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TOWARD DEVELOPMENT OF A GREAT LAKES RELEVANT BWMS TESTING PROTOCOL: Evaluating a Most Probable Number Method for Assessing the Viability of Great Lakes Protists

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1 ABSTRACT

To support type approval testing of ballast water management systems we evaluated freshwater viability assessments for protists from the Duluth-Superior harbor of western Lake Superior using the most probable number (MPN) method. Tests were performed using varying temperatures and growth media and were compared to standard microscopic methods for determining live organism densities. Tests were also performed focusing on growth series derived from harbor water, and during an actual land-based test of a treatment system being evaluated for efficacy. We determined that growth of protists during MPN experiments was especially favored under higher temperatures and a growth medium comprising a 50 % solution of Bold Modified Basal Media. This medium also supported the growth of the greatest number of protist taxa. Based on microscopic analysis of live protists use of a treatment system during land-based testing reduced protist densities from 554 – 3000 cells/mL in the untreated water to 12 – 52 cells/mL after treatment. Corresponding assessments using the MPN method estimated respective densities of 1651 – 6060 cells/mL and 0 – 2.8 cells/mL, indicating that MPN likely overestimated viable cells in ambient harbor samples while it underestimated cell densities in treated samples. As asserted in the MPN protocols we confirmed that MPN-estimated protist densities were similar to densities in the protist size class that includes only cells strictly 10 – 50 µm in minimum dimension; protist densities including cells <10 µm were much higher than MPN estimates. However, based on all evaluations of freshly acquired samples containing a wide range of starting densities there was no correlation between MPN- and microscopy-determined densities, regardless of size class. Based on all testing, certain protist taxa were poorly favored during MPN grow-out periods (e.g., the chrysophyte *Mallomonas*), while others (e.g., free-living centric diatoms) tended to thrive, though there was substantial variability in taxonomic selectivity among tests. These findings contribute important freshwater data to the field of efficacy testing of ballast water treatment systems.

2 PROJECT BACKGROUND AND OBJECTIVES

2.1 BACKGROUND

The introduction of aquatic invasive species (AIS) in the Laurentian Great Lakes has been challenging native biological communities for decades, particularly since the opening of the St. Lawrence Seaway in 1959 (Mills et al. 1994). Invasive species have caused millions of dollars in damage to the Great Lakes (First 2019; Rothlisberger et al. 2012) and have had irreversible effects on Great Lakes ecosystems. Recognizing ballast water as one of the known vectors contributing to the introduction and spread of invasive species, the first federal regulations were developed pursuant to the Nonindigenous Aquatic Nuisance Prevention and Control Act of 1990 and the National Invasive Species Act of 1996 which required ships to manage their ballast water (e.g., ballast water exchange). More broadly, the International Maritime Organization (IMO) adopted the International Convention for the Control and Management of Ships' Ballast Water and Sediments in 2004 (IMO 2004) although that Convention did not enter into force until 2017.

A major focus area of the Lake Superior Research Institute's (LSRI) Great Waters Research Collaborative (GWRC) is providing unbiased, independent data in support of the accelerated development of technologies with potential for preventing the introduction and/or controlling the spread of nonindigenous organisms within the Laurentian Great Lakes. Currently, ballast water management

systems (BWMSs) undergo type approval testing following either United States Coast Guard (USCG) or IMO protocols to assess the effectiveness of these systems in meeting desired performance standards for reducing the potential for the spread of nonindigenous organisms. Differences in language and definitions between IMO and the USCG performance standards has caused some controversy regarding appropriate methods and their approval for use in type approval testing of BWMSs (Lindholm 2019; Cohen et al. 2017).

