BENCH-SCALE TECHNICAL REPORT

FRESHWATER VERIFICATION OF THE B-QUA QUICK BALLAST WATER MONITORING KIT

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ABSTRACT

This technical report presents findings from bench-scale verification tests evaluating the performance of the B-QUA Quick Ballast Water Monitoring Kit, hereafter B-QUA, in freshwater. B-QUA was developed by LuminUltra Technologies Ltd. of New Brunswick, Canada.

Researchers began conducting the bench-scale evaluation in October 2019 and ending in February 2020 at the Lake Superior Research Institute (LSRI) of the University of Wisconsin-Superior (UWS) in Superior, Wisconsin, USA. The monitoring kit utilizes adenosine triphosphate (ATP) and size fractionation to quantify living organisms in marine, brackish, and freshwater. The measurement of ATP is one of the indicative analyses to test for gross compliance with the D-2 ballast water management standard under the International Maritime Organization's (IMO) Ballast Water Management (BWM) Convention, which applies to countries outside of the United States, including Canada (IMO, 2015).

Two phases of testing were done. Phase I testing was completed in two water types using cultured organisms in the three regulated size classes, utilizing the pathogen indicator organisms *Escherichia coli* and *Enterococcus faecium*, the algae *Haematococcus pluvialis* and the zooplankton *Ceriodaphnia dubia* and *Daphnia magna*. Phase II testing was completed using naturally occurring Great Lakes organisms in the Duluth-Superior Harbor of Lake Superior in two of the regulated size classes.

Phase I testing showed high correlation of B-QUA's luminometer output (i.e., cATP values) with microscopic counts for the algae *Haematococcus pluvialis* (≥10 µm to <50 µm size class) and the zooplankton *Ceriodaphnia dubia* and *Daphnia magna* (>50 µm size class) in both water types. However, the B-QUA system was unable to detect *E. coli* or *E. faecium* (<10 µm size class) at levels above the D2 regulatory value in either water type in Phase I. Phase II correlation between B-QUA cATP values and microscopic counts was good for natural assemblages of phytoplankton and zooplankton in Duluth-Superior harbor water.

KEY WORDS

Compliance Monitoring Tool, Ballast Water, ATP

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ACKNOWLEDGEMENTS

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This work was supported by the United States Maritime Administration (United States Department of Transportation; Washington, D.C) and the United States Environmental Protection Agency's Great Lakes Restoration Initiative.

1 INTRODUCTION

A major focus area of the Lake Superior Research Institute's Great Waters Research Collaborative (LSRI-GWRC) is providing unbiased, independent data in support of the accelerated development of technologies having the potential for preventing the introduction and/or controlling the spread of non-indigenous organisms within the Laurentian Great Lakes. This report details the results of the LSRI-GWRC bench-scale evaluation of the B-QUA Quick Ballast Water Monitoring Kit, hereafter B-QUA. Developed by LuminUltra Technologies Ltd. of New Brunswick, Canada, the B-QUA monitoring kit is the result of six years of research and development with LuminUltra's partners in France (Aqua-tools) and Switzerland (SGS International).

The B-QUA utilizes adenosine triphosphate (ATP) and size fractionation to quantify living organisms in marine, brackish, and freshwater.

This technical report presents the findings from the freshwater verification of the B-QUA, which took place from October 2019 to February 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 ATP analysis results from the B-QUA correspond to detailed standard laboratory/microscopic analysis of freshwater laboratory-cultured organisms in the three regulated size classes?

2. Does increased turbidity and total suspended solids affect the ability of the B-QUA to detect ATP in a water sample?

3. Do ATP results from the B-QUA correspond to detailed microscopic analysis of organisms in the ≥10 to <50 µm and >50 µm size classes from Western Lake Superior water?

4. What percentage of organisms in the ≥10 to <50 µm size class are lost through a 10 µm filter? Does decreasing the filter size effectively capture Great Lakes protists in this size class?

2 TEST METHODS

2.1 TEST PLAN AND SOPS

A Test Quality Assurance Plan (TQAP) and standard operating procedures (SOPs) were used to implement all test activities (LSRI, 2019). These procedures facilitate consistent conformance to technical and quality system requirements and increase data quality. 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 LuminUltra Technologies Ltd. prior to the start of the device verification activities. The SOPs followed throughout testing are described in the methods section and listed in the References section of this report.

2.2 BALLAST WATER COMPLIANCE MONITORING TOOL DESCRIPTION

The B-QUA evaluated by LSRI-GWRC is a commercially available ballast water discharge compliance monitoring tool. The B-QUA consists of a Pelican™ case containing all equipment, reagents, and consumables needed to quantify living aquatic organisms in a water sample via the measurement of ATP (Figure 1). The components of the B-QUA allow the user to concentrate the sample and isolate the three size classes of regulated organisms via filtration, extract the cellular ATP with an enzyme via cell lysis, homogenize the sample, react the homogenized extract with luminase enzyme, and analyze the individual fractions for ATP with the use of a luminometer.

Figure 1. Pelican case containing all equipment needed for B-QUA analysis.

The sample is filtered using a 500 mL Nalgene© reusable polysulfone filter funnel (Figure 2) and a glass fiber filter suitable for the organism size fraction in question. A syringe and two filters are used for the

bacteria size fraction. Filtering speed is increased using a Nalgene© hand-operated vacuum pump (Figure 2). The LuminUltra/Aqua Tools IKA© ULTRA TURRAX© Tube Drive grinder (Figure 3) is used to achieve homogenization of the filter and sample.

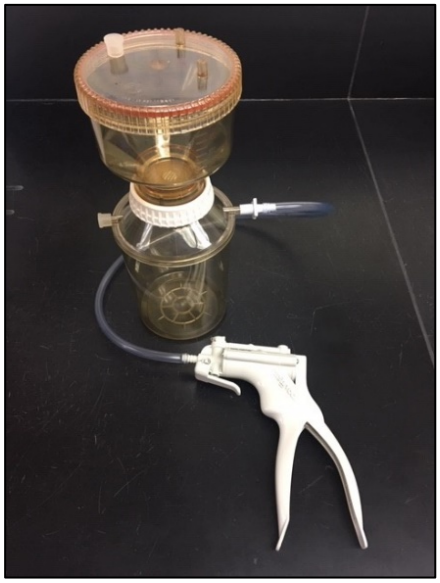


Figure 2. Nalgene© filter funnel and hand pump.

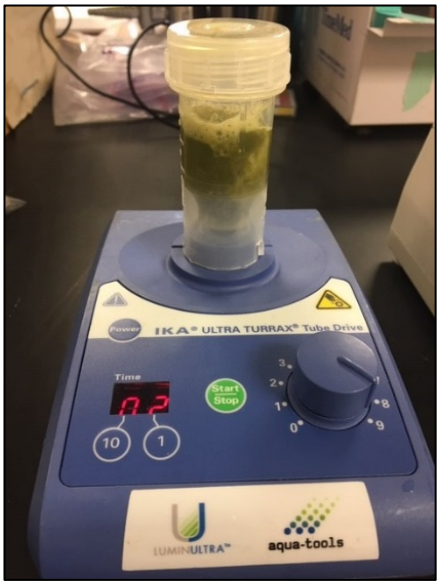


Figure 3. LuminUltra/Aqua tools IKA© ULTRA TURRAX© tube drive grinder with beads tube attached.

The bioluminescent reaction obtained through the mixture of the homogenous sample and the Luminase enzyme is measured with the LuminUltra PhotonMaster™ Luminometer (Figure 4, left). Readings from the luminometer output are expressed as relative light units (RLU). RLU values ≤10 are below the low detection limit (LuminUltra, 2018). In addition, sample values less than the negative control (tube + Luminase + Ultralute) and negative RLU values once the sample result is subtracted from the negative control indicate that the sample is in the low detection range of the device (LuminUltra,

2018). The RLU output is sent to the PhotonMaster™ Bluetooth© Module (PBM) (Figure 4, right) which also provides the power and operation functions for the luminometer.

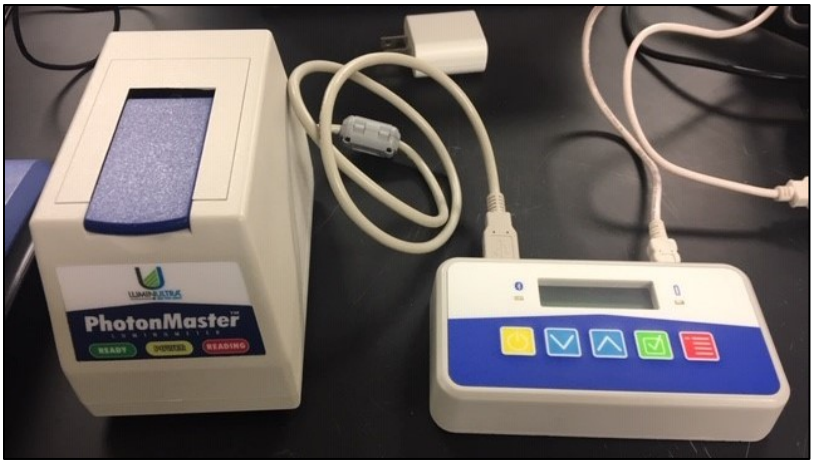


Figure 4. LuminUltra's PhotonMaster™ Luminometer (left) and PhotonMaster™ Bluetooth© Module (right).

The RLU values are converted to ATP concentrations using the supplied formulas for each regulated size class and/or using the provided LumiCapture MS Excel File. LuminUltra and SGS have established compliance limits according to the IMO Convention for all three regulated size classes. Results are interpreted using the following categories: Most Likely Compliant [to the ballast water discharge standard], Signal Close to the Limit [ballast water discharge standard], Most Likely Not Compliant.

2.3 EXPERIMENTAL WATER PREPARATION

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

Laboratory Water (LW): The LW is municipal water from the City of Superior, Wisconsin (sourced from Lake Superior), that 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., <3 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. In Phase I testing with bacteria, LW was autoclaved to sterilize it prior to using as a blank.

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. 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. In Phase I testing with bacteria, LW-TMH was autoclaved to sterilize it prior to using as a blank.

2.4 BALLAST WATER COMPLIANCE MONITORING TOOL TRAINING

The B-QUA was delivered via UPS and received on April 24, 2019. Carine Magdo, Business Development Manager of Ballast Water Monitoring Solutions, provided training to GWRC staff via a web conference on May 2, 2019. Michael Thomas, Inside Sales Representative from LuminUltra, provided hands-on training on the operation of the monitoring system to LSRI staff members, Lana Fanberg, Heidi Schaefer, and Christine Polkinghorne on May 29, 2019. In addition to the training sessions, LuminUltra provided GWRC staff with written instructions and a video of how to conduct testing with the compliance monitoring tool.

2.5 EXPERIMENTAL DESIGN AND VERIFICATION METHODS

2.5.1 PHASE I

Phase I was conducted using known densities of laboratory-cultured freshwater organisms to compare the B-QUA analysis results to traditional laboratory/microscopic analysis. Freshwater organisms used encompassed all three of the regulated size classes including two types of bacteria (i.e., organisms <10 µm), a green alga (i.e., organisms ≥10 µm and <50 µm), and two sizes of zooplankton (i.e., organisms ≥50 µm). Testing was done in two water types to represent high transparency and low transparency conditions to determine whether increased turbidity and total suspended solids affect the ability of B-QUA to detect ATP in a water sample. With all organisms tested, the goal was to have samples that were above and below the D-2 ballast water discharge standard.

When using the B-QUA kit for analysis for all three size classes, GWRC followed the *Test Kit Instructions* provided by LuminUltra. Prior to sample analysis each day, an ATP Standard Calibration was performed and the RLU value of the Luminase plus UltraCheck1 was verified to be >5,000 prior to analyzing any samples. In addition, several negative controls (i.e., empty tube, tube plus Luminase, and tube plus Luminase and Ultralute) were analyzed and verified to be <20 RLU prior to sample analysis each day.

Sample measurement values (as RLU) obtained from the Luminometer were entered into B-QUA's "LuminUltra _data analysis_VI" Excel file to obtain cATP (ATP concentration) values to be used for development of graphs. Experimental blank samples were LW or LW-TMH water without test organisms that were processed and analyzed exactly as the samples containing organisms were. For the bacteria testing in Phase I, LW and LW-TMH were autoclaved prior to use to sterilize the water. IDEXX HPC for Quanti-tray was used prior to conducting Phase I testing with bacteria to verify that the water was sterile.

2.5.1.1 BACTERIA SAMPLE PREPARATION AND ENUMERATION (<10 µm SIZE CLASS)

Water was prepared as described in Section 2.3 and autoclaved to produce sterile test water. *Escherichia coli* (ATCC#259220) and *Enterococcus faecium* (ATCC#35667) stocks used in B-QUA tests

were prepared November 8, 2019 using log phase cultures grown at 37°C for four to six hours in Tryptic Soy Broth or Brain-Heart-Infusion broth, respectively. Log-phase cultures were frozen in cryovials at -70 to -80°C with a 1:1 ratio of sterile glycerol and enumerated at a later time. To prepare bacteria samples for the B-QUA tests, frozen stocks were brought to room temperature prior to preparing dilutions of *E. coli* or *E. faecium* in sterile test water to produce triplicate samples with concentrations of 0, 50 and 500 most probable number (MPN) *E. coli* or *E. faecium* per 100 mL. Samples were enumerated according to LSRI SOP SA/56 – *Detection and Enumeration Total Coliforms and E. coli Using IDEXX Colilert®* (LSRI, 2016) or LSRI SOP SA/62 – *Detection and Enumeration of Enterococcus using IDEXX Enterolert®* (LSRI, 2017a). Both the Colilert® and Enterolert® tests use Defined Substrate Technology® (DST) in which the bacteria metabolize the enzymes in the specific media causing the sample to fluoresce. Results are given as MPN, a common method of obtaining quantitative data on concentrations of discrete items from positive/negative (incidence) data, and in this case correlates well with colony forming units (CFU). Both tests have a detection limit of 1 MPN/100 mL. After a 100 mL subsample was collected for enumeration via Colilert® or Enterolert® method, samples were immediately analyzed via the B-QUA kit.

The B-QUA bacterial samples were mixed well by inverting about 25 times, and 100 mL was filtered through a 2.7 µm and 0.7 µm filter placed in series on a syringe. Then, 1 mL of Ultralyse7 was passed through the 0.7 µm filter into a 9 mL ultralute tube. The ultralute dilution tube was slowly mixed by inverting approximately 3 times, and 100 µL of luminase and 100 µL from the ultralute dilution tube was added to a luminometer tube, mixed, and immediately read by the luminometer. The result was recorded as RLU and converted to $cATP_{BACT}$ (pg/100 mL) using the "LuminUltra _data analysis_VI" Excel file with the calibration check result. In instances where B-QUA sample analysis resulted in $cATP_{BACT}$ values below the levels of blank samples for all bacterial densities, positive controls were prepared by diluting the stock to a lesser extent, thus creating higher MPN/100 mL.

2.5.1.2 ALGAL ENUMERATION (≥10 µm to <50 µm SIZE CLASS)

Water was prepared as described in Section 2.3 and was then spiked with *H. pluvialis* cultures to produce triplicate samples of algae with nominal concentrations of 0, 10, 50, 100, 500, and 1,000 cells/mL. Algae samples were analyzed microscopically by staining a subsample of *H. pluvialis* cells from each sample with the vital stain SYTOX® Green. The LSRI SOP GWRC/11 - *Assessing Bench-Scale Dose Effectiveness of Potential Ballast Water Treatment Processes on Selenastrum capricornutum* (LSRI, 2017b) was followed for staining and counting. Counting was conducted by enumerating the live and dead cells within a known area using a compound microscope equipped with epifluorescence able to excite samples at 450-490 nm under 400x magnification.

