BENCH-SCALE TECHNICAL REPORT

FRESHWATER VERIFICATION OF THE MICROWISE BALLASTWISE COMPLIANCE MONITORING DEVICE

MICHAEL NAGEL, CHRISTINE POLKINGHORNE*, KELSEY PRIHODA, MEAGAN ALIFF, KIMBERLY BEESLEY, LANA FANBERG, STEVE GEBHARD, HOLLY WELLARD KELLY, SAMANTHA MCCLUNG, MARYLEE MURPHY, DESIREE NIEWINSKI, EUAN REAVIE, HEIDI SAILLARD, HEIDI SCHAEFER, AND MATTHEW TENEYCK

Lake Superior Research Institute, University of Wisconsin-Superior, Belknap and Catlin Ave., P.O. Box 2000, Superior, WI, USA

Reviewed and Approved by:

KELSEY PRIHODA	Digitally signed by Kelsey R. Prihoda Date: 2021.05.04 09:00:16 -05'00'	5/4/21
Researcher, LSRI and GWRC Program Manager	**Signature**	**Date**

Cleared for Issue by:

MATTHEW TENEYCK	Matthew TenEyck Digitally signed by Matthew TenEyck Date: 2021.05.03 16:19:58 -05'00'	5/3/21
Director, LSRI	**Signature**	**Date**

*Correspondence author. E-mail: cpolking@uwsuper.edu. Web Address: uwsuper.edu/gwrc.

RECORD OF REVISIONS

Revision No. (Effective Date)	Revision Description

ABSTRACT

This technical report presents findings from freshwater verification tests evaluating the performance of the MicroWISE BallastWISE compliance monitoring device, hereafter BallastWISE. BallastWISE was developed by MicroWISE, located in Ebeltoft, Denmark.

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. BallastWISE utilizes separate chambers to enumerate organisms in each of two regulated size classes, ≥10 and <50 µm (nominally protists) and ≥50 µm (nominally zooplankton). Cameras and optical chambers capture video and track motility through software analysis for the zooplankton size class. Fluorescence microscopy evaluates chlorophyll containing organisms in addition to motility tracking in the protist size class.

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 *Eucyclops* spp. and *Daphnia magna*. Phase II testing was completed using naturally occurring Great Lakes organisms in the Duluth-Superior Harbor of 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 also calculated from Phase I data.

Phase I testing showed BallastWISE was effective at quantifying single-celled protists to within about 20% of the microscopic counts, but undercounted colonial protists. Colonial protist entity counts were close to microscopic entity counts suggesting that individuals within the colonies were not resolved. High total suspended solids (TSS) and (DOC) may slightly reduce BallastWISE sensitivity to protists. BallastWISE overcounted zooplankton in both species tested in both high and low TSS/DOC by between 150% and 420%. Phase II testing from the Duluth-Superior Harbor showed BallastWISE counts of natural assemblages of protists strictly in the ≥10 and <50 µm size class to be slightly below microscopic counts by about 35% and with high precision. Zooplankton were overestimated by BallastWISE by roughly 40% and with considerably more variation compared to microscopic counts. Phase III testing showed low BallastWISE accuracy and precision in untreated protist and zooplankton samples. This may have been caused by organism densities higher than the device's effective upper limit of detection in the zooplankton samples, but further investigation would be needed to determine the cause of low accuracy and precision in protist analysis. BallastWISE accurately measured treated protist samples as 0 cells/mL in agreement with strict microscopic counts, but overcounted treated zooplankton samples in 2 out of 3 tests, possibly due to the method of treatment. A number of operational issues made enumeration of zooplankton unreliable, but improvements (e.g., software updates, guidance on device operation) from the developer over the period of this assessment have already improved performance. BallastWISE shows promise as a useful device for detecting and measuring protists and zooplankton in the Great Lakes as additional improvements are made.

KEY WORDS

Compliance Monitoring Device, Ballast Water, Automated Microscope, Fluorescence

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

LSRI-GWRC would like to thank MicroWISE (Ebeltoft, Denmark) for their application to our laboratory-based testing program and for providing the BallastWISE system and the expendable supplies for analysis. Pia Haecky, MicroWISE CEO, and Nicholas Blackburn, MicroWISE Software Development, provided operational training support prior to the start of testing and were also instrumental in helping to troubleshoot technical/operational issues that occurred during testing.

This work was supported by the United States Maritime Administration (United States Department of Transportation; Washington, D.C).

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 technical report details the results of the LSRI-GWRC bench-scale evaluation of the MicroWISE BallastWISE compliance monitoring device (hereafter BallastWISE). Development of BallastWISE by MicroWISE (Ebeltoft, Denmark) was supported by Danish Maritime Fund, The Danish Innovation Fund, The Danish Market Development Fund, and The Ministry of Environment and Food of Denmark.

BallastWISE utilizes optical chambers, video cameras, fluorescence microscopy, and software analysis to quantify living organisms in marine, brackish, and freshwater. This is applicable to both the ≥10 and <50 µm (hereafter protists) and ≥50 µm (hereafter zooplankton) size class of the D-2 standard, which states that ships conducting ballast water management must discharge fewer than 10 viable organism per mL that are ≥10 and <50 µm in minimum dimension and fewer than 10 viable organisms per m^3 that are ≥50 µm in minimum dimension.

A BallastWISE prototype participated in the 2019 Great Lakes Ballast Monitoring Practicums (Ram et al., 2019), and determined that a large portion of the plankton community in the zooplankton size class can be close to the 50 µm size limit. Results of the Practicum prompted a performance upgrade of the BallastWISE system on detection accuracy by increasing camera resolution and decreasing chamber volume to improve performance under Great Lakes conditions.

The freshwater verification of the BallastWISE device 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 BallastWISE 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 BallastWISE analysis compared to detailed microscopic analysis of freshwater laboratory-cultured organisms in the protist and zooplankton size class, both in single-celled and colonial protists?
3. Do results from sample analysis by the BallastWISE 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 the BallastWISE correlate to detailed microscopic analysis of freshwater organisms in the protist and zooplankton size classes in uptake and

treated discharge samples collected during land-based ballast treatment technology at Montreal Pier Facility (Superior, WI)?

To better answer these questions quantitatively, BallastWISE 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), LSRI MicroWISE BallastWISE 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 the quality assurance and quality control (QA and QC) requirements. It was approved by both LSRI-GWRC and MicroWISE prior to the start of the device verification activities. The SOPs followed throughout testing are described in the Section 2 and listed in the Section 8 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 BallastWISE device evaluated by LSRI-GWRC is a commercially available ballast water discharge compliance monitoring device. BallastWISE (Figure 1) consists of a waterproof hard plastic Peli® case (38 x 31 x 18 cm) housing the measuring chambers and video cameras. Two measuring chambers, one for each of the two size classes (protist and zooplankton), are connected by silicone tubing to peristaltic pumps. Video is recorded by 6 MP machine vision industrial cameras with LED illumination. The BallastWISE device is operated with a computer that connects to the device via a USB cable. Minimum computer requirements are 8GB RAM, quad core CPU, hard drive write speed >120 MB/s, and BallastWISE software. MicroWISE provided the computer used for this evaluation (Figure 2), which was housed in a similar, smaller Peli case.

Figure 1. BallastWISE case with tubing and sample chambers.

Figure 2. BallastWISE computer and software.

A container holding a water sample is placed near the device and the intake tubing is inserted into the sample. The outflow tubing is placed in a second container to capture the analyzed sample. The BallastWISE software is used to input the sample information and desired size class(es) to be analyzed. The device can analyze both size classes simultaneously or individually. If zooplankton size class samples were concentrated before the analysis, the concentration factor (initial volume divided by the final volume) can be input to the software to account for the increased organism density. The device pumps a small volume of the sample into the measuring chamber and then pauses, allowing the sample to settle. The protist size class chamber (Figure 3) analyzes approximately 1 mL of sample over 40 chamber volumes and takes approximately 20 minutes to complete. The zooplankton size class chamber (Figure 4) analyzes approximately 800 mL of sample over 20 chamber volumes and takes approximately 30 minutes to complete. In the protist size class, phototrophic organisms are first evaluated using an imaging PAM (Pulse Amplitude Modulation) method. A low intensity flash and a high intensity flash are each followed by a camera image capture to measure fluorescence signals. In both size classes, video tracks and records organism movement. The sample is then discharged from the chamber and a new sample portion is analyzed. A still image or live feed of the camera is displayed on the software whenever a sample portion is being analyzed by the device. A running estimate of organism density is displayed with color coding for compliancy expectedness (green: very low risk, yellow: low risk, red: high risk) and is updated after each chamber refill. Very low risk is defined by the BallastWISE device as <13 organisms/m^3 or cells/mL, low risk as ≥13 to <30 organisms/m^3 or cells/mL, and high risk as ≥30

organisms/m³ or cells/mL. Very low risk is slightly above the D-2 discharge standard due to limits on the measurement and sampling accuracy of the device.

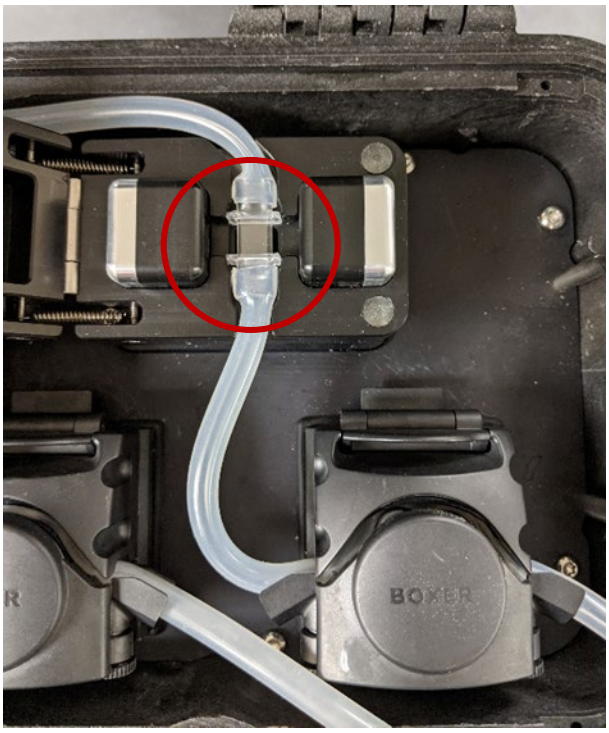


Figure 3. BallastWISE protist sample chamber.

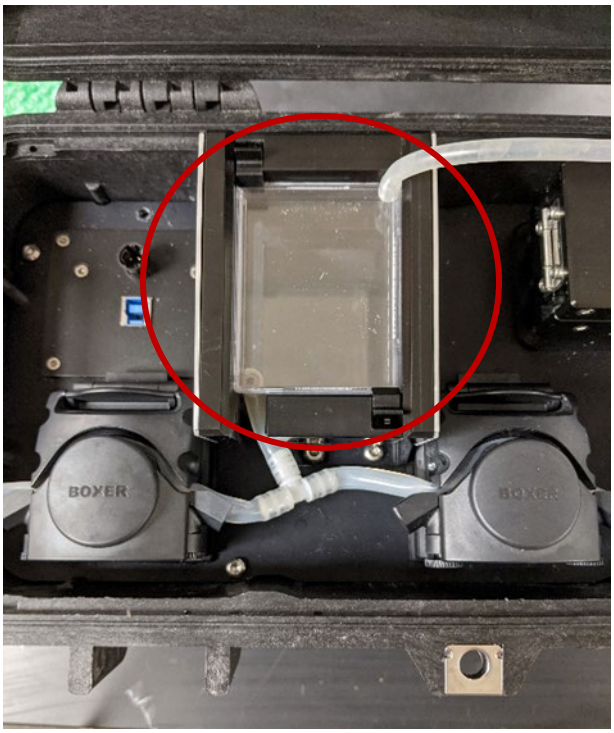


Figure 4. BallastWISE zooplankton sample chamber.

The software saves a file of the analysis report, an image of any motion tracked in the sample, an image of any fluorescence captured, and a histogram of organism sizes contained in the sample.

2.3 BALLAST WATER COMPLIANCE MONITORING DEVICE RECEIPT AND TRAINING

The BallastWISE device and accompanying computer were shipped via FedEx and received by LSRI on July 13th, 2020. MicroWISE provided LSRI-GWRC staff with instruction manuals for the operation of the device. Additional information and instruction were obtained from MicroWISE via email as required over the duration of the evaluation.

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 BallastWISE analysis results to traditional laboratory/microscopic analysis. Freshwater organisms used represented two of the regulated size classes including a single-celled alga and a colonial alga (i.e., protists), and two types of zooplankton (i.e., organisms ≥50 µm). 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 BallastWISE to detect organism motility and chlorophyll in a

sample. Three replicates for each of the size classes and water types with sample densities below the D-2 ballast water discharge standard and one or more sample densities above the D-2 ballast water discharge standard were prepared and analyzed.

LSRI-GWRC staff followed the *BallastWISE Users Guide* (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. Although BallastWISE is capable of analyzing both size classes simultaneously, each size class was analyzed individually for this evaluation.

2.4.1.1 EXPERIMENTAL WATER PREPARATION

Phase I of the BallastWISE 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): LW is municipal water from the City of Superior, Wisconsin (sourced from Lake Superior and is accessed via hot and cold taps located in the LSRI testing lab) 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 chemistry limits for LW and LW-TMH can be found 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
	mg/L	LW	Less than detection - 2

Parameter	Units	Water Type	Acceptable Range for Initiating Bench-Scale Testing
Dissolved Organic Carbon (DOC)		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

Experimental water was prepared as described in Section 2.4.1.1 and was spiked with stock mixtures of *H. pluvialis* or *S. quadricauda* cultures (approximately 10,000 cells/mL) to produce triplicate samples of protists with nominal concentrations within the ranges of 0, <10, 10-30, and 75-150 live 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 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 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, "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 of protists was verified to be within the target ranges by employing a microscopic blind count.

The BallastWISE samples were analyzed as described in Section 2.2, as directed in the *BallastWISE Users Guide* (2020). Samples were placed on a magnetic stir plate during analysis to maintain organism suspension. The system was rinsed with the sample before each analysis and with an additional Milli-Q rinse between samples of different concentrations.

2.4.1.3 ZOOPLANKTON ENUMERATION

Experimental water was prepared as described in Section 2.4.1.1 and *Daphnia magna* or *Eucyclops* spp. were added to the water. Organisms were individually tested at concentrations of 0, 5, 10, 15, and 50

organisms/m³ 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. Organisms for three replicates were counted by one analyst and verified by a second analyst before being added to the water for analysis.

