMO D-2 ballast water discharge standard.

LSRI staff followed the instructions in *the FastBallast Device Handbook* (Chelsea Technologies Ltd., 2020) during all stages of analysis. Before each trial, experimental blank samples of LW or LW-TMH were analyzed, in the same manner as samples containing organisms, to ensure proper device operation.

2.4.1.1 EXPERIMENTAL WATER PREPARATION

Phase I of the FastBallast verification was conducted in LSRI laboratories equipped with adequate ventilation, electrical connections, and climate control. Two experimental water types were prepared as follows:

Laboratory Water (LW): The LW was 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 and was passed through an activated carbon column in order to remove the majority of the chlorine. The remaining residual chlorine was removed through injection of sodium sulfite, resulting in 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 (Table 1). 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 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.

The acceptance ranges for water chemistry of LW and prepared LW-TMH are shown in Table 1.

Table 1. Reference limits for 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 - 2

Parameter	Units	Water Type	Acceptable Range for Initiating Bench-Scale Testing
		LW-TMH	4.4 - 6.8
Non-Purgeable Organic Carbon (NPOC)	mg/L	LW	Less than detection - 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

Performance checks were conducted on each water type for water quality and chemistry measurements (Section 2.4.5). Water was spiked with stock mixtures of *Haematococcus pluvialis* or *Scenedesmus quadricauda* protist cultures (approximately 10,000 cells/mL) to produce samples of protists with nominal concentrations within the ranges of 0, <10, 10-30, and 75-150 cells/mL. Both the *H. pluvialis* and *S. quadricauda* samples were stained with FDA (fluorescein diacetate)/CMFDA (5-chloromethylfluorescein diacetate) and counted following LSRI SOP *GWRC/30 – Procedure for Protist Sample Analysis* (LSRI, 2020c) using a compound microscope and epifluorescence. Microscopic counts included cells strictly ≥10 to <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 Program criteria (US EPA, 2010), "strictly" refers to organisms that range from ≥10 to <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 total "allowable" method as the individual cells within each colony were <10 µm in minimum dimension. Each test concentration of protists was verified to be within the target ranges by employing a microscopic blind count.

Triplicate 1-L subsamples were collected from each target concentration and analyzed using the FastBallast device according to the provided *FastBallast Device Handbook* (2020), as described above. Data was promptly recorded on prepared datasheets, along with notes from the analysis day. In addition to the FastBallast analysis, each sample was enumerated using traditional laboratory/microscopic analysis (LSRI, 2020c).

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 Lake Superior. For the assessment of the protist size class, a minimum of two 20-L of water was 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 (LSRI, 2020c). Then, 10-15 L samples, targeting the following live density ranges; 0, 5-20, 30-50, 51-150 live cells/mL following the ETV protocol strictly ≥10 µm and <50 µm size range were prepared using harbor water filtered through a Whatman 934-AH filter (1.5-µm particle retention) to dilute the original protist sample. Experimental blanks were prepared by filtering harbor water through a Whatman 934-AH filter to remove all plankton and the majority of suspended solids.

Triplicate subsamples of the blank sample and each targeted cell density range were collected and analyzed for live organisms in protist size class with the FastBallast device and by traditional microscopic techniques. LSRI-GWRC staff followed the instructions in the *FastBallast Device Handbook* (2020) during all stages of analysis with the device, including filtration of all samples with the 41 µm filter prior to analysis. Before each trial, experimental blank samples of filtered Duluth-Superior Harbor water were analyzed to ensure proper device operation. Blank samples were processed and analyzed in the same manner as samples containing organisms.

Total live density was conducted on the whole water samples following LSRI SOP *GWRC/30*. Protists were enumerated using the "strictly" and "allowable" methods described in Section 2.4.1.2. FastBallast results were compared to the "strictly" count and to the total "allowable" counts. Each test concentration was verified to be within the designated ranges by a microscopic blind count. A detailed taxonomic analysis of community composition of this size class was completed on preserved samples (LSRI, 2020c).

2.4.3 PHASE III

Phase III testing was conducted at the Montreal Pier Test Facility during the land-based evaluation of an ozone-based BWT technology (currently in development). The technology delivers 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 FastBallast and following GWRC's standard operating procedures for microscopic analysis of organisms in the protist size class (LSRI, 2020c). Each trial included analysis of untreated uptake water and treated discharge water.

Samples were enumerated using the "strictly" and total "allowable" methods for the assessment described in Section 2.4.1.2. FastBallast results were compared to the "strictly" count and the total of the "strictly" and total "allowable" counts.

2.4.4 STATISTICAL ANALYSIS

FastBallast results from datasheets associated with the day of analysis were proofed and verified against the printed reports from FaBtest. Level 1, level 2, standardized cell fluorescence (SCF) values, and the results with a pass/fail status are reported in the results tables below. The level 2 results were averaged

and compared against the mean microscopic count associated with the test date and sample target concentration. Samples that indicated the use of a 30-µm filter, were compared to the same sample without the 30-µm filter and the level 2 result that was highest was presented in this report and used for data analysis.

The data were entered into Microsoft Excel for data analysis. The program was used to calculate the coefficient of variance (CV) for the FastBallast results. CV is a measure of precision which shows variability in a sample in relation to the sample mean. The data was graphed using MS Excel by plotting microscopic counts on the x-axis and the FastBallast 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 data were also analyzed for the probability (on a scale of 0 to 1) to detect an exceedance of the IMO D-2 discharge standard to test device accuracy (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.

Accuracy was further quantified by a binary pass/fail value. This was accomplished by creating a confusion matrix, a 2x2 table developed by enumerating the four outcomes of the binary pass/fail value (true positive, false positive, true negative, and false negative). Accuracy was calculated as the number of all correct predictions (true positive + true negative) divided by the total number of predictions.

Precision was further quantified by binary pass/fail values. Using the confusion matrix developed for accuracy determination, precision was calculated as the number of true positive predictions divided by the total number of positive predictions (true positive + false positive).

The detection/quantification limits of the FastBallast 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, and the minimum concentration was determined at which the known value (microscopy results) can be quantified with a signal-to-noise ratio of 10:1.

During all three phases of FastBallast verification, device reliability was determined by calculating the percent completeness of the combined dataset. Percent completeness was 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 and/or total number of times that the device operated as designed without interruption (i.e., non-scheduled maintenance, non-scheduled calibration, or repair) was also reported.

2.4.5 WATER QUALITY AND WATER CHEMISTRY

Water quality measurements were made throughout all three phases of the FastBallast 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, 2017a). 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 each sample was filtered it was dried in an oven and brought to a constant weight. TSS values were determined based on the weight of particulates collected on the filter and the volume of water filtered. The residue from the TSS analysis was ignited to a constant weight at 550°C in a muffle furnace. The concentration of POM was determined by the difference of the dry weight of the particulates on the filter before and after ignition (i.e., the mass lost to combustion).

%T sample analysis 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 and filtered sample aliquots. Deionized 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.

NPOC/DOC analysis 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.

DO analysis 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 to Measure Dissolved Oxygen in Water Samples* (LSRI, 2017b). Temperature was measured using a Fisher digital thermometer that was calibrated quarterly following LSRI SOP GLM/17 – *Procedures for Thermometer Verification and Calibration* (LSRI, 1995). Specific conductivity was measured using an Oakton Model CON 150 Conductivity/TDS/Temperature Meter that was calibrated on a monthly basis following LSRI SOP*GLM/28- Procedures for Calibrating and Using the Oakton CON 150 Conductivity/TDS/Temperature Meter* (LSRI, 2021a). Its accuracy was also verified daily prior to sample analysis using a Daily Check Standard (0.0100M potassium chloride). pH analysis 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 pH Meter Calibration and pH Measurement for Ballast Treatment Systems Utilizing pH as the Active Substance* (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 III testing, DO,

temperature, pH and conductivity 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 Multiparameter Water Quality Sondes* (LSRI, 2017c).

2.5 TEST PLAN DEVIATIONS

During testing with the FastBallast device 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 encountered during FastBallast freshwater verification tests.

Test	Description and Root Cause of Deviation or Quality Control Failure	Description of Corrective Action(s)	Describe the Impact on the Project/Test	Data Need to be Qualified?
Phase I and II 4 November 2020, 19 November 2020, 10 December 2020, 11 December 2020	Triplicate samples of each target concentration of protists were analyzed only one time rather than in triplicate, as was indicated in test plan. **The device developer asked that the triplicate samples be analyzed in triplicate. **Root Cause:** Due to issues with the stirrer and the blanks going to Level 2 for analysis, the analysis time was too great to analyze all of the samples in one day.	No corrective action taken. In an email with Mary Burkitt Gray on 8 October 2020, GWRC was given permission to run each triplicate sample only one time rather than in triplicate because there was low variability between the triplicate measurements.	Minimal effect, triplicate samples were already a part of the test plan. Analyzing them in triplicate had been added to the test plan at the request of the developer.	No
Phase I, *H. pluvialis* LW-TMH 19 November 2020	The unfiltered percent transmittance sample from. the LW-TMH stock solution produced a result of 23.6 %T which is less than the acceptable range for unfiltered LW-TMH (25.5-35.5%). **Root cause:** Using a new method to prepare LWTMH 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.	No