The B-QUA algae sample was mixed well, and 500 mL was filtered through a 10 µm filter using a filter funnel and hand pump. Any bacteria present in the sample would be <10 µm and should pass through the filter. Sterile lab water was used to rinse the sides of the funnel and sample bottle to ensure the entire sample was captured on the filter. The filter was added to a beads tube along with 5 mL of Ultralyse30 and 1 mL of Ultralute and homogenized using the ULTRA TURREX®Tube Drive Grinder three times for two minutes at position nine (6000 rpm). The homogenized beads tube with sample was allowed to settle for at least ten minutes before a 100 µL of supernatant was transferred to a 5 mL

ultralute tube. The ultralute dilution tube was slowly mixed by inverting approximately three times. Then, 100 µL of luminase and 100 µL from the ultralute dilution tube was added to a luminometer tube, mixed, and immediately read by the luminometer. The result was recorded as RLU and converted to $cATP_{10\text{-}50}$ (pg/mL) using the calibration check result.

2.5.1.3 ZOOPLANKTON ENUMERATION (>50 µm SIZE CLASS)

Daphnia magna and *Ceriodaphnia dubia* were used for the >50 µm size class comparison. They were individually tested at the following concentrations: 0, 5, 10, 15, 50, and 100 organisms per sample. Organisms were ≤24 hours old and collected the day of analysis. Three replicates of each concentration were counted out and a second person counted them before they were added to the water for analysis.

The B-QUA zooplankton sample was mixed well, and 1000 mL was filtered through a 50 µm filter using a filter funnel and hand pump. Sterile lab water was used to rinse the sides of the funnel and sample bottle to ensure the entire sample was captured on the filter. The filter was added to a beads tube along with 5 mL of Ultralyse30 and 1 mL of Ultralute and homogenized using the ULTRA TURREX®Tube Drive Grinder three times for two minutes at position nine (6000 rpm). The homogenized beads tube with sample was allowed to settle for at least ten minutes before a 100 µL of supernatant was transferred to a 5 mL ultralute dilution tube. The ultralute dilution tube was slowly mixed by inverting approximately three times, then 100 µL of luminase and 100 µL from the ultralute dilution tube was added to a luminometer tube, mixed, and immediately read by the luminometer. The result was recorded as RLU and converted to $cATP_{50}$ (pg/m^3) using the calibration check result.

2.5.2 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 organisms in the ≥10 µm to <50 µm and >50 µm size classes with the B-QUA kit and by following the methods required by the ETV Protocol. When using the B-QUA kit for analysis for both size classes, GWRC followed the *Test Kit Instructions* provided by LuminUltra. Prior to sample analysis each day, an ATP Standard Calibration was performed and the RLU value of the Luminase plus UltraCheck1 was verified to be >5,000 prior to analyzing any samples. In addition, prior to sample analysis each day the following negative controls were analyzed and verified to be <20 RLU: empty tube, tub plus Luminase, and tube plus Luminase and Ultralute.

Sample analysis values (as RLU) obtained from the Luminometer were entered into B-QUA's "LuminUltra _data analysis_VI" Excel file to obtain cATP values to be used for development of graphs. The data generated from this portion of the evaluation was used to correlate Great Lakes protist and zooplankton community densities with ATP concentrations. As in Phase I, experimental blanks were prepared and analyzed with each set of samples. To prepare the Phase II experimental blanks, Duluth-Superior Harbor water was collected each day and filtered through a 934-AH Whatman 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.

Protist communities within the Laurentian Great Lakes are smaller than many of their marine counterparts, therefore, an additional analysis was conducted during Phase II to determine the percentage of organisms in the ≥10 µm to <50 µm size class that are lost through the B-QUA 10 µm filter. The results from analysis using filtration at 10 µm was compared to results from a 5 µm filter was done at the same time. A 5 µm filter was chosen as it is the next smallest size filter which corresponds to the type and manufacturer of the 10 µm filter used in the kit.

2.5.2.1 PROTIST ENUMERATION

For the assessment of the ≥10 µm to <50 µm size class, two 20 L carboys of water were collected from the nearby Duluth-Superior Harbor and filtered through a 35-µm plankton net to remove the larger organisms. Total live density and a detailed taxonomic analysis of the community composition of this size class was completed on the filtered whole water samples using the methods in LSRI SOP GWRC/30 – *Procedure for Protist Analysis* (LSRI, 2017c). Appendix 1 shows the detailed taxonomic categories that the organisms in this size class were identified to. Following the density and community composition determination, two sets of dilutions (in triplicate) were created which targeted the following live densities: 0, 10, and 100 cells/mL, for a total of four densities (including the starting density). Dilutions were created using filtered harbor water (934-AH Whatman filters, 1.5-µm particle retention).

2.5.2.2 ZOOPLANKTON ENUMERATION

For the assessment of the ≥50 µm size class, GWRC filtered 1.98 m^3 of Duluth-Superior Harbor water through a 35 µm plankton net and determined total live density and a general taxonomic categorization of the zooplankton community captured in the net. GWRC zooplankton analysts also enumerated any protists >50 µm in minimum dimension, but live/dead status and taxonomic determination was not completed. GWRC used methods in LSRI SOP GWRC/3 – *Determination of Zooplankton Density in Ballast Water Samples, Section "Analysis of Live Uptake Samples"* (LSRI, 2017d) for these analyses. Appendix 2 shows the taxonomic categories that the organisms in this size class were identified to. Following the density and general taxonomic categorization, two sets of dilutions (in triplicate) were created which targeted the following live densities: 0, 10, and 100/m^3 for a total of four densities (including the starting density). Dilutions were created using filtered harbor water (934-AH Whatman filters, 1.5-µm particle retention).

2.5.2.3 STATISTICAL ANALYSIS

Following calculation of the cATP values for organisms in each size class and water type using B-QUA's "LuminUltra _data analysis_VI" Excel file, cATP values and organism densities determined by standard count methods described above were entered into Microsoft Excel. The program was used to calculate coefficient of variance (CV) values for cATP values and organism densities at each target density. CV is a measure of precision and is calculated as the standard deviation divided by the mean multiplied by 100. The CV shows variability in a sample in relation to the sample mean. Microsoft Excel was also used to develop graphs of organism densities determined by standard count methods described above versus cATP values. Linear trendlines were fitted to the data and the R-squared value, a measure of how closely the data are fitted to the regression line were calculated in Excel.

2.5.2.4 FILTER LOSS STUDY

The second portion of the Phase II evaluation determined the percentage of organisms in the ≥10 µm to <50 µm size class are lost through the B-QUA 10 µm filter and compared that to the efficiencies of a 5 µm filter. A portion of Duluth-Superior Harbor water that was filtered through the 35 µm plankton net was filtered with the B-QUA 10 µm filter and a second portion was filtered with a 5 µm filter. The filtrate was preserved with Lugol's solution and analyzed at a later date for protist densities as well as community composition (Appendix 1). GWRC's protist analyst generalized the size of the dominant taxa found in the filtrate to determine the typical size of organisms that are passing through both filter sizes. Live analysis and ATP determination was not completed on these samples.

2.5.3 WATER QUALITY

Water quality measurements were made throughout the duration of the B-QUA 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), total alkalinity, total hardness, dissolved oxygen (DO), temperature, specific conductivity, and/or 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, 2017e). Briefly, accurately measured sample volumes (± 1%) were vacuum filtered through 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).

%T sample analysis was conducted according to LSRI SOP SA/69 – *Determining Percent Transmittance (%T) of Light in Water at 254 nm* (LSRI, 2019). For analysis of the filtered aliquot, an appropriate volume of sample was filtered through a glass fiber filter (i.e. Whatman 934-AH). A Perkin Elmer Lambda 35 UV-Vis spectrophotometer was used to measure %T of the unfiltered and filtered sample aliquots. Deionized water was used as a reference to adjust the spectrophotometer to 100%T, and then each unfiltered and filtered sample aliquot was measured in a pre-rinsed sample cuvette with a 1 cm path length.

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

concentrated HCl. The standard was used to generate a calibration curve which was then used to determine the concentration of organic carbon in the samples.

DO analysis was conducted using a Hach LDO HQ30d Dissolved Oxygen meter and LDO101 electrode, which was calibrated daily following LSRI SOP GLM/30 – *Calibrating, Maintaining and Using the HQ30d and HQ40d Meter and LDO101 Optical Electrode to Measure Dissolved Oxygen in Water Samples* (LSRI, 2017f). 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 110 Conductivity/TDS/Temperature Meter that is calibrated on a monthly basis following LSRI SOP GLM/26 - *Procedures for Calibrating and Using the Oakton CON 110 Conductivity/TDS/Temperature Meter* (LSRI, 2011). Its accuracy was also verified daily prior to sample analysis using a Daily Check Standard (0.0100M potassium chloride). pH analysis was conducted using an Orion 3 Star meter and Orion 8157BNUMD pH probe. Both instruments were calibrated daily following LSRI SOP GLM/05 – *Procedure for pH Meter Calibration and pH Measurement for Ballast Treatment Systems Utilizing pH as the Active Substance* (LSRI, 1992). A check buffer of 8.00 was also measured after calibration to verify the accuracy of the calibration.

2.6 DEVIATIONS

During the course of conducting testing with the B-QUA, there were several deviations that occurred from the TQAP. Those deviations are listed in Table 1 along with corrective actions that were taken as a response to the deviation and perceived impact of the deviation on the test results.

Table 1. Deviations Encountered during B-QUA Freshwater Verification.

Test	Description and Root Cause of Deviation or Quality Control Failure	Description of Corrective Action(s)	Describe the Impact on the Project/Test	Do the Data Need to be Qualified? (Y/N)
LW-TMH *H. pluvialis*	One less blank sample was analyzed due to insufficient number of filters available	No corrective action necessary.	One less blank sample, however, the two blank samples that were run (mean 149 RLU) were within the range of other LW-TMH samples previously analyzed (mean 168 RLU)	No
LW-TMH *H. pluvialis*	Stock LW-TMH was outside the range acceptable for test initiation. Root cause: New LW-TMH preparation method was developed and conducted leading to more complete suspension of solids and dissolution of solutes	No change needed. If %T continues to be lower than historical data in future tests, the target ranges may need to be reviewed to reflect the new LW-TMH preparation method.	Minimal, all other water quality parameters were within the target range for test initiation.	No

Test	Description and Root Cause of Deviation or Quality Control Failure	Description of Corrective Action(s)	Describe the Impact on the Project/Test	Do the Data Need to be Qualified? (Y/N)
	causing lower %T than was historically observed.			
Initial LW *E. coli* and *Enterococcus*	Expired Ultralyse 7 was used for multiple samples	During the test, a sample was run with Ultralyse 7 that had not passed expiration date. For further testing, expiration dates will be checked on all solutions prior to use.	Due to bacterial contamination in the stock blank water, tests will be repeated. There is no data from the expired Ultralyse 7 included in this report.	Yes, the test was repeated
Initial LW *E. coli* and *Enterococcus*	Stock Lab Water %T filtered and unfiltered, NPOC and DOC were outside of the range acceptable for test initiation. Root cause: believed to be bacterial contamination, possibly due to growth in carboy as it sat for several days after bleaching or from one or more of the brown jugs water was autoclaved in, as the autoclave may not have reached the appropriate pressure and temperature during the cycle due to damaged seal which was discovered shortly after	The seal on the autoclave was repaired. In subsequent tests, both the water and the carboys were autoclaved. Once water was added to the carboys, a sterility check was performed prior to starting testing. The sterility check was conducted by using IDEXX HPC for QuantiTray to verify that the water was sterile.	The bacteria tests in LW and LW-TMH were repeated	Yes, the test was repeated
LW and LW-TMH *E. coli* and *Enterococcus*	Less blanks were run than listed in the test plan between the two organisms. Only three total blanks were run for each test (as opposed to three for each organism) as the same water was used for both organisms.	None needed as the same water was used for both organisms so doing more blanks was not necessary.	Three less blanks were run for the LW and LWTMH tests.	No
Harbor Water ZP and PP	The test plan called for sterile filtered water to be used in making the dilutions. Harbor water was filtered through 934 AH glass microfiber filters	None taken as it was determined that sterile water was not needed for dilution preparation due to the filtering step of the B-QUA system not collecting any bacteria.	Sterile filtered water was not used for dilutions. No impact on the test as sterile filtered water was not necessary for proper testing.	No

Test	Description and Root Cause of Deviation or Quality Control Failure	Description of Corrective Action(s)	Describe the Impact on the Project/Test	Do the Data Need to be Qualified? (Y/N)
	instead (1.5 µm particle size filtration).	Bacteria would go through the filter used in the B-QUA analysis and thus not be measured during analysis.		

3 B-QUA OPERATIONAL PERFORMANCE

During the testing period, no operational issues occurred with the B-QUA.

4 RESULTS

Findings from the B-QUA Phase I and Phase II tests are presented in the following subsections. In result tables with cATP values reported, the values have been highlighted to align with what the B-QUA analysis indicates regarding compliance with IMO's *International Convention for the Control and Management of Ships' Ballast Water and Sediments* Regulation D-2 Ballast Water Performance Standard (2004). Regulation D-2 specifies that ships conducting ballast water management shall discharge:

- <10 viable organisms/m^3 ≥50 µm in minimum dimension
- <10 viable organisms/mL <50 µm and ≥10 µm in minimum dimension
- Indicator microbes not to exceed:
    - Toxigenic *Vibrio cholerae* (O1 and O139) <1 cfu/100 mL (note that *Vibrio cholerae* were not included as part of this verification)
    - *Escherichia coli* <250 cfu/100 mL
    - *Intestinal Enterococci* <100 cfu/100 mL

Figure 5 shows the ATP ranges used to determine compliance for each size class that were provided with B-QUA at the initiation of the project. During the final review of the report, the developer provided Figure 6 with updated guidelines for compliance based on ATP values. The new table was based on data collected after GWRC testing with B-QUA had begun. The acceptance range for the ≥50 µm size class has higher ATP values associated with each compliance level. The ranges for the ≥10 µm to <50 µm and bacteria size classes did not change. The ATP ranges used to determine compliance for each size class in this report are shown in Figure 6 as they represent improvements made during the ongoing development with the B-QUA device.

Figure 5. Figure from B-QUA Excel file indicating how compliance is determined from cATP values. This table was provided at the time of test initiation with the B-QUA system.

BALLAST WATER FRACTION	Most likely compliant	Signal close to the limit	Most likely not compliant
≥50µm $cATP_{50}$ (pg/m^3)	< 10 000	10 000 to 750 000	> 750 000
≥10 and <50µm $cATP_{10\text{-}50}$ (pg/ml)	< 500	500 to 1 500	> 1 500
Bacteria $cATP_{bact}$ (pg/100ml)	< 1 000	1 000 to 5 000	> 5 000

Figure 6. Figure from B-QUA Excel file indicating how compliance is determined from cATP values. This table was provided by the developer during the review of the report.