Preliminary trials of the BallastWISE device showed that cultured zooplankton were capable of evading the draw of the intake tubing which could result in systematic undercounting of these samples. At the recommendation of MicroWISE, the volume of water that the device required to run an analysis while leaving a minimum volume of unanalyzed sample was determined. This volume was determined by running BallastWISE five times using blank LW samples, measuring the volume of water used, and calculating the mean of those volumes. The mean volume was determined to be 780 mL. Organisms were added to 780 mL of LW or LW-TMH for the remainder of Phase I zooplankton testing. The concentration factor of these samples was calculated by dividing 1 m³ by 780 mL (1282). The system was rinsed with the sample before each analysis and with an additional Milli-Q rinse between samples of different concentrations.

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. The water was analyzed for live organisms in the protist and zooplankton size classes with BallastWISE and by following the methods required by the ETV Protocol LSRI-GWRC staff followed the *BallastWISE Users Guide* (2020) during all stages of analysis with the device. Before each trial, experimental blank samples of filtered Duluth-Superior Harbor water were analyzed to ensure proper device operation. 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 samples containing organisms. Although BallastWISE is capable of analyzing both size classes simultaneously, each size class was analyzed individually for this evaluation.

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 (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. Triplicate subsamples were prepared and analyzed microscopically for the blank sample and each targeted cell density range. Total live density was conducted on the whole water samples following LSRI, 2020c. Protists were enumerated using the “strictly” and “allowable” methods described in Section 2.4.1.1. BallastWISE results were compared to the “strictly” count and to the total of “strictly” and “allowable” counts, however, BallastWISE is specified to measure organisms in the “strictly” ≥10 µm and <50 µm range. Each test concentration was verified to be within the designated ranges by a microscopic blind count. A

detailed taxonomic analysis of the community composition of this size class was completed on preserved samples (LSRI, 2020c).

2.4.2.2 ZOOPLANKTON ENUMERATION

For the assessment of the zooplankton size class, 1.04 m^3 Duluth-Superior Harbor water was filtered through a 35-µm plankton net and collected into three 20 L carboys. Total live densities and general taxonomic categorization were determined following LSRI SOP *GWRC/25 – Procedure for Zooplankton Analysis* (LSRI, 2021a). Four, 10-15 L sample densities of zooplankton (0, 5-20, 30-50, and >50 live organisms/m^3) were diluted from the initial concentrated sample using filtered harbor water (934-AH Whatman filters, 1.5-µm particle retention). Each sample dilution was verified by microscopic blind counts (LSRI, 2017a). Three replicate samples were collected from each of the four densities and analyzed with BallastWISE. The concentration factor for Phase II zooplankton testing was 1000.

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. Ozone was analyzed before and after microscopic/BallastWISE analysis to ensure concentrations returned to non-detectible concentrations. Untreated uptake and treated discharge samples were collected during three trials of the treatment technology evaluation and were analyzed using BallastWISE and following GWRC's standard operating procedures for microscopic analysis of organisms in the protist (LSRI, 2020c) and zooplankton (LSRI, 2021a) size classes. LSRI-GWRC staff followed the *BallastWISE Users Guide* (2020) during all stages of analysis. Although BallastWISE is capable of analyzing samples in both size classes simultaneously, each size class was analyzed individually for this evaluation.

2.4.3.1 PROTIST ENUMERATION

Protists were enumerated using the "strictly" and "allowable" methods for the assessment of the protist size class, described in Section 2.4.1.2. Samples were prepared and analyzed microscopically for each sample. Total live density was conducted on the whole water samples following LSRI, 2020c. BallastWISE results were compared to the "strictly" count and to the total "strictly" and "allowable."

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 and using BallastWISE. A concentration factor of 1000 was used while conducting this analysis. The BallastWISE results were later adjusted to the final concentration factor when the initial and final sample volumes were determined.

2.4.4 STATISTICAL ANALYSIS

BallastWISE results from Phase I and II and the corresponding microscopic counts were used for statistical analysis utilizing Microsoft Excel. The software was used to calculate the coefficient of variance (CV) for the BallastWISE results. CV is a measure of precision and shows variability in a sample in relation to the sample mean. The data were graphed using Microsoft Excel by plotting microscopic organism counts on the x-axis and the BallastWISE 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. Data were also analyzed for the probability (on a scale of 0 to 1) to detect an exceedance of the D-2 discharge standard to test device accuracy (First, 2018). The binary regression needed for the probability charts was performed using IBM SPSS Statistics and plotted graphically using Microsoft Excel. Phase I data were used to calculate the limit of detection (LOD) for each species of protist tested and for the combined data collected from zooplankton analyses (Tamburri, 2020). BallastWISE was analyzed for reliability by two evaluations. First, a total count of all BallastWISE trials was conducted and each trial was evaluated as either resulting in a successful or failed analysis. The percentage of successful trials was presented as an overall reliability rating on a per analysis basis. Second, the total amount of time spent performing successful and failed trials was determined using the start and end times of each trial. The percentage of time spent on successful trials was presented as an overall reliability rating by time spent.

2.4.5 WATER QUALITY

Water quality measurements were made throughout the duration of the BallastWISE 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 each sample was filtered it was dried in an oven and brought to constant weight. TSS values were determined based on the weight of particulates collected on the filter and the volume of water filtered. The residue from the TSS analysis was ignited to a constant weight at 550°C in a muffle furnace. The concentration of POM was determined by the difference of the dry weight of the particulates on the filter before and after ignition (the mass lost to combustion). Mineral matter concentration is 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 to Measure Dissolved Oxygen in Water Samples* (LSRI, 2017c). 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 is calibrated on a monthly basis following GLM/28- *Procedures for Calibrating and Using the Oakton CON 150 Conductivity/TDS/Temperature Meter* (LSRI, 2021b), respectively. Its 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 the Calibration and Operation of pH Meters Utilizing Automatic Temperature Compensation (ATC)* (LSRI, 1992). A check buffer of 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, 2021c) which was calibrated prior to each test cycle following LSRI SOP *FS/39 – Calibration of YSI EXO2 Multiparameter Water Quality Sondes* (LSRI, 2017d).

2.5 TEST PLAN DEVIATIONS

During testing with the BallastWISE 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 BallastWISE freshwater verification.

Test and Date	Description and Root Cause of Deviation or Quality Control Failure	Description of Corrective Action(s)	Describe the Impact on the Project/Test	Data Qualified? (Y/N)
Phase II Protist, Zooplankton 23 October 2020	According to the test plan, in cases where the same measuring chamber is used for a dilution series, the lowest concentration should be analyzed first with BallastWISE so as to not carry over from a higher dilution to a lower dilution. This was not always done because it could mean that nearly all the samples would be analyzed prior to determining that the device was not functioning optimally. **Root Cause:** When using the device, it was determined that it was necessary to run a higher concentration sample to ensure the device was functioning correctly before proceeding with analysis.	No corrective action taken, as this was a consciously made decision to run one of the highest dilutions early in analysis order to help determine that the device was functioning. The chamber was flushed between dilutions to eliminate carry over.	Minimal effect, no carry over was observed when moving from higher concentration samples to lower concentration samples.	No
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**: Parameter was overlooked in the test plan.	Better review of Test Plan. Summarize data ASAP so it is more apparent if parameters are overlooked.	Minimal, all other water quality parameters were measured.	No
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	No corrective action taken. The microscopically determined concentration of zooplankton was close to the target range and when working	Minimal effect, the concentrations were slightly out of range each concentration was distinct from the other. Test data excluded from final	No

Test and Date	Description and Root Cause of Deviation or Quality Control Failure	Description of Corrective Action(s)	Describe the Impact on the Project/Test	Data Qualified? (Y/N)
	assemblages of organisms, there is inherent variability.	with samples from the environment, there will be some variability when making dilutions.	analysis due to bubble formation in the BallastWISE system.	
Phase II Protist 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 Harbor Water that was collected and analyzed on the same day.	No
Phase III 22 October 2020	For the Phase III testing, only uptake samples and treatment discharge samples were analyzed with compliance monitoring device. 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
Phase II Zooplankton 23 October 2020	The microscopically determined concentration of the zooplankton in the 30-50 org/m^3 dilution was determined to be 54 org/m^3. **Root Cause:** When working with natural assemblages of	No corrective action taken. The microscopically determined concentration of zooplankton was close to the target range and when working	Minimal effect, the concentration was slightly out of range but was different from the next dilution in the series (126 live org/m^3)	No

Test and Date	Description and Root Cause of Deviation or Quality Control Failure	Description of Corrective Action(s)	Describe the Impact on the Project/Test	Data Qualified? (Y/N)
	organisms, there is inherent variability.	with samples from the environment, there will be some variability when making dilutions.		
Phase I LW-TMH HP 19 November 2020	The unfiltered percent transmittance sample from the LWTMH 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 LWTMH without updating the acceptable ranges for parameters using the data we have accrued since the new method was implemented.	The method for preparing LWTMH has changed in the last year and SOP AT/46 was created. However, we have not re-evaluated our data since adopting the new LWTMH 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 I LW-TMH DM 4 December 2020	The unfiltered percent transmittance sample from the LWTMH stock solution produced a result of 24.9 %T which is outside the acceptable range for unfiltered LWTMH (25.5-35.5%). **Root cause:** Using a new method to prepare LWTMH without updating the acceptable ranges for parameters using the data we have accrued since the new method was implemented.	The method for preparing LWTMH has changed in the last year and SOP AT/46 was created. However, we have not re-evaluated our data since adopting the new LWTMH 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 I LW-TMH SQ	The unfiltered percent transmittance sample from the LWTMH stock solution produced a result of 25.1	The method for preparing LWTMH has changed in the last year and SOP AT/46	Minimal, all other water quality parameters were within the target	No

Test and Date	Description and Root Cause of Deviation or Quality Control Failure	Description of Corrective Action(s)	Describe the Impact on the Project/Test	Data Qualified? (Y/N)
12 December 2020	%T which is outside the acceptable range for unfiltered LWTMH (25.5-35.5%). **Root cause:** Using a new method to prepare LWTMH without updating the acceptable ranges for parameters using the data we have accrued since the new method was implemented.	was created. However, we have not re-evaluated our data since adopting the new LWTMH 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.	range for test initiation.	

3 BallastWISE Operational Performance

During the testing period, several operational issues were encountered and have been categorized as either mechanical issues or software issues.

3.1 Mechanical Performance Issues

The most recurrent operational issue while operating the BallastWISE system was the formation of bubbles in the zooplankton size class sample chamber. These bubbles caused large increases in estimated organism density when environmental movement caused the bubbles to fluctuate. When this occurred, large numbers of movement tracks were erroneously counted around the edges of the bubbles (Figures 5 and 6). Chamber placement within its holder effected the formation of bubbles. The chamber is raised slightly in the back-right corner to allow trapped air to leave from the exhaust hose. If the chamber is lying more horizontal, air does not fully leave the chamber and causes bubbles. If the intake hose was placed so that there was extra length after the pump, the extra length could force the sample chamber up in the corner, causing bubbles to form. The peristaltic pump slowly pushed the hose through the pump causing extra length and bubbles to form unless the hose was reset after several samples were analyzed. After reviewing the report, the developer has noted that this can be remedied by adjusting the tension on retainer springs on the guides on both sides of the pump head. A software update was provided by MicroWISE after Phase II and III testing but before Phase I testing that slowed the filling procedure which eliminated most, but not all, bubble issues. After reviewing the report, the developer has noted that small bubbles alone should rarely be a problem for the analysis.

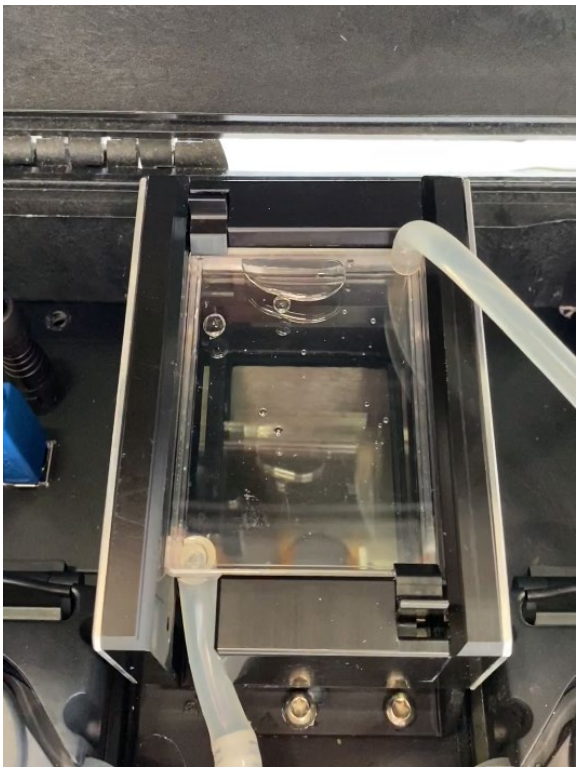


Figure 5. Bubbles in the zooplankton sample chamber.

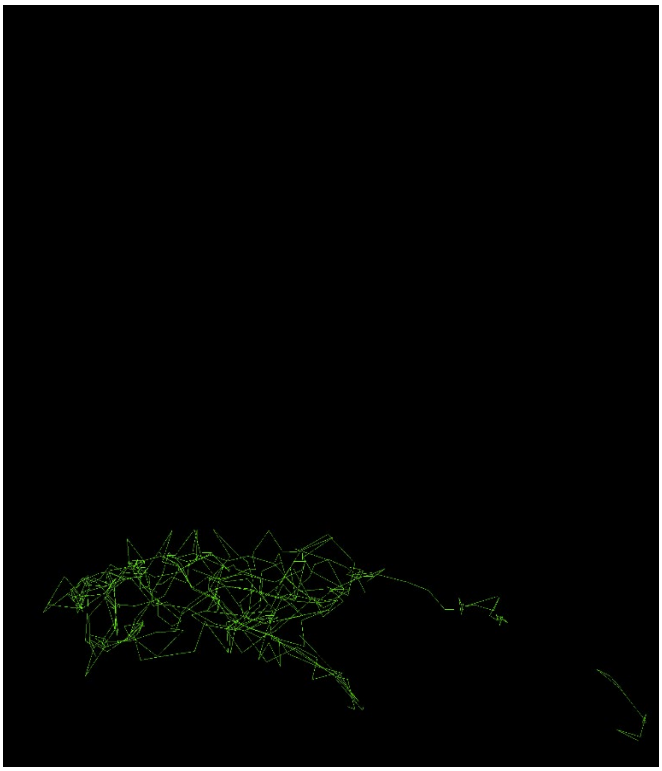


Figure 6. Motion tracking of a bubble in the BallastWISE zooplankton sample chamber.