Regulation D-2 of the IMO International Convention for the Control and Management of Ships' Ballast Water and Sediments (Ballast Water Management Convention) specifies the performance standard that ballast water discharges must have <10 viable organisms per mL in the size category of ≥10 µm and <50 µm in minimum dimension (IMO 2017), herein organisms in this size category are referred to as "protists." The term "viable" is defined as "organisms that have the ability to successfully generate new individuals in order to reproduce the species" (IMO 2008). The USCG has its own standards, numerically the same as IMO, but the USCG requires ballast water discharges to contain <10 living (rather than viable) organisms per mL. To date most protist evaluations supporting efficacy testing of shipboard BWMSs for purposes of USCG type-approval testing have followed epifluorescence microscopy methods as described in the EPA Generic Protocol for the Verification of Ballast Water Treatment Technology (USEPA 2010, herein referred to as the Environmental Technology Verification Program [ETV] Method). Assessment of living cells is well-suited to such protocols that employ fluorescent probes (also called stains; Reavie et al. 2010; Steinberg et al. 2010, 2011). This approach has provided good estimates of live cells in freshwater samples, and few false positives and negatives, from the Laurentian Great Lakes based on multiple experimental and applied GWRC assessments (Reavie et al. 2010, and many subsequent BWMS evaluations). Other researchers have observed problems with false negatives while applying vital stains in more eutrophic fresh waters (Hansen et al. 2023). In December 2018, the president signed into law the Vessel Incidental Discharge Act (VIDA) that specifies that U.S. regulation of organisms in ballast water is to change from "living" to "viable" organisms using test methods based on best available science, if any. The only protist assessment method currently approved for USCG type approval testing is the Stain-Motility (SM) method, which employs the use of fluorescent probes fluorescein diacetate (FDA) and 5-chloromethylfluorescein diacetate (CMFDA) to detect cell metabolic activity, an assumed characteristic of living organisms. Motility in cells is also regarded as a living trait regardless of fluorescence. SM provides good evidence of living and not necessarily reproductively capable protists, but the USCG has not yet approved any methods to assess the viability of organisms in treated ballast water during type approval testing.

Requiring BWMS type-approval testing for organism viability is preferable given the possibility that some ballast treatment types may make some protists non-viable despite remaining alive after treatment. For example, if the viable protist density in post-treatment water is 9 cells/mL while the live density is 11 cells/mL, the latter value would erroneously indicate an exceedance of the discharge criterion of 10 cells/mL (IMO 2014). Hence, fluorescent stain methods may be unsuitable for type approval testing of BWMSs designed to render organisms non-viable, such as UV treatment technologies (Cullen & MacIntyre 2016). New methods for assessing viability must be based on the best available science and provide data that are as accurate and precise as the current approved method (USCG 2019; final policy letter signed in 2022 [USCG 2022]). The IMO recognized and approved the Most Probable Number (MPN) Dilution Culture + Motility method in 2016 (IMO 2016) for use in enumerating viable organisms for type approval of BWMSs. The MPN procedure is used to estimate microbial densities by adding a

volume of each of several serial dilutions of a sample to replicate tubes of culture medium and, following incubation, recording the number of tubes showing growth (McCrady 1915). A density estimate is based on growth in the inoculum containing at least one viable organism at the beginning of incubation. Assumptions include reproductive ability under incubation of all viable protists in the sample, and no clumps or aggregates of organisms. Otherwise the MPN procedure may underestimate true microbial cell density (Jarvis et al. 2010).

Many institutions have defended the MPN method (sometimes referred to as the "alternative method" or "viability assessment") via public comments and scientific documentation posted online in response to the Draft Policy Letter on the Coast Guard's process to evaluate proposed type approval testing methods and protocols for BWMSs that render organisms nonviable (USCG 2019). Although data intended to support the method's use in salt and/or brackish water are available (Cullen 2018), data validating and supporting the use of MPN-based methodologies in freshwater, particularly the Laurentian Great Lakes, are limited. Given the absence of an approved viability assessment method for USCG type approval tests in the Great Lakes, additional validation of a method to assess viability will be essential for the forward movement and type approval of BWMSs using lower dose UV-based technologies. Generally, UV treatment is a preferred technology in the Great Lakes as there are no hazardous chemicals to transport that could be corrosive to the uncoated ballast tanks of laker vessels or of potential risk to the crew. Power consumption for the operation of UV-based BWMSs is reduced as UV dose is reduced. The UV dose required to break down cell membranes and damage DNA, thereby eliminating organism ability to reproduce, is much lower than the dose required to kill organisms. Therefore, UV-based BWMSs operating with the intent to render organisms nonviable is preferable over a killing mechanism because of the reduced power requirements of such systems.