Fraction	ATP Concentration			
	Unit	Most Likely Compliant	Signal Close to Limit	Most Likely Not Compliant
> 50 um	pg/m^3	< 150,000	150,000 to 750,000	> 750,000
10-50 um	pg/mL	< 500	500 to 1,500	> 1,500
Bacteria	pg/100 mL	< 1,000	1,000 to 5,000	> 5,000

4.1 PHASE I

Phase I testing with bacteria was conducted on two occasions. The data from the initial test is not included in this report because, upon discussion with LuminUltra representatives, it was determined that there must have been bacterial contamination (heterotrophic bacteria other than *E. coli* or *E. faecium*) in the test water that obscured the results from the samples that were spiked with *E. coli* or *E. faecium*. Although the blanks and negative and positive controls analyzed with the samples were within acceptable ranges using the standard enumeration methods (i.e., Colilert® and Enterolert®), all samples in both water types and with both bacteria species had high RLU values when analyzed on the luminometer, including the experimental blanks that were not spiked with bacteria. No patterns were discernable when the data was analyzed. Prior to repeating the Phase I microbial tests, all test water was autoclaved and then was verified to be sterile prior to testing to ensure there would be no interference of heterotrophic bacteria other than *E. coli* or *E. faecium* with the B-QUA analysis and the target organisms (*E. coli* and *E. faecium)*.

4.1.1 *ESCHERICHIA COLI* (<10 µm SIZE CLASS)

4.1.1.1 VERIFICATION TESTS IN LABORATORY WATER

Results of *E. coli* enumeration using Colilert® compared to results of B-QUA analysis of aliquots of the same sample are shown in Table 2 for LW tests. Using the Colilert® method of analysis, all three samples prepared in LW for the 0 MPN/100 mL target concentration (i.e., experimental blanks) were <1 MPN/100 mL. The 50 MPN/100 mL samples had an average of 97.5 MPN/100 mL resulting from Colilert® analysis, while the nominally 500 MPN/100 mL samples had an average concentration of >2190.7 MPN/100 mL. The goal in choosing target *E. coli* concentrations was to have samples below, near and above the discharge standard. While the target of 500 MPN/100 mL was exceeded, the sample was above the discharge standard (<250 MPN/100 mL), so the goal was achieved.

All calibration standards and negative controls analyzed using B-QUA met the guidelines provided by LuminUltra. Nearly all of the *E. coli* samples analyzed had RLU values less than the negative control (tube + Luminase + Ultralute), therefore, all samples were at or near the low detection range of the device. The positive control (173,290 MPN/100 mL) was analyzed in triplicate using the Colilert® method. When this sample was analyzed in triplicate using B-QUA, RLU values ranged from 11 – 53; only two of three analysis replicates were greater than the negative control. This indicates that the low detection range of the device for this single-species sample is at or near 173,290 MPN/100 mL *E. coli*. The calculated $cATP_{BACT}$ values indicated all of the samples were most likely compliant with discharge regulations. Due to the samples being at or below the device detection range, the coefficient of variation was not able to be calculated for any of the B-QUA analyses.

Table 2. Results of Organism Counts and B-QUA Analysis using *E. coli* in LW.

Sample Description	Mean Organism Count (MPN/100 mL)	Count CV	RLU	$cATP_{BACT}$ (pg/100 mL)	Mean (pg/100 mL)	B-QUA CV
Empty Tube (Neg. Control)	---	---	0	---	---	---
Tube + Luminase (Neg. Control)	---	---	8	---	---	---
Tube + Luminase + Ultralute (Neg. Control)	---	---	12	---	---	---
ATP Calibration	---	---	27,131 25,265	---	---	---
<1 MPN/100 mL (Blank, Rep. 1)	<1	0	6	0	0	NC
<1 MPN/100 mL (Blank, Rep. 2)			16	1		
<1 MPN/100 mL (Blank, Rep. 3)			3	0		
72.3 MPN/100 mL	97.5	28.8	5	0	0	NC
96.0 MPN/100 mL			6	0		
124.6 MPN/100 mL			16	1		
1,732.9 MPN/100 mL	>2,190.7	18.1	8	0	1	NC

Sample Description	Mean Organism Count (MPN/100 mL)	Count CV	RLU	$cATP_{BACT}$ (pg/100 mL)	Mean (pg/100 mL)	B-QUA CV
>2,419.6 MPN/100 mL			8	0		
>2,419.6 MPN/100 mL			20	3		
173,290 MPN/100 mL (sample analyzed in triplicate)	173,290	NC	53	16	6	NC
			15	1		
			11	0		

Green highlighting indicates the sample is most likely compliant according to B-QUA interpretation guidelines.

4.1.1.2 VERIFICATION TESTS IN AMENDED LABORATORY WATER

For verification conducted in LW-TMH, results of *E. coli* enumeration using Colilert® compared to results of B-QUA analysis of aliquots of the same sample are shown in Table 3. All calibration standards and negative controls analyzed using B-QUA met the guidelines provided by LuminUltra. As in the LW tests, target ranges for the triplicate samples were 0, 50 and 500 MPN/100 mL. In LW-TMH, all three samples prepared for the 0 MPN/100 mL analysis were <1 MPN/100 mL. The 50 MPN/100 mL samples had an average of 54.6 MPN/100 mL, while the nominally 500 MPN/100 mL samples had an average concentration of 1,193.2 MPN/100 mL. Again, the actual concentration exceeded the nominal concentration, but the goal of having a sample with a concentration above the discharge standard was achieved. With the exception of one sample, all *E. coli* in LW-TMH samples analyzed with the B-QUA system had luminometer readings that were less than the low detection limit (i.e., RLU ≤10), which indicates that the limit of detection for *E. coli* is >1,193.2 MPN/100 mL. The $cATP_{BACT}$ values indicate all of the samples are most likely compliant with discharge regulations. Coefficient of variation was not able to be calculated due to the luminometer readings being below the low limit of detection.

Table 3. Results of Organism Counts and B-QUA Analysis using *E. coli* in LW-TMH.

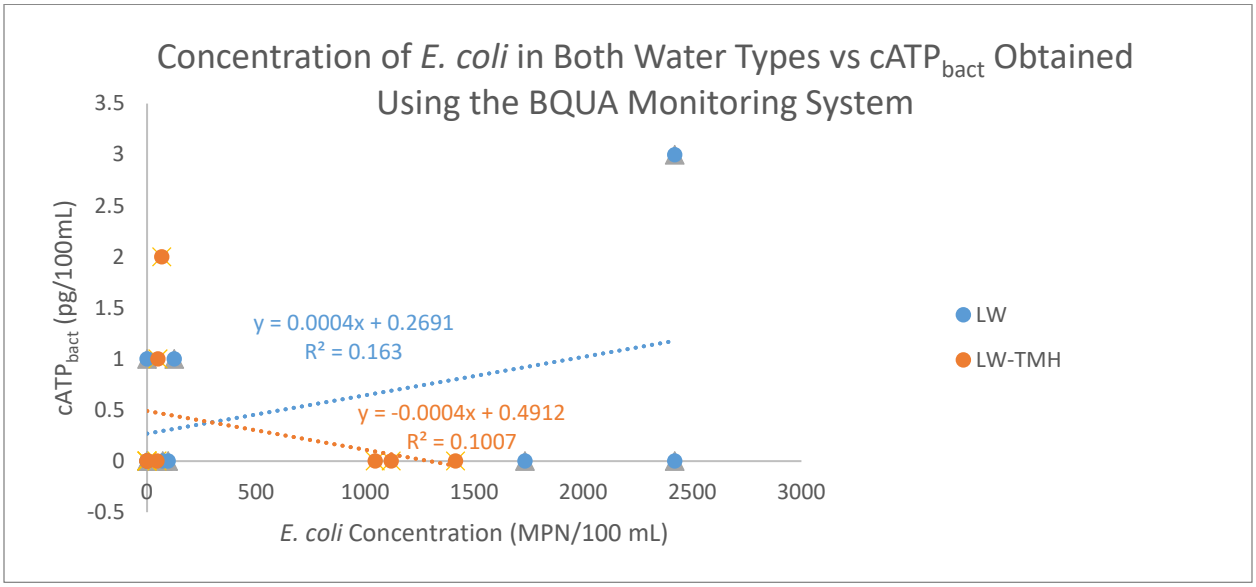
Sample Description	Mean Organism Count (MPN/100 mL)	Count CV	RLU	$cATP_{BACT}$ (pg/100 mL)	Mean (pg/100 mL)	B-QUA CV
Empty Tube (Neg. Control)	---	---	3	---	---	---
Tube + Luminase (Neg. Control)	---	---	5	---	---	---
Tube + Luminase + Ultralute (Neg. Control)	---	---	8	---	---	---
ATP Calibration	---	---	17,808	---	---	---
<1 MPN/100 mL (Blank Rep. 1)	<1	0	4	0	0	NC
<1 MPN/100 mL (Blank Rep. 2)			5	0		
<1 MPN/100 mL (Blank Rep. 3)			3	0		
67.7 MPN/100 mL	54.6	21.2	11	2	1	NC
45.7 MPN/100 mL			6	0		
50.4 MPN/100 mL			10	1		
1,046.2 MPN/100 mL	1,193.2	16.3	4	0	0	NC
1,413.6 MPN/100 mL			5	0		

Sample Description	Mean Organism Count (MPN/100 mL)	Count CV	RLU	$cATP_{BACT}$ (pg/100 mL)	Mean (pg/100 mL)	B-QUA CV
1,119.9 MPN/100 mL			7	0		

Green highlighting indicates the sample is most likely compliant according to B-QUA interpretation guidelines.

The data shown in Tables 2 and 3 are shown graphically in Figure 7. The *E. coli* concentrations determined using Colilert® (LSRI SOP SA/56) are on the x-axis and the B-QUA analysis results for the <10 µm size class are on the y-axis. The correlation values (R^2) for the analyses in LW and LW-TMH both are both less than 0.16, demonstrating poor agreement between the two methods of analysis.

Figure 7. Concentration of *E. coli* vs $cATP_{bact}$ Obtained via B-QUA Analysis in LW and LW-TMH.



4.1.2 *ENTEROCOCCUS FAECIUM* (< 10 µm SIZE CLASS)

4.1.2.1 VERIFICATION TESTS IN LABORATORY WATER

Results of *Enterococcus* enumeration using Enterolert® (LSRI SOP SA/62) compared to results of B-QUA analysis of aliquots of the same sample are shown in Table 4 for LW tests. Target ranges for the triplicate samples were 0, 50 and 500 MPN/100 mL. In LW, all three experimental blank samples were <1 MPN/100 mL, which is the detection limit for the Enterolert® method. The 50 MPN/100 mL samples had an average of 25.2 MPN/100 mL, while the nominally 500 MPN/100 mL samples had an average concentration of 137.0 MPN/100 mL. While the concentration of the highest samples was lower than the target concentration, the samples were all above the ballast water discharge standard for *Enterococci* (<100 MPN/mL), which was the goal.

All calibration standards and negative controls analyzed using B-QUA met the quality control guidelines provided by LuminUltra. Apart from one sample (Blank, Rep. 2), all of the *E. faecium* samples in LW analyzed for the <10 µm size fraction with the B-QUA system had luminometer readings that were

below the negative control (12 RLU). The positive control (2,111.0 MPN/100 mL via the Enterolert® method) was also below the low limit of detection (i.e., BQUA $cATP_{BACT}$ measurement of 0 pg/100 mL), which indicates that the device cannot detect *E. faecium* in single-species samples at concentrations ≤2,111 MPN/100 mL. The $cATP_{BACT}$ values indicate all of the samples are most likely compliant with discharge regulations. Coefficient of variation was not determined for these readings because they were below the device's low limit of detection.

Table 4. Results of Organism Counts and B-QUA Analysis using *E. faecium* in LW.

Sample Description	Mean Organism Count (MPN/100 mL)	Count CV	RLU	$cATP_{BACT}$ (pg/100 mL)	Mean (pg/100 mL)	B-QUA CV
Empty Tube (Neg. Control)	---	---	0	---	---	---
Tube + Luminase (Neg. Control)	---	---	8	---	---	---
Tube + Luminase + Ultralute (Neg. Control)	---	---	12	---	---	---
ATP Calibration	---	---	27,131 25,265	---	---	---
<1 MPN/100 mL (Blank, Rep. 1)	<1	0	6*	0	0	NC
<1 MPN/100 mL (Blank, Rep. 2)			16*	1		
<1 MPN/100 mL (Blank, Rep. 3)			3*	0		
53.8 MPN/100 mL	25.2	98.9	3	0	0	NC
13.1 MPN/100 mL			3	0		
8.6 MPN/100 mL			5	0		
129.6 MPN/100 mL	137.0	5.2	5	0	0	NC
137.6 MPN/100 mL			5	0		
143.9 MPN/100 mL			4	0		
2,110.0 MPN/100 mL	---	---	6	0	---	---

*** Only three total replicates of the Blank were analyzed for LW microbe testing, as the same LW was used for the *E. coli* and *E. faecium* testing. The RLU values reported in this table are the same as those reported in Table 2. Green highlighting indicates the sample is most likely compliant according to B-QUA interpretation guidelines.**

4.1.2.2 VERIFICATION TESTS IN AMENDED LABORATORY WATER

Results of *Enterococcus* enumeration using Enterolert® compared to results of B-QUA analysis of aliquots of the same sample are shown in Table 5 for LW-TMH tests. As in the LW test with *Enterococcus,* target ranges for the triplicate samples were 0, 50 and 500 MPN/100 mL. In LW-TMH, all three samples prepared for the 0 MPN/100 mL analysis were <1 MPN/100 mL using the Enterolert® method of analysis. LW-TMH 50 MPN/100 mL samples had an average of 55.6 MPN/100 mL while the nominally LW-TMH 500 MPN/100 mL samples had an average concentration of 603.5 MPN/100 mL.

All calibration standards and negative controls analyzed using B-QUA met the quality control guidelines provided by LuminUltra. All *Enterococcus* samples analyzed with the B-QUA analysis in LW-TMH had RLU

≤10, which is below the low detection limit. For this single-species sample, the *E. faecium* low detection limit is >770.1 MPN/100 mL. The $cATP_{BACT}$ values indicate all of the samples are most likely compliant with discharge regulations. Coefficient of variation was not calculated for the B-QUA analysis because the measurements were below the B-QUA low detection limit.