3.2 SOFTWARE AND COMPUTER ISSUES

The BallastWISE device occasionally could not be detected by the attached laptop, reporting “Error 1 occurred at Unable to find Arduino”. Restarting the system or switching USB ports could remedy this error. This error could also be caused by using characters that were not allowed in the sample name such as “?” or “@”. When analyzing the protist size class, the device reported several "ImagingControl3” errors that caused the device to end any ongoing analysis. Insufficient memory or storage errors occurred several times throughout testing which caused the BallastWISE software to crash. In the case of the storage errors, data needed to be removed from the hard drive to make room for the files created while running a sample. The BallastWISE software occasionally crashed without any error messages, but it is unclear if this was a fault of the software or the laptop on which it was running.

4 RESULTS

Findings from the BallastWISE Phase I, Phase II, and Phase III tests are presented in the following subsections. In result tables with BallastWISE cell or organism counts reported, the values have been highlighted to align with the BallastWISE analysis indication 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). Green highlighting indicates very low risk

(within D-2 regulations), yellow highlighting indicates low risk and red highlighting indicates high risk (above D-2 regulations), Section 2.2. 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

Preliminary Phase I trials raised questions about organism mortality caused by the BallastWISE device, specifically in the zooplankton size class. Additionally, bubble formation issues as discussed in Section 3.1 resulted in these trials being omitted from the final analysis. GWRC staff received guidance from MicroWISE on tubing placement and received a software update which slowed the filling procedure. The operational changes reduced, but did not eliminate, bubble formation in subsequent trials. Further investigation of organism mortality revealed that deaths may have been caused by the stir plate used to mix samples as they were being analyzed. Eliminating the stir plate and using a minimal volume of water to prevent organisms from evading the intake tubing reduced organism mortality. The stir plate was initially used because the *BallastWISE Users Guide* (2020) pictured its use during analysis. Correspondence with MicroWISE revealed that they no longer used a stir plate for samples when analyzing the zooplankton size class.

Phase I testing with zooplankton and protists occurred on eight separate occasions (i.e., one for each species in LW and LW-TMH) after implementing the changes to device operating procedures described above.

4.1.1 HAEMATOCOCCUS PLUVIALIS

Results from BallastWISE and microscopic counts (LSRI, 2020c) from LW and LW-TMH samples containing *H. pluvialis* are shown in Table 3. Phase I microscopic cell counts and BallastWISE cell counts for *H. pluvialis in LW and LW-TMH.* A subsample of *H. pluvialis* (Figure 7) was measured and cells were found to have an average diameter 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 counts of 0 cells/mL in LW and LW-TMH by both analysis methods. In LW, the final microscopic cell count averages for each range were 0, 5.56, 23.4, and 103 cells/mL and BallastWISE counts averaged 0, 2.71, 24.39, and 119.27 cells/mL. The coefficient of variation for the BallastWISE counts ranged from 6.3 to 173.2 with the highest CV in the <10 cells/mL samples. The CV was not calculable for the blank samples as the mean was 0 cells/mL. In the LW-TMH, the final microscopic cell count averages were 0, 3.92, 19.9, and 89.0 cells/mL and the BallastWISE counts averaged 0, 4.74, 20.66, and 77.24 cells/mL. The coefficient of variation for the BallastWISE counts ranged from 2.6 to 65.5 with the highest CV in the <10 cells/mL samples. The CV was not calculable for the blank samples as the mean was 0 cells/mL.

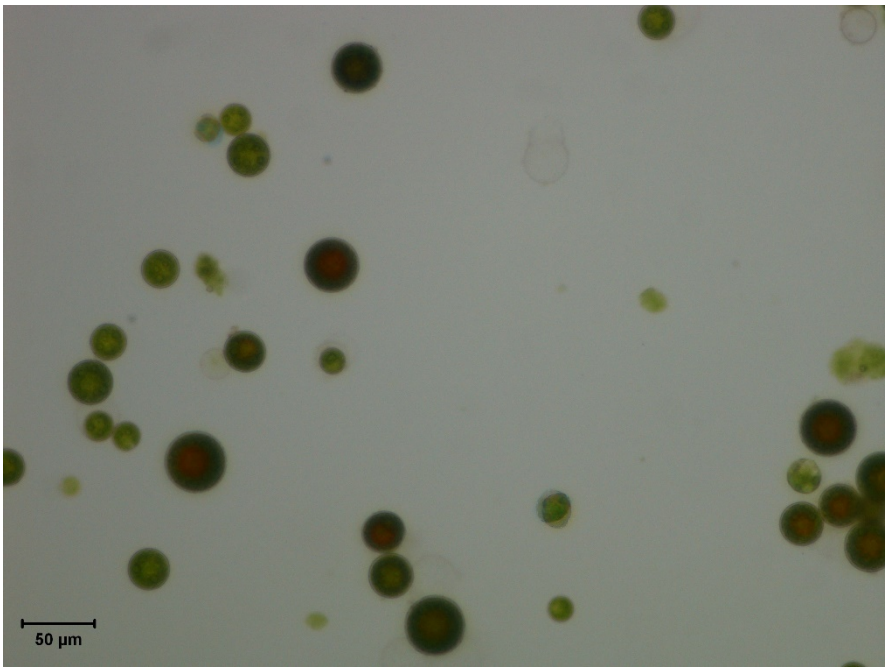


Figure 7. *H. pluvialis* cells.

Table 3. Phase I microscopic cell counts and BallastWISE cell counts for *H. pluvialis in LW and LW-TMH.*

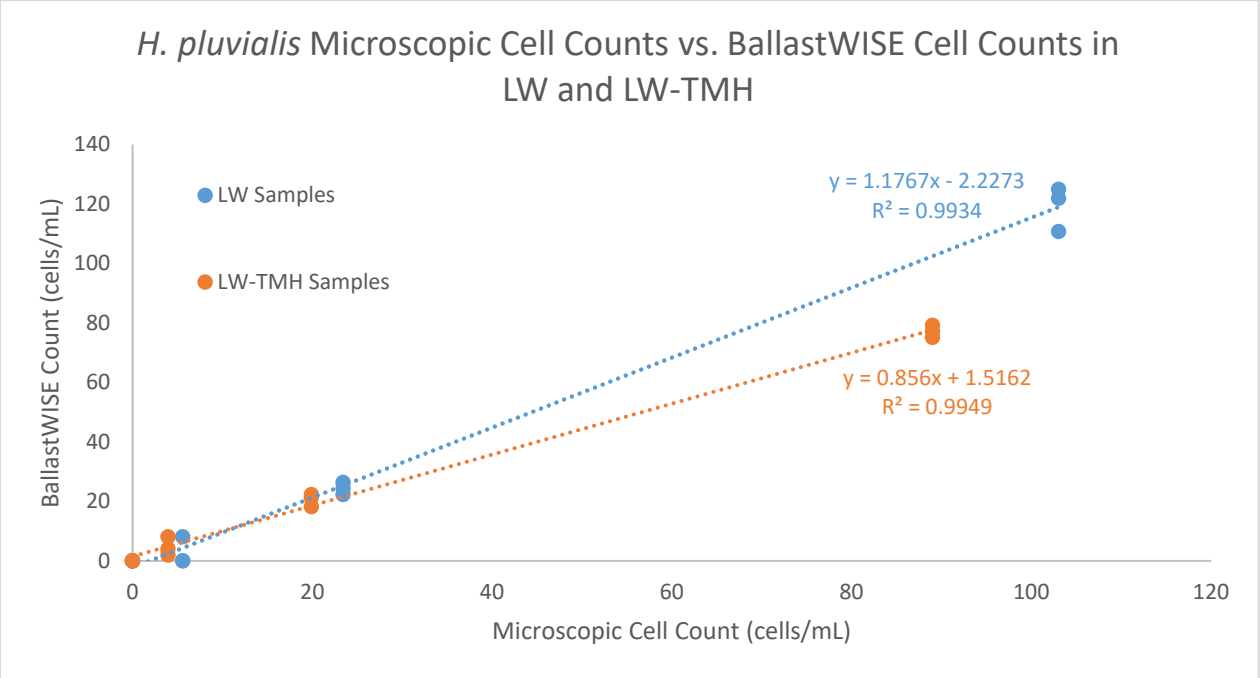
Sample Description	LW Samples			LW-TMH Samples		
	Microscopic Cell Count (cells/mL)	BallastWISE Count (cells/mL)	Mean (CV)	Microscopic Cell Count (cells/mL)	BallastWISE Count (cells/mL)	Mean (CV)
0 cells/mL (Blank)	0	0 ^{VL} 0 ^{VL} 0 ^{VL}	0 (NA)	0	0 ^{VL} 0 ^{VL} 0 ^{VL}	0 (NA)
<10 cells/mL	5.56	0 ^{VL} 0 ^{VL} 8.13 ^{VL}	2.71 (173.2)	3.92	8.13 ^{VL} 2.033 ^{VL} 4.065 ^{VL}	4.74 (65.5)
10-30 cells/mL	23.4	24.39 ^{L} 26.42 ^{L} 22.36 ^{L}	24.39 (8.3)	19.9	18.29 ^{L} 22.36 ^{L} 21.34 ^{L}	20.66 (10.3)
75-150 cells/mL	103	110.8 ^{H} 125 ^{H} 122 ^{H}	119.27 (6.3)	89.0	79.27 ^{H} 75.2 ^{H} 77.24 ^{H}	77.24 (2.6)

Colored highlighting indicates device results for compliance or non-compliancy as described in the BallastWISE user manual. Green indicates very low risk (^{VL}), yellow indicates low risk (^{L}), and red indicates high risk (^{H}).

The data shown in Table 3. Phase I microscopic cell counts and BallastWISE cell counts for *H. pluvialis in LW and LW-TMH.* are shown graphically in Figure 8. The R^2 value for the LW and LW-TMH analyses were

both >0.9 indicating a high level of precision for the device. Analyses in both LW and LW-TMH were close to the expected values obtained by microscopic counts.

Figure 8. *H. pluvialis* microscopic cells counts vs. BallastWISE cell counts in LW and LW-TMH.



4.1.2 *SCENEDESMUS QUADRICAUDA*

The dimensions of a subsample of *S. quadricauda* were measured and results are displayed in Table 4. Cells were found to have an average length of 18.0 µm (14-27 µm cell size range) while colonies had an average length of 22.7 µm (14-32 µm). Colony length including the spikes was an average of 40.1 µm (23-51 µm) with spines counted. The majority of colonies consisted of 2 or 4 cells, but colonies observed during the evaluation of the device ranged from 1-8 cells. Several examples of these colonies are shown in Figure 9.

Table 4. *S. quadricauda* cell/colony lengths with and without spines.

Cell Length -one from colony (µm)	Cell Width -one from colony (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 Colony
17	6	25	47	17	34	4
27	9	32	50	27	37	4 (8, possibly dividing)
14	7	27	49	14	26	4
18	7	28	51	18	35	4

Cell Length -one from colony (µm)	Cell Width -one from colony (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 Colony
16	7	14	23	16	34	2
17	9	16	32	17	36	2
17	9	17	29	17	33	2

Results of counts done on *S. quadricauda* samples in LW and LW-TMH determined by microscopic counts as well as using the BallastWISE system 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. All blank samples analyzed had microscopic counts of 0 cells/mL in both LW and LW-TMH. In LW, the final cell count averages for each range were 0, 6.8, 14.2, and 89.4 cells/mL and BallastWISE counts averaged 0, 0.67, 8.03, and 38.28 cells/mL. The coefficient of variation for the BallastWISE counts ranged from 20.6 to 86.6 with the highest CV in the <10 cells/mL samples. The CV was not calculable for the blank samples as the mean was 0 cells/mL.

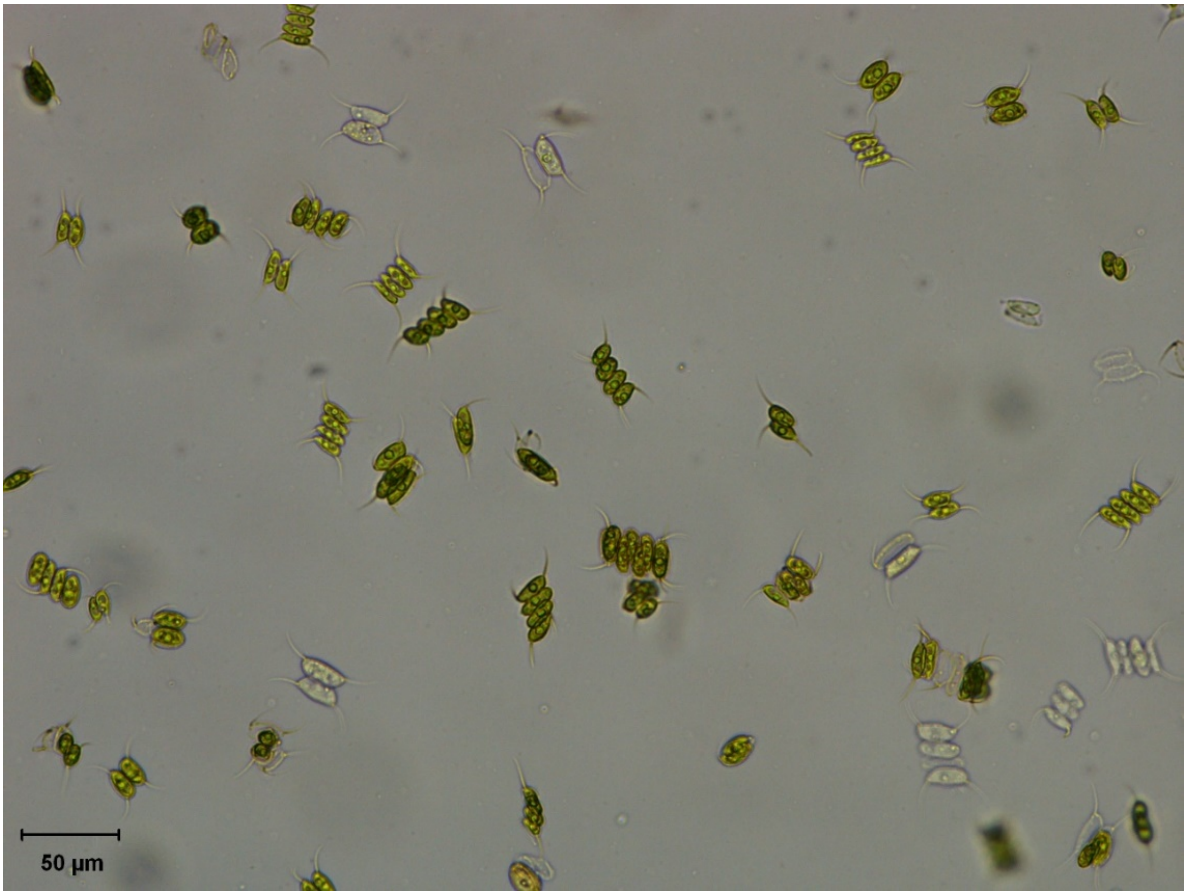


Figure 9. Cultured *S. quadricauda* colonies.