GWRC previously participated in a project for the validation of a freshwater MPN-based method led by the Naval Research Laboratory (First 2019), which included testing across a variety of water salinities. GWRC activities included comparisons of microscopic (stain) and MPN-based methods using freshwater surrogate protist taxa (autotroph *Haematococcus pluvialis* and heterotroph *Tetrahymena* sp.) and ambient communities from the vicinity of Superior, Wisconsin in 2018 and 2019. The MPN-based method estimated viable protist concentrations to be less than or equal to ~50 % of concentration estimates of the microscopy-based method. It is expected that viable organisms would be a subset of living organisms, but in a healthy ambient community we may also assume the difference between protist densities from MPN- and microscopy-based methods is negligible. Additional knowledge gained from the previous assessment was used to guide activities for this follow-up project and can be summarized as follows: (1) An initial, microscopy-based estimate of concentrations of protists ≥10 and <50 µm should be used to inform the starting points used for the MPN method. (2) Approaches to facilitate shipboard analyses, simplify sample handling, or allow for automated sample analysis (e.g., a rapid analysis for chlorophyll) should be proposed and developed. (3) Compile data on protist community composition and cultivability at a test location to inform the proportion of non-cultivable species. (4) Methods should be developed to increase the capture efficiency during filtration. (5) The contribution of heterotrophs to the total community should be included in analyses. (6) Confidence intervals should be reported with MPN estimates of viable protist concentrations, including a sum of both autotrophs and heterotrophs, to provide realistic uncertainty estimates of the MPN-based method. For the purposes of this manuscript, the combined use of the autotroph (via fluorescence after

incubation) and heterotroph (via microscopy) assessments to derive estimated protist densities is referred to as the MPN method.

In the assessment herein we aimed to answer the following questions, at least as they pertain to fresh waters in western Lake Superior:

- What is the most suitable growth medium for grow-out of incubated samples during MPN assessment?
- What is the effect of water condition (largely temperature) on grow-out performance?
- How do estimates of protist cell density compare between MPN and standard microscopic methods?
- Using IMO criteria for cell densities (10 – 50 µm in minimum cell dimension) the protist size criterion usually excludes a large proportion of the Great Lakes protist community (Reavie & Cangelosi 2020). Do MPN-facilitated estimates of protist densities better match with strict IMO criteria or actual protist numbers?
- Ideally all viable protist taxa are equally able to reproduce during the grow-out process of MPN evaluation. Are certain protist taxa favored or disfavored under MPN procedures?

The GWRC portion of the previous MPN assessment (First 2019) addressed a series of hypotheses that will be revisited with this study.

- *The MPN method is an acceptable analytical approach for mixed-species viability measurements*. The previous assessment suggested that there are several non-growable freshwater autotrophs that could result in a significant underestimation bias of the MPN Method. The number of non-growable autotrophs is also likely impacted by seasonality of communities within the Laurentian Great Lakes. Use of source water collected over varying seasons and surface water temperatures, including the type and/or concentration of the growth medium, are methodological variations that should help to address these outstanding questions.
- *The precision of the MPN and ETV Methods is similar*. The ETV Method (microscopy and stains) appears to be more precise at lower organism densities compared to MPN. At higher densities (~500 cells/mL) both methods appear to have similar precision. It is noteworthy that lower densities approaching the regulatory ballast water discharge standard of 10 cells/mL is where the most measurement precision is required.
- *The combination of separate measurements of living autotrophs and living heterotrophs is statistically valid*. The portion of the MPN values resulting from the autotroph measurements had a large amount of variability. Some way to combine the heterotroph and autotroph measurements, and still capture the uncertainty that results from the probability-based autotroph method, still needs to be developed.
- *The proposed MPN viable cell detection method (chlorophyll fluorescence) is reliable across a range of water conditions*. Given the limited period for the previous study there was not much variation in water quality, so it has not yet been possible to characterize a range of conditions.
- *Not accounting for the non-growable phototrophic species present in source waters is problematic for meeting regulatory limits*. Excluding non-growable phototrophic organisms (i.e., protists that may be viable but have specific ecological tolerances that prevent them from reproducing under the specific conditions provided during MPN growth assays) was likely one of the greatest contributors to the underestimation of the MPN method. This is problematic from a

regulatory perspective, as underestimating the discharge density could result in a BWMS falsely meeting the discharge standard. While this hypothesis has been confirmed, the current study provides additional details on environmental reasons and taxa that may be causing this problem.

The Viability Assessment Method Development Project (Research Area 2 – Project 2) is one of several projects within GWRC's Great Lakes Ballast Water Research and Development Plan (R&D Plan) that was designed for the forward movement toward developing a Great Lakes Relevant BWMS Testing Protocol. This project summary specifically provides data that may be used in the proposal and submission of a freshwater viability assessment method for treated Great Lakes ballast water samples. Briefly, this project aimed to address the following objectives: evaluate viability assessment methods for BWMS efficacy; determine whether these methods are suitable for the mixed protist assemblages in the Great Lakes; and assess the feasibility of implementing these methods during land-based evaluation of BWMSs.