Test	Description and Root Cause of Deviation or Quality Control Failure	Description of Corrective Action(s)	Describe the Impact on the Project/Test	Data Need to be Qualified?
Phase I, *S. quadricauda* LW-TMH 12 December 2020	The unfiltered percent transmittance 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 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, data had not been reevaluated 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.	No
Phase II 10 August 2020	The test plan called for NPOC and DOC to be analyzed on the stock water, however, only DOC was analyzed. **Root cause**: Parameter was overlooked in the test plan.	Better review of Test Plan. Summarize data ASAP so it's more apparent if parameters are overlooked.	Minimal, all other water quality parameters were measured.	No
Phase II 11 August 2020	The test plan called for TSS, POM, MM, NPOC, DOC, and %T to be analyzed on the stock water, however, only TSS, POM and MM were analyzed. **Root cause**: Parameters were overlooked in the test plan. Facility Validation-5 was conducted that day and the Harbor water sample was only analyzed for TSS, POM, and MM.	Better review of Test Plan. Summarize data ASAP so it's more apparent if parameters are overlooked.	Minimal, all other water quality parameters were measured.	No
Phase II 25 September 2020	%T was not analyzed within 24 hours of collection. **Root cause**: Parameter analysis requirements were 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	No

Test	Description and Root Cause of Deviation or Quality Control Failure	Description of Corrective Action(s)	Describe the Impact on the Project/Test	Data Need to be Qualified?
			Harbor Water that was collected and analyzed on the same day.	
Phase III 8-9 October 2020, 15 October 2020, 22 October 2020	For the Phase III testing, only uptake samples and treatment discharge samples were analyzed with all 3 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, 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.	No

3 FASTBALLAST OPERATIONAL PERFORMANCE

During the testing period there were several operational issues that occurred within the FaBtest software as well as the FastBallast device. There were frequent issues with the connection between FaBtest and the device when FastBallast was shut down. Several times when the FastBallast device was shut down, the software also needed to be shut down and restarted rather than automatically recognizing the FastBallast device. Discrepancies were also seen between the report in the FaBtest software located in the files on the ToughPad and the printed report. Either the SCF value was incorrect or the numeric value associated with the pass/fail status was incorrect (Figure 5). The developer has indicated that this discrepancy may have been caused by the software version installed on the device which has been updated since the analysis occurred. Finally, when the software prompted the use of a 30 µm filter, LSRI-GWRC staff processed the sample according to Section 2.5 of the device handbook. On most of the occasions, even after filtering with the 30-µm filter and running a second test, a second message prompting the use of the 30-µm filter appeared.

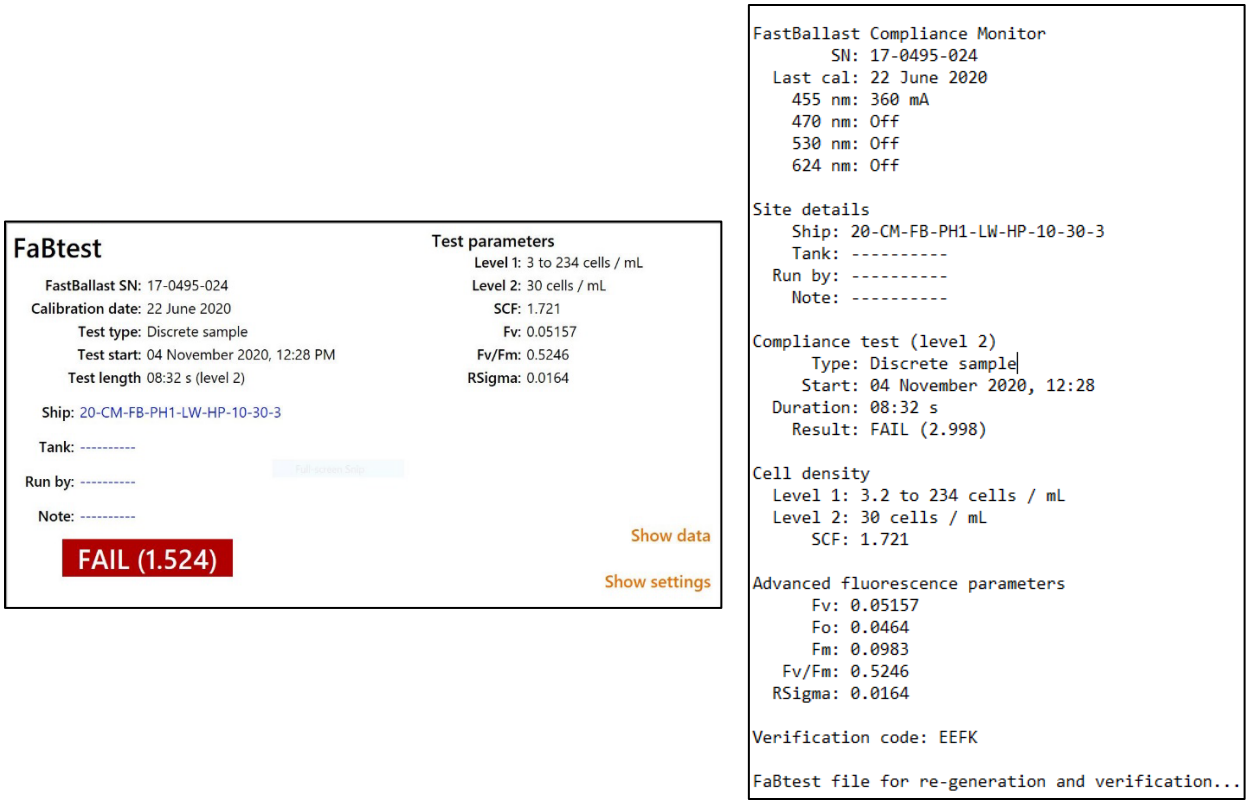

Figure 5. Screenshots of discrepancies between FaBtest and printed report on November 4th, 2020. FaBtest (left) shows a FAIL (1.524) and printed report (right) shows a FAIL (2.998).

During the initial test date (August 4th, 2020) multiple blanks and zero samples were failing while using a Milli-Q water rinse. After corresponding and consulting with the developer on August 5th, 2020, a regimen that consisted of drying the chamber and stirrer, rinsing with isopropyl alcohol (IPA), drying a second time, rinsing with Milli Q, and drying a third time was adopted. On December 10th a single rinse with cold tap water was used per developer instructions, after having issues with samples reporting a result of 0-1 cells/mL in a higher concentration sample.

One occasion occurred in which the ToughPad would not respond to touch after a couple water droplets were on the screen. Wiping the screen with lens cleaning solution and a KimWipe resolved this issue. During one occasion of verifying the printed reports, the ToughPad would not respond to touch, however this was resolved by removing and reinserting the battery.

Several issues occurred with the FastBallast stir units. The original stir unit sent was difficult to plug into the FastBallast device due to the markings on the stirrer cable and instrument not lining up and possibly damaged components within the plug that were not visible. The stirrer also had a loose wire and the wiring needed to be propped up for the stir unit to run (Figure 6, left). The second stir unit, received October 15, 2020, worked at first but on at least two occasions would not turn back on after performing the IPA rinse between samples (as prompted by the developer's instructions). This may have been due to loose wiring as on December 11th, 2020 the wires disconnected from the connection port (Figure 6, right).

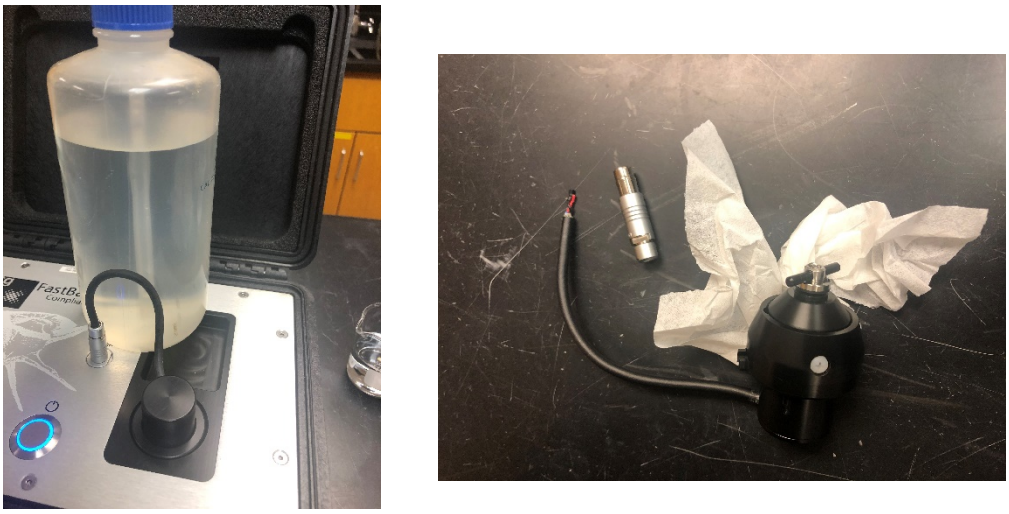

Figure 6. Photographs depicting stirrer issues. The photo on the left is of the loose wires that needed to be propped up on the second stirrer. The photo on the right shows the broken stirrer when the wires disengaged.

4 RESULTS

Findings from the FastBallast Phase I, Phase II, and Phase III tests are presented in the following subsections. Throughout the results section, the outcome of level 1 and level 2 analysis and standardized cell fluorescence (SCF) values are displayed in addition to the concentrations determined microscopically using the FDA/CMFDA staining process (LSRI, 2020c). In result tables with FastBallast cell counts reported, the pass/value indications have been highlighted to align with what the FastBallast analysis indicates regarding compliance with IMO's D-2 discharge standards. Results highlighted in green indicate FastBallast sample analysis output was below the D-2 discharge level and samples highlighted in red are above the D-2 discharge level, according to the FastBallast output. The D-2 Regulation specifies that ships conducting ballast water management shall discharge <10 viable organisms/mL ≥10 µm and <50 µm in minimum dimension (2004).

4.1 PHASE I

Phase I testing with protists occurred on four separate occasions (i.e., one for each species in LW and LW-TMH).