In the LW-TMH, the final cell count averages were 0, 2.6, 16.2, and 91.2 cells/mL and the BallastWISE counts averaged 0, 2.371, 10.16, and 31.5 cells/mL. The coefficient of variation for the BallastWISE

counts ranged from 9.7 to 65.5 with the highest CV in the <10 cells/mL samples. The CV was not calculable for the blank samples as the mean was 0 cells/mL.

The number of entities and the number of colonies plus the number of individual cells for each sample was estimated. In LW, the final estimated entity count average for each range was 0, 2.6, 5.2, and 32.7 entities/mL and in the LW-TMH, the estimated averages were 0, 0.9, 5.6, and 31.7 entities/mL. In both LW and LW-TMH, BallastWISE results were nearer to the estimated number of entities/mL than to the number of cells/mL.

Table 5. Microscopic counts of *S. quadricauda* cells and colonies in LW and LW-TMH.

Sample Description	LW Samples				LW-TMH Samples			
	Microscopic Organism Count (cells/mL)	Estimated Number of Entities (entities/mL)	BallastWISE Cells Counted (cells/mL)	Mean (CV)	Microscopic Organism Count (cells/mL)	Estimated Number of Entities (entities/mL)	BallastWISE Cells Counted (cells/mL)	Mean (CV)
0 cells/mL (Blank)	0	0	0 [VL] 0 [VL] 0 [VL]	0 (NA)	0	0	0 [VL] 0 [VL] 0 [VL]	0 (NA)
<10 cells/mL	6.8	2.6	1.016 [VL] 0 [VL] 1.016 [VL]	0.67 (86.6)	2.6	0.9	4.065 [VL] 2.033 [VL] 1.016 [VL]	2.371 (65.5)
10-30 cells/mL	14.2	5.2	6.098 [VL] 12.2 [VL] 6.098 [VL]	8.03 (43.3)	16.2	5.6	13.21 [L] 6.098 [VL] 11.18 [L]	10.16 (36.0)
75-150 cells/mL	89.4	32.7	29.47 [L] 40.65 [H] 44.72 [H]	38.28 (20.6)	91.2	31.7	28.46 [L] 31.5 [H] 34.55 [H]	31.5 (9.7)

Colored highlighting indicates device results for compliance or non-compliancy as described in the BallastWISE user manual. Green indicates very low risk ([VL]), yellow indicates low risk ([L]), and red indicates high risk ([H]).

Figure 10 shows the microscopic cell count versus the BallastWISE cell count in both water types. Figure 11 shows the microscopic entity count versus the BallastWISE cell count in both water types. The R^2 values for all data sets were >0.9 indicating good precision of the device. *S. quadricauda* cell counts by BallastWISE were well below microscopic cell counts in both LW and LW-TMH. When BallastWISE results were compared to microscopic entity counts instead, BallastWISE results were very near the expected values in both LW and LW-TMH.

Figure 10. Microscopic *S. quadricauda* cell counts vs. BallastWISE cell counts in LW and LW-TMH.

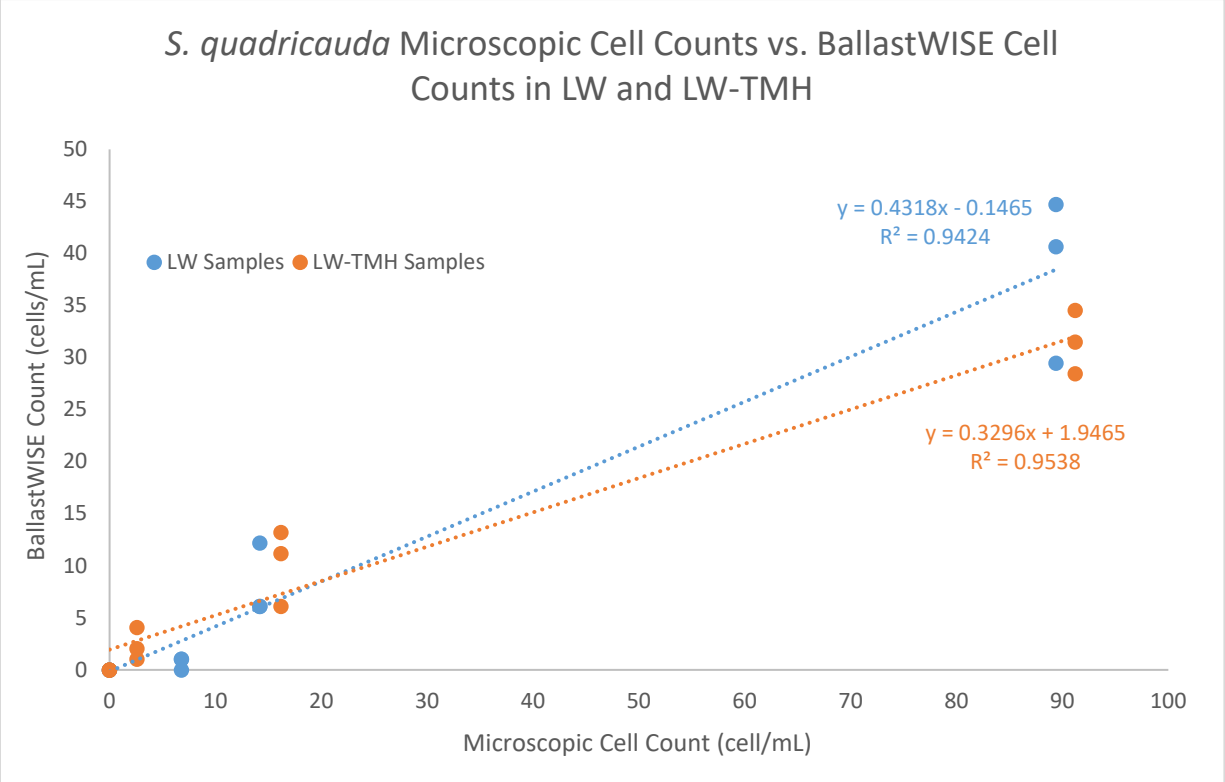
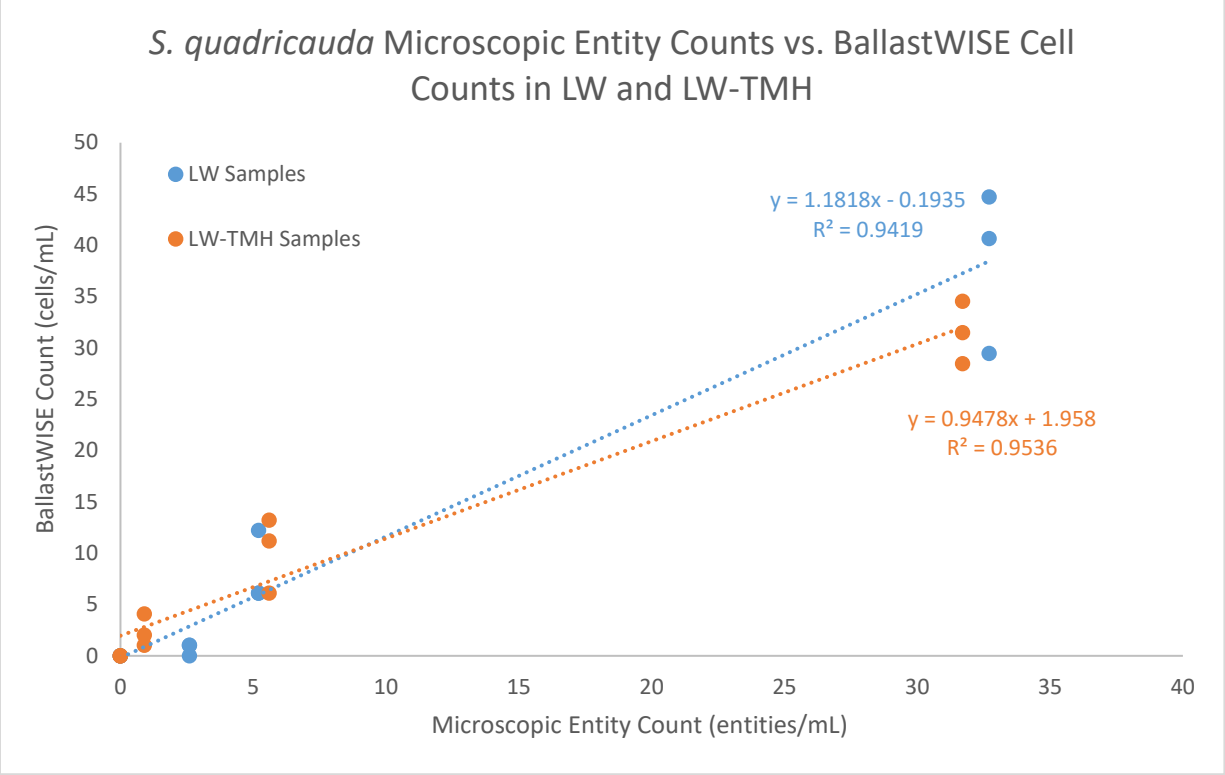


Figure 11. Microscopic *S. quadricauda* entity counts vs. BallastWISE cell counts in LW and LW-TMH.



4.1.3 DAPHNIA MAGNA

Results from the BallastWISE analysis of LW and LW-TMH samples containing *D. magna* <48 hours of age are shown in Table 6. The size of *D. magna* was on average 1045 µm ± 109 µm. No CV values are provided for the organism counts for the zooplankton samples because all samples were counted by one analyst and the count was verified by a second analyst, which resulted in all samples having the same target density of organisms. The number of organisms added to each sample were 0, 5, 10, 15, and 50 organisms/m³. All blank samples had counts of 0 organisms/m³ in both water types. BallastWISE organism count averages in LW were 0, 9.45, 14.76, 24.21, and 76.76 organisms/m³. BallastWISE organism count averages in LW-TMH were 0, 16.54, 27.16, 43.70, and 126.37 organisms/m³. The coefficients of variance ranged from 13.9 to 39.0 in LW and 18.3-49.4 LW-TMH and generally decreased with increasing organisms. The CV was not calculable for the blank samples as the mean was 0 organisms/m³.

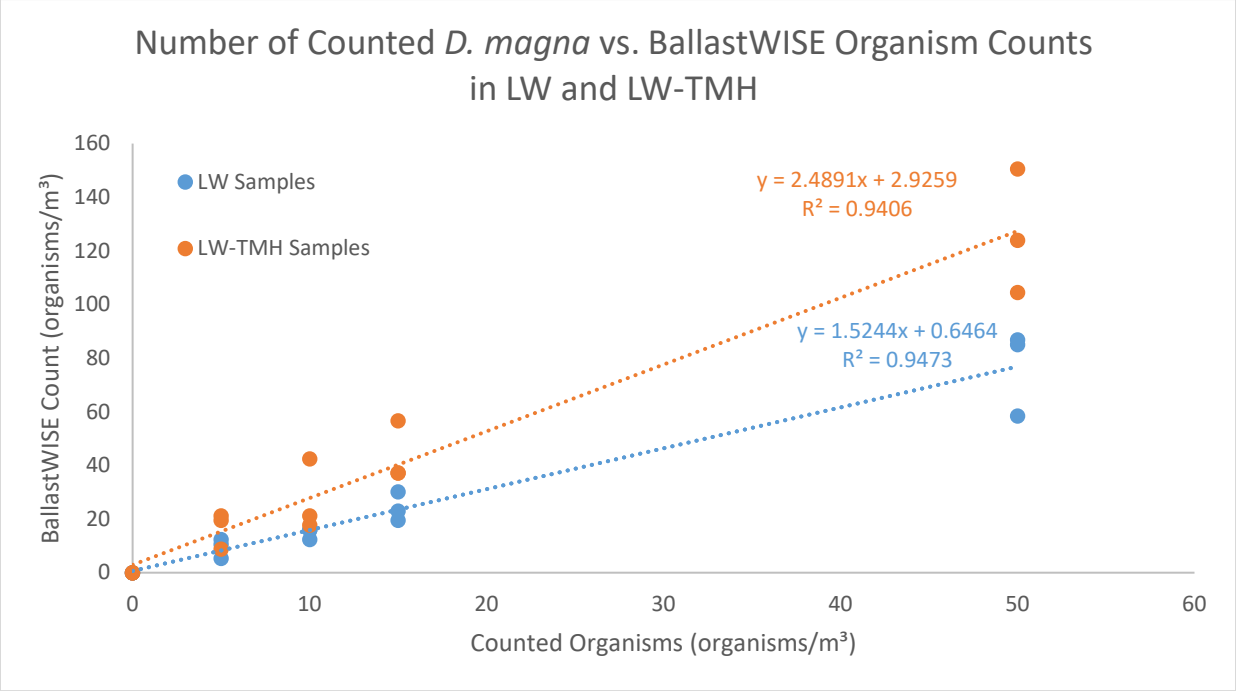
Table 6. Visual organism counts and BallastWISE counts using *D. magna* in LW and LW-TMH.

Sample Description	Visual Organism Count (organisms/m³)	LW Samples		LW-TMH Samples	
		BallastWISE Count (organisms/m³)	Mean (CV)	BallastWISE Count (organisms/m³)	Mean (CV)
0 organisms/m³ (Blank)	0	0 VL	0 (NA)	0 VL	0 (NA)
		0 VL		0 VL	
		0 VL		0 VL	
5 organisms/m³	5	12.4 VL	9.45 (39.0)	8.857 VL	16.54 (40.6)
		5.314 VL		19.49 L	
		10.63 VL		21.26 L	
10 organisms/m³	10	15.94 L	14.76 (13.9)	42.51 H	27.16 (49.4)
		12.4 VL		17.71 L	
		15.94 L		21.26 L	
15 organisms/m³	15	19.49 L	24.21 (22.3)	37.2 H	43.70 (25.8)
		23.03 L		37.2 H	
		30.11 H		56.69 H	
50 organisms/m³	50	58.46 H	76.76 (20.7)	124 H	126.37 (18.3)
		86.8 H		150.6 H	
		85.03 H		104.5 H	

Colored highlighting indicates device results for compliance or non-compliancy as described in the BallastWISE user manual. Green indicates very low risk (VL), yellow indicates low risk (L), and red indicates high risk (H).