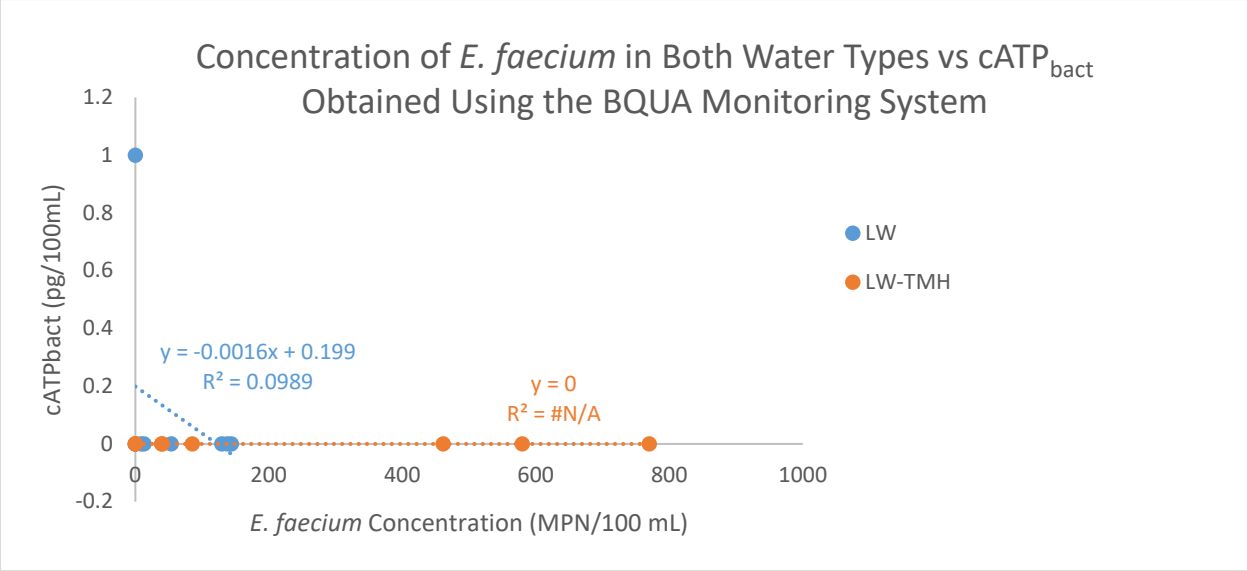
Table 5. Results of Organism Counts and B-QUA Analysis using *E. faecium* in LW-TMH.

Sample Description	Mean Organism Count (MPN/100 mL)	Count CV	RLU	$cATP_{BACT}$ (pg/100 mL)	Mean (pg/100 mL)	B-QUA CV
Empty Tube (Neg. Control)	---	---	3	---	---	---
Tube + Luminase (Neg. Control)	---	---	5	---	---	---
Tube + Luminase + Ultralute (Neg. Control)	---	---	8	---	---	---
ATP Calibration	---	---	17,808	---	---	---
<1 MPN/100 mL (Blank, Rep. 1)	<1	0	4*	0	0	NC
<1 MPN/100 mL (Blank, Rep. 2)			5*	0		
<1 MPN/100 mL (Blank, Rep. 3)			3*	0		
85.5 MPN/100 mL	55.6	47.8	4	0	0	NC
39.3 MPN/100 mL			7	0		
40.4 MPN/100 mL			5	0		
770.1 MPN/100 mL	603.5	25.8	5	0	0	NC
579.4 MPN/100 mL			5	0		
461.1 MPN/100 mL			6	0		

*** Only three total replicates of the Blank were analyzed for LW-TMH microbe testing, as the same LW-TMH was used for the *E. coli* and *E. faecium* testing. The RLU values reported in this table are the same as those reported in Table 3. Green highlighting indicates the sample is most likely compliant according to B-QUA interpretation guidelines.**

The data shown in Tables 4 and 5 are shown graphically in Figure 8. The *Enterococcus* concentrations determined by analysis using Enterolert® are on the x-axis and the B-QUA analysis results for the <10 µm size class are on the y-axis. The correlation values (R^2) for the analysis in LW is less than 0.10, demonstrating poor agreement between the two methods of analysis. No correlation could be calculated for the analysis conducted in LW-TMH because the B-QUA analysis was not able to detect *E. faecium* in any of the samples.

Figure 8. Concentration of *E. faecium* vs $cATP_{bact}$ Obtained via B-QUA Analysis in LW and LW-TMH.



4.1.3 HAEMATOCOCCUS PLUVIALIS (≥10 µm to <50 µm SIZE CLASS)

Results of counts done on *H. pluvialis* samples in LW and LW-TMH (i.e., amended LW) following LSRI SOP GWRC/11 (i.e., staining and fluorescence microscopy) as well as using the B-QUA system are shown in Table 6. A subsample of *H. pluvialis* was measured and cells were found to have an average size of 20.95 µm (17.8-22.4 µm cell size range). Target concentrations of the *H. pluvialis* in both water types were 0 (experimental blank), 10, 50, 100, 500 and 1,000 cells/mL. In LW, the averages of the *H. pluvialis* cell counts for the samples were 0, 11, 57, 86.7, 511.7, and 921.7 cells/mL. The CV values from organism counts ranged from 0-31.5. All quality control samples analyzed using B-QUA during both LW and LW-TMH experiments met the guidelines provided by LuminUltra. All samples analyzed, including the experimental blanks that contained no organisms, had RLU values greater than the low detection range of the B-QUA device. The calculated $cATP_{10\text{-}50}$ values measured with the B-QUA system for LW samples increased with increasing organism counts. Coefficient of variation values for B-QUA from 5.9 to 80.4 with the highest CV values being in the 100 cell/mL and the 1000 cell/mL samples. The blank CV was excluded because the developer has indicated that the blank is less than the background noise of the device. In LW-TMH, the averages of the *H. pluvialis* cell counts for the samples were 0, 8, 43.3, 101.3, 587.7, and 1,095 cells/mL. The $cATP_{10\text{-}50}$ values measured with the B-QUA system increased with increasing organism counts with the exception of the 43.3 to 101.3 cell/mL sample where the $cATP_{10\text{-}50}$ values were 16 pg/mL and 14 pg/mL, respectively. When compared to the $cATP_{10\text{-}50}$ values for LW, samples created in LW-TMH had $cATP_{10\text{-}50}$ values that were approximately two to three times lower, on average, than the value measured in the respective LW samples. The $cATP_{10\text{-}50}$ values indicate all of the samples, with the exception of one of the LW samples, are most likely compliant with discharge regulations. The 940 cell/mL LW sample had a cATP value indicating the signal is close to the discharge regulation limit. Coefficient of variation values for B-QUA from 7.5 to 62.3 the highest CV values was on the nominal 100 cell/mL samples. The CV values from organism counts ranged from 0-22.

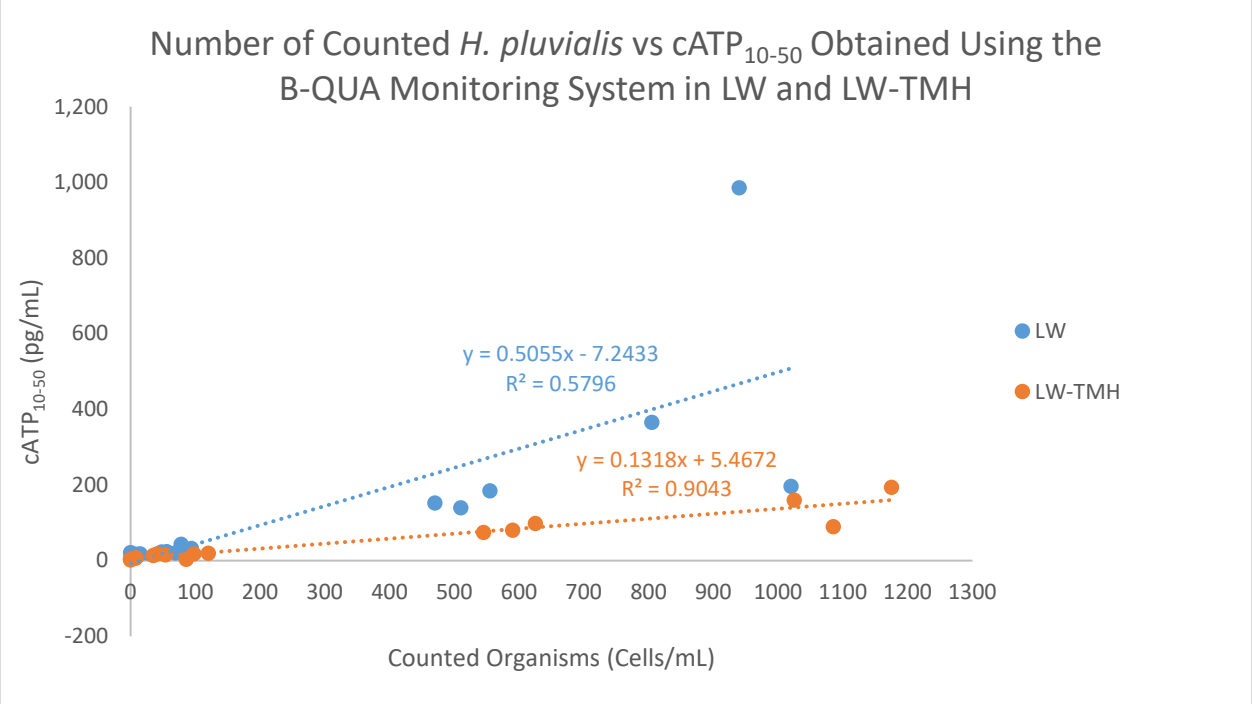
Table 6. Results of Organism Counts and B-QUA Analysis using *H. pluvialis* in LW and LW-TMH. NM = Not Measured.

Sample Description	LW Samples					LW-TMH Samples				
	Microscopy Organism Count (cells/mL)	Mean (CV)	RLU	B-QUA cATP (pg/mL)	Mean (CV)	Microscopy Organism Count (cells/mL)	Mean (CV)	RLU	B-QUA cATP (pg/mL)	Mean (CV)
Empty Tube (Neg. Control)	---	---	2	---	---	---	---	2	---	---
Tube + Luminase (Neg. Control)	---	---	2	---	---	---	---	2	---	---
Tube + Luminase + Ultralute (Neg. Control)	---	---	3	---	---	---	---	8	---	---
ATP Calibration	---	---	23,867 24,402	---	---	---	---	27,166 25,774	---	---
0 cells/mL (Blank)	0	0 (0)	232	6	11 (86.7)	0	0 (0)	120	2	3 (47.1)
	0		209	5		0		177	4	
	0		862	22				NM		
10 cells/mL	15	11 (31.5)	731	18	17 (5.9)	8	8 (0)	321	7	8 (7.5)
	9		694	17		8		327	8	
	9		652	16		8		333	8	
50 cells/mL	69	57.7 (18.4)	823	21	23 (6.7)	41	43.3 (22.4)	789	18	16 (12.5)
	56		961	24		35		609	14	
	48		924	23		54		699	16	
100 cells/mL	94	86.7 (9.3)	1,337	33	33 (31.5)	98	101.3 (17.0)	826	18	14 (62.3)
	88		893	23		120		904	20	
	78		1,736	44		86		189	4	
500 cells/mL	555	511.7 (8.3)	7,218	185	159 (14.5)	590	587.7 (6.8)	3,604	81	85 (14.7)
	510		5,450	140		625		4,418	99	
	470		5,962	153		545		3,349	75	
1000 cells/mL	1,020	921.7 (11.8)	7,698	197	516 (80.4)	1,085	1,095 (6.9)	4,010	90	148 (35.8)
	940		38,440	986		1,175		8,626	194	
	805		14,280	366		1,025		7,118	160	

Green highlighting indicates the sample is most likely compliant, yellow highlighting indicates the signal is close to the compliance limit according to B-QUA interpretation guidelines.

The data shown in Table 6 are shown graphically in Figure 9. The *H. pluvialis* concentrations determined by analysis following LSRI SOP GWRC/11 are on the x-axis and the B-QUA analysis results for the ≥10 µm and <50 µm size class are on the y-axis. The correlation values for the analyses in LW-TMH was >0.9 indicating good agreement between the two methods of analysis. The correlation value for the analyses in LW as <0.6. The lower correlation in LW was largely due to the high variability in the nominal 1000 cell/mL samples. The increased TSS and DOC in the LW-TMH samples resulted in a much lower slope for the regression line than in the LW samples (Figure 9).

Figure 9. *H. pluvialis* Counts vs $cATP_{10-50}$ Obtained via B-QUA Analysis in LW and LW-TMH.



4.1.4 *DAPHNIA MAGNA* (>50 µm SIZE CLASS)

Results from the B-QUA analysis of LW and LW-TMH samples containing *D. magna* <24 hours of age are shown in Table 7. No CV values are provided for the organism counts for the zooplankton samples because all of the samples were counted by one individual and the count was verified by a second individual, which resulted in all samples having the same density (i.e., the target density) of organisms. *D. magna* size was on average 1045 µm ± 109 µm. All negative controls and the ATP calibration standards analyzed during LW and LW-TMH verification met guidelines provided by LuminUltra. In LW, all samples measured were greater than the B-QUA low detection range. Mean B-QUA measurements generally increased with increasing organism count. The exception to this was the 10 organism/sample and 15 organism/sample replicates. Coefficient of variation generally decreased with increasing organism number/sample. The experimental blank samples had a mean RLU of 154, which translates to a mean $cATP_{50}$ pg/m^3 value of 2,505 with very low variability (CV=0.4). The experimental blanks were LW

or LW-TMH with no *D. magna* added. Analysis followed the same procedure as the samples containing *D. magna*. In LW-TMH, all samples measured were greater than the B-QUA low detection range. As the number of *D. magna* increased in the samples, the mean $cATP_{50}$ increased. The CV was highest in the samples containing five *D. magna*/replicate and decreased with increasing concentration as seen in LW. The experimental blanks had a mean RLU of 168, which calculated to an average of 3,352 pg/m^3 $cATP_{50}$. The $cATP_{50}$ values in LW indicated that the blank samples and one of the samples with five organisms were most likely compliant with discharge standards, the samples with 5-15 organisms had signals close to the limit and the samples with 50-100 organisms were most likely not compliant with discharge standards. The compliance determinations in the LW-TMH samples indicated samples containing 0 to 10 organisms were most likely compliant, samples containing 15 organisms had a signal close to the limit and the samples with 50-100 organisms were most likely not compliant with discharge standards. When comparing mean $cATP_{50}$ values measured in LW and LW-TMH, the samples with lower organism densities (i.e., 5, 10, and 15 organisms per sample) had lower average $cATP_{50}$ in LW-TMH than in LW. At the higher organism densities (i.e., 50 and 100 organisms per sample), the $cATP_{50}$ values were similar between water types. This could mean that dissolved organic carbon may suppress the instrument's ability to detect ATP, especially when zooplankton are sparse in a sample (i.e., densities near the ballast water discharge standard). This suppression was not observed in samples that are well above the D-2 Regulation (i.e., 100 organisms/m^3).

Table 7. Results of Organism Counts and B-QUA Analysis using *D. magna* in LW and LW-TMH.