4.1.1 HAEMATOCOCCUS PLUVIALIS

Results from FastBallast and microscopic analysis (LSRI, 2020c) from LW and LW-TMH samples containing *H. pluvialis* are shown in Tables 2 and 3, respectively. A subsample of *H. pluvialis* was measured (Figure 7) and cells were found to have an average size of 20.95 µm (17.8-22.4 µm cell size range). Target concentrations of the *H. pluvialis* in both water types were 0 (experimental blank), <10, 10-30 and 75-150 cells/mL. All blank samples analyzed had microscopic counts of 0 cells/mL. In LW, the

final microscopic cell count averages for each range were 0, 5.56, 23.4, and 103 cells/mL. The FastBallast count averages for each range in LW were 0, 5.5, 19.7, and 71.1 cells/mL (Table 3). In LW-TMH, the final microscopic cell count averages were 0, 3.92, 19.9, and 89 cells/mL, while FastBallast counts were <1, 1.20, 5.87, 84.1 cells/mL (Table 4). Results from FastBallast were comparable to the microscopic counts in each target concentration (Tables 3 and 4) with the exception of the 10-30 cells/mL samples in LW-TMH. For this sample set, the FastBallast device detected an average concentration of 5.87 cells/mL indicating a pass of the D-2 discharge standard of <10 cells/mL while microscopic counts yielded a concentration of 19.9 cells/mL (Table 4). FastBallast correctly assigned risk categories to all samples in LW, and to all but the 10-30 cells/mL samples in LW-TMH. The CV for the FastBallast counts ranged from 4.89 to 37.2 with the highest CV for LW in the 10-30 cells/mL sample and the highest CV for LW-TMH in the 75–150 cells/mL sample. The CV was not calculable for the blank samples as the mean was 0 or <1 cells/mL.

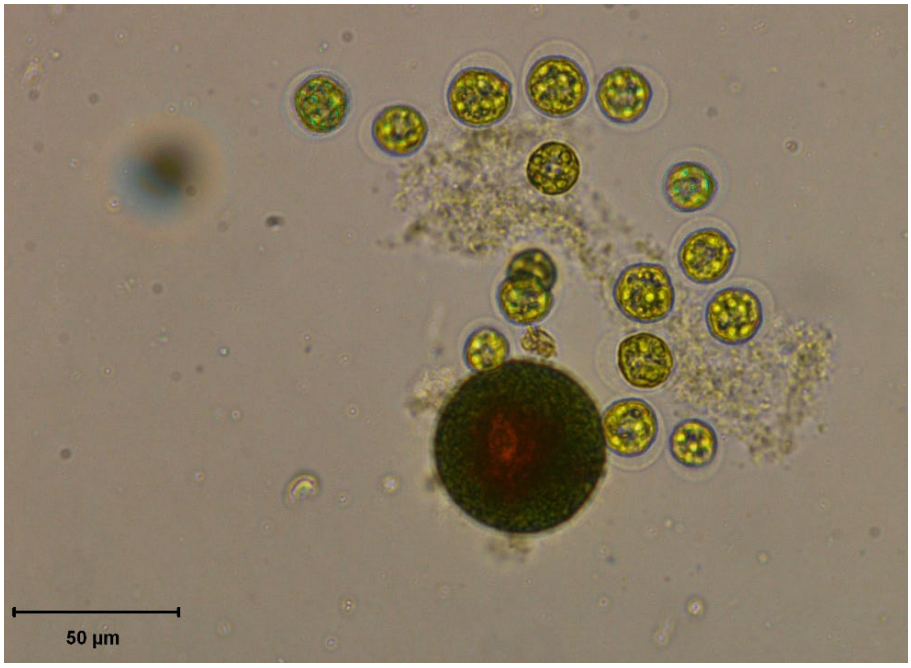

Figure 7. Photograph of *Haematococcus pluvialis*.

Table 3. Phase I FastBallast analysis and microscopic cell counts for live H. pluvialis in LW.

Sample Description	FastBallast Results					Microscopic Mean Count (cells/mL)
	Level 1 (cells/mL)	Level 2 (cells/mL)	SCF	Result	Mean Count (CV) (cells/mL)	
0 cells/mL (Blank)	0 to 0	NA	NA	Pass (0.003564)	0	0
	0 to 0	NA	NA	Pass (0.005575)		
	0 to 0	NA	NA	Pass (0.001072)		
<10 cells/mL	1 to 48	5.8*	1.826	Pass (0.5791)	5.5 (24.3)	5.56
	0 to 34	3.7*	2.052	Pass (0.3654)		
	1 to 43	6.9*	1.385	Pass (0.6872)		
10-30 cells/mL	3 to 225	15.4	3.214	Fail (1.539)	19.7 (37.2)	23.4
	3 to 232	13.7	3.716	Fail (1.373)		
	3 to 234	30	1.721	Fail (2.998)		
75-150 cells/mL	14 to 993	68.9	3.171	Fail (6.89)	71.1 (9.73)	103.0
	17 to 1220	80.5	3.333	Fail (8.054)		
	15 to 1105	64	3.796	Fail (6.405)		

*Values that indicate the use of a 30-µm filter, as prompted by FastBallast.

Table 4. Phase I FastBallast analysis and microscopic cell counts for live *H. pluvialis* in LW-TMH.

Sample Description	FastBallast Results					Microscopic Mean Count (cells/mL)
	Level 1 (cells/mL)	Level 2 (cells/mL)	SCF	Result	Mean Count (CV) (cells/mL)	
0 cells/mL (Blank)	0 to 3	NA	NA	Pass (0.07259)	<1	0
	0.2 to 15	<1	3.255	Pass (<0.001)		
	0.1 to 11	<1	2.406	Pass (<0.001)		
<10 cells/mL	0 to 16	2	1.773	Pass (0.1995)	1.20 (29.0)	3.92
	0 to 15	1.1	3.06	Pass (0.1056)		
	0.2 to 16	<1	3.624	Pass (<0.001)		
10-30 cells/mL	1 to 96	5.5*	3.846	Pass (0.5474)	5.87 (4.89)	19.9
	1 to 68	5.9*	2.543	Pass (0.5852)		
	1 to 91	6.2*	3.221	Pass (0.6183)		
75-150 cells/mL	7 to 500	86.9	1.265	Fail (8.694)	84.1 (35.3)	89.0
	7 to 537	119	0.994	Fail (11.9)		
	7 to 514	46.5	2.429	Fail (4.654)		

*Values that indicate the use of a 30-µm filter, as prompted by FastBallast.

The data shown in Tables 3 and 4 are shown graphically in Figure 8. The *H. pluvialis* concentrations determined by microscopic analysis (LSRI, 2020c) are on the x-axis and the FastBallast analysis results are on the y-axis. The trials using LW to simulate high transparency conditions are shown in blue, while trials using LW-TMH used to simulate low transparency conditions are shown in orange. There was a strong positive correlation between the FastBallast counts and the microscopy counts ($R^2 > 0.98$) in both water types. A slightly stronger correlation was noted in the LW water ($R^2 = 0.9976$).

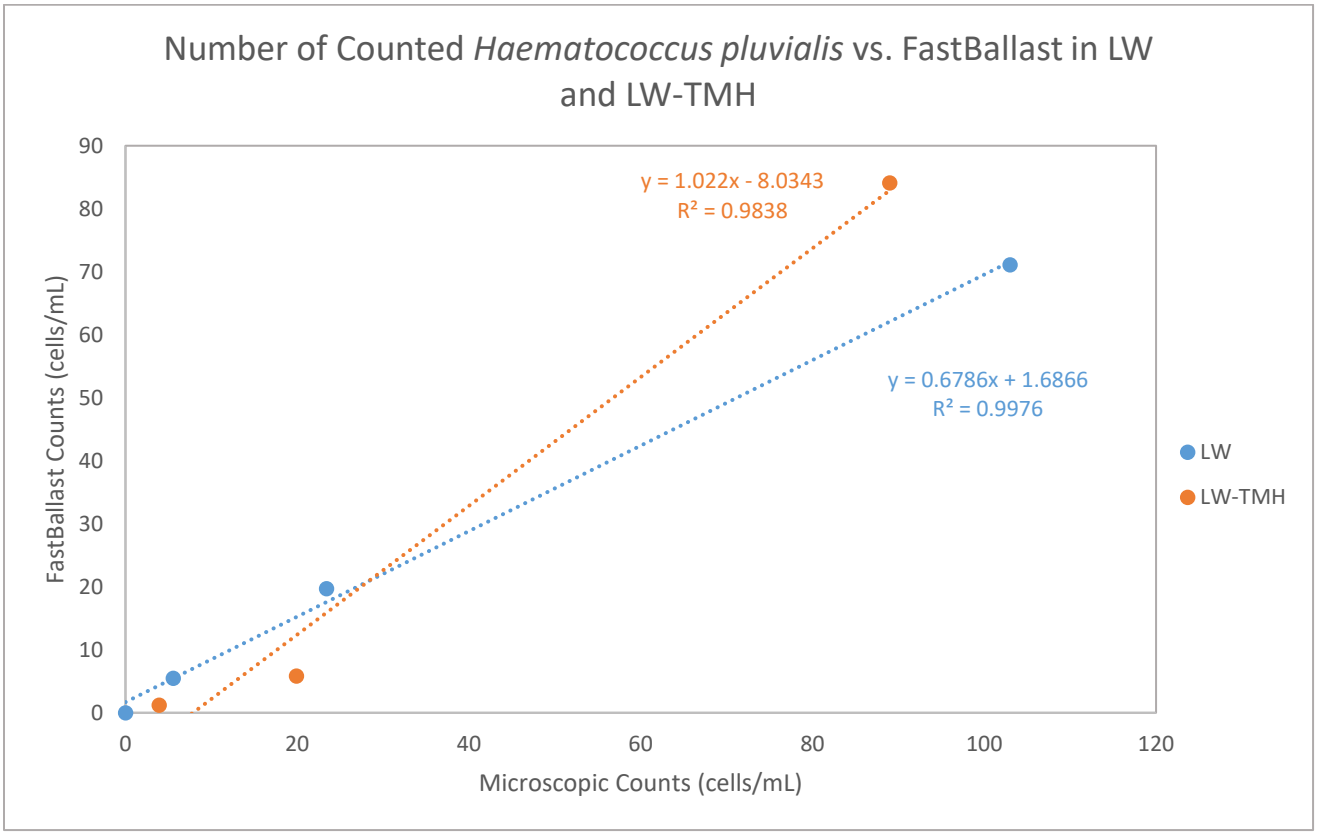

Figure 8. *H. pluvialis* counts vs. FastBallast counts in LW and LW-TMH.