Data from the *D. magna* testing in both LW and LW-TMH are displayed graphically in Figure 12. The R^2 values for the LW and LW-TMH analyses were both >0.9 indicating high precision of BallastWISE. Both BallastWISE analyses overestimated the number of organisms at each density with the LW estimated at approximately 50% higher than the visually counted value and the LW-TMH estimated at approximately 125% higher than the visually counted value.

Figure 12. Visual *D. magna* counts vs. BallastWISE organism counts in LW and LW-TMH.



4.1.4 *EUCYCLOPS* SPP.

Results from the BallastWISE analysis of LW and LW-TMH samples containing mixed age *Eucyclops* are shown in Table 7. The average length of the *Eucyclops* was 0.93 mm (range 0.75-1.02 mm). No CV values are provided for the organism counts for the zooplankton samples because all of the samples were counted by one analyst and the count was verified by a second analyst, 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, 5, 10, 15, and 50 organisms/m³. All blank samples had counts of 0 organisms/m³ in both LW and LW-TMH. BallastWISE organism count averages in LW were 0, 2.36, 0, 60.81, and 196.60 organisms/m³. BallastWISE organism count averages in LW-TMH were 0, 10.63, 27.75, 46.64, and 88.58 organisms/m³. The coefficients of variance ranged from 13.9-173.2 in LW and 17.8-88.2 LW-TMH and generally decreased with increasing organisms. The CV was not calculable for the blank samples as the mean was 0 organisms/m³.

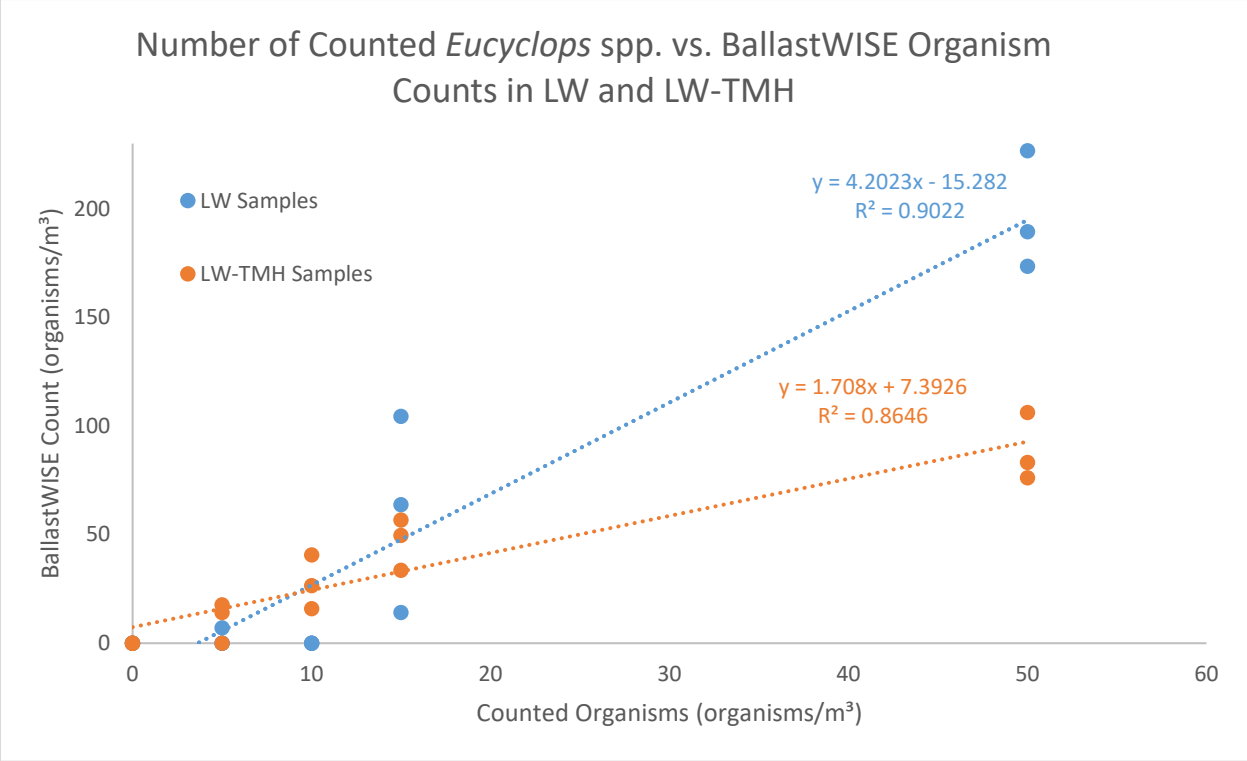
Table 7. Visual organism counts and BallastWISE counts using *Eucyclops* spp. in LW and LW-TMH.

Sample Description	Visual Organism Count (organisms/m³)	LW Samples		LW-TMH Samples	
		BallastWISE Count (organisms/m³)	Mean (CV)	BallastWISE Count (organisms/m³)	Mean (CV)
0 organisms/m³ (Blank)	0	0 VL	0 (NA)	0 VL	0 (NA)
	0	0 VL		0 VL	
	0	0 VL		0 VL	
5 organisms/m³	5	0 VL	2.36 (173.2)	14.17 L	10.63 (88.2)
	5	0 VL		0 VL	
	5	7.086 VL		17.71 L	
10 organisms/m³	10	0 VL	0 (NA)	15.94 L	27.75 (44.8)
	10	0 VL		26.57 L	
	10	0 VL		40.74 H	
15 organisms/m³	15	63.77 H	60.81 (74.4)	33.66 H	46.64 (25.3)
	15	14.17 L		56.69 H	
	15	104.5 H		49.6 H	
50 organisms/m³	50	189.5 H	196.60 (13.9)	76.17 H	88.58 (17.8)
	50	173.6 H		83.26 H	
	50	226.7 H		106.3 H	

Colored highlighting indicates device results for compliance or non-compliancy as described in the BallastWISE user manual. Green indicates very low risk (VL), yellow indicates low risk (L), and red indicates high risk (H).

Data from the *Eucyclops* testing in both LW and LW-TMH are displayed graphically in Figure 13. The R^2 values for the LW and LW-TMH analyses were >0.85 indicating good precision of BallastWISE. BallastWISE underestimated the number of organisms at or below the D-2 limit and overestimated the number of organisms above the D-2 limit in LW. BallastWISE overestimated the number of organisms in all samples containing organisms in LW-TMH.

Figure 13. Visual *Eucyclops* spp. counts vs. BallastWISE organism counts in LW and LW-TMH.



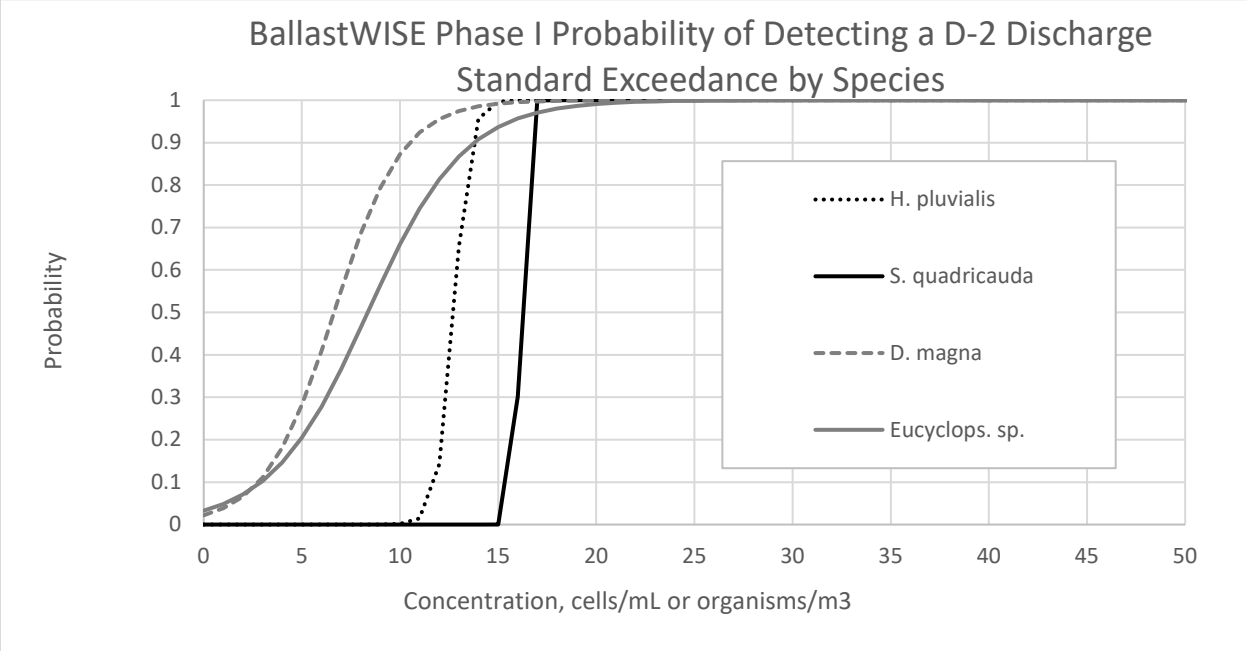
4.1.1 STATISTICS

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 as seen in Figure 14. 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). In general, the probability curves for each species used in Phase One rose sharply at densities between 10 and 20 cells/mL or organisms/m^3. For all species, the probability of detecting an exceedance at 20 cells/mL or organisms/m^3 was at or near 1. The probability of detecting an exceedance (of 10 cells/mL) for *H. pluvialis* was 0.0, but increased to >0.95 at 14 cells/mL. The probability of detecting an exceedance (of 10 cells/mL) for *S. quadricauda* was 0.0, but increased to >0.95 at 17 cells/mL. The probability of detecting an exceedance (of 10 organisms/m^3) for *D. magna* was 0.87, but increased to >0.95 at 12 organisms/m^3. The probability of detecting an exceedance (of 10 organisms/m^3) for *Eucyclops* spp. was 0.66, but increased to >0.95 at 16 organisms/m^3.

Phase I data were used to calculate LOD values for BallastWISE at each test concentration for each species of protist and for the combined zooplankton species. The signal-to-noise ratio (S/N) was determined at each LOD value. S/N is used to determine if the random noise from errors is too high to evaluate the LOD at a given concentration. The concentration at which the S/N ratio was approximately 10 was used to determine LOD (Tamburri, 2020). None of the zooplankton sample concentrations produced a S/N ratio <2.5, indicating that there was too much error to calculate the LOD. *H. pluvialis*

produced a S/N of 8.2 in the 10-30 cells/mL sample range. The LOD calculated at this concentration was 7.5 cells/mL. The highest S/N ratio produced during *S. quadricauda* testing was 5.4 in the 75-100 cells/mL sample range. The LOD calculated at this concentration was 17.7 cells/mL.

Figure 14. BallastWISE Phase I probability of detecting a D-2 discharge standard exceedance by species.



4.1.2 WATER CHEMISTRY

Water quality measurements taken during Phase I testing with BallastWISE are shown in Table 8. The LW samples are shown without shading while the LW-TMH sample rows have been shaded to differentiate between the water types. The measurements were within historical ranges for each of the experimental water types.

Table 8. Water quality measurements made during Phase I testing with BallastWISE.

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	24.5	7.06	4.9	144.2
D. magna	LW-TMH	24.8	7.06	5.3	147.2
Eucyclops spp.	LW	25.0	6.96	5.2	133.1
Eucyclops spp.	LW-TMH	24.7	7.04	5.3	145.9

Water chemistry measurements taken during Phase I testing with BallastWISE 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 for all established parameters except for % Transmittance which was out of range 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). The acceptance range for & Transmittance will be revised once enough data has been gathered.

Table 9. Water chemistry data collected in water used for Phase I BallastWISE 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	0.9J	1.2J	<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	1.0J	1.0J	<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	98.4	98.8	0.9J	0.8J	<1.25	<1.25
D. magna	LW-TMH	22.6	27.5	24.9	9.7	6.3	8.8	13.8
Eucyclops spp.	LW	<1.25	98.6	98.6	1.1J	0.77J	<1.25	<1.25
Eucyclops spp.	LW-TMH	21.2	28.2	25.6	9.6	5.9	8.3	12.9

***Values are outside the acceptable range. J values indicate that the data point is between the Limit of Detection (LOD) and the Limit of Quantification (LOQ).**

4.2 PHASE II

Results from Phase II testing of the protist and zooplankton size classes in Duluth-Superior Harbor water using the BallastWISE compliance monitoring device alongside 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 high number of filamentous protist forms. This caused difficulty creating a cell density that was below the discharge standard. The trial was later repeated without these difficulties. The results of the total live density analysis of the "strictly" and total "allowable" and "strictly" organisms in the protist size class using microscopic analysis (LSRI, 2020c) along with the results of BallastWISE analysis of the samples are shown in Table 10. Appendix 1 shows the detailed taxonomic assessment and community composition counts for the Duluth-Superior Harbor Water sample used for the protist sample dilutions. The experimental blanks and dilution water were verified through microscopic analysis using vital stain to have a live density of 0 cells/mL. The ambient harbor density of protists on the day of the verification test was 276.3 cells/mL. Microscopic cell counts for the samples were 0, 10.0, 34.9, and 106.9 using the "strictly" cell definition and 0, 25.2, 105.4, and 265.0 cells/mL using the total "allowable" and "strictly" cell definitions. CV values for both microscopic count methods were comparable and ranged from 4.9 to 24.6. All cell densities were within targeted ranges. BallastWISE cell count averages were 0.68, 7.45, 29.13, and 69.8 cells/mL. BallastWISE count coefficient of variance values ranged from 7.9 to 170 with the highest variance in the 0 cells/mL targeted density samples. BallastWISE cell counts increased with increasing microscopic counts and were generally lower than the microscopic counts. BallastWISE properly designated all samples above the discharge standard as either high risk or low risk and all samples below the discharge standard were properly designated as very low risk.

Table 10. Native protist counts and BallastWISE analysis in Duluth-Superior Harbor water in Phase II.