3 METHODS

3.1 EXPERIMENTAL DESIGN

3.1.1 Viability Assessment Method Identification, Refinement and Scrutiny

3.1.1.1 *Literature Review and Information Request*

In January 2021, GWRC hosted a meeting with the R&D Plan Data Working Group, involving stakeholder group members and organizations who may have data or information that would be helpful identifying established or potential emerging methods for determining viability in treated ballast water. No new methods were identified during the request for information at the working group, as well as in comments to the R&D plan and public comments regarding the VIDA draft policy letter. GWRC staff then conducted a significant data and literature review to search for alternative methods to the existing ETV method that could potentially be used for assessing viability in treated ballast water. Evaluation methods presented herein follow GWRC's findings on recommended methods for viability assessment of protists. Four test types (sections 3.1.1.2 – 3.1.1.5) were performed.

3.1.1.2 *MPN Method Development and Optimization Growth Experiments*

Growth experiments (Table 1) were used to identify a freshwater medium that would provide the necessary nutrients to maximize the number of Great Lakes taxa that will grow to a detectable concentration, measured via an increase of *in-vivo* chlorophyll-a (>0.025 µg/L) and taxonomic microscopic assessment. Experiments were conducted using ambient densities of protists. From April to July 2021, growth experiments employing the autotroph method were conducted using various media prepared with filter-sterilized water collected at the Montreal Pier Facility in Superior, Wisconsin. Culturing media included those suggested for freshwater in the IMO approved MPN + Motility method (IMO 2019) and Wright's Chu (WC) media (Table 2). While the selected media have been commonly used to support monocultures of non-diatom algae, a silica component was also added to each medium due to the presence of the abundant diatom community found in Great Lakes waters. To simulate environmental effects on growth, additional parameters such as source water quality, filtration, light, and temperature were considered during experiments. Greater details of the MPN methods used are provided in Appendix A.

3.1.1.3 *Selenastrum Tests*

We evaluated the autotroph method using a freshwater organism known to be amenable to culturing. We used the standard test organism *Selenastrum capricornutum* (synonymous under the current taxonomic assignment *Raphidocelis subcapitata* and the interim name *Pseudokirchneriella subcapitata*). *Selenastrum* tests were conducted using 50%BBM media and sterile EPA media, which is the media typically used for optimal growth of *Selenastrum*. Three small scale experiments (trials S1, S2, S3; Table 1) were conducted using a starting concentration of approximately 1000 cells/mL of *Selenastrum*, as quantified by counting via hemacytometer and selective dilution (*Assessing Bench-Scale Dose Effectiveness of Potential Ballast Water Treatment Processes on Selenastrum capricornutum*, SOP GWRC/11, LSRI 2017a). Tests were conducted under continuous light incubating at 6 °C and 25 °C. The first trial in test tubes used 10^{-0} (undiluted) through 10^{-4} dilutions, measuring fluorescence at day 0, 14, and 21. The second trial was conducted in flasks with undiluted sample and media to verify growth. The third trial in test tubes used 10^{-0} (undiluted) through 10^{-4} dilutions while shaking at 100 rpm, measuring fluorescence at day 0 and 14.

3.1.1.4 *MPN Method Validation Using Ambient Harbor Water*

Following on findings from the growth experiments, experimental parameters (e.g., media, incubation time) for the Great Lakes MPN method were identified and a laboratory validation experiment was conducted using harbor water collected at GWRC's Montreal Pier Facility located in Superior, Wisconsin. This independent validation was conducted using ambient densities of protists to evaluate the accuracy and precision of the MPN method, using the ETV method as a reference. Laboratory tests were to include at least five unique taxa amenable to laboratory culture and were representative of ambient Great Lakes protist communities. Growth optimization replicates (June – July) and control uptake replicates from land-based trials (September and October, section 3.1.1.5), which were analyzed using the same media and methods as the Superior harbor water validation trials, were combined when comparing method results.