4.1.2 *SCENEDESMUS QUADRICAUDA*

The dimensions of a subsample of *S. quadricauda* were measured (Table 5 and Figure 9) and individual cells were found to have an average length 18 µm (14-27 µm) while colonies had an average length of 22.7 µm (14-32 µm) without spines and 40.1 µm (23-51 µm) with spines. The majority of colonies consisted of 2 or 4 cells, however colonies observed during the evaluation of the device ranged from 1-8 cells. Cells had an average width of 7.7 µm.

Under fluorescence with FDA/CMFDA stain *S. quadricauda* appeared red in color versus green, which was discussed to potentially be an alternate vital signal. Chlorophyll glows red under fluorescent activation with blue light, so the red color we observed indicated a strong chlorophyll signal, potentially

overwhelming the green metabolic signal that would come from the stain. It is also possible some unknown condition prevented the prescribed function of FDA/CMFDA in *S. quadricauda*. Whatever the case, the chlorophyll signal suggested the cells were healthy and so use of that signal was deemed an appropriate alternative for identifying live cells. A test of heat killed organisms confirmed that the *S. quadricauda* cells no longer fluoresced after death.

Table 5. *Scenedesmus quadricauda* cell and colony measurements.

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

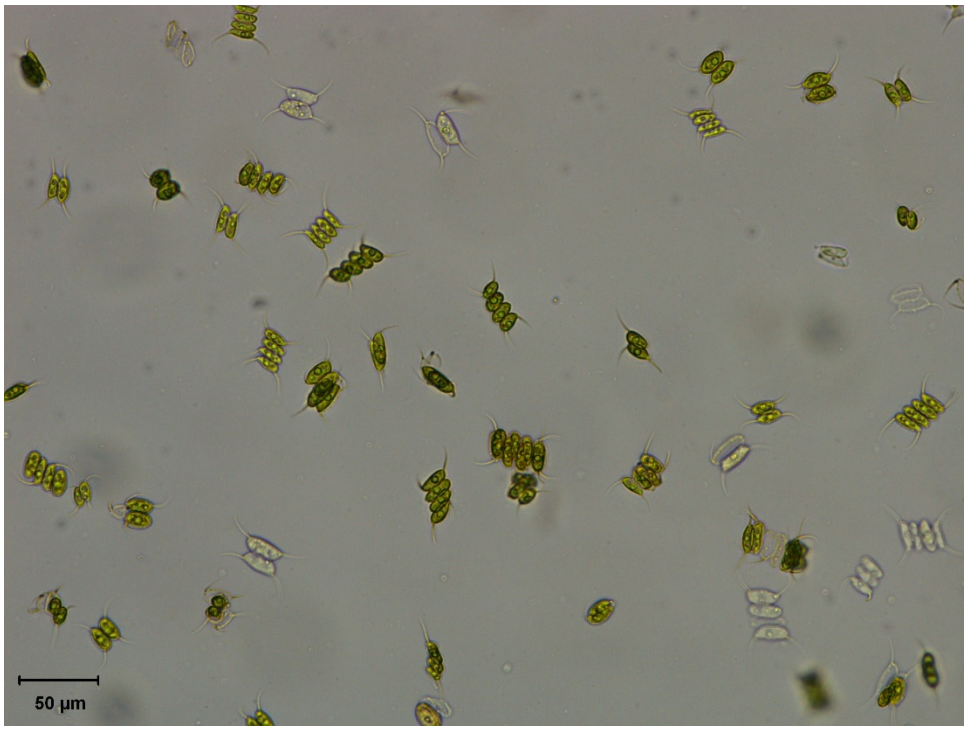

Figure 9. Photograph of *Scenedesmus quadricauda*.

Results of counts of *S. quadricauda* samples in LW and LW-TMH determined microscopically as well as using the FastBallast device are shown in Table 6 and 7, respectively. Target concentrations of the *S. quadricauda* in both water types were 0 (experimental blank), <10, 10-30 and 75-150 cells/mL. All blank samples analyzed had microscopic counts of 0 cells/mL. In LW, the final microscopic cell count averages for each range were 0, 6.8, 14.2, and 89.4 cells/mL while FastBallast count averages for each range in LW were 0, 0, 0, and 23.2 cells/mL (Table 6). In LW-TMH, the final microscopic cell count averages for each range were 0, 2.6, 16.2, and 91.2 cells/mL and FastBallast counts averages were <1, <1, <1, and, 6.6 cells/mL (Table 7). It is possible that the spines on the *S. quadricauda* resulted in some of the colonies being filtered out in the 41 µm filtration step.

The cells counted in the <10 cells/mL target range in LW water samples had a 0.382 (13/34) of colony entities to total number of cells ratio, while the 75-150 cells/mL target range samples had 0.367 (164/447) ratio of entities to total cells. These ratios were used to calculate the estimated number of entities in the LW and LW-TMH water samples. Tables 6 and 7 show that only cells in the 75-150 cells/mL target range were detected by the FastBallast device and no cells were detected by the FastBallast device in all other target ranges. The risk was correctly assigned in the blank and <10 cells/mL samples in both water types and in the 75-150 cells/mL samples in LW. Low risk was assigned in the 10-30 cells/mL samples in both water types and in the 75-150 cells/mL samples in LW-TMH, while the microscopic count for these samples was >10 cells/mL and above the IMO D-2 discharge standard. In both LW and LW-TMH, the counts produced by FastBallast were more similar to the estimated number of entities than the number of individual cells counted. The CV was only calculable in the 75-150 cells/mL samples for both water types due to the average result of 0 or <1 in all other samples. The CV for LW was 31.0 and for LW-TMH was 3.96.

Table 6. Results of FastBallast analysis and microscopic cell counts of *S. quadricauda* in LW.

Sample Description	FastBallast Results					Microscopic Counts	
	Level 1 (cells/mL)	Level 2 (cells/mL)	SCF	Result	Mean Count (CV) (cells/mL)	Mean (cells/mL)	Estimated Number of Entities
0 cells/mL (Blank)	0 to 0	NA	NA	Pass (0.005664)	0	0	0
	0 to 1	NA	NA	Pass (0.01499)			
	0 to 0	NA	NA	Pass (0.005988)			
<10 cells/mL	0 to 0	NA	NA	Pass (0.002188)	0	6.8	2.6
	0 to 0	NA	NA	Pass (0.001741)			
	0 to 0	NA	NA	Pass (0.001769)			
10- 30 cells/mL	0 to 0	NA	NA	Pass (0.001652)	0	14.2	5.2
	0 to 0	NA	NA	Pass (0.002749)			
	0 to 0	NA	NA	Pass (0.001476)			
75- 150 cells/mL	2 to 147	16.6	1.945	Fail (1.657)	23.2 (31.0)	89.4	32.7
	2 to 163	33.2	1.077	Fail (3.321)			
	2 to 139	19.8	1.544	Fail (1.98)			

Table 7. Results of FastBallast analysis and microscopic cell counts of *S. quadricauda* in LW-TMH.

Sample Description	FastBallast Results					Microscopic Counts	
	Level 1 (cells/mL)	Level 2 (cells/mL)	SCF	Result	Mean Count (CV) (cells/mL)	Mean (cells/mL)	Estimated Number of Entities
0 cells/mL (Blank)	0.1 to 10	<1	NA	Pass (<0.001)	<1	0	0
	0.2 to 15	<1	NA	Pass (<0.001)			
	0 to 11	NA	NA	Pass (0.2455)			
<10 cell/mL	0.1 to 4	NA	NA	Pass (0.09216)	<1	2.6	0.9
	0 to 12	<1	NA	Pass (<0.001)			
	0 to 14	<1	NA	Pass (<0.001)			
10- 30 cells/mL	0 to 17	<1	NA	Pass (<0.001)	<1	16.2	5.6
	0 to 15	<1	NA	Pass (<0.001)			
	0 to 16	<1	NA	Pass (<0.001)			
75- 150 cells/mL	1.4 to 102	6.4*	3.516	Pass (0.6406)	6.6 (3.96)	91.2	31.7
	1.1 to 83	6.5*	2.837	Pass (0.6455)			
	1.2 to 85	7*	2.692	Pass (0.6957)			

*Values that indicate the use of a 30-µm filter, as prompted by FastBallast.

The data in Tables 6 and 7 are shown graphically in Figure 10. The *S. quadricauda* concentrations determined by following microscopic analysis (LSRI, 2020c) are on the x-axis and the FastBallast analysis results for the ≥10 µm and <50 µm size class are on the y-axis. The trials using LW to simulate high transparency conditions are shown in blue, while the trials using LW-TMH to simulate low transparency conditions are shown in orange. The data displays a strong linear correlation in both water types (R^2 > 0.97); however, these results are due to the FastBallast device only reporting counts within the 75- 150 cells/mL target range.