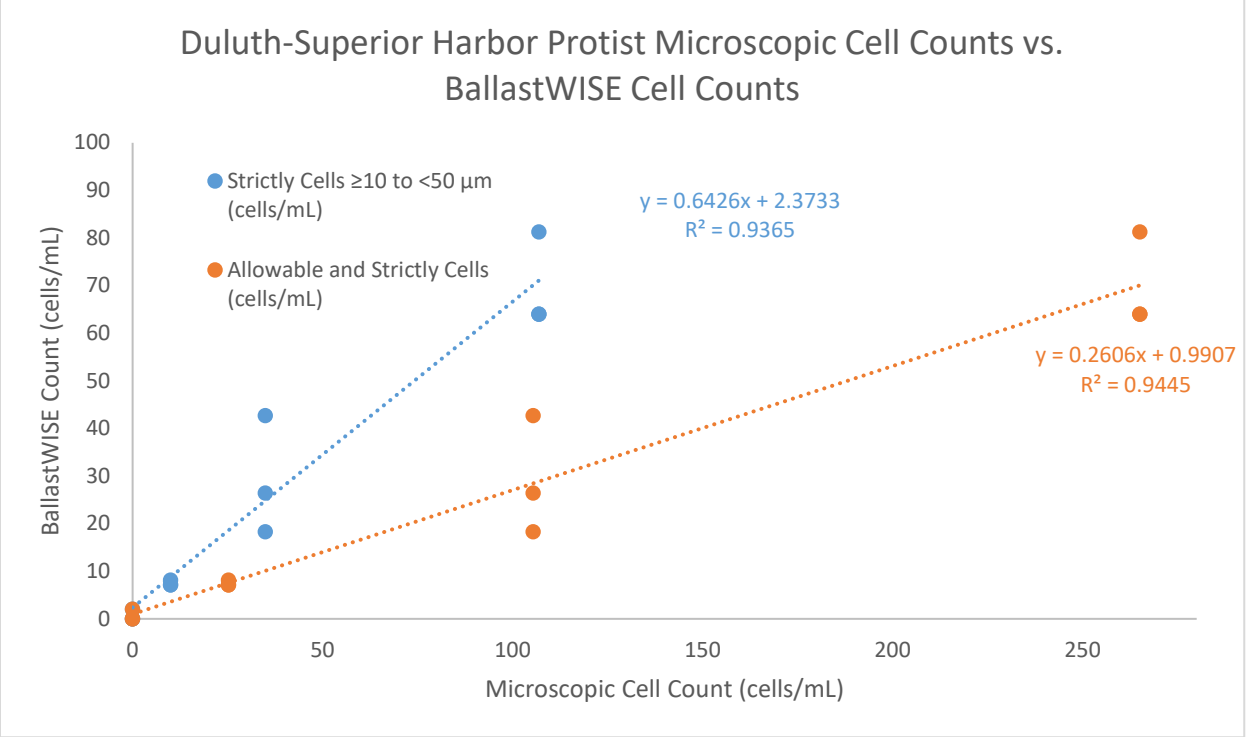
Sample Description	Microscopic Counts		BallastWISE Count (cells/mL)	Mean (CV)
	Strictly Cells ≥10 and <50 µm (cells/mL) (CV)	Total Allowable and Strictly (cells/mL) (CV)		
0 cells/mL (Blank)	0	0	0 VL 2.033 VL 0 VL	0.68 (170)
5-20 cells/ mL	10.0 (24.6)	25.2 (16.4)	7.114 VL 8.13 VL 7.114 VL	7.45 (7.87)
30-50 cells/ mL	34.9 (10.8)	105.4 (9.4)	26.42 L 18.29 L 42.68 H	29.13 (42.63)

Sample Description	Microscopic Counts		BallastWISE Count (cells/mL)	Mean (CV)
	Strictly Cells ≥10 and <50 µm (cells/mL) (CV)	Total Allowable and Strictly (cells/mL) (CV)		
51-150 cells/ mL	106.9 (4.9)	265.0 (11.8)	64.02 [H]	69.8 (14.3)
			64.02 [H]	
			81.3 [H]	

Colored highlighting indicates device results for compliance or non-compliancy as described in the BallastWISE user manual. Green indicates very low risk ([VL]), yellow indicates low risk ([L]), and red indicates high risk ([H]).

The information in Table 10 is displayed graphically in Figure 15 and shows the microscopic cell counts versus the BallastWISE cell count results. Both measuring methods for protists were undercounted by BallastWISE, but the R^2 values were both >0.9 indicating that the device had a high level of precision. BallastWISE measurements were more closely associated with microscopic counts in the "strictly" protist size range. BallastWISE is specified to measure the shortest dimension of each cell which corresponds to the strict measurement procedure.

Figure 15. Microscopic counts of native phytoplankton vs. BallastWISE counts in Phase II.



4.2.2 ZOOPLANKTON

Phase II testing for zooplankton occurred on three separate occasions. The first trial was discarded due to a mathematical error causing zooplankton counts to be outside the targeted ranges. The second trial was discarded due to bubbles in the BallastWISE device causing high levels of inaccuracy. Input from the

developer was implemented for the third trial which greatly reduced the presence of bubbles in the device and which is implemented in software version 5.1 onwards. The results of the live density analysis of organisms in the zooplankton size class using microscopic analysis along with the results of BallastWISE analysis of the samples are shown in Table 11. Appendix 2 shows the detailed taxonomic assessment and community composition counts for the Duluth-Superior Harbor Water sample used for the zooplankton sample dilutions. The experimental blanks (and dilution water) were verified through microscopic analysis to have a live organism density of 0 organisms/m^3. The ambient harbor density of zooplankton on the day of the verification test was 102 live organisms/m^3. Microscopic counts for the targeted sample densities were 0, 11, 54, and 126 live organisms/m^3. The 30-50 organisms/m^3 targeted density was slightly over the set range but was deemed acceptable for the purpose of this analysis (See Deviation from October 23, 2020 in Table 2). BallastWISE organism counts averaged 0, 14.39, 32.58, and 180 organisms/m^3 with coefficients of variance ranging from 50.8 to 91.1 indicating a higher amount of variability than in Phase II protist testing. In the targeted 30-50 organisms/m^3 sample, the BallastWISE results for the three replicates were 9.091, 22.73, and 65.91 organisms/m^3, placing each in a different risk rating category.

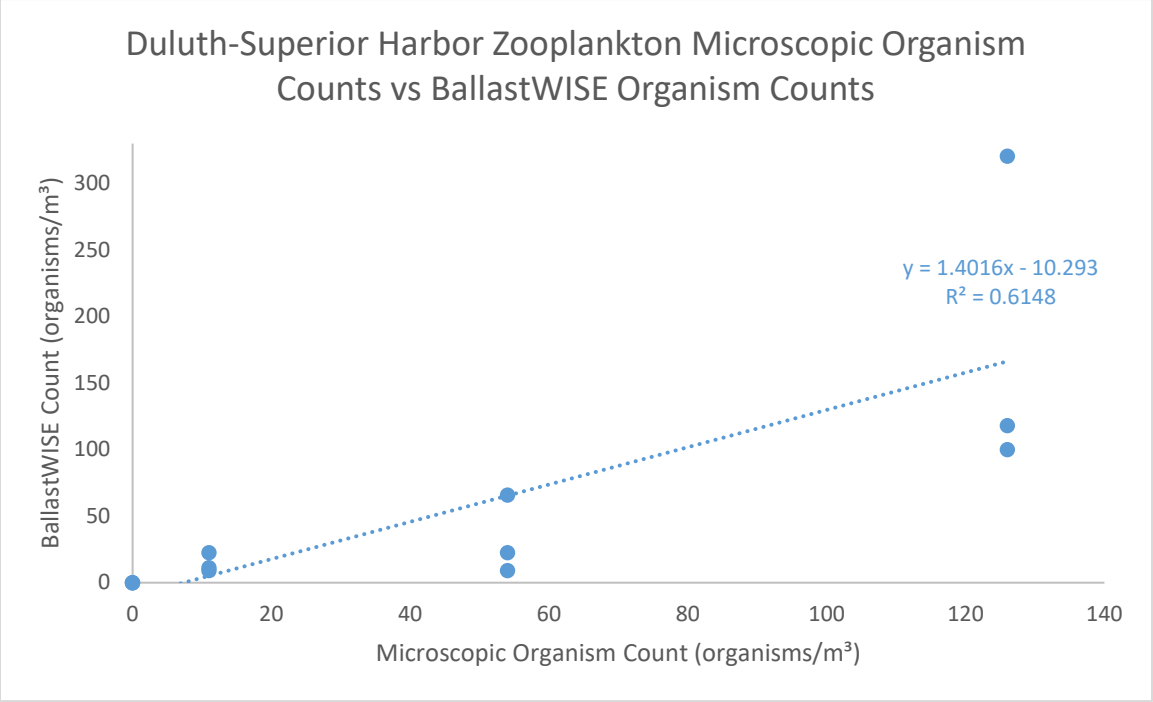
Table 11. Native zooplankton counts and BallastWISE analysis in Duluth-Superior Harbor water in Phase II.

Sample Description	Microscopic Organism Count (live organisms/m^3)	BallastWISE Count (organisms/m^3)	Mean (CV)
0 organisms/m^3 (Blank)	0	0 ^{VL} 0 ^{VL} 0 ^{VL}	0 (NA)
5-20 organisms/m^3	11	9.091 ^{VL} 11.36 ^{VL} 22.73 ^{L}	14.39 (50.8)
30-50 organisms/m^3	54	9.091 ^{VL} 22.73 ^{L} 65.91 ^{H}	32.58 (91.1)
51-150 organisms/m^3	126	320.5 ^{H} 100 ^{H} 118.2 ^{H}	180 (68.2)

Colored highlighting indicates device results for compliance or non-compliancy as described in the BallastWISE user manual. Green indicates very low risk (^{VL}), yellow indicates low risk (^{L}), and red indicates high risk (^{H}).

The information in Table 11 is displayed graphically in Figure 16 shows the microscopic live organism count versus the BallastWISE counted organism results. Although the linear regression produced a line close to expected outcomes in this range of data, the R^2 value of the line was only 0.605 and may have been caused by the higher variance among these samples than in Phase II protist testing.

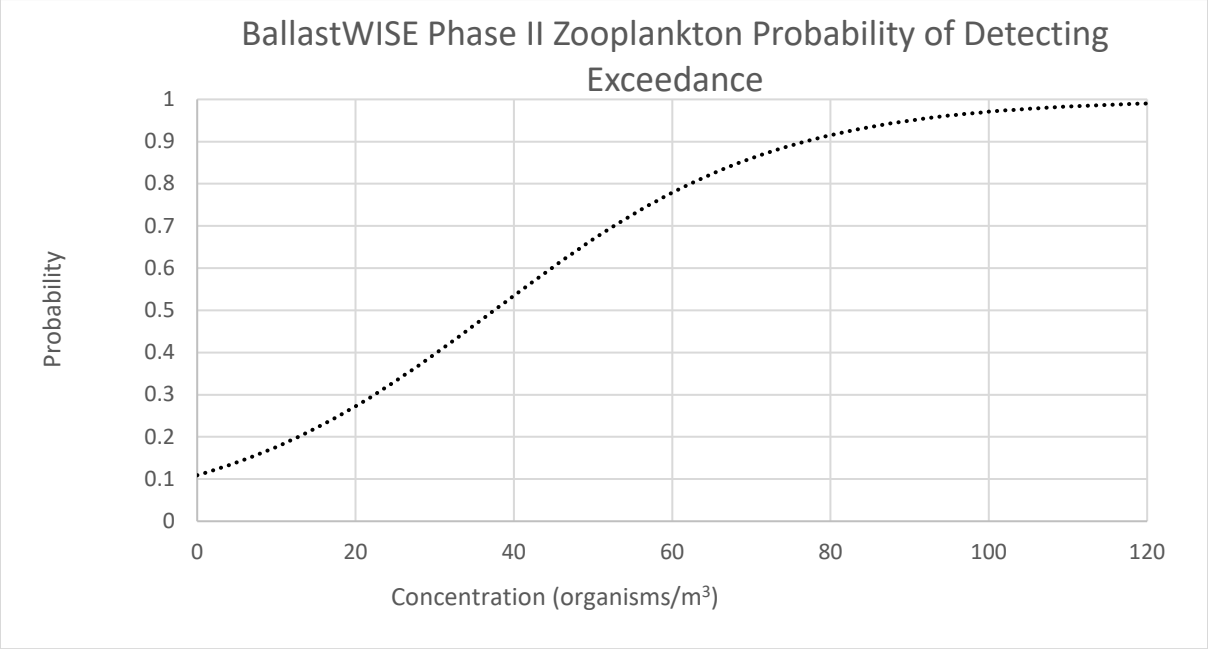
Figure 16. Microscopic counts of native zooplankton vs. BallastWISE counts in Phase II.



4.2.3 STATISTICS

The probability of detecting an exceedance based on the organism concentration of a sample was calculated for Phase II zooplankton data and is shown in Figure 17. The concentration of organisms at which BallastWISE had a >0.95 probability of detecting an exceedance was 95 organisms/m³. To calculate a more accurate/precise curve, considerably more data would be necessary. Protist data could not be analyzed because the parameter covariance matrix could not be computed by the statistical software.

Figure 17. BallastWISE Phase II zooplankton probability of detecting exceedance.



4.2.4 WATER CHEMISTRY

Water quality measurements taken during Phase II testing with BallastWISE are shown in Table 12. There were no requirements for the water quality parameters, however, the measurements were within historical ranges for the Duluth-Superior Harbor.

Table 12. Water quality measurements made during Phase II of BallastWISE testing.

Water Type	Temperature (°C)	pH	Dissolved Oxygen (mg/L)	Conductivity (µS/cm)	Turbidity (FNU)
Harbor Water Prior to Filtration (Protist Dilution Water)	14.7	7.06	9.6	131.9	1.42
Protist Source Water	13.7	7.06	9.6	173.3	31.9
Harbor Water Prior to Filtration (Zooplankton Dilution Water)	6.3	7.02	11.7	206.5	16.2
Zooplankton Source Water	6.9	7.29	11.7	217.5	15.3

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

Table 13. Water chemistry measurements made during Phase II BallastWISE testing.

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 (Protist Dilution Water)	5.7	49.3	45.6	7.5	7.0	1.1	4.6
Protist Source Water	7.1	50.4	45.2	6.8	6.6	1.3	5.8
Harbor Water Prior to Filtration (Zooplankton Dilution Water)	4.5	39.2	35.5	9.0	8.5	<1.25	NC
Zooplankton Source Water	8.5	38.1	32.8	9.4	8.9	1.4	7.1

NC = Not Calculable

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. Control discharge sample analysis was omitted from the BallastWISE assessment because the BWT technology had a short treatment time. Results from Phase III testing of the protist and zooplankton size classes in Duluth-Superior Harbor water using the BallastWISE compliance monitoring device alongside traditional microscopic enumeration methods are discussed below.