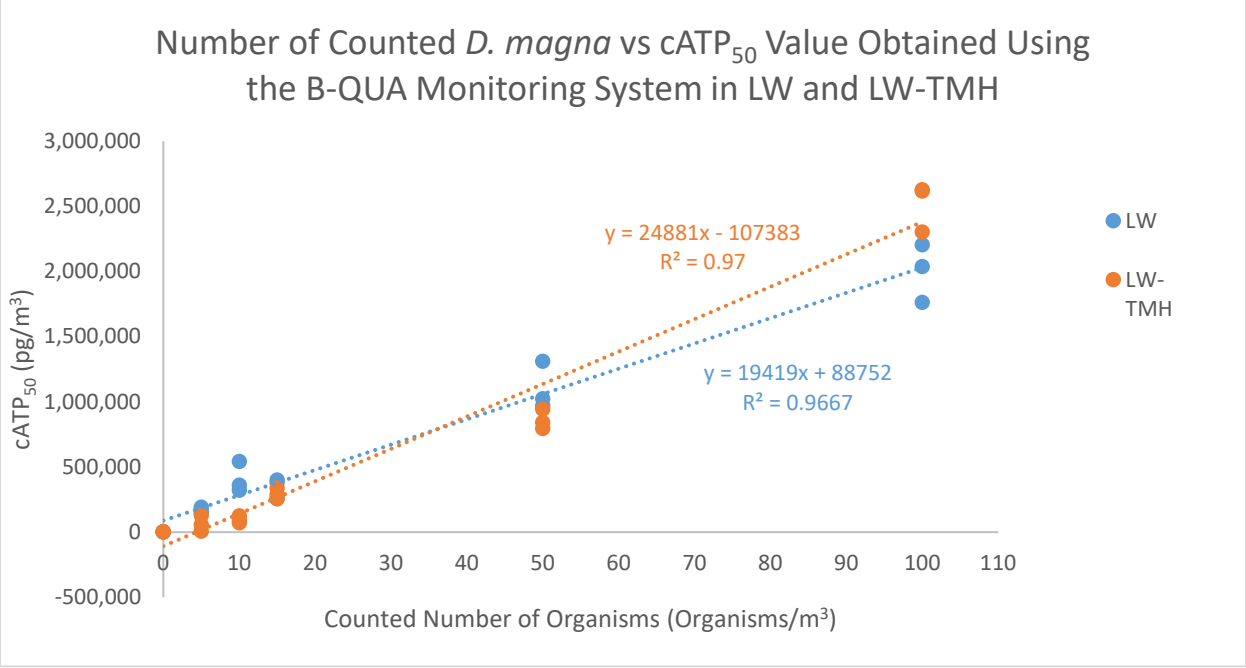
LW Samples				LW-TMH Samples			
Sample Description	RLU	B-QUA cATP (pg/m^3)	Mean (CV)	Sample Description	RLU	B-QUA cATP (pg/m^3)	Mean (CV)
Empty Tube (Neg. Control)	1	---	---	Empty Tube (Neg. Control)	0	---	---
Tube + Luminase (Neg. Control)	0	---	---	Tube + Luminase (Neg. Control)	2	---	---
Tube + Luminase + Ultralute (Neg. Control)	4	---	---	Tube + Luminase + Ultralute (Neg. Control)	0	---	---
ATP Calibration	30,607	---	---	ATP Calibration	25,564	---	---
0 (Blank)	154	2,499	2,505 (0.4)	0 (Blank)	140	2,793	3,352 (19.5)
0 (Blank)	155	2,516		0 (Blank)	204	4,070	
0 (Blank)	154	2,499		0 (Blank)	160	3,192	
5 organisms/m^3	8,745	145,650	165,434 (14.6)	5 organisms/m^3	397	7,920	63,993 (93.9)
5 organisms/m^3	11,545	192,306		5 organisms/m^3	6,389	127,460	
5 organisms/m^3	9,507	158,347		5 organisms/m^3	2,837	56,598	
10 organisms/m^3	32,586	542,909	408,490 (28.9)	10 organisms/m^3	3,630	72,418	95,573 (28.6)
10 organisms/m^3	19,310	321,693		10 organisms/m^3	6,304	125,764	
10 organisms/m^3	21,661	360,867		10 organisms/m^3	4,438	88,538	
15 organisms/m^3	15,452	257,408	346,382 (22.4)	15 organisms/m^3	12,804	255,439	294,035 (14.3)
15 organisms/m^3	24,051	400,692		15 organisms/m^3	14,430	287,877	
15 organisms/m^3	22,872	381,046		15 organisms/m^3	16,982	338,790	
50 organisms/m^3	61,458	1,023,999	1,102,242	50 organisms/m^3	39,940	796,800	860,573

LW Samples				LW-TMH Samples			
Sample Description	RLU	B-QUA cATP (pg/m^3)	Mean (CV)	Sample Description	RLU	B-QUA cATP (pg/m^3)	Mean (CV)
50 organisms/m^3	78,776	1,312,566	(16.7)	50 organisms/m^3	47,256	942,754	(8.7)
50 organisms/m^3	58,227	970,161		50 organisms/m^3	42,214	842,166	
100 organisms/m^3	105,891	1,764,380	2,002,942 (11.1)	100 organisms/m^3	115,504	2,304,297	2,516,810 (7.3)
100 organisms/m^3	132,349	2,205,246		100 organisms/m^3	131,261	2,618,648	
100 organisms/m^3	122,384	2,039,200		100 organisms/m^3	131,704	2,627,486	

Green highlighting indicates the sample is most likely compliant, yellow highlighting indicates the signal is close to the compliance limit, red highlighting indicates that the sample is most likely not compliant according to B-QUA interpretation guidelines.

Data from the *D. magna* testing in both LW and LW-TMH are graphically displayed in Figure 10. Correlation values for B-QUA results compared to actual concentrations of *D. magna* in both water types were >0.97 suggesting high agreement between methods. There was much less impact of high DOC on the slope of the regression lines with *D. magna* analysis than was observed with *Haematococcus* analysis (Figure 10, Figure 9).

Figure 10. *D. magna* Counts vs cATP$_{50}$ Obtained via B-QUA Analysis in LW and LW-TMH.



4.1.5 *CERIODAPHNIA DUBIA* (>50 µm SIZE CLASS)

Results from the B-QUA analysis of LW and LW-TMH samples containing *C. dubia* <24 hours of age are shown in Table 8. The average size of *C. dubia* was 324 µm ± 12 µm. All negative control and ATP calibration standard checks met the guidelines provided by LuminUltra. All measured samples had RLU values greater than the low detection range of the B-QUA device. In both water types, mean B-QUA measurements increased with increasing organism count. In LW, CV ranged from 4.7 to 11.1. The experimental blanks, which did not contain *C. dubia*, had a mean RLU of 103 in LW and a mean calculated $cATP_{50}$ pg/m^3 value of 2,250 with a coefficient of variation of 9.3. In LW-THM, CV ranged from 9.0 to 23.8. The experimental blanks, which were LW-TMH without the addition of *C. dubia* had a mean RLU of 168 and a mean calculated $cATP_{50}$ pg/m^3 value of 3,352 with a coefficient of variation of 19.5. Experimental blanks were LW or LW-TMH to which no *C. dubia* were added. The blank samples were processed and analyzed in the same manner as samples containing *C. dubia*.

Similar to the *D. magna* experiment, samples with lower organism densities (i.e., 5, 10, 15, and 50 organisms per sample) had lower average $cATP_{50}$ in LW-TMH than in LW. At the highest organism densities (i.e., 100 organisms per sample), the $cATP_{50}$ value was still lower in LW-TMH samples, but this difference was less pronounced. Again, this suggests that dissolved organic carbon may suppress instrument response to ATP when organisms are sparse in a sample and ATP concentration is low (i.e., densities near the ballast water discharge standard). Compliance determinations in both LW and LW-TMH indicated that the blank samples and those with 5-15 organisms were most likely compliant while all of the remaining samples, 50-100 organisms, had $cATP_{50}$ values that indicated signals close to the discharge limit.

Table 8. Results of Organism Counts and B-QUA Analysis using *C. dubia* in LW and LW-TMH.

LW Samples				LW-TMH Samples			
Sample Description	RLU	B-QUA cATP (pg/m^3)	Mean (CV)	Sample Description	RLU	B-QUA cATP (pg/m^3)	Mean (CV)
Empty Tube (Neg. Control)	4	---	---	Empty Tube (Neg. Control)	0	---	---
Tube + Luminase (Neg. Control)	2	---	---	Tube + Luminase (Neg. Control)	2	---	---
Tube + Luminase + Ultralute (Neg. Control)	1	---	---	Tube + Luminase + Ultralute (Neg. Control)	0	---	---
ATP Calibration	23,192 25,688	---	---	ATP Calibration	25,564 25,821 23,844 11,653	---	---
0 (Blank)	103	2,243	2,250 (9.3)	0 (Blank)	140	2,793	3,352 (19.5)
0 (Blank)	113	2,463		0 (Blank)	204	4,070	
0 (Blank)	94	2,045		0 (Blank)	160	3,192	
5 organisms/m^3	1166	25,619	26,081 (11.1)	5 organisms/m^3	762	15,051	16,532 (15.3)
5 organisms/m^3	1067	23,442		5 organisms/m^3	764	15,090	
5 organisms/m^3	1328	29,181		5 organisms/m^3	985	19,455	
10 organisms/m^3	2653	58,318	62,948 (8.6)	10 organisms/m^3	1163	22,971	20,844 (10.9)
10 organisms/m^3	3565	60,871* 70,758		10 organisms/m^3	1069	21,114	
10 organisms/m^3	3116	61,844		10 organisms/m^3	934	18,448	
15 organisms/m^3	4454	88,408	84,027 (4.7)	15 organisms/m^3	1685	33,281	40,261 (23.8)
15 organisms/m^3	4171	82,790		15 organisms/m^3	2392	51,163	
15 organisms/m^3	4075	80,884		15 organisms/m^3	1699	36,340	
50 organisms/m^3	17,324	343,924	335,361 (7.8)	50 organisms/m^3	7206	154,129	174,722 (15.8)
50 organisms/m^3	15,405	305,825		50 organisms/m^3	4711	206,180	
50 organisms/m^3	17,949	356,333		50 organisms/m^3	3744	163,858	
100 organisms/m^3	26,418	524,473	490,470 (6.0)	100 organisms/m^3	6528	285,702	311,582 (9.0)
100 organisms/m^3	23,930	475,077		100 organisms/m^3	7802	341,459	
100 organisms/m^3	23,768	471,861		100 organisms/m^3	7028	307,584	

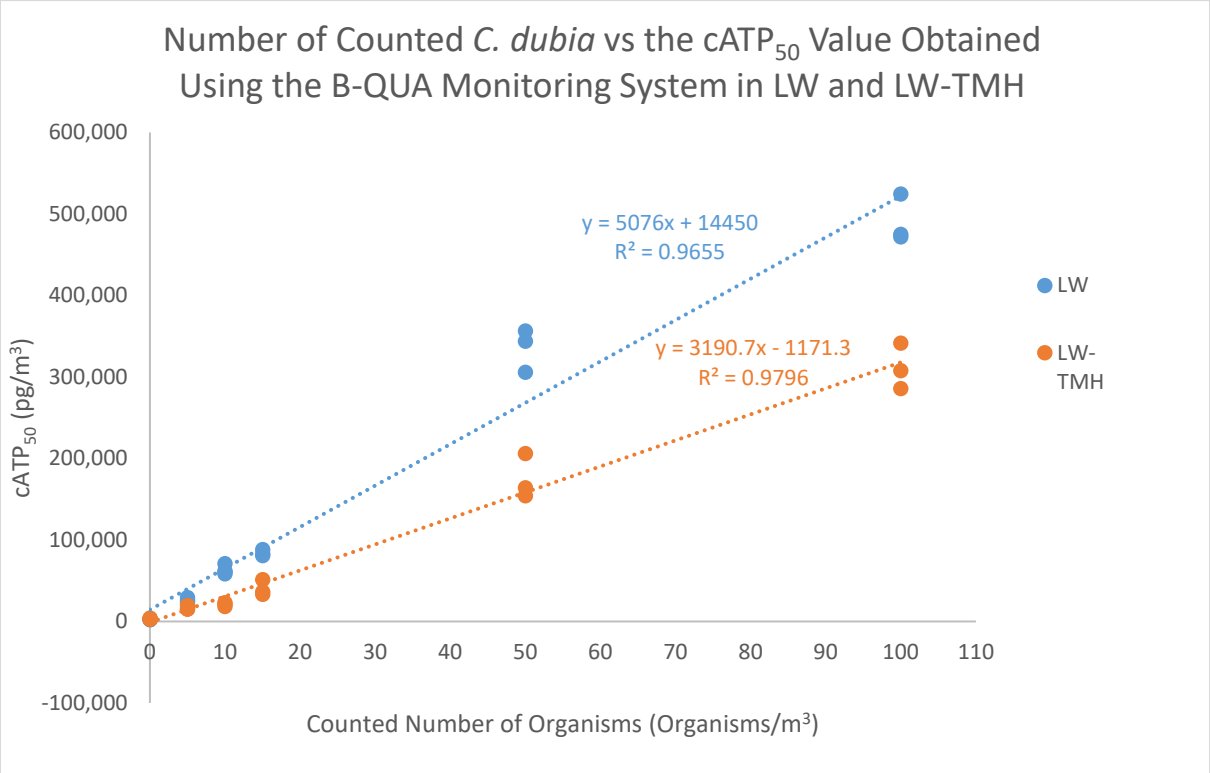
*** Duplicate sample, duplicate is included in average.**

Green highlighting indicates the sample is most likely compliant, yellow highlighting indicates the signal is close to the compliance limit according to B-QUA interpretation guidelines.

The comparison of *C. dubia* concentrations from visual counts in both LW and LW-TMH to B-QUA analysis of the >50 µm size class are shown graphically in Figure 11. The correlation value between the two analysis methods was >0.96 in both water types, showing a high degree of agreement between

methods. With *C. dubia* analysis in both LW and LW-TMH, there was a lower response of the instrument overall with increased DOC (Figure 11).

Figure 11. *C. dubia* Counts vs $cATP_{50}$ Obtained via B-QUA Analysis in LW and LW-TMH.



4.1.6 PHASE I WATER QUALITY AND CHEMISTRY

Water quality measurements taken during Phase I testing with B-QUA are shown in Table 9. The LW samples are shown without shading while the LW-TMH sample rows have been shaded to differentiate between the water types. There were no requirements for the water quality parameters, however, the measurements were within historical ranges. It is interesting to note that the pH of the LW-TMH water increased from 7.51 to 9.54 following autoclaving, likely due to carbon dioxide in the water off-gassing due to heat and pressure.

Table 9. Water Quality Measurements made during Phase I Testing with the B-QUA.

Organism(s)	Size Class	Water Type	Temperature (°C)	pH	Dissolved Oxygen (mg/L)	Conductivity (µS/cm)
E. coli and *E. faecium*	<10 µm	LW (post-autoclave	18.3	8.18	8.9	146.5
E. coli and *E. faecium*	<10 µm	LW-TMH (pre-autoclave)	24.3	7.51	8.2	183.5
	<10 µm	LW-TMH (post-autoclave)	NM	9.54	7.9	183.1
H. pluvialis	≥10 µm-<50 µm	LW	23.8	7.24	4.2	138.4
H. pluvialis	≥10 µm-<50 µm	LW-TMH	27.0	7.53	7.1	137.2
D. magna	>50 µm	LW	25.1	7.19	7.5	141.9
C. dubia	>50 µm	LW	23.7	7.48	8.4	127.9
D. magna and *C. dubia*	>50 µm	LW-TMH	25.6	7.57	7.0	154.1

Water chemistry measurements taken during Phase I testing with B-QUA are shown in Table 10. 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. As was noted in the water quality measurements, autoclaving the water for use in the bacteria testing caused changes in the water chemistry as well. In the LW samples used for *E. coli* and *E. faecium* testing, the parameters were all within the acceptable ranges (Table 11) for water chemistry, but following autoclaving the %T, NPOC, and DOC all were outside of the established parameters. Since the measured parameters were in the acceptable range prior to being autoclaved, and the parameters were established for non-autoclaved water, the water was acceptable for use in testing.

Table 10. Water Chemistry Data Collected in Water used for Phase I B-QUA Testing.