3.1.1.5 *MPN Method Feasibility During Land-based Biological Efficacy Tests*

Land-based feasibility tests were conducted coincident with biological efficacy trials for the Great Lakes Ballast Water R&D Plan's (TenEyck & Junemann 2022) Research Area 1 – Project 2 and followed GWRC's Quality Assurance Project Plan for Land-Based Tests (LSRI/GWRC/QAPP/LB/1, GWRC 2019). GWRC land-based tests take place at the purpose-built GWRC Montreal Pier Facility located in the freshwater port of the Duluth-Superior Harbor on Lake Superior. The overall physical configuration and sampling methodology is consistent with requirements detailed in the EPA ETV Program's Generic Protocol for the Verification of Ballast Water Treatment Technology (USEPA 2010). The facility mimics a commercial vessel's BWMS, with the benefit that it can be operated with greater control than would be possible onboard a vessel. This testing used samples collected from uptake and discharge streams during testing of the Optimarin UV-based BWMS (Polkinghorne et al. 2022).

3.2 Sampling and Test Methods

3.2.1 Harbor Water Sample Collection for Media Preparation and Growth Tests

Ambient harbor water (HW) samples and source water for growth experiments and the validation trial were collected from the Duluth-Superior harbor via a sump pump and hose located dockside at GWRC's Montreal Pier facility. Source water for media preparation was collected in a 19-L carboy one to two

days prior to test initiation to filter-sterilize the source water and prepare media prior to experiments. HW samples collected for biological analysis were collected in sterile 1-L polypropylene bottles and transported to the LSRI laboratory for analysis. Samples were also collected in bottles for total suspended solids, ultra violet transmittance (UVT), and organic carbon analysis. At the time of collection, water quality conditions (temperature, pH, dissolved oxygen, specific conductivity, turbidity, salinity and chlorophyll-a) were measured using a calibrated YSI Multiparameter Water Quality Sonde (LSRI 2021a) and recorded on a pre-printed datasheet (section 3.3.1).

Protist densities were measured in the source water the morning of experiment start days (growth tests, HW validation tests, land-based tests) to determine required dilutions. Protist count data were represented two ways: (1) cells with minimum visible dimension strictly meeting the 10 – 50 µm criterion, as originally designated as a ballast water performance standard for the protist size class (IMO 2014); and (2) all cells in entities (e.g., single cells, colonies, filaments) that were larger than 10 µm in any visible dimension. The latter data type includes smaller or narrower taxa, which are deemed necessary to consider because these small-celled taxa tend to dominate Great Lakes phytoplankton communities (Reavie & Cangelosi 2020). Counts within the 10^{-1} dilutions of each medium were verified by the protist analyst and within the IMO-specified range (targeting 1 cell/mL in the mid-range dilution) to begin an experiment.

3.2.2 MPN Method Optimization Growth Test Methods

3.2.2.1 *Growth Media Preparation*

For all growth experiments HW was sterilized through 0.22-µm filters to prepare filter-sterilized source water (FSW) which was used for preparing each growth medium. The use of source water to prepare media minimizes shock to ambient organisms and provides the natural components present in the water that may encourage growth. Media used for the growth optimization tests included:

- BBM –Bold Basal Media Modified with f/2 Vitamins, Canadian Phycological Culture Collection (University of Waterloo, ON, Canada)
- ISO – International Organization for Standardization method 8692 media (ISO; (ISO 2004))
- MBF – Bold Modified Basal Freshwater Media (Sigma Aldrich)
- WC – Wright's Chu, WC (ed) media, Canadian Phycological Culture Collection (University of Waterloo, ON, Canada)

Each medium was prepared according to manufacturer's instructions. All media was filter-sterilized after media components and silica (1 mL/L of a 28.42 g/L stock $NaSiO_3 \cdot 9H_2O$) were added to the FSW (Table 2). For each medium, 50-mL tubes (growth test 1 only), dilution tubes, and autotroph tubes were prepared and stored at HW sample collection temperature in an incubator until test initiation.

3.2.2.2 *Autotroph Method Growth Optimization Test Initiation Methods and Parameters*

A total of four growth tests were conducted in a trial-and-error manner using a mixed assemblage of organisms present in ambient harbor water. The testing parameters are summarized in Table 1 and described below. Though an ideal experimental protocol would have included all tested media across all environmental conditions, the considerable effort associated with the tests and uncertainty around the most suitable approaches necessitated modifications as tests proceeded.