4.3.1 PROTISTS

The results of the total live density analysis of organisms in the protist size class using microscopic analysis and the results of BallastWISE analysis of the samples are shown in Table 14. Two methods, “strictly” and total “strictly” and “allowable” were used for protist enumeration and are described in Section 2.5.1.1. Total untreated uptake protist cell counts ranged from 354.9 to 665.7 cells/mL and the ranges of counts for cells strictly ≥10 and <50 µm were 152.9 to 300.4 cells/mL. BallastWISE counts of untreated uptake protist cells ranged from 41.6 to 100.6 cells/mL, well below the expected values. In treated protist samples, total cell counts ranged from 0 to 34.4 cells/mL and cells strictly ≥10 and <50

µm ranged from 0 to 0.4 cells/mL. All BallastWISE counts for treated protist samples were 0 cells/mL. The three uptake/treated discharge sample represent three different cycles of testing and should not be compared to one another or used to calculate means or CVs. Only comparisons between the microscopic counts and BallastWISE counts should be made for each cycle.

Table 14. Microscopic protist counts and BallastWISE analysis of uptake and treated samples in Phase III.

Sample Description	Microscopic Count Cells Total "Allowable" (cells/mL) (CV)	Microscopic Count Cells Strictly ≥10 µm (cells/mL) (CV)	BallastWISE Count (cells/mL)
Phase III-1 Uptake	665.7 (7.5)	300.4 (9.2)	41.7 [H]
Phase III-2 Uptake	645 (17.4)	252 (1.6)	100.6 [H]
Phase III-3 Uptake	354.9 (8.8)	152.9 (9.1)	56.91 [H]
Phase III-1 Treated	34.4	0.4	0 [VL]
Phase III-2 Treated	0	0	0 [VL]
Phase III-3 Treated	0	0	0 [VL]

Colored highlighting indicates device results for compliance or non-compliancy as described in the BallastWISE user manual. Green indicates very low risk ([VL]), yellow indicates low risk ([L]), and red indicates high risk ([H]).

4.3.2 ZOOPLANKTON

The results of the live density analysis of organisms in the zooplankton size class using microscopic analysis and the results of BallastWISE analysis of the samples are shown in Table 15. Appendix 3 shows the detailed taxonomic assessment and community composition counts for the Duluth-Superior Harbor water untreated uptake sample. Phase III zooplankton microscopic counts of uptake samples ranged from 4.4 x 10^4 to 1.5 x 10^5 live organisms/m³ and BallastWISE analysis ranged from 735.8 to 1.9 x 10^4 live organisms/m³. Microscopic counts in treated samples ranged from 3.9 to 17.3 live organisms/m³ and BallastWISE analysis ranged from 10.3 to 143.3 live organisms/m³. BallastWISE results show undercounts for untreated uptake samples and overcounts for samples treated with the BWT system. BallastWISE counts for the untreated uptake samples were repeated several times because the device ended the analysis before completing a full sampling procedure. Developer feedback revealed that this was due to the device counting 200 individual organisms at which point it is statistically unnecessary to continue counting. When this occurred and multiple trials were available for inclusion in this analysis, the first trial ran on a day was included. The three uptake/treated discharge samples represent three different cycles of testing and should not be compared to one another or used to calculate means or CVs. Only comparisons between the microscopic counts and BallastWISE counts should be made for each cycle.

Table 15. Microscopic zooplankton counts and BallastWISE counts uptake and treated samples in Phase III.

Sample Description	Microscopic Count (organisms/m³)	BallastWISE Count (organisms/m³)
Phase III-1 Uptake	1.5×10^5	1.98×10^4 H
Phase III-2 Uptake	$1.2x\ 10^5$	735.8 H
Phase III-3 Uptake	4.4×10^4	1169.4 H
Phase III-1 Treated	13.2	143.3 H
Phase III-2 Treated	17.3	10.3 VL
Phase III-3 Treated	3.9	50 H

Colored highlighting indicates device results for compliance or non-compliancy as described in the BallastWISE user manual. Green indicates very low risk (VL), yellow indicates low risk (L), and red indicates high risk (H).

4.3.3 STATISTICS

Probabilities of detecting an exceedance could not be calculated for Phase III data because the parameter covariance matrix could not be computed by the statistical software.

4.3.4 WATER CHEMISTRY

Water quality measurements taken during Phase III testing with BallastWISE are shown in Table 16. There were no requirements for the water quality parameters, however, the measurements were within historical ranges of the Duluth-Superior Harbor.

Table 16. Water quality measurements made during Phase III of BallastWISE testing.

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 Phase III testing in Duluth-Superior Harbor Water to provide the developer with data to show how naturally occurring total suspended solids may impact BallastWISE test results. The values obtained during the Phase II testing are shown in Table 17 and are within historical ranges measured in the Duluth-Superior Harbor.

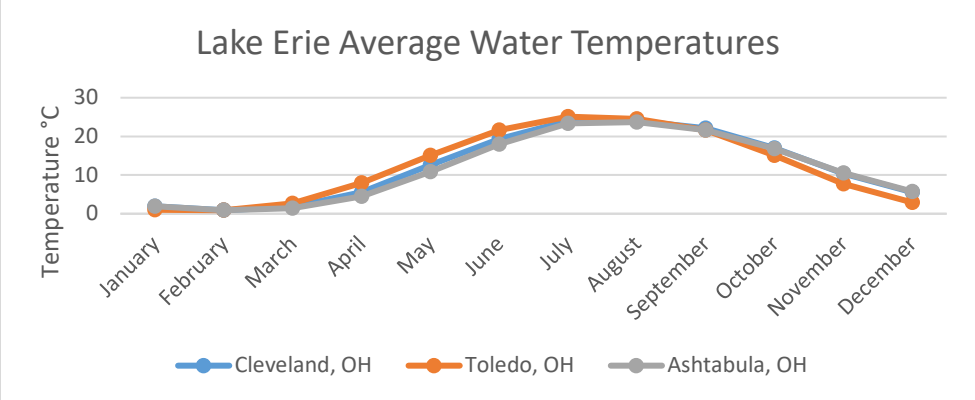
Table 17. Water chemistry measurements made during Phase III testing with BallastWISE.

Test Date	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	<1.25	NC

5 DEVICE USABILITY AND COMPATABILITY WITH GREAT LAKES CONDITIONS

BallastWISE was found to produce fewer tracking errors, particularly those caused by air bubbles, when the device was operated in a level, vibration free environment. Meeting ideal conditions could be challenging in a vessel where engine vibrations or slight movement could interfere with BallastWISE results. Further, the cold average temperatures of the Great Lakes (Figure 18) might cause condensation on the exterior of the BallastWISE sample chambers if the device is operated in a warm environment such as the engine room of a vessel. The impact of condensation was not determined by this evaluation but could possibly interfere with the recording of fluorescence or motion tracking.

Figure 18. Ten-year average water temperatures measured at Lake Erie ports.



6 PERSONNEL RESPONSIBILITIES

All GWRC staff who were directly involved in data collection and analysis during the BallastWISE verification have completed hands-on and competency training on the procedures for which they were assigned and have read the BallastWISE Compliance Monitoring Device Validation 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.

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.

7.2 ZOOPLANKTON TESTING

A summary of QC counts for Phase I zooplankton testing can be found in Table 18. During testing with *D. magna* and *Eucyclops* spp., data quality was ensured by having a second individual conduct counts on a minimum of 10% of the samples. This minimum was exceeded in both tests with 100% of the samples having quality assurance counts conducted. The relative percent difference (RPD) met the data quality objectives (DQO) for all samples in the *D. magna* and *Eucyclops* spp. testing.

Table 18. Average relative percent difference (RPD) of samples counted for *D. magna* and *Eucyclops* spp. tests conducted during BallastWISE Phase I tests.

Test Species	Test Date	Percent of Samples with QC counts	DQO	Relative Percent Difference between counts
D. magna	2 December 2020	100%	RPD ≤10%	0%
D. magna	4 December 2020	100%		0%
Eucyclops spp.	1 December 2020	100%		0%
Eucyclops spp.	3 December 2020	100%		0%

7.3 WATER CHEMISTRY

The DQO for water chemistry analyses conducted during the evaluation of the BallastWISE 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.

Table 19. Data quality objectives (DQOs), criteria, and performance measurement results from water chemistry analyses conducted during BallastWISE 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 ± 0.1%
			NPOC: 13.6%	%NPOC: 10.1 ± 7.6%

Data Quality Indicator	Evaluation Process/Performance Measurement	Data Quality Objective	Performance Measurement Result	
			DOC: 11.1%	%DOC: 13.2 ± 4.7%
			POM: 10.7%	POM: 0.0 ± 0.0%
			TSS: 10.7%	TSS: 0.0 ± 0.0%
Bias, Filter Blanks	%T method blanks were prepared by filtering Milli-Q samples (one per analysis date).	>98% average %T	Number of %T Method Blanks Analyzed: 18	Method Blanks (%T): 99.9 ± 0.6%
	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: 19	Method Blanks (TSS): <1.25 ± 0
			Number of POM Method Blanks Analyzed: 19	Method Blanks (POM): <1.25 ± 0
	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: 37	Blanks (NPOC): <0.48 ± 0
	DOC method blanks were prepared by filtering Milli-Q samples (one per analysis date).	<1.6 mg/L average DOC	Number of DOC Method Blanks Analyzed: 20	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: 20.4%	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: 2.2 ± 1.6%
			POM: 100%	POM: 2.7 ± 1.9%
			NPOC: 100%	NPOC: 8.1 ± 2.6%
		A least one per 10 samples <10% average D	Percentage (vs total samples) Check Standards:	NPOC 10 mg/L Standard % Difference
			NPOC/DOC: 76%	3.7 ± 2.3%

Data Quality Indicator	Evaluation Process/Performance Measurement	Data Quality Objective	Performance Measurement Result
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 Sections 2 and 8 were used for all water chemistry and water quality analyses.
Completeness	Percentage of valid (i.e., collected, handled, analyzed correctly and meeting DQOs) water chemistry samples measured out of the total number of water chemistry samples collected. Performance is measured by percent completeness (%C).	>90% C	TSS: 100%
			%T Filtered: 96%
			%T Unfiltered: 96%
			NPOC: 79%*
			DOC: 96%
Sensitivity	The limit of detection (LOD) and limit of quantification (LOQ) for each analyte and analytical method utilized was determined annually unless a reporting limit was used based on the amount filtered as was the case with TSS/POM.	Not Applicable	TSS/POM RL: 1.25 mg/L based on filtering 800 mL of sample
			NPOC/DOC LOD: 0.48 mg/L
			NPOC/DOC LOQ: 1.6 mg/L
			Determined 7 February 2020

***Completeness NPOC**: NPOC samples were not collected for Phase II zooplankton testing due to overlap of sample collection and overlooking the parameter in the test plan.

8 CONCLUSIONS AND DISCUSSION

The LSRI-GWRC freshwater verification of the BallastWISE device met the stated objectives, as outlined in the Test Plan (LSRI, 2020). The reported deviations do not impact LSRI-GWRC's ability to draw conclusions on BallastWISE performance during this verification. BallastWISE was operated in accordance with the developer's instructions and operated reliably during all reported tests with the exceptions noted in Section 3.

Results from this verification indicate potential effectiveness of BallastWISE for monitoring of ballast water in Great Lakes vessels for organisms on the protist and zooplankton size classes. To determine the effectiveness of the BallastWISE system, a series of questions were addressed through experimentation.

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

In the protist size class, *H. pluvialis* BallastWISE results for both LW and LW-TMH samples closely matched expected results from microscopic counts. All samples were appropriately categorized as very low, low, or high risk for discharge by the device and all blank samples were properly analyzed as containing no organisms (Table 3). The <10 cells/mL targeted density samples had high coefficients of variance (e.g., 65.5 and 173.2), but low sample means often have high CV values (First, 2018). The CV of BallastWISE counts for the higher targeted densities ranged from 2.6 to 10.3 and R^2 values for both test waters were >0.99 indicating a high level of precision for this organism in LW and LW-TMH. BallastWISE was an effective and reliable device for counting *H. pluvialis* in both low and high TSS and DOC environments. BallastWISE had a near 0 probability to detect an exceedance at the D-2 discharge standard with this data set (Figure 14), but achieved a very high probability (>0.95) of detecting an exceedance just above the D-2 discharge standard at 14 cells/mL.

The second organism tested in the protist size class, *S. quadricauda,* was selected because it is most commonly found in a colonial form like many of the protist species found in the Duluth-Superior Harbor. BallastWISE was also precise when analyzing this protist, but not to the same level as with *H. pluvialis* with slightly higher CV values and a slightly lower R^2 (Table 5). The BallastWISE cell counts were significantly lower than microscopic counts, however, with values approximately half of what was expected. As a result, several of the BallastWISE counts for the targeted 10-30 cells/mL samples were incorrectly designated as very low risk for discharge instead of low risk. If BallastWISE results are instead compared to a total entity count which enumerates all colonies as one entity and all lone cells as one entity, the BallastWISE results match quite closely. This would suggest that BallastWISE is not able to distinguish between a single cell and a colony of cells for the protist *S. quadricauda.* The probability of detecting an exceedance did not become likely (>0.5 probability) until a concentration of 17 cells/mL, but this higher value may partly be due to the inability of the device to distinguish between cells and colonies of protists (Figure 14).

In the zooplankton size class, *D. magna* and *Eucyclops* spp. densities were both greatly overestimated by BallastWISE in LW and LW-TMH samples (Table 6 and Table 7). *Eucyclops* spp. counts in LW were nearly four times the visual counts and roughly double in the LW-TMH, even as small numbers of *Eucyclops* spp. were left uncounted in the unanalyzed sample portion. In *D. magna* testing, the LW-TMH samples were overestimated by a larger amount than the LW samples. *D. magna* and *Eucyclops* movement tracks were often broken into many segments when the organisms moved quickly and can be seen in Figure 18 and Figure 19. This effect was even more pronounced in the *Eucyclops* as their movement is marked by short bursts of rapid movement followed by brief pauses. The egg sacs of gravid *Eucyclops* were often counted as separate organisms, causing organism counts to be inflated. The double tracking effect on *Eucyclops* can be seen in Figure 19. Despite these issues, the device was likely to detect an exceedance in both species at the D-2 discharge standard (Figure 14). In single taxon testing with cultured organisms, BallastWISE was more accurate at detecting exceedances in zooplankton than in protists but was fairly precise when measuring both size class.