Organism(s)	Water Type	TSS (mg/L)	%T Filtered	%T Unfiltered	NPOC (mg/L)	DOC (mg/L)	POM (mg/L)	MM (mg/L)
E. coli and *E. faecium*	LW (pre-autoclave)	<1.25	98.1	98.1	0.9	0.8	<1.25	<1.25
	LW (post-autoclave)	<1.25	85.9	85.7	7.2	7.1	<1.25	<1.25
E. coli and *E. faecium*	LW-TMH	19.2	29.1	26.6	8.0	5.0	7.8	11.4
H. pluvialis	LW	<1.25	97.9	98.3	0.74	0.73	<1.25	<1.25
H. pluvialis	LW-TMH	19.8	26.5	24.6	9.3	5.70	7.1	12.7
D. magna	LW	<1.25	99.3	99.4	0.82	0.76	<1.25	<1.25
C. dubia	LW	<1.25	97.8	97.8	0.98	0.80	<1.25	<1.25
D. magna and *C. dubia*	LW-TMH	17.8	30.0	27.2	8.5	5.95	7.6	10.2

Table 11. Reference Limits for Water Types Prepared for GWRC Bench-Scale Evaluations.

Parameter	Units	Water Type	Acceptable Range for Initiating Bench-Scale Testing
Total Suspended Solids (TSS)	mg/L	LW	Less than reporting limit
		LW-TMH	11.9 - 30.3
Particulate Organic Matter (POM)	mg/L	LW	Less than reporting limit
		LW-TMH	4.1 - 12.1
Dissolved Organic Carbon (DOC)	mg/L	LW	Less than detection - 2
		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)

4.2 PHASE II

Results from Phase II testing of the ≥10 to <50 µm and ≥50 µm size classes in Duluth-Superior harbor water using the B-QUA compliance monitoring tool alongside traditional microscopic enumeration methods are discussed below. During Phase II testing, the water used for experimental blank samples and dilution water was harbor water filtered through a 934-AH Whatman filter (1.5 µm particle retention). The blank samples were processed analyzed in the same manner as samples containing organisms.

4.2.1 PHYTOPLANKTON

The results of the total live density analysis of organisms in the ≥10 µm to <50 µm size class using the methods in LSRI SOP GWRC/30 – *Procedure for Protist Analysis* (i.e., staining combined with fluorescence microscopy) along with the results of B-QUA analysis of the samples are shown in Table 12. Appendix 1 shows the detailed taxonomic assessment of the organisms in this size class were identified to. 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 403 cells/mL. The nominal 100 cell/mL dilution had an average of 104 cells/mL resulting from microscopic analysis. The sample that was targeted to have protist densities below the ballast water discharge standard had an average of 6.9 cells/mL. The CV for microscopic analyses ranged from 14.0 to 95.3. The high CV of 95.3% was from the low-density sample; four replicates were analyzed on this sample and counts ranged from 3 to 17 cells/mL.

All negative controls and the ATP calibration standard analyzed using B-QUA met the acceptability guidelines provided by LuminUltra. All samples, including the experimental blank, were well above the B-QUA low detection range. The RLU of the blanks ranged from 167 – 201, which was similar to LW-TMH experimental blanks in Phase I. The mean $cATP_{10\text{-}50}$ values increased with increasing concentration of phytoplankton in the samples. The $cATP_{10\text{-}50}$ values for all organism counts indicated that the samples were most likely compliant with discharge standards. Coefficient of variation of samples analyzed with the B-QUA system ranged from 4.2 to 14.8.

Table 12. Results of Native Phytoplankton Counts and B-QUA Analysis in Duluth-Superior Harbor Water.

Sample Description	Mean Organism Count (cells/mL)	Count CV	RLU	$cATP_{10\text{-}50}$ (pg/mL)	Mean (pg/mL)	B-QUA CV
Empty Tube (Neg. Control)	---	---	0	---	---	---
Tube + Luminase (Neg. Control)	---	---	3	---	---	---
Tube + Luminase + Ultralute (Neg. Control)	---	---	3	---	---	---
ATP Calibration	---	---	21,517	---	---	---
0 (Blank)	0	0	167	5	5.3	10.8
0 (Blank)			169	5		
0 (Blank)			201	6		
3 cells/mL	6.9	95.3	252	7	6.8	7.4
3 cells/mL			257	7		
5 cells/mL			251	7		
17 cells/mL			213	6		
103 cells/mL	104	14.0	413	12	10	14.8
119 cells/mL			328	9		

90 cells/mL			364	10		
497 cells/mL	403	25.0	886	25	24	4.2
297 cells/mL			808	23		
416 cells/mL			860	24		

Green highlighting indicates the sample is most likely compliant according to B-QUA interpretation guidelines.

Figures 12 and 13 show the data displayed in Table 12 graphically. Figure 12 graphs all phytoplankton concentrations displayed in Table 12 while Figure 13 graphs only the phytoplankton concentrations in the three lowest dilutions. While the correlation between the microscopic counts and the B-QUA analysis was high when all four dilutions were graphed in Figure 12 (R^2=0.9487), the correlation was much lower when the three lowest dilutions were graphed in Figure 13 (R^2=0.7541). This difference may be due to the high CV in the microscopic counts in the second dilution of phytoplankton.

Figure 12. All Concentrations of Counted Native Phytoplankton vs $cATP_{10\text{-}50}$ Obtained via B-QUA Analysis.

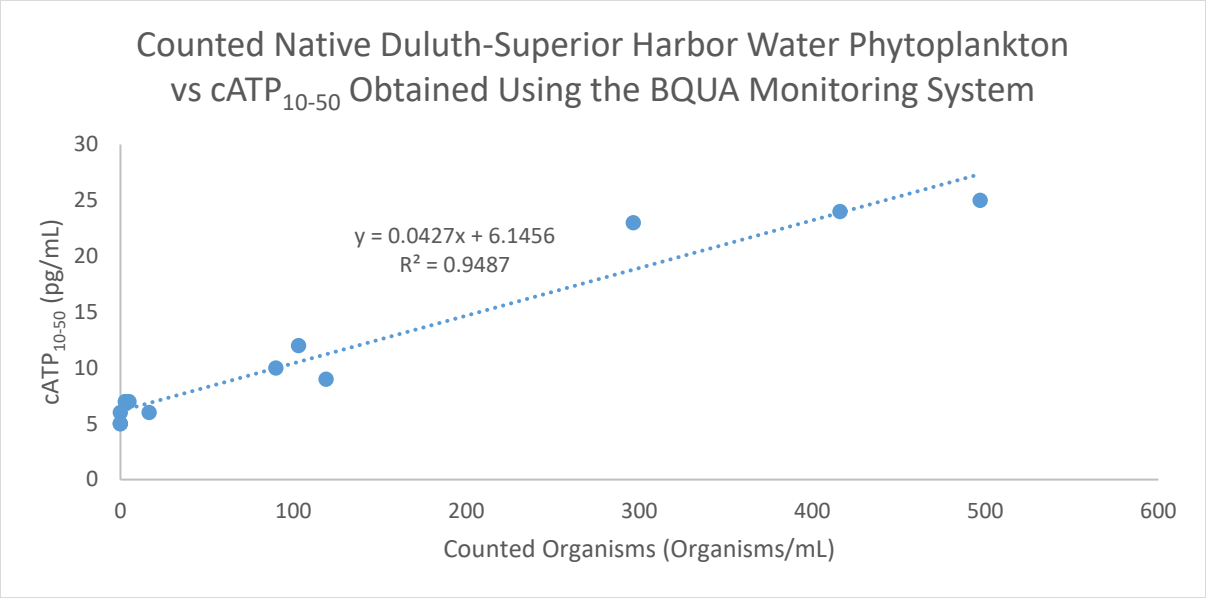
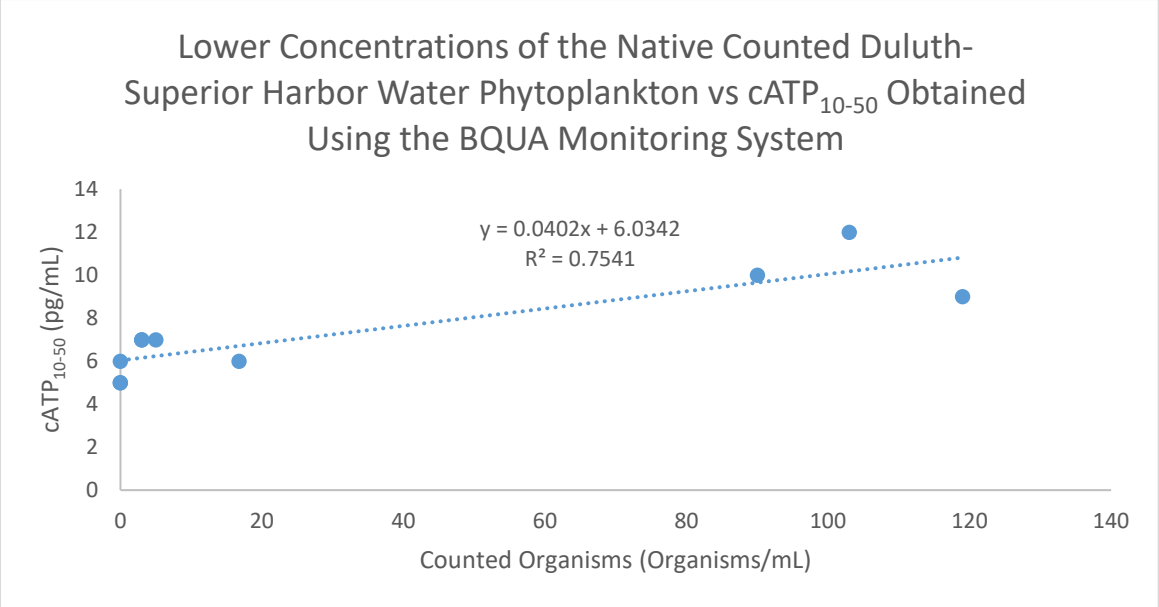


Figure 13. Lower Concentrations of Counted Native Phytoplankton vs $cATP_{10-50}$ Obtained via B-QUA Analysis.



4.2.2 ZOOPLANKTON

The results of the live density analysis of organisms in the ≥50 µm size class using the methods in LSRI SOP GWRC/25 – *Procedure for Zooplankton Analysis* along with the results of B-QUA analysis of the samples are shown in Table 13. Due to the length of time (>2 hours per count) required to conduct microscopic zooplankton analysis, only one count was done per dilution so no CV for microscopic counts is shown in Table 13. Appendix 2 shows the community composition counts for the dilutions of zooplankton analyzed in the Duluth-Superior Harbor Water. The experimental blanks (and dilution water) were verified through microscopic analysis to have a live organism density of 0/m^3. The ambient harbor density of protists on the day of the verification test was 9,782 organisms/m^3. The nominal 100 organisms/m^3 dilution had 99 organisms/m^3 resulting from microscopic analysis. The sample that was targeted to have protist densities below the ballast water discharge standard had 7 organisms/m^3.

All negative controls and the ATP calibration standard analyzed using B-QUA met the acceptability guidelines provided by LuminUltra. All samples, including the experimental blank, were well above the B-QUA low detection range. The RLU of the blanks ranged from 103 - 248, which was similar to the results obtained from analysis of experimental blanks during Phase II protist verification and similar to LW-TMH experimental blanks in Phase I. Mean $cATP_{50}$ values increased with increasing concentrations of native zooplankton in the samples (Table 13). The blank sample which had no zooplankton present, as verified by the microscopic count method, was analyzed five times with the B-QUA system rather than three times due to the high variability among the analyses. The $cATP_{50}$ values measured with B-QUA indicate that the blank samples and samples with 7 organisms/m^3 were most likely compliant with discharge standards, the samples with 99 organisms/m^3 had signals close to the discharge limit and the samples with 9,782 organisms/m^3 were most likely not compliant. Coefficients of variance in the

samples containing zooplankton ranged from 7.3 to 9.3 while the sample that did not have zooplankton had a CV=59.3.

Table 13. Results of Native Zooplankton Counts and B-QUA Analysis in Duluth-Superior Harbor Water.

Sample Description	RLU	$cATP_{50}$ (pg/m³)	Mean (pg/m³)	B-QUA CV
Empty Tube (Neg. Control)	1	---	---	---
Tube + Luminase (Neg. Control)	0	---	---	---
Tube + Luminase + Ultralute (Neg. Control)	3	---	---	---
ATP Calibration	13,089 20,405	---	---	---
0 (Blank)	248	9,546	4,883	59.3
	231	5,699		
	103	3,896		
	105	2,549		
	112	2,724		
7 organisms/m³	823	31,950	32,730	9.3
	929	36,081		
	777	30,158		
99 organisms/m³	8326	324,298	299,517	7.6
	7575	295,036		
	7169	279,216		
9,782 organisms/m³	476045	18,548,508	20,116,004	7.3
	551248	21,478,719		
	521530	20,320,786		

Green highlighting indicates the sample is most likely compliant, yellow highlighting indicates the signal is close to the compliance limit, red highlighting indicates that the sample is most likely not compliant according to B-QUA interpretation guidelines.

4.2.3 WATER CHEMISTRY

Water chemistry analysis was conducted during the Phase II testing in Duluth-Superior Harbor Water in order to provide the developer with data to show how naturally occurring turbidity and total suspended solids may impact B-QUA test results. The values obtained during the Phase II testing are shown in Table 14 and are within historical ranges measured in the Duluth-Superior Harbor.

Table 14. Water Chemistry Measurements made during Phase II Testing with B-QUA.

Organism(s)	Water Type	TSS (mg/L)	%T Filtered	%T Unfiltered	NPOC (mg/L)	DOC (mg/L)	POM (mg/L)	MM (mg/L)

Phytoplankton and Zooplankton	Filtered Harbor Water (Dilution Water)	<1.25	16.6	10.1	22.0	19.9	<1.25	<1.25
Phytoplankton and Zooplankton	Concentrated Algae in Harbor Water	4.1	17.8	9.2	22.6	21.1	1.4	2.7

4.2.4 FILTER LOSS STUDY

The density of protists (entities ≥10 to <50 µm) lost through a 5 µm and 10 µm filter are shown in Table 15. The sample prior to filtration had an average protist density of 400 cells/mL and was dominated by filamentous forms of protists (i.e., blue-green algae) whose long chains do not normally pass through filters of this size.

After filtration, there was very little difference in the density of organisms present in the filtrate, i.e., for both filters the filter loss was similar and was very low (between 0 and 2 cells/mL). Filter loss is a function of community composition, particularly in Great Lakes samples, ideally this experiment would be repeated during different times of the year in order to determine the impact of community composition on filter loss.

Table 15. Results of the Filter Comparison Study. Data presented shows the number of organisms in the sample filtrate.

Sample-Rep	Live Cell Density: ≥ 10 µm in any cell dimension but < 10 µm in minimum dimension	Live Cell Density: ≥ 10 and < 50 µm in all visible cell dimensions	Total Live Density	Classification of Organisms Present
5 µm- 1	0	0	**0**	**Nothing**
5 µm-2	0	1	**1**	**Cryptomonas**
5 µm-3	0	2	**2**	**Round microflagellates**
10 µm-1	0	1	**1**	**Cryptomonas**
10 µm-2	0	0	**0**	**Nothing**
10 µm-3	0	0	**0**	**Nothing**

5 QUALITY ASSURANCE/QUALITY CONTROL – DATA QUALITY OBJECTIVES

5.1 BACTERIA TESTING

Data quality objectives for precision, bias and accuracy and completeness (i.e., method blanks, duplicate agreements, and quantitative positive and negative controls) were all within acceptable limits for the bacteria test involving *E. coli* and *E. faecium* (Table 16).