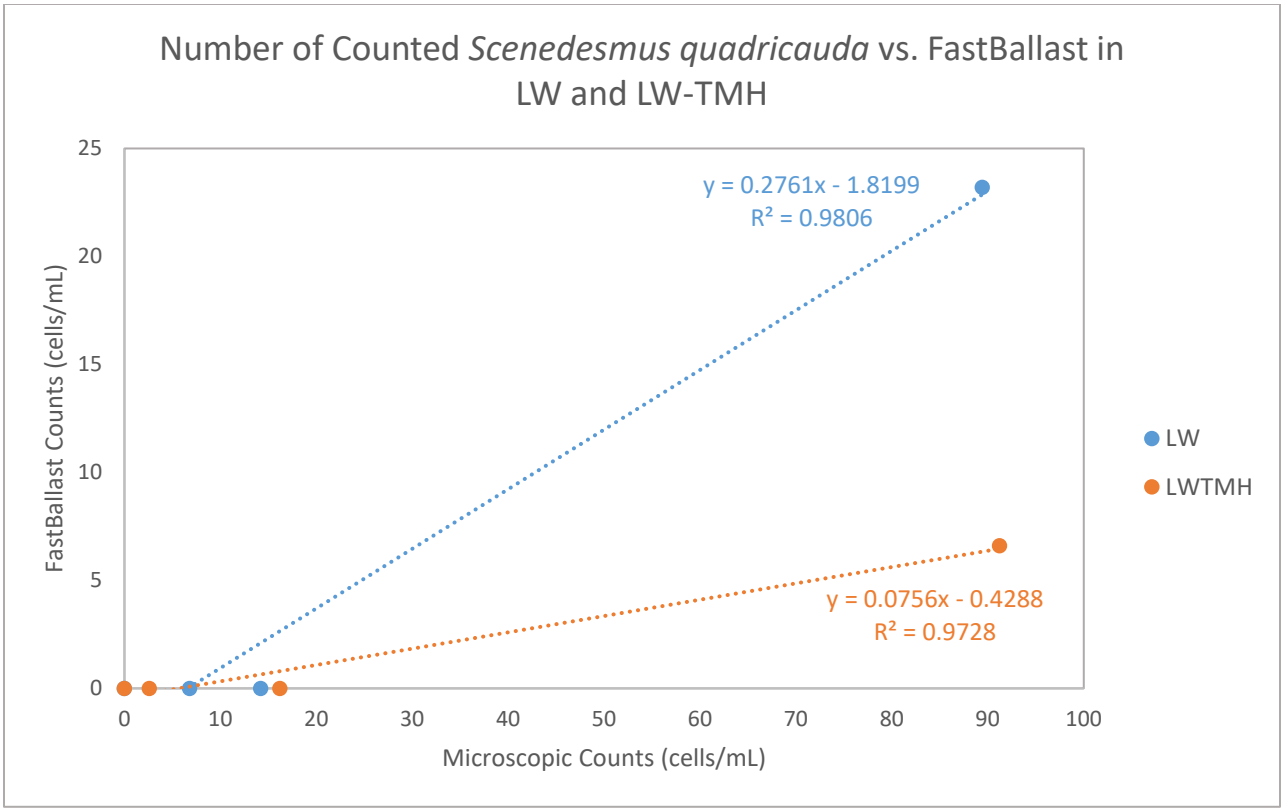

Figure 10. *S. quadricauda* Microscopic Counts vs. FastBallast Counts in LW and LW-TMH.

4.1.3 STATISTICS

All data from Phase I were combined and analyzed by individual species to determine overall probabilities of detecting an exceedance of the D-2 discharge standard based on a sample's concentration (Figure 11). 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). The probability of detecting an exceedance for *H. pluvialis* was 95% when a sample contains 22 cells/mL, but only 0.3% with a sample concentration of 21 cells/mL. *S. quadricauda* only has a 50% probability of detecting exceedance when a sample has a concentration of 90 cells/mL.

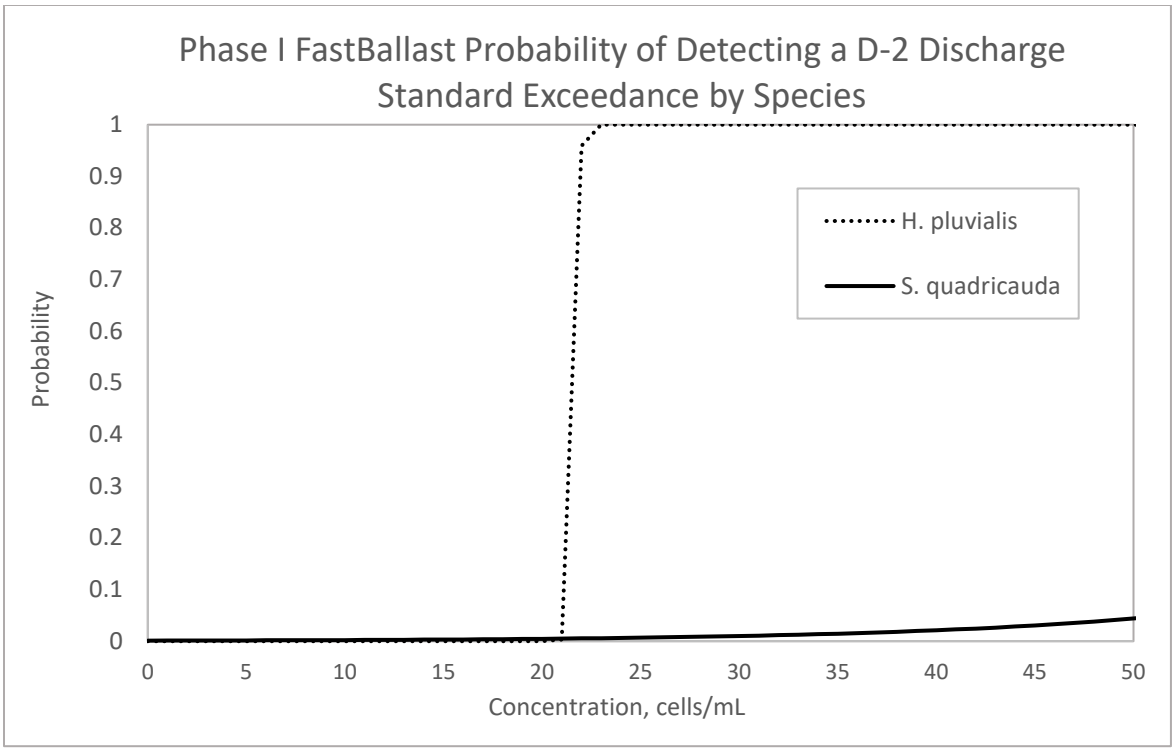

Figure 11. FastBallast Phase I probability of detecting exceedance of D-2 discharge standard.

The accuracy and precision calculations were combined for both water types and species measured for Phase I. Determined by the confusion matrix, accuracy was calculated at 75% for Phase I by calculating the (true positive + true negatives) divided by the total samples. Precision was also determined by confusion matrix and calculated at 66.7%, by taking the true positives divided by (true positives + false positives).

The detection/quantification limits (LOD) of FastBallast were also determined as the capability to discriminate between measurement responses representing different levels of a variable of interest. An attempt was made to determine the quantification limit as described in Section 2.4.4. The LOD was calculated by multiplying the standard deviation of all replicates and corresponding Student t-values, 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. S/N can range from 1 to 10, typically within 3 to 5. A S/N below 2.5 indicates that the random error of the measurements is too high. The *H. pluvialis* were routinely detected at a lower concentration than *S. quadricauda.* The *H. pluvialis* concentrations used for analysis were: 0 (blank), <10, 10-30, and 75-150 cells/mL. There were three replicates for each water type, LW and LW-TMH. The blank and <10 cell/mL samples resulted in a LOD that was too high, therefore it was necessary to do the analysis at a higher concentration. The 10-30 cells/mL data resulted in a LOD of 31.85 cells/mL and a S/N of 1.4. Similarly, the *S. quadricauda* S/N ratio was below one until the 75-150 cells/mL data was analyzed, resulting in an

LOD of 35.83. When using both organisms in both water types the LOD was 101.7 cells/mL with a S/N of 1.2—at lower concentrations the S/N ration was below 1.

4.1.4 WATER QUALITY AND CHEMISTRY

Water quality measurements taken during Phase I testing with FastBallast are shown in Table 8. There were no requirements for the water quality parameters, however, the measurements were within LSRI historical ranges.

Table 8. Water quality measurements made during Phase I testing with *H. pluvialis* and *S. quadricauda*.

Organism	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

Water chemistry measurements taken during Phase I testing with FastBallast are shown in Table 9. Samples of stock water solution were collected prior to addition of organisms. The LW samples are shown without shading while the LW-TMH sample rows have been shaded to differentiate between the water types. All LW and LW-TMH samples were within acceptable limits (Table 1) for all established parameters except for % Transmittance which was out of range due to a change in LW-TMH preparation method (see Table 1). The acceptance range for Percent Transmittance will be revised once enough data has been gathered.

Table 9. Water chemistry data collected in water used for Phase I *H. pluvialis* and *S. quadricauda* 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	1.2	<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	1.0	<2.50	<2.50
S. quadricauda	LW-TMH	21.5	27.7	25.1*	9.3	6.4	8.5	13.0

*Indicates values out of the acceptance range.

4.2 PHASE II

Results from Phase II testing of the protist size classes in Duluth-Superior harbor water using the FastBallast device and microscopic analysis (LSRI, 2020c) are shown in Table 10. A previous trial of Phase II was conducted but not included in the discussion, due to target concentration ranges not being achieved (Appendix 1). During Phase II testing, the water used for experimental blank samples and

dilution water was harbor water filtered through a 934-AH Whatman filter (1.5 µm particle retention). The blank samples were processed and analyzed in the same manner as samples containing organisms.

Appendix 2 shows the detailed taxonomic assessment of the source water used for Phase II testing. The filtered harbor water (dilution water) was verified through microscopic analysis using vital stain to have a live density of 0 cells/mL (LSRI, 2020c). Microscopic counts are presented in the total "allowable" cell density and strictly (ETV Protocol) cell density.