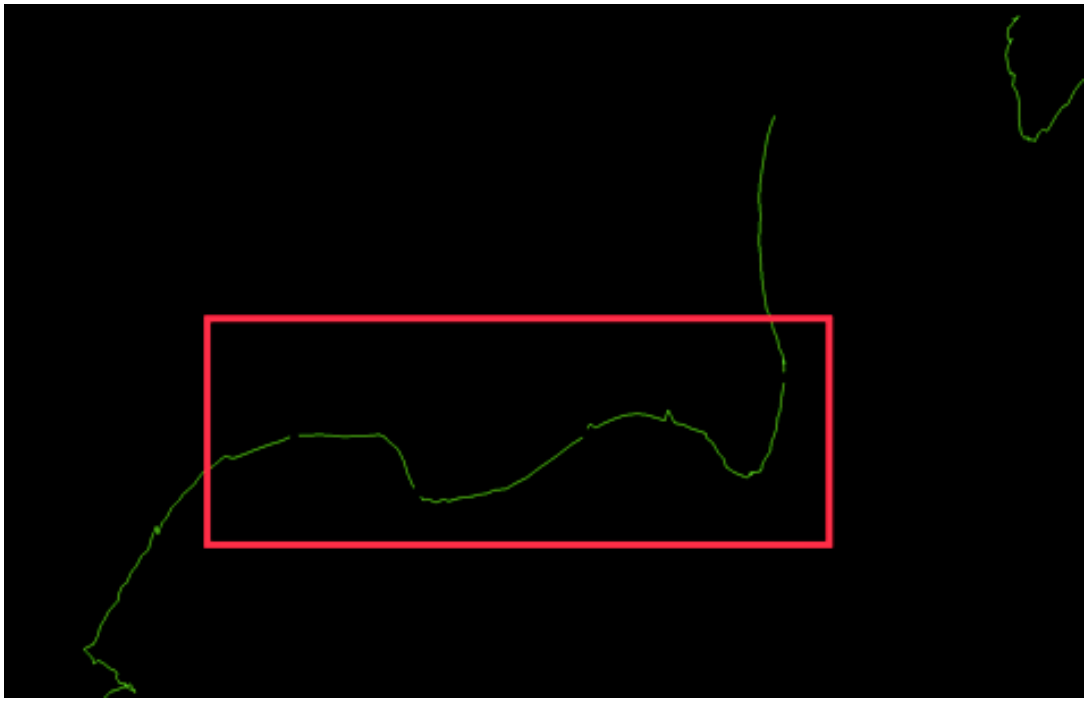


Figure 19. *D. magna* movement track broken into several segments by BallastWISE software.

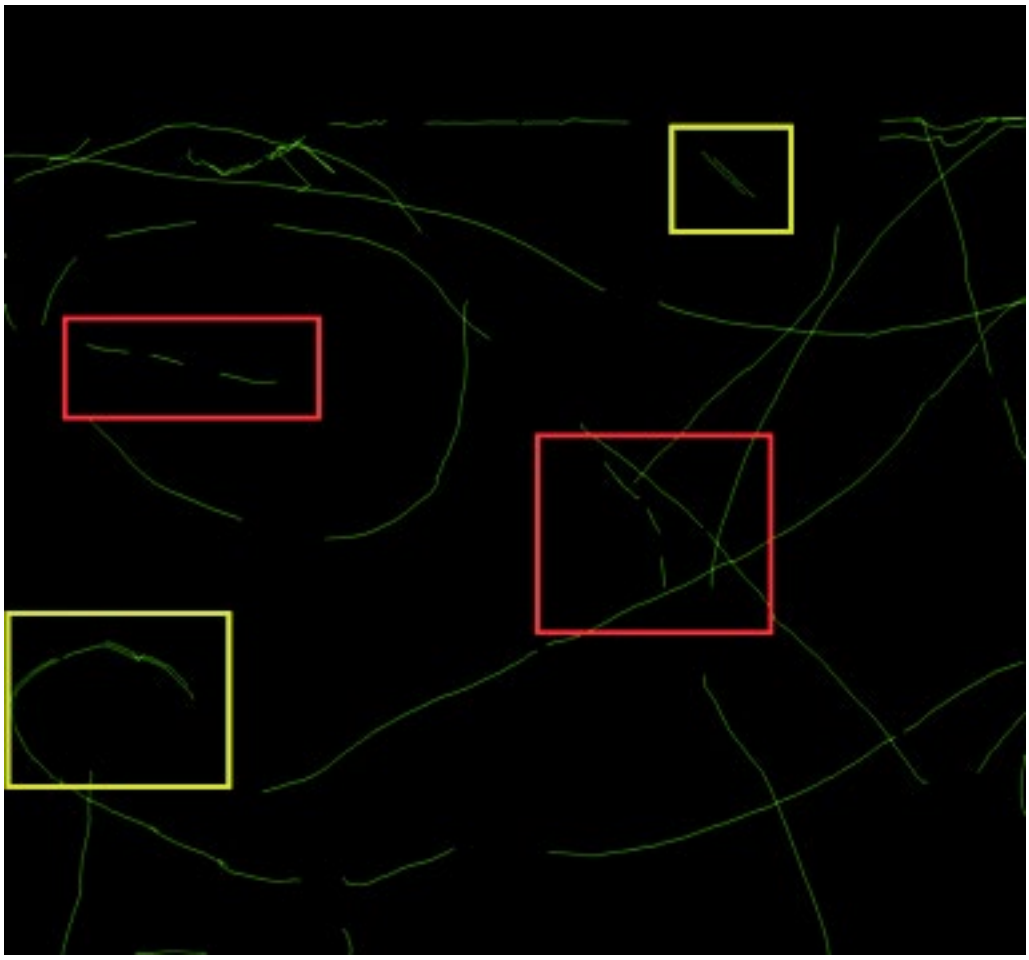


Figure 20. Broken movement tracks (red highlighting) and doubled movement tracks (yellow highlighting) of *Eucyclops* spp. in LW.

For both organisms in the protist size class, the smaller sample chamber performed very well with no mechanical issue and fewer software issues than the sample chamber for the zooplankton size class. More software errors occurred while measuring for the larger size class and the formation of bubbles was only a concern in this sample chamber. Bubbles most often increased BallastWISE organism counts when nearby movement or vibration caused the bubbles to fluctuate and trigger the BallastWISE software to track that movement. This effect was minimized in LSRI's land-based facilities but could be of greater concern for shipboard use. Overall reliability of the BallastWISE device was found to be 85.9% when the total number of trials performed by the device were compared to the number of trials that failed due to the performance of the device itself and not user error. When the total trials are converted into the time spent performing each analysis, the reliability increases slightly to 90.5%. The increase in reliability is due to many of the failed analyses being aborted by the operator before completion when an issue was detected.

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

In Phase I testing, BallastWISE slightly overestimated *H. pluvialis* and *S. quadricauda* density in LW and slightly underestimated in LW-TMH at the highest densities while the lowest densities were accurate. Closer examination of the fluorescence images taken by the device showed that the images taken in the LW samples were sharper and brighter than the images taken in the LW-TMH samples. This suggests that the increased TSS and/or DOC may slightly hinder the ability of BallastWISE to detect the fluorescence of *H. pluvialis* and *S. quadricauda,* although the effect appears to be relatively small. See Appendix 4 for full size images for comparison.

BallastWISE often broke *Eucyclops* spp. tracks into multiple segments, essentially counting the same organism multiple times. After cursory visual analysis, it appears that this occurred less in the LW-TMH samples than in the LW samples, possibly due to the increased TSS and/or DOC altering the movement of the organisms or the ability of the BallastWISE software to track their movement. *D. magna* counts were overestimated by a higher percent in LW-TMH samples than in LW samples, while the opposite was true in *Eucyclops* spp., but no observations were made to explain this effect. The developer has indicated that the upper velocity limit for tracking, an internal software setting based on a number of parameters, is specified at 22 mm/s. Some large species or individuals, like *Eucyclops* spp., can occasionally exceed the upper velocity limit. This would result in an overestimation of the concentration of organisms if velocity exceedances caused segmented movement tracks.

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

Phase II testing of protists collected from the Duluth-Superior Harbor were well below microscopic counts when measuring a total “allowed” and “strictly” cell count for entities in the protist size class (Table 10). When BallastWISE results were compared to a count measuring strictly ≥10 and <50 µm, the results look much better with averages just below the microscopic counts. This is also in accordance with BallastWISE measurement specifications, which state that measurements are based on the minimum dimensions of cells. Coefficients of variance were much lower in this size class than the zooplankton size class and the R^2 values were high indicating good precision. In Phase I testing of colonial protists, results suggested that BallastWISE had trouble distinguishing between individual cells and colonies and the same may be happening in the natural assemblages collected for Phase II. BallastWISE may also not be quantifying protist under the “allowable” measurement definition which is the appropriate method for the protist size class under the D-2 discharge standard, but does not accurately quantify the protists assemblages in western Lake Superior. However, risk classification was correct in the majority of instances.

In Phase II testing with assemblages collected from the Duluth-Superior Harbor in the zooplankton size class, fair correlation was seen between BallastWISE results and microscopic analysis (Table 11). BallastWISE counts for zooplankton had a large amount of variance and the R^2 value of the linear regression was only 0.6148 even as the linear regression itself was near to the expected values from microscopic counts. At the time of Phase II testing, it had not yet been determined that the stir plate used during analysis caused mortality among the zooplankton in the sample. This may have affected the number of live zooplankton entering the sample chamber and decreasing the final organism counts produced by the device.

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

Protist testing in Phase III showed high variability in BallastWISE counts of untreated uptake samples. This may be due to the assemblages of the protists collected during sampling events. Variable numbers of "allowable" size protists and colonial protists could cause unexpectedly high or low values from BallastWISE. Risk was correctly classified in all cases.

Phase III untreated zooplankton uptake samples analyzed by BallastWISE were well below the microscopic counts of the same samples, but this may be due to a limit on the number of organisms the device is able to count. BallastWISE stops counting after 200 organisms have entered the sample zooplankton sample chamber since it is statistically impossible to achieve an acceptable D-2 discharge at that point. All uptake samples were correctly classified as high risk. As in Phase II, it had not yet been determined that the stir plate used during analysis caused mortality among the zooplankton in the sample. This most likely did not affect the untreated zooplankton results as they were all above the effective detection capabilities of the device, although it is possible the treated samples resulted in lower than expected values if organisms were injured or killed by the stirring.

Treatment with the BWT technology did not appear to affect BallastWISE operation in the protist size class as all samples measured at 0 cells/mL which was near expected value from microscopic analysis. In treated zooplankton samples, BallastWISE results were higher than expected when compared to microscopic counts. Treated samples were correctly assigned to the low risk category. Analysis of BallastWISE software tracks shows short straight lines oriented in the same direction in the protist size class and can be seen in Figure 20. The source of these tracks is unknown, but they were seen in all three trials in Phase III in the zooplankton size class. A possible explanation is that they were caused by the ozone nanobubbles created by the BWT technology. The Phase I *H. pluvialis*, Phase II and Phase III testing were conducted with the version 5.0 software that was installed on the device when it was received. Phase I *S. quadricauda*, *D. magna* and *Eucyclops* tests were conducted with version 5.1 software. Version 5.1 has an increased velocity threshold to reject particles and bubbles moving with internal

currents. It is unknown how using the version 5.1 software would have affected the results of Phase III testing.

Figure 21. Phase III-2 treated sample unidentified BallastWISE tracks.

This compliance monitoring device verification report demonstrates that BallastWISE has potential to be a useful and reliable device for evaluating ballast water discharges in the Laurentian Great Lakes. Currently, operational issues such as bubble formation and software performance hinder the reliability of the device. Additionally, the current procedures used by the device result in underestimates of the colonial protists commonly found in the Great Lakes. Modifications made during this analysis (e.g., slowing the sample chamber filling procedure) have already produced more reliable results from BallastWISE and further updates could presumably be made to increase performance.

Although the color coding of results given by BallastWISE was helpful and intuitive, the current colors may be difficult for individuals with color blindness to differentiate. MicroWISE might consider altering the colors or applying a secondary indication to increase accessibility of the device.

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. (2015). Guidance on ballast water sampling and analysis for trial use in accordance with the BWM Convention and Guidelines (G2). Rev. 1.

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.

Lake Superior Research Institute. (2020a). MicroWISE BallastWISE 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. (2017d). SOP/FS/39 v.2. Procedure for Calibration of YSI EXO2 Multiparameter Water Quality Sondes.

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

Lake Superior Research Institute. (2021a). SOP/GWRC/25 (Draft). 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. (2021b). SOP/GLM/28. Procedures for Calibrating and Using the Oakton CON 150 Conductivity/TDS/Temperature Meter.

Lake Superior Research Institute. (2017c). 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.

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.

MicroWISE. (2020). *BallastWISE Users Guide* v.2.1.

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.

Tamburri, M.N., et al. (2020). Protocol for the verification of ballast water compliance monitoring devices. *ICES Techniques in Marine Environmental Sciences*, Vol. 63.

USEPA. (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. Phase II taxonomic characterization of the organisms in the protist size class.

Taxonomy	Minimum Dimension <10 µm (cells/mL)	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

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

BallastWISE Phase II Zooplankton Size Class Taxonomy								
	Starting Density Sample (1:20 Dilution)		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	11	11	16	14	4	4	3	2
Daphnia	-	-	-	-	1	1	-	-
Copepods								
Calanoids	1	1	1	0	-	-	-	-
Cyclopoids	3	3	2	1	1	1	1	0
Nauplii	1	0	4	2	2	2	-	-
Other Organisms								
Planaria	1	1	-	-	-	-	-	-
Protista >50 µm	1	1	3	3	3	3	-	-
Rotifers								
Bdelloid	1	1	2	2	-	-	-	-
Dicranophoridae	-	-	1	1	-	-	-	-
Colurella	1	1	-	-	-	-	-	-
Conochilus	1	1	1	1	-	-	-	-
Gastropus	1	1	-	-	-	-	-	-
Kellicottia	1	1	1	1	1	0	-	-
Keratella	23	22	34	33	8	8	3	3
Monostyla	-	-	2	2	-	-	-	-
Polyarthra	15	10	21	13	8	6	2	2
Synchaeta	48	45	58	53	30	28	4	4
Tricocerca	3	3	-	-	1	1	-	-
Total	**112**	**102**	**146**	**126**	**59**	**54**	**13**	**11**
Percent Live	**91%**		**86%**		**92%**		**85%**	

Appendix 3. Phase III taxonomic characterization of the organisms in the zooplankton size class.

BallastWISE Phase III Untreated Uptake Sample Zooplankton 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 3. Phase III taxonomic characterization of the organisms in the zooplankton size class.

BallastWISE Phase III Treated Discharge Sample Zooplankton Size Class Taxonomy			
	Phase III-1	**Phase III-2**	**Phase III-3**
Taxonomy	**Live Organisms /m^3**	**Live Organisms /m^3**	**Live Organisms /m^3**
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**

Appendix 4. BallastWISE *Haematococcus pluvialis* fluorescence microscopy images in LW and LW-TMH.

BallastWISE fluorescence image of *H. pluvialis* in LW.

BallastWISE fluorescence image of *H. pluvialis in LW-TMH.*