Table 16. Data Quality Objective Summary for B-QUA Tests using *E. coli* and *E. faecium*.

Data Quality Indicator	Evaluation Process/ Performance Measurement	Data Quality Objective	Performance Measurement Result
Precision	Samples (10%) are analyzed in duplicate – with performance measured R_{log} not greater than precision criterion (PC)	R_{log} not greater than 0.4043 for *E. coli and* not greater than 0.5597 for *Enterococcus* spp.	***E. coli:*** 4 of 20 of (20%) samples were analyzed in duplicate; Average R_{log} = 0.1149
			E. faecium: 4 of 20 of (20%) samples were analyzed in duplicate; Average R_{log} = 0.1658
Bias, Operator	Samples (10%) are counted by two separate analysts with performance measured by average relative percent difference (RPD) of all second counts.	<20% average RPD	***E. coli*:** 16 of 31 (51.6%) samples counted by 2nd analyst; Average RPD=0.1%
			E. faecium: 17 of 32 (53.1%) samples counted by 2nd analyst; Average RPD=1.8%
Bias, Positive Control	Qualitative positive control samples (American Type Culture Collection) are analyzed on each analysis date or IDEXX-QC samples are analyzed as a quantitative positive control at least once per ballast water treatment system test.	Results must be greater than the limit of detection.	***E. coli:*** Qualitative Positive controls >1 MPN/100 mL n=1
			E. faecium: Qualitative Positive controls >1 MPN/100 mL n=1
Bias, Negative Control	Qualitative negative control samples (American Type Culture Collection) are analyzed on each analysis date or IDEXX-QC samples are analyzed as a negative control at least once per ballast water treatment system test.	Results must be less than the limit of detection.	***E. coli:*** Qualitative Negative controls <1 MPN/100 mL, n=2
			E. faecium: Qualitative Negative controls <1 MPN/100 mL, n=3
Bias, Method	Sterilized water (similar matrix sample) analyzed using same method as samples on each analysis date.	Results must be less than the limit of detection.	***E. coli:*** All method blanks <1 MPN/100 mL, n=6
			E. faecium: All method blanks <1 MPN/100 mL, n=6
Bias, Diluent Blank	One per analysis day, diluent (e.g., sterile deionized water) blank run analyzed using same media as samples	Results must be less than the limit of detection.	***E. coli:*** Blank <1 MPN/100 mL, n=1
			E. faecium: Blank <1 MPN/100 mL, n=1

Data Quality Indicator	Evaluation Process/ Performance Measurement	Data Quality Objective	Performance Measurement Result
Accuracy	IDEXX-QC samples are analyzed as a quantitative positive control at least once per ballast water treatment system test.	*E. coli:*	***E. coli:*** All quantitative analyses within IDEXX acceptance ranges (n=1)
		19-461 MPN/100 mL	07 Feb. 2020; 166 MPN/100 mL
		E. faecalis:	***E. faecalis:*** All quantitative analyses within IDEXX acceptance ranges (n=1)
		53-179 MPN/100 mL	07 Feb. 2020; 105 MPN/100 mL
Representativeness	All samples are collected, handled, and analyzed in the same manner.	Not Applicable – Qualitative.	All bacterial samples were collected, handled, and analyzed in the same manner (using the appropriate LSRI/GWRC SOPs).
Comparability	Routine procedures are conducted according to appropriate SOPs to ensure consistency between tests.	Not Applicable – Qualitative.	The LSRI/GWRC SOPs listed in Section 2.5.1.1 were used for all bacterial analyses conducted.
Completeness	Percentage of valid (i.e., collected, handled, analyzed correctly and meet DQOs) bacterial samples measured out of the total number of bacterial samples collected. Performance is measured by percent completeness (%C).	>90% C.	***E. coli:*** 29 of 31 samples = 93.5% Completeness
			E. faecium: 32 of 32 samples = 100% Completeness
Sensitivity	The limit of detection (LOD) for the analytical method used is reported.	Dependent upon the analytical technique used. Adjusted for volume used.	***E. coli:*** **LOD:** <1 MPN/100 mL
			E. faecium: **LOD:** <1 MPN/100 mL

5.2 ALGAE TESTING

No quality assurance counts were conducted during the *H. pluvialis* testing. No requirement for quality assurance counts were specified in the B-QUA test plan (LSRI, 2019).

5.3 ZOOPLANKTON TESTING

During testing with *D. magna,* 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 of the tests, with 100% of the samples having quality assurance counts conducted (Table 17). The RPD met the DQO for all samples in

the *D. magna* tests. A reference test conducted on October 15, 2019 with *D. magna* resulted in an LC_{50} within the acceptable range, indicating that the organisms used for the B-QUA test were healthy.

Table 17. Average Relative Percent Difference (RPD) of Samples Counted for *D. magna* Tests Conducted during B-QUA Phase I Tests.

Test Date	Percent of Samples with QA counts	DQO	Relative Percent Difference between counts
3 October 2019	100%	RPD ≤ 10%	0%
8 November 2019	100%		0%

During testing with *C. dubia,* 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 (Table 18). The RPD was 0% for all of the samples counted in duplicate. A reference test conducted on October 8, 2019 with *C. dubia* resulted in an LC_{50} within the acceptable range, indicating that the organisms used for the B-QUA test were healthy.

Table 18. Average Relative Percent Difference (RPD) of Samples Counted for *C. dubia* Tests Conducted during B-QUA Phase I Tests.

Test Date	Percent of Samples with QA counts	DQO	Relative Percent Difference between counts
30 October 2019	100%	RPD ≤ 10%	0%
8 November 2019	100%		0%

5.4 WATER CHEMISTRY AND WATER QUALITY

The data quality objectives (DQO) for water quality and chemistry analyses conducted during the evaluation of the B-QUA 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 and Water Quality Analyses Conducted during B-QUA 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: 30.0%	%TF: 0.6 ± 0.3%
			%TU: 30.0%	%TU: 0.3 ± 0.6%
			NPOC: 30.0%	%NPOC: 1.1 ± 0.4%%
			DOC: 30.0%	%DOC: 1.3 ± 1.2%

Data Quality Indicator	Evaluation Process/Performance Measurement	Data Quality Objective	Performance Measurement Result	
			POM: 30.0%	POM: 2.3%
			TSS: 30.0%	TSS: 0.4%
Bias, Filter Blanks	%T method blanks were prepared by filling a sample bottle with deionized water, then filtering, and analyzing as a sample (one per analysis date)	> 98% average %T	Number of %T Method Blanks Analyzed: 7	Method blanks (%T): 99.6 ± 0.5%
	TSS/POM method blanks prepared by filtering deionized water samples from a sample bottle and then filtering, drying, weighing, ashing and weighing the blank as a sample (one per analysis date)	< 0.63 mg/L average TSS/POM	Number of TSS Method Blanks Analyzed: 7	Method Blanks (TSS): <0.63 ± 0 mg/L
			Number of POM Method Blanks Analyzed: 7	Method Blanks (POM): <0.63 ± 0 mg/L
	NPOC procedural blanks were prepared by acidifying a volume of deionized water to 0.2% with concentrated hydrochloric acid	< 0.70 mg/L average NPOC	Number of NPOC Procedural Blanks Analyzed: 22	Procedural Blanks (NPOC): <0.70 ± 0 mg/L
	DOC method blanks were prepared by filtering a deionized water sample from a sample bottle (one per analysis date)	< 0.70 mg/L average DOC	Number of DOC Method Blanks Analyzed: 7	Method Blanks (DOC): <0.70 ± 0 mg/L
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: 15.0%	NPOC/DOC Spike Recovery= 104.0 ± 8.2
	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 an Independent Reference Standard:	Independent Reference Standard Percent Difference
			TSS: 100%	TSS: 2.9 ± 1.9%
			POM: 100%	POM: 3.1 ± 2.1%
			NPOC: 114%*	NPOC: 4.5 ± 2.6%
		A least one per 10 samples < 10% average D	Percentage (vs total samples) Check Standards:	Check Standards Percent Difference
			NPOC/DOC: 105%*	NPOC: 4.5 ± 2.6%
Represent-ativeness	All samples were collected, handled, and analyzed in the same manner.	Not Applicable – Qualitative.	All water chemistry/quality samples were collected, handled, transported and analyzed in the same manner using the appropriate SOPs.	

Data Quality Indicator	Evaluation Process/Performance Measurement	Data Quality Objective	Performance Measurement Result
Comparability	Routine procedures were conducted according to appropriate SOPs to ensure consistency between tests.	Not Applicable – Qualitative.	The SOPs listed in the methods and references section were used for all water chemistry and water quality analyses.
Completeness	Percentage of valid (i.e., collected, handled, analyzed correctly and meeting DQOs) water chemistry samples measured out of the total number of water chemistry samples collected. Performance is measured by percent completeness (%C).	> 90% C	TSS: 100%
			%T, Filtered: 100%
			%T, Unfiltered: 100%
			NPOC: 100%
			DOC: 100%
Sensitivity	The limit of detection (LOD) and limit of quantification (LOQ) for each analyte and analytical method utilized was determined annually unless a reporting limit was used based on the amount filtered as was the case with TSS/POM.	Not Applicable	TSS/POM RL: 1.25 mg/L based on filtering 800 mL of sample
			NPOC/DOC LOD: 0.70 mg/L
			NPOC/DOC LOQ: 2.3 mg/L
			Determined 7 February 2019

*A spike recovery failed and was re-analyzed. The original spike analysis was not counted but the reference and check standards analyzed were, explaining why these percentages are greater than 100%.

6 CONCLUSIONS AND DISCUSSION

The LSRI-GWRC freshwater verification of the B-QUA Quick Ballast Monitoring Kit met the stated objectives, as outlined in the Test Plan (LSRI, 2019). The reported deviations do not impact LSRI-GWRC's ability to draw conclusions on B-QUA performance during this verification. B-QUA was operated in accordance with the developer's instructions and operated reliably during all reported tests.

Results from this verification, although limited in scope to non-treated water, indicate potential effectiveness of B-QUA for monitoring of ballast water in Great Lakes vessels for planktonic organisms only. To determine the effectiveness of the B-QUA system, a series of questions were addressed through experimentation.

Objectives 1 and 2: Do ATP analysis results from the B-QUA correspond to standard laboratory/microscopic analysis of freshwater laboratory-cultured organisms in the three regulated size classes? Does increased turbidity and total suspended solids affect the ability of the B-QUA to detect ATP in a water sample?

When *E. coli* and *E. faecium,* the indicator organisms for the <10 µm size class, were examined using standard laboratory analysis compared to the B-QUA analysis, the majority of the samples had RLU values that were less than the negative control (tube + Luminase + Ultralute). The B-QUA system was not able to detect ATP present in nearly all of the bacteria samples analyzed. Consequently, there was

no difference between the experimental blanks and the samples that did contain bacteria, including samples containing concentrations of either indicator organism as high as 2,000 MPN/100 mL. The results of testing in the <10 µm size class did not differ from low TSS, low turbidity water to high TSS, high turbidity samples. Although the B-QUA system did not quantify the *E. coli* and *E. faecium* in the laboratory samples with concentrations of either bacteria of 2,000 MPN/100 mL, the initial test conducted, using test water contaminated with a high concentration of other heterotrophic bacteria, registered high $cATP_{BACT}$ values for all water samples, including the experimental blanks. The testing completed as part of this assessment used two of the bacteria indicator species in the <10 µm size class, as the IMO D-2 Regulation is based on these two species in addition to *Vibrio cholerae*. According to the developer, B-QUA was not designed to test single-species samples at the concentrations tested in this study. Additionally, according to the developer, the B-QUA compliance monitoring tool is designed to determine gross non-compliance in the <10 µm size class. The results reported in this paper show that B-QUA cannot detect *E. coli* concentrations as high as 173,290 MPN/100 mL (Table 2). A possible next step in this freshwater verification would be to first determine the B-QUA detection limit for single-species *E. coli* and *E. faecium* samples, and then spike *E. coli* and *E. faecium* into surface water samples and develop a detection limit for these discharge standard organisms in the presence of other ambient bacteria.

Organisms in the ≥10 µm to <50 µm size class and the >50 µm size class had good correlation between microscopic count methods and B-QUA counts in both low TSS, low turbidity water and high TSS, high turbidity samples. The impact of higher TSS and DOC values was different depending on the organism. In LW-TMH for both *H. pluvialis* and *C. dubia,* cATP values measured by BQUA were lower than comparable concentrations in LW suggesting that TSS and/or DOC can decrease the sensitivity of BQUA, particularly in samples with relatively low ATP concentrations. In *D. magna* the BQUA cATP measurements were lower in LW-TMH samples than in LW samples in all except the blank and highest density sample causing the slope for the regression line to be higher in LW-TMH than LW. Since cATP values measured for the >50 µm size class were much higher in general than for the ≥10 µm to <50 µm size class, the impact of decreased sensitivity due to high TSS and/or high DOC would be most important in the ≥10 µm to <50 µm size class and for densities close to the ballast water discharge standard.

Determination of compliance with the discharge standards in the Phase I testing of the ≥10 µm to <50 µm size class indicated all concentrations up to a nominal concentration of 1000 organisms/mL in both types of water were most likely with compliant with the discharge limit with the exception of one sample in LW (Table 6). This B-QUA result differs from the microscopic counts of the *H. pluvialis* in the 50, 100, 500 and 1000 organisms/mL ranges. This result indicates that it may be necessary to develop interpretation guidelines for B-QUA that are specific to freshwater protists.

Although the correlation for the >50 µm size class was high, the blank samples had high cATP values. For both organisms in the ≥50 µm size class, blank values exceeded cATP values for the highest concentrations of *H. pluvialis* (1000 cells/mL) while the blank samples for the <10 µm and ≥10 µm to <50 µm size classes had very low cATP values in both water qualities. Analysis of samples for the >50 µm size class within the high TSS, high DOC water did have lower relative cATP values than the low TSS, low DOC

water, but correlation values between microscopic counts and B-QUA analysis were high in both water types.

The compliance assessment by B-QUA for the organisms in the ≥50 µm size class agreed well with visual counts for *D. magna* in both water types (Table 7) indicating samples in lab water containing 5-15 organisms were near the compliance limit and those with 50 or 100 organisms being most likely non-compliant. In LW-TMH the *D. magna* samples containing 0-10 organisms were most likely compliant, those with 15 organisms were near the compliance limit and those with 50-100 organisms were most likely non-compliant. However, the B-QUA compliance assessment with *C. dubia* was not in agreement with visual counts of the organisms. All analysis with 0-15 *C. dubia* were indicated as being most likely compliant while the samples with 50-100 *C. dubia* were indicated as being near the compliance limit (Table 8). The difference between the results of the *D. magna* tests and *C. dubia* tests could be due to the size difference in the organisms, with *C. dubia* being approximately 1/3 as large as the *D. magna*. This result indicates that it may be necessary to refine the B-QUA interpretation guidelines for smaller, freshwater zooplankton.

Objective 3: Do ATP results from the B-QUA correspond to detailed microscopic analysis of organisms in the ≥10 to <50 µm and >50 µm size classes from Western Lake Superior water?