The ambient harbor density of protists on the day of the Phase II verification test was 685.6 cells/mL total "allowable" and 276.3 cells/mL strictly ≥10 and <50 µm in all visible cell dimensions (i.e., 60% of the protist population did not fit the strict ETV Protocol definition of the size class based on minimum dimension). The targeted concentrations were prepared using strictly cell density as an initial concentration. The average microscopic counts in the strictly ≥10 and <50 µm size category were 0.03, 10.0, 34.9, and 106.9 cells/mL and the FastBallast average counts were 104.0, 98.3, 180.9, and 468.3 cells/mL (Table 10). Milli-Q water blanks analyzed between the different concentrations of samples all gave passing (0 to 0 cell/mL) results when analyzed on the FastBallast device. The trial on September 28th showed that the FastBallast measurements were 4-10 times greater than the microscopic counts.

Data in Table 10 is shown graphically in Figure 12. The microscopic counts are shown on the x-axis while the counts reported by FastBallast are shown on the y-axis. While not a predictor of device accuracy, there was a strong positive correlation (R^2 = 0.9841) between the FastBallast counts and the strictly microscopic counts shown on water from the Duluth- Superior Harbor. The FastBallast counts were closer to the total allowable microscopic counts, however the correlation was slightly weaker (R^2 = 0.9653) than the strictly ≥10 and <50 µm microscopic counts.

Table 10. Results of FastBallast analysis and ambient protist microscopic counts in Duluth-Superior Harbor Water.

Sample Description	FastBallast Results					Microscopic Counts	
	Level 1 (cells/mL)	Level 2 (cells/mL)	SCF	Result	Mean Count (CV) (cells/mL)	Mean Total "Allowable" (cells/mL)	Mean Strictly ≥10 µm and <50 µm (cells/mL)
0 cells/mL (Blank)	1.8 to 131	93.1	0.3088	Fail (9.313)	104.0 (8.52)	0	0.03
	1.7 to 123	104.0	0.261	Fail (10.4)			
	1.6 to 120	114.8	0.2293	Fail (11.48)			
5-20 cells/mL	3.7 to 269	73.8	0.8024	Fail (7.383)	98.3 (17.7)	25.2	10.0
	3.7 to 247	112.6	0.4821	Fail (11.26)			
	3.5 to 253	108.5	0.5128	Fail (10.85)			
30-50 cells/mL	8.3 to 602	184.8	0.7171	Fail (18.48)	180.9 (23.3)	105.4	34.9
	8.8 to 637	127.5	1.099	Fail (12.75)			
	8.1 to 591	230.5	0.564	Fail (23.05)			
51-150 cells/mL	16.7 to 1216	478.3	0.5595	Fail (34.54)	468.3 (2.23)	265.0	106.9
	16.6 to 1205	453.9	0.5843	Fail (45.39)			
	16.6 to 1207	472.8	0.5614	Fail (47.28)			

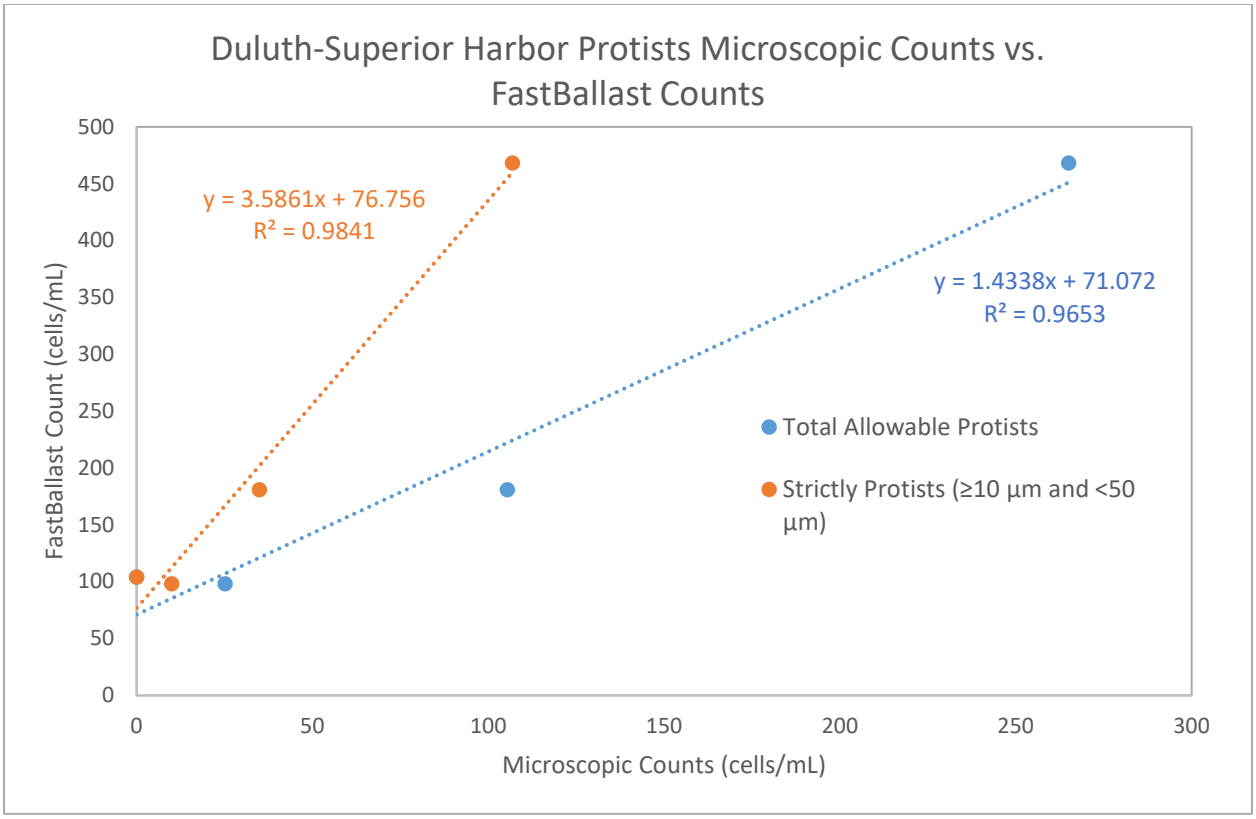

Figure 12. Average protists strictly and total allowable counts vs. FastBallast protists counts.

4.2.1 STATISTICS

Figure 13 shows the probability of FastBallast detecting an exceedance of the D-2 discharge standard based on a sample's concentration using results from Phase II. 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). There is a 50% chance FastBallast will detect an exceedance when a sample contains 0 cells/mL and a 100% chance when there are 3 cells/mL.

The accuracy and precision calculations were determined by the confusion matrix as described in Section 4.1.3 and accuracy was calculated at 50% and precision was not calculable for Phase II results.

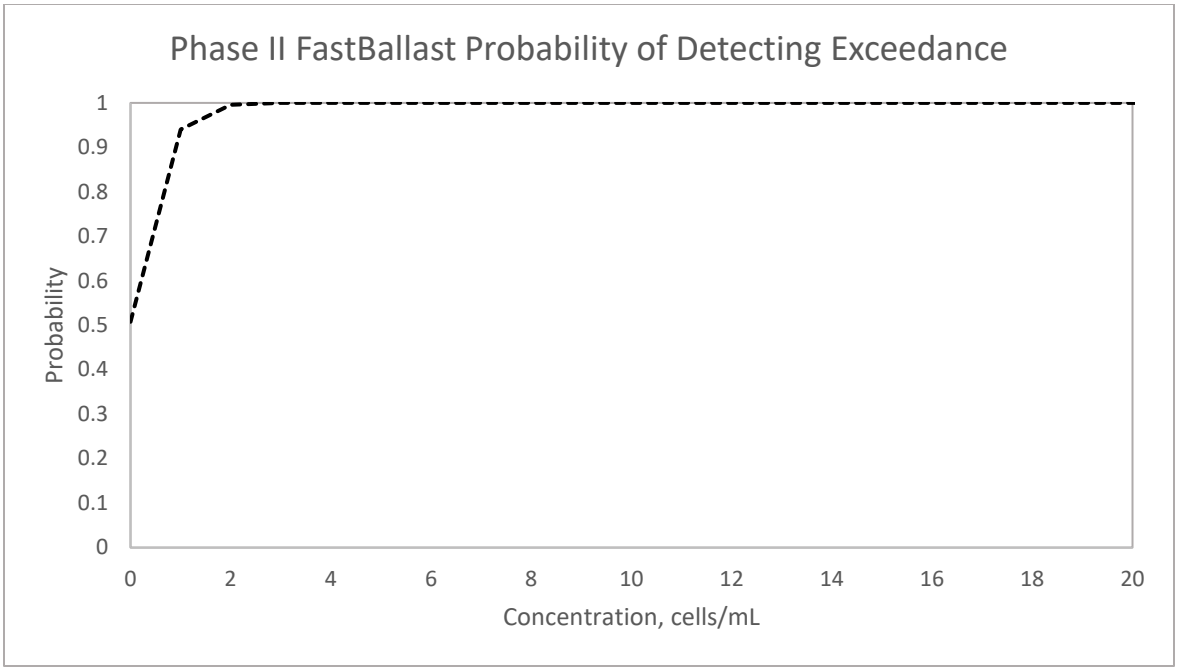

Figure 13. FastBallast Phase II probability of detecting exceedance of D-2 discharge standard.

4.2.2 WATER QUALITY AND CHEMISTRY

Water quality measurements taken during Phase II testing with FastBallast are shown in Table 11. There were no requirements for the water quality parameters, however, the measurements were within historical ranges.

Table 11. Water quality measurements made during Phase II of FastBallast testing. Dilution water measurements were taken prior to filtration.

Water Type	Temperature (°C)	pH	Dissolved Oxygen (mg/L)	Conductivity (µS/cm)	Turbidity (FNU)
Harbor Water Prior to Filtration (Dilution Water)	14.7	7.89	9.4	131.9	1.42
Source Water	13.7	7.06	9.6	173.3	31.2

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

Table 12. Water chemistry measurements made during Phase II testing with FastBallast. Dilution water measurements were taken prior to filtration.

Water Type	TSS (mg/L)	%T Filtered	%T Unfiltered	NPOC (mg/L)	DOC (mg/L)	POM (mg/L)	MM (mg/L)
Harbor Water Prior to Filtration (Dilution Water)	5.7	49.3	45.6	7.5	7.0	1.1	4.6
Source Water	7.1	50.4	45.2	6.8	6.6	1.3	5.8

4.3 PHASE III

Phase III testing occurred on three individual testing events. The BWT technology produced ozone impregnated nanobubbles which are highly oxidative and eliminate microscopic organisms in the treated water. Ozone was analyzed prior to analysis of samples using FastBallast to ensure concentrations of ozone were below the limit of detection. The control discharge sample analysis was omitted from the FastBallast assessment because the in-tank BWT technology had a short treatment time, and the control discharge density would not have been substantively different from that of the uptake density.

In the uptake water, the microscopic strictly count results were 300.4, 252, and 152.9 cells/mL, and the FastBallast results were 648.8, 655.5, and 447.5 cells/mL. The counts from the FastBallast device were closer in range to the live protist total "allowable" counts in the uptake water than they were to the strictly counts (Table 13). The FastBallast counts were on average 2.5 times greater than the count using the strictly ≥10 and <50 µm in all visible cell dimensions method in the uptake water. FastBallast was reading minimal cell counts and ending at a Level 1 measurement in the treatment discharge water of all samples, which is consistent with the microscopic protists counts. In all uptake and treatment discharge samples, the risk level indicated by FastBallast agreed with the microscopic assessment (i.e., a 'fail' is associated with protist densities higher than 10 cells/mL in the strictly ≥10 and <50 µm size class).

Table 13. Results of FastBallast analysis and ambient protist microscopic counts in Phase III.

Trial	FastBallast Results				Microscopic Counts	
	Level 1 (cells/mL)	Level 2 (cells/mL)	SCF	Result	Total "Allowable" (cells/mL)	Strictly ≥10 µm and <50 µm (cells/mL)
Phase III-1 Uptake	30.3 to 2202	648.8	0.7467	Fail (64.88)	665.7	300.4
Phase III-2 Uptake	35.6 to 2591	655.5	0.8697	Fail (65.55)	645.0	252.0

Trial	FastBallast Results				Microscopic Counts	
	Level 1 (cells/mL)	Level 2 (cells/mL)	SCF	Result	Total "Allowable" (cells/mL)	Strictly ≥10 µm and <50 µm (cells/mL)
Phase III-3 Uptake	20.5 to 1494	447.5	0.7345	Fail (44.75)	354.9	152.9
Phase III-1 Treatment	0 to 2	NM	NM	Pass (0.03708)	34.4	0.4
Phase III-2 Treatment	0 to 1	NM	NM	Pass (0.01206)	0	0
Phase III-3 Treatment	0 to 1	NM	NM	Pass (0.01651)	0	0

4.3.1 WATER QUALITY AND CHEMISTRY

Water quality measurements taken during the uptake of Phase III testing with FastBallast are shown in Table 14. There were no requirements for the water quality parameters, however, the measurements were within historical ranges measured in the Duluth-Superior Harbor.

Table 14. Water quality measurements made during Phase III testing with FastBallast in Duluth-Superior harbor water.

Water Type	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

Water chemistry analysis was conducted during the uptake of Phase III testing in Duluth-Superior Harbor Water in order to provide the developer with data to show how naturally occurring turbidity and total suspended solids may impact FastBallast test results. The values obtained during the Phase III testing are shown in Table 15 and are within historical ranges measured in the Duluth-Superior Harbor.

Table 15. Water chemistry measurements made during Phase III testing with FastBallast in Duluth-Superior harbor water.

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

4.3.2 STATISTICS

A logistics regression was not performed on Phase III data since there were not enough data points for an accurate analysis. The accuracy and precision calculations were determined by the confusion matrix as described in Section 4.1.3 and both the accuracy and precision for Phase III was 100%.

5 PERSONNEL RESPONSIBILITIES

All LSRI-GWRC staff who were directly involved in data collection and analysis during the FastBallast verification have completed hands-on and competency training on the procedures for which they were assigned and have read the FastBallast Compliance Monitoring Tool Verification Plan in its entirety prior to testing. Staff have completed the Great Waters Research Collaborative *Conflict of Interest Disclosure Form* prior to the start of test activities.

6 DEVICE USABILITY AND COMPATIBILITY WITH GREAT LAKES CONDITIONS

FastBallast was determined to be 100% reliable based on the calculation that all planned samples were measured and analysis was not interrupted once the measurement began. FastBallast had issues with delays in between samples, with the stir unit or connection to the software, that make the instrument more difficult to use but this did not affect reliability. Due to some of the issues experienced during testing we would recommend that the developer update instructions in the manual regarding the stirrer connection as well as rinsing the stirrer between samples. The manual should address if the stir unit should be unplugged or just turned off during analysis. Unplugging between every sample seemed to be hard on the wiring but when turning off and on there were delays with the tablet recognizing the FastBallast device. Due to the issues experienced with the discrepancies between the FaBtest software located in the files on the ToughPad and the printed report, the software should ensure that both reports display the same results.

The results from Phase II indicate a potential interference related to the harbor water matrix, which was not observed in Phase I testing with laboratory-created experimental water. The Phase II samples were dominated by blue-green algae and diatoms, both of which were taxonomic groups that were not included in Phase I experiments. Further experimentation would be needed to determine the cause of this apparent matrix effect, and whether it is associated with a particular taxon (i.e., blue-green algae) or with a physical-chemical condition of the water itself.

7 QUALITY ASSURANCE/QUALITY CONTROL – DATA QUALITY OBJECTIVES

7.1 PROTIST TESTING

Quality control (QC) counts were not conducted during the protist testing due to COVID-19 restrictions.

Data produced from the FaBtest software were recorded on a datasheet as it was collected during testing and proofed by a second analyst from the printed reports given by FaBtest to account for any errors in recording or discrepancies found within the software output. The printed reports were also verified for accuracy within the software during proofing. All datasheets were stored in a dedicated three-ring binder, as well as scanned and uploaded to secure LSRI Egnyte Cloud storage.

7.2 WATER CHEMISTRY

The data quality objectives (DQO) for water quality and chemistry analyses conducted during the evaluation of the FastBallast are summarized in Table 16. Data quality objectives were met for all measures of precision, bias, and accuracy. The percent completeness exceeded the required percentage for all parameters.

Table 16. Data Quality Objectives (DQOs), criteria, and performance measurement results from water chemistry analyses conducted during FastBallast 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: 11.1%	%TF: 0.2 ± 0.2%
			%TU: 11.1%	%TU: 0.1 ± 0.0%
			NPOC: 11.8%	%NPOC: 8.6 ± 10.2%
			DOC: 11.1%	%DOC: 11.3 ± 4.8%
			POM: 10.5%	POM: 0.0 ± 0.0%
			TSS: 10.5%	TSS: 0.0 ± 0.0%
Bias, Method Blanks	%T method blanks were prepared by filtering Milli-Q samples (one per analysis date).	>98% average %T	Number of %T Method Blanks Analyzed: 9	Method Blanks (%T): 99.7 ± 0.3%
	TSS/POM method blanks were prepared by filtering Milli-Q samples from a 1L sample bottle (one per analysis date) and then drying, weighing, ashing and weighing the filter.	<1.25 mg/L average TSS/POM	Number of TSS Method Blanks Analyzed: 10	Method Blanks (TSS) = <1.25 ± 0
			Number of POM Method	Method Blanks (POM) = <1.25 ± 0

Data Quality Indicator	Evaluation Process/Performance Measurement	Data Quality Objective	Performance Measurement Result	
			Blanks Analyzed: 10	
	NPOC blanks were prepared by acidifying a volume of Milli-Q to 0.2% with concentrated hydrochloric acid.	<0.48 mg/L average NPOC	Number of NPOC Blanks Analyzed: 24	Blanks (NPOC) = <0.48 ± 0
	DOC method blanks were prepared by filtering Milli-Q samples from a 125 mL glass sample bottle (one per analysis date).	<1.6 mg/L average DOC	Number of DOC Method Blanks Analyzed: 11	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: 23.5%	NPOC/DOC Spike Recovery = 98.9 ± 3.3
	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: 2.4 ± 1.0%
			NPOC: 100%	NPOC: 8.2 ± 3.1%
		A least one per 10 samples <10% average D	Percentage (vs total samples) Check Standards:	NPOC 10 mg/L Standard % Difference
			NPOC/DOC: 69%	3.2 ± 2.2%
Representativeness	All samples were collected, handled, and analyzed in the same manner.	Not Applicable – Qualitative.	All water chemistry/quality samples were collected, handled, transported and analyzed in the same manner using the appropriate SOPs.	
Comparability	Routine procedures were conducted according to appropriate SOPs to ensure consistency between tests.	Not Applicable – Qualitative.	The SOPs listed in the methods and references section were used for all water chemistry and water quality analyses.	
Completeness	Percentage of valid (i.e., collected, handled, analyzed correctly and meeting DQOs) water chemistry samples measured out of the total number of water chemistry samples	>90% C	TSS: 100%	
			%T Filtered: 95%	
			%T Unfiltered: 95%	
			NPOC: 89%	
			DOC: 95%	

Data Quality Indicator	Evaluation Process/Performance Measurement	Data Quality Objective	Performance Measurement Result
	collected. Performance is measured by percent completeness (%C).		
Sensitivity	The limit of detection (LOD) and limit of quantification (LOQ) for each analyte and analytical method utilized was determined annually unless a reporting limit was used based on the amount filtered as was the case with TSS/POM.	Not Applicable	TSS/POM Reporting Limit: 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 7 February 2020

8 CONCLUSIONS AND DISCUSSION

The LSRI-GWRC freshwater verification of the FastBallast device met the stated objectives, as outlined in the TQAP (LSRI, 2020a). The reported deviations do not impact LSRI-GWRC's ability to draw conclusions on FastBallast performance during this verification process. FastBallast was operated in accordance with the developer's instructions and operated reliably during all reported tests with the exceptions noted in Section 3. All samples intended to be measured were measured, which shows that FastBallast is a reliable device, as defined by the completeness of dataset, for measuring protists in low and high transparency water types.