In Phase II with natural assemblages of both the ≥10 to <50 µm and >50 µm size classes using standard microscopic enumeration and B-QUA analysis, good correlation was seen between the two methods (Table 12 and 13, respectively). Although the correlation for the ≥50 µm size class was high, the blank samples had high cATP values. In fact, there were high blank cATP values in the ≥50 µm size class in both the Phase I and Phase II testing indicating that the filters may possibly be entrapping heterotrophic bacteria that would result in measurable RLU in samples containing no zooplankton. This may be particularly relevant in the case of ballast water samples where the ballast tanks could contain mineral debris or pieces of shells with entrapped microorganisms that could be detected by B-QUA if they are caught on the membrane and homogenized in the beads tubes with the sample.

In the >50 µm size class, the cATP values for samples with both 0 and 7 organisms/m^3 both indicated that the cATP signal was most likely compliant, those with 99 organisms/m^3 were close to the discharge standard while the sample with 9,782 organisms/m^3 indicated that the sample was most likely not compliant with the discharge standard. The ≥10 to <50 µm size class had low cATP values in the blank samples suggesting increased sensitivity of B-QUA to freshwater protists than to zooplankton. All samples in Phase II testing with the ≥10 to <50 µm size class, which had organism concentrations up to an average of 403 cells/mL were indicated by the B-QUA system to be most likely compliant with discharge standards. Like the Phase I results, this indicates that it may be necessary to refine the B-QUA interpretation guidelines for freshwater protists.

It is interesting to note that results from Phase II testing with the ambient assemblage in Duluth-Superior harbor water have similar results to those obtained in Phase I testing with the *H. pluvialis* and *C. dubia* in LW-TMH. In LW-TMH, samples with an average density of 101.3 cells/mL *H. pluvialis* resulted in a cATP range of 4-20 pg/mL, with an average of 14 pg/mL. In harbor water, the ambient protist

assemblage of 104 cell/mL resulted in a cATP range from 9-12 pg/mL with an average of 10 pg/mL. With *C. dubia in* LW-TMH, an average of 100 organisms/sample resulted in a cATP range from 285,702 to 341,459 pg/m^3 with an average of 311,582 pg/m^3. Similarly, in harbor water the ambient assemblage of 99 organisms/m^3 produced an BQUA cATP measurement range of 279,216 to 324,298 pg/m^3 with an average of 299,517 pg/m^3. The similarity in the measurements of Phase I and Phase II samples shows that the work done in Phase I is relevant to real-world samples.

Objective 4: What percentage of organisms in the ≥10 to <50 µm size class are lost through a 10 µm filter? Does decreasing the filter size effectively capture Great Lakes protists in this size class?

In the samples associated with this study it appears that there was very little difference in what was lost by the 5 µm filter compared to the 10 µm filter, although the ambient assemblage was dominated by filamentous forms of algae that would not normally be a concern in terms of filter loss. These results should be considered preliminary, and this experiment should be repeated with ambient assemblages at various times of the year in order to more accurately quantify loss through the 10 µm filter.

Ideally, GWRC would have used a 7 µm filter for comparison, if the manufacturer carried it. The 7 µm filter (10 µm on diagonal) is more appropriate for Great Lakes organisms. The 5 µm may have been retaining some organisms, particularly single-celled organisms, that were below the regulated size class. Conversely, using the 10 µm filter may cause the loss of some of the organisms that are close to the 10 µm minimum.

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Appendix 1. Taxonomic Characterization of Phase II Organisms in the ≥10 µm and <50 µm Size Class.

Sample Name	497 cells/mL	
Analyst	MNA	
Water Filtered	200	
Conc. Sample volume	15	
Squares Counted	100	
Taxonomy	**min. dimension < 10 µm**	**min. dimension > 10 µm**
Blue Greens		
***Anabaena* - like**	97	25
***Oscillatoria* - type**		
coccoid		
***Microcystis* - like coccoid**	350	NA
other filamentous cells	8	
filamentous-nocells (length)		
Merismodedia		
Greens		
***Scenedesmus* - type desmid**	2	
Pediastrum		
coccoid	6	9
single spindle	1	NA
filamentous - cells		
other colonial (non-coccoid)		
other colonial (spindle)		
euglenoid		1
Cryptophytes (and other small flagellates)		
***Cryptomonas/ Chroomonas* - types**		3
round microflagellates		74
irregular microflagellates	2	4
Diatoms		

Totals	<10 total	>10 total
Blue Greens	455	25
Greens	9	10
Cryptophytes	2	81
Diatoms	162	49
Chrysophytes	0	24
Dinoflagellates	0	0
Protozoans and Animals	0	11
Unknown	0	0
All Taxa	628	200
Total Cells Counted:	828	

Chain ***(Aulacoseira, Melosira, S. binderanus*****)**	24	31
Asterionella	127	
Tabellaria		
Diatoma		
Centric nonchain ***(Cyclotella, Stephanodiscus)***	3	14
Fragilarioid (ribbon colony)	6	
Synedra - like (includes nitzschioid)		
naviculoid (or other single pennate)	1	4
Rhizosolenia	1	
Chrysophytes		
Mallomonas		
Synura		24
Dinobryon **(and *Kephyrion)***		
Dinoflagellates		
round/teardrop/ pointy		
Ceratium (Eiffel Tower)		
Protozoans and Animals		
ciliate	NA	3
round or oval protozoan		8
irregular protozoan		
Keratella	NA	
Polyarthra	NA	
Egg		
Unknown Entities/Cells		
round/oval "could be anything"		
irregular "could be anything"		
Taxonomy	**min. dimension < 10 µm**	**min. dimension > 10 µm**

Sample Name	497 cell/mL Dup	
Analyst	EMR	
Water Filtered	200	
Conc. Sample volume	15	
Squares Counted	100	
Taxonomy	**min. dimension < 10 µm**	**min. dimension > 10 µm**
Blue Greens		
***Anabaena* - like**	28	
***Oscillatoria* - type**	12	
coccoid		
***Microcystis* - like coccoid**	130	NA
other filamentous cells		
filamentous-nocells (length)		
Merismodedia		
Greens		
***Scenedesmus* - type desmid**		
Pediastrum		
coccoid	9	1
single spindle	1	NA
filamentous - cells		
other colonial (non-coccoid)		
other colonial (spindle)		
euglenoid		1
Cryptophytes (and other small flagellates)		
***Cryptomonas/ Chroomonas* - types**	3	9
round microflagellates	58	2
irregular microflagellates		
Diatoms		

Totals	<10 total	>10 total
Blue Greens	170	0
Greens	10	2
Cryptophytes	61	11
Diatoms	164	73
Chrysophytes	0	1
Dinoflagellates	0	1
Protozoans and Animals	0	3
Unknown	0	1
All Taxa	405	92
Total Cells Counted:	497	

Chain ***(Aulacoseira, Melosira, S. binderanus*****)**	46	53
Asterionella	77	
Tabellaria	2	
Diatoma		
Centric nonchain ***(Cyclotella, Stephanodiscus)***	20	18
Fragilarioid (ribbon colony)	18	
Synedra - like (includes nitzschioid)	1	1
naviculoid (or other single pennate)		1
Rhizosolenia		
Chrysophytes		
Mallomonas		1
Synura		
Dinobryon **(and *Kephyrion)***		
Dinoflagellates		
round/teardrop/ pointy		1
Ceratium (Eiffel Tower)		
Protozoans and Animals		
ciliate	NA	3
round or oval protozoan		
irregular protozoan		
Keratella	NA	
Polyarthra	NA	
Egg		
Unknown Entities/Cells		
round/oval "could be anything"		1
irregular "could be anything"		
Taxonomy	**min. dimension < 10 µm**	**min. dimension > 10 µm**

Sample Name	297 cells/mL	
Analyst	EMR	
Water Filtered	200	
Conc. Sample volume	15	
Squares Counted	100	
Taxonomy	min. dimension < 10 µm	min. dimension > 10 µm
Blue Greens		
Anabaena - like	2	12
Oscillatoria - type		
coccoid		
Microcystis - like coccoid	19	NA
other filamentous cells		
filamentous-nocells (length)		
Merismodedia		
Greens		
Scenedesmus - type desmid		
Pediastrum		
coccoid	4	
single spindle	2	NA
filamentous - cells		
other colonial (non-coccoid)		
other colonial (spindle)		
euglenoid		
Cryptophytes (and other small flagellates)		
Cryptomonas/ Chroomonas - types	4	7
round microflagellates	38	13
irregular microflagellates		1

Totals	<10 total	>10 total
Blue Greens	21	12
Greens	6	0
Cryptophytes	42	21
Diatoms	176	26
Chrysophytes	0	1
Dinoflagellates	0	0
Protozoans and Animals	0	2
Unknown	0	2
All Taxa	245	64
Total Cells Counted:	309	

Diatoms		
Chain ***(Aulacoseira, Melosira, S. binderanus*****)**	35	13
Asterionella	99	
Tabellaria		
Diatoma		
Centric nonchain ***(Cyclotella, Stephanodiscus)***	14	13
Fragilarioid (ribbon colony)	28	
Synedra - like (includes nitzschioid)		
naviculoid (or other single pennate)		
Rhizosolenia		
Chrysophytes		
Mallomonas		1
Synura		
Dinobryon **(and *Kephyrion)***		
Dinoflagellates		
round/teardrop/ pointy		
Ceratium (Eiffel Tower)		
Protozoans and Animals		
ciliate	NA	2
round or oval protozoan		
irregular protozoan		
Keratella	NA	
Polyarthra	NA	
Egg		
Unknown Entities/Cells		
round/oval "could be anything"		2
irregular "could be anything"		
Taxonomy	**min. dimension < 10 µm**	**min. dimension > 10 µm**

Sample Name	297 cells/mL Dup	
Analyst	MNA	
Water Filtered	200	
Conc. Sample volume	15	
Squares Counted	100	
Taxonomy	**min. dimension < 10 µm**	**min. dimension > 10 µm**
Blue Greens		
Anabaena **- like**	14	15
Oscillatoria **- type**		
coccoid	5	
Microcystis **- like coccoid**	8	NA
other filamentous cells	13	
filamentous-nocells (length)	86	
Merismodedia		
Greens		
Scenedesmus **- type desmid**		
Pediastrum		
coccoid	3	7
single spindle		NA
filamentous - cells		
other colonial (non-coccoid)		
other colonial (spindle)	4	
euglenoid		
Cryptophytes (and other small flagellates)		
Cryptomonas/ Chroomonas **- types**	2	2
round microflagellates	1	56
irregular microflagellates	2	

Totals	<10 total	>10 total
Blue Greens	126	15
Greens	7	7
Cryptophytes	5	58
Diatoms	102	113
Chrysophytes	2	33
Dinoflagellates	0	0
Protozoans and Animals	0	13
Unknown	0	0
All Taxa	242	239
Total Cells Counted:	481	

Diatoms		
Chain ***(Aulacoseira, Melosira, S. binderanus*****)**	10	101
Asterionella	60	
Tabellaria		
Diatoma		
Centric nonchain ***(Cyclotella, Stephanodiscus)***		11
Fragilarioid (ribbon colony)	31	
Synedra - like (includes nitzschioid)		
naviculoid (or other single pennate)	1	1
Rhizosolenia		
Chrysophytes		
Mallomonas		1
Synura		32
Dinobryon **(and *Kephyrion)***	2	
Dinoflagellates		
round/teardrop/ pointy		
Ceratium (Eiffel Tower)		
Protozoans and Animals		
ciliate	NA	5
round or oval protozoan		8
irregular protozoan		
Keratella	NA	
Polyarthra	NA	
Egg		
Unknown Entities/Cells		
round/oval "could be anything"		
irregular "could be anything"		
Taxonomy	**min. dimension < 10 µm**	**min. dimension > 10 µm**

Sample Name	416 cells/mL	
Analyst	EMR	
Water Filtered	200	
Conc. Sample volume	15	
Squares Counted	100	
Taxonomy	**min. dimension < 10 µm**	**min. dimension > 10 µm**
Blue Greens		
***Anabaena* - like**	12	6
***Oscillatoria* - type**		
coccoid		
***Microcystis* - like coccoid**	140	NA
other filamentous cells		
filamentous-nocells (length)	45	
Merismodedia		
Greens		
***Scenedesmus* - type desmid**		
Pediastrum		
coccoid	7	
single spindle	2	NA
filamentous - cells		
other colonial (non-coccoid)		
other colonial (spindle)		
euglenoid		2
Cryptophytes (and other small flagellates)		
***Cryptomonas/ Chroomonas* - types**	5	11
round microflagellates	73	15
irregular microflagellates		3

Totals	<10 total	>10 total
Blue Greens	197	6
Greens	9	2
Cryptophytes	78	29
Diatoms	181	42
Chrysophytes	0	5
Dinoflagellates	0	1
Protozoans and Animals	0	5
Unknown	0	0
All Taxa	465	90
Total Cells Counted:	555	

Diatoms		
Chain ***(Aulacoseira, Melosira, S. binderanus*****)**	46	29
Asterionella	124	
Tabellaria		
Diatoma		
Centric nonchain ***(Cyclotella, Stephanodiscus)***	8	13
Fragilarioid (ribbon colony)		
Synedra - like (includes nitzschioid)	3	
naviculoid (or other single pennate)		
Rhizosolenia		
Chrysophytes		
Mallomonas		1
Synura		
Dinobryon **(and *Kephyrion)***		4
Dinoflagellates		
round/teardrop/ pointy		1
Ceratium (Eiffel Tower)		
Protozoans and Animals		
ciliate	NA	5
round or oval protozoan		
irregular protozoan		
Keratella	NA	
Polyarthra	NA	
Egg		
Unknown Entities/Cells		
round/oval "could be anything"		
irregular "could be anything"		
Taxonomy	**min. dimension < 10 µm**	**min. dimension > 10 µm**

Appendix 2. Taxonomic Characterization of the Phase II Organisms in the >50 µm Size Class. No organisms were present in the 0/m³ sample.

		B-QUA 10/m³		B-QUA 100/m³		B-QUA Starting Density ZP Sample	
Taxonomic Group	**Taxa**	**Total Organisms #/m³**	**Live Organisms #/m³**	**Total Organisms #/m³**	**Live Organisms #/m³**	**Total Organisms #/m³**	**Live Organisms #/m³**
Cladocerans	Bosmina			6.1	4.0	298.2	227.2
	Chydoridae			0.5	0.5	5.7	5.7
	Daphnia					5.7	2.8
Copepods	Calanoids			0.5	0.0	22.7	5.7
	Cyclopoids	0.5	0.5	0.5	0.0	45.4	11.4
	Harpacticoid					2.8	2.8
	Nauplii			2.0	0.5	108.1	36.0
Other Organisms	Chironomid					2.8	2.8
	Planaria					8.5	8.5
	Protista >50			3.5	3.5	180.2	180.2
	Tardigrade			1.0	0.5		
Rotifers	Bdelloid					108.1	72.1
	Collotheca					36.0	36.0
	Colurella			0.5	0.5		
	Conochilus			1.0	0.5	36.0	36.0
	Gastropus					36.0	36.0
	Kellicottia			0.5	0.0	108.1	108.1

	Keratella	4.0	4.0	44.4	42.9	4,505.3	3,964.7
	Monostyla			1.0	0.5	72.1	72.1
	Polyarthra	1.5	0.5	10.1	2.5	2,270.7	1,477.7
	Synchaeta	2.0	2.0	45.5	42.9	3,856.6	3,496.1
	Testudinella					36.0	0.0
Total		**8.08**	**7.07**	**117.17**	**98.99**	**11,745.29**	**9,782.17**
Percent Live		**88%**		**84%**		**83%**	