To determine the effectiveness of the FastBallast device, a series of questions were addressed through experimentation:

Objectives 1: Do results from sample analysis by the FastBallast correlate to detailed microscopic analysis of freshwater laboratory-cultured organisms in the protist size class, both in single-celled and colonial protists?

The FastBallast results from the single celled protist *H. pluvialis* were closely related to the microscopic counts in both LW and LW-TMH. All results from FastBallast read below the microscopic counts and the experimental blanks read as 0 or <1 cells/mL. The risk was correctly categorized for all but the 10–30 cells/mL in LW-TMH. The coefficients of variation ranged from 9.73 to 37.2 in LW and from 4.89 to 35.3 in LW-TMH, and the R^2 calculated from linear regression was >0.98 for both water types indicating a high level of precision and accuracy. The probability of detecting an exceedance for *H. pluvialis* was 95% when a sample contains 22 cells/mL, but only 0.3% with a sample concentration of 21 cells/mL. All samples intended to be measured in the *H. pluvialis* trials were measured, which shows additional support that FastBallast is an effective and reliable device for measuring the single celled protist *H. pluvialis* in low and high transparency water types.

The FastBallast results from the colonial protist *S. quadricauda* were not closely related to the microscopic counts in either LW or LW-TMH. Results from FastBallast in three of the four targeted ranges did not detect any cells in either water type, and cells were only detected in the 51-150 cells/mL range. This is unusual given that FastBallast functions by detecting chlorophyll in samples and the chlorophyll signal was very clear with the microscopy method (LSRI, 2020c). Within the 51-150 cells/mL samples, the counts from FastBallast were more closely related to the estimated number of entities rather than the number of individual cells. The coefficient of variation in LW in the highest target range was 31.0 and 3.96 in LW-TMH, indicating a high level of precision in the countable results within the 51-150 cells/mL target range. The R^2 calculated from linear regression was >0.97 in both water types and when comparing FastBallast results to both the number of cells and the estimated number of entities. These results however, are based on a 2-point curve due to FastBallast only detecting cells in one target range, showing a low level of accuracy from the device. *S. quadricauda* only had a 50% probability of detecting exceedance when a sample had a concentration of 90 cells/mL.

Some of the colonies of *S. quadricauda* were 47- 51 µm in length when the spines were included (Table 4). Due to the initial filtration step with a 41-µm filter for the FastBallast device, this could cause some colonies to be filtered out of the sample, decreasing the counts detected by FastBallast. If the additional 30-µm filtration step was recommended, this would filter out more of the larger colonies and decrease the number of cells/colonies detected by FastBallast. Four of the seven colonies counted by microscopic analysis (LSRI, 2020c) were >41 µm with the spines, and six out of the seven colonies counted were >30 µm, with the spines.

Objective 2. Does water quality, specifically turbidity, transparency and organic carbon content impact the results of FastBallast analysis compared to detailed microscopic analysis of freshwater laboratory-cultured organisms in the protist size class, both in single-celled and colonial protists?

During Phase I testing with LW water to simulate high transparency, low turbidity and low carbon content, and LW-TMH to simulate low transparency and higher turbidity and carbon content, FastBallast slightly underestimated the concentration of cells in both water types and organisms measured compared to microscopic counts. Correlations displayed in LW were slightly stronger ($R^2 > 0.98$) than the correlations displayed in LW-TMH ($R^2 > 0.97$). These correlations suggest that the decreased transparency and increased turbidity and carbon content may slightly effect readings detected by FastBallast, however the data display a minimal effect.

Objective 3. Do results from sample analysis by the FastBallast correlate to detailed microscopic analysis of freshwater organisms in the protist size class collected from western Lake Superior?

During Phase II testing of the FastBallast device, results were compared against microscopic counts of ambient assemblages of protists from samples collected in the Duluth Superior Harbor. All samples analyzed, including the blanks, resulted in a Fail as a risk assessment in

Phase II. The coefficients of variation ranged from 2.23 to 23.3, and the R^2 value was 0.984 from a linear regression. Although the data display a strong positive correlation, the y-intercept of the regression equation indicates the FastBallast results are skewed toward false positives. The two regression equations indicate that if microscopic counts were 0 cell/mL, then the result from FastBallast would be 71 cells/mL (considering the entire protist population) or 77 cells/mL (considering only those protists with cells ≥10 µm in minimum dimension). Based on confusion matrix analysis, FastBallast only had a 50% accuracy for the samples measured and the precision was not calculable. There was a 50% chance FastBallast would detect an exceedance when a sample contained 0 cells/mL and a 100% chance when there were 3 cells/mL, as calculated from Phase II data. The counts reported by FastBallast were 4 to 10 times greater than the microscopic counts, and the experimental blank read above the D-2 discharge standard reading 104.0 cells/mL, while the microscopic count averaged at 0.03 cells/mL. The developers have commented that the blank results from FastBallast suggest that some contamination with live cells was present in the samples, although the microscopic analysis does not support that statement. The developers also noted that if there were cells just below 10 µm in the smallest dimension with a SCF value typical of cells that are larger than 10 µm, FastBallast would count them, but the analysts conducting microscopic analysis of the strictly ≥10 µm and <50 µm would exclude those cells from their count. The mean total "allowable" microscopic counts do more closely concur with the FastBallast results than the strictly ≥10 µm counts.

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

Phase III counts reported by FastBallast on uptake samples were 2.5 times greater than the microscopy counts. As observed during Phase II, the analysis by FastBallast was more similar to the total "allowable" protist count, rather than the strictly ≥10 to <50 µm count in uptake samples. The treatment samples read as 0 to 1 or 0 to 2 cells/mL which was consistent with the microscopic counts. Risk was correctly categorized in all of the uptake and treated discharge samples from the ozone-based treatment technology.

These tests indicated that colonial protists in ambient assemblages may be underestimated by the FastBallast device and the device detects cells outside of the strictly ≥10 to <50 µm size class. Morphology of protists in the ambient assemblage may play a role in the effectiveness of the device. It is important to note that within the Great Lakes, it is not uncommon for a substantial proportion of the protist population to consist of large entities/filaments comprised of cells that are <10 µm in minimum dimension (Reavie & Cangelosi, 2020). In fact, this is typical of freshwater protists in general (Kim et al., 2016).

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Appendix 1. Data from August 11th, 2020 trial of Phase II which were not used in data analysis due to low microscopic concentrations.

Sample Description	Level 1 (cells/mL)	Level 2 (cells/mL)	SCF	Result	FastBallast Mean Count (CV) (cells/mL)	Microscopic Mean Count Total (cells/mL)	Microscopic Mean Count Strictly (cells/mL)
0 cells/mL (Blank)*	1 to 47.7	15.5	0.6760	Fail (1.546)	14.9 (6.62)	1.0 (0.0- 2.5)	0.8 (0.0- 2.5)
	1 to 42.3	14.8	0.6390	Fail (1.478)			
	1 to 40.7	14.5	0.6590	Fail (1.450)			
5-20 cells/mL	0 to 36	12.0	0.6660	Fail (1.197)	11.1 (17.3)	29.7 (6.7- 75.1)	8.0 (5.5-11.7)
	0 to 33	12.8	0.5649	Fail (1.276)			
	1 to 37	8.4	0.9767	Pass (0.8427)			
30-50 cells/mL	1 to 49	11.5	0.9410	Fail (1.15)	11.3 (6.21)	23.4 (6.7 -32.0)	4.0 (0.9- 5.0) 2
	1 to 50	10.4	1.062	Fail (1.04)			
	1 to 48	12.1	0.8730	Fail (1.211)			
51-150 cells/mL*	1 to 79.7	19.7	0.9010	Fail (1.971)	18.8 (14.3)	66.9 (32.5- 133.3)	14.1 (2.2- 22.5)
	1 to 77.7	19.5	0.8800	Fail (1.953)			
	1 to 75	16.5	1.0540	Fail (1.649)			

***Triplicate Subsamples were run in triplicate, except the third sample in the 51-150 cells/mL sample was run in duplicate.**

Appendix 2. Phase II taxonomic characterization of the organisms in the protist size class.

Taxonomy	"Allowable" Minimum Dimension <10 µm (cells/mL)	"Strictly" Minimum Dimension >10 µm (cells/mL)
Blue Greens		
Other filamentous cells	42.6	-
Filamentous-no cells (length)	186.3	-
Merismopedia	16.8	-
Greens		
Scenedesmus	4.2	-
Coccoid	3	-
Single spindle	0.3	NA
Filamentous - cells	0.3	-
Cryptophytes (and other small flagellates)		
Cryptomonas/Chroomonas	0.3	0.6
Round microflagellates	-	2.4
Diatoms		
Chain *(Aulacoseira, Melosira, S. binderanus*)	159.9	40.2
Asterionella	1.5	-
Centric nonchain *(Cyclotella, Stephanodiscus)*	1.2	48.9
Fragilarioid (ribbon colony)	4.8	-
Naviculoid (or other single pennate)	0.9	0.6