On the start day of each growth test, a subsample of HW was analyzed in triplicate via the ETV method (USEPA 2010) to determine initial protist densities in the HW. Triplicate subsamples of HW were prepared to conduct the autotroph portion of the MPN method using each medium. The first growth trial included filtering a 50-mL subsample of HW through a sterile Swinnex filter unit (25-mm, 7-µm mesh filter) to concentrate protists and remove smaller organisms and retain organisms in the ≥10 and <50 µm size class. The filter was then resuspended in 50 mL of the appropriate medium (BBM, MBF, ISO) and vortexed at low speed to resuspend the organisms. Samples were serially diluted and added to autotroph/MPN tubes as described in section 3.3.2.1.1.

Growth trial 1 used freshwater media of full strength BBM, ISO, and MBF media. HW Samples were collected in early spring, soon after ice-out, therefore water temperature was quite low and autotroph tubes were incubated at 6.1 °C. Initial protist densities from 10^{-1} dilution aliquot assessments were approximately half of what was expected based on HW assessments. Microscopic examination of filtrate and filters post-vortexing did not reveal any notable protists larger than 10 µm, so the loss is unexplained. Starting densities among replicate tubes for each medium were similar, so we proceeded acknowledging that the loss of protists should not affect experimental goals. To avoid this discrepancy subsequent trials did not use filtration. Trials 2 – 4 instead used a dilution series prepared from straight HW subsamples using the associated media and five autotroph tubes for each dilution (Figure 1). Dilutions were prepared by directly pipetting 1 mL of HW sample into dilution tubes. Method blanks for the autotroph method were prepared for each sterile medium to determine blank-corrected fluorescence changes and determination of growth over 14 days. Media blanks were also analyzed via the ETV and heterotroph methods (section 3.3.2.1.2) to verify no organisms were present. To confirm starting conditions, an aliquot from each triplicate HW sample in media, or 10^{-1} dilution was composited and analyzed for protist density via ETV and heterotroph methods, and the remaining sample was preserved to determine initial community composition (at a convenient time later) via microscopic taxonomic assessment. Fluorescence was measured on all autotroph tubes and then incubated and subsequently analyzed for fluorescence on days 14 and 18. At test end, tubes that were scored positive for growth were composited, preserved and analyzed for community composition as described in section 3.3.2.2.2.

After speaking with a representative at the University of Waterloo who produces the BBM, it was noted that lower strengths of the BBM media were often used for growing mixed assemblages of organisms. Therefore, in growth trial 3, half-strength BBM (50%BBM), ISO and MBF media were evaluated. WC media was also evaluated in this trial as it is a freshwater medium often used for diatom growth. Samples were incubated at 18.8 °C, and fluorescence was measured at day 0 and day 14. Dilutions 10^{-1} through 10^{-4} were used due to HW protist density being lower than expected upon test initiation.

Growability results of previous trials steered the decision process for growth trial 4 which evaluated 50%BBM and ISO again to determine if results could be reproduced. Growth was also evaluated in 10%BBM. Samples were incubated at 19.8 °C to match July HW water temperatures, and fluorescence was measured at day 0 and day 14. Dilutions 10^{-1} through 10^{-4} were used for 50%BBM and dilutions 10^{-2} through 10^{-4} were used for ISO and 10%BBM.

3.2.2.3 *Determination of MPN Method Parameters*

Growth was assessed in autotroph tubes that scored positive after the incubation period. Growth was scored by the blank-corrected fluorescence of a tube being greater than four times the standard

deviation of the average fluorescence values of the initial method blanks. Tubes that exhibited growth were composited per each medium (1 mL per positive tube) and assessed for community composition to obtain a final cell density and determine the taxa present.

Data from the community composition samples were standardized to account for the dilutions of the autotroph tubes and to compare densities across media and trials. Cell densities derived from microscopic analysis of combined, growth-positive tubes were calculated as follows. Where the density of the community composition was calculated based on the sample given to the analyst, the dilution factor was presented as a decimal (10^{-1} = 0.1, 10^{-2} = 0.01, etc.); 5.9 mL is the total volume of media and sample present within each autotroph tube, and the volume of community composition accounted for each sample scored positive (1 mL per sample). Briefly, because different dilutions were combined prior to microscopic community analysis, conversion of the cell density in the merged sample to a value representing the various dilutions was needed. Selective growth of taxa was assessed by comparing taxa present within the harbor water sample upon collection with taxa in community composition samples at test termination.

3.2.3 MPN Method Validation Tests Using Ambient Harbor Water

Growth tests with multiple media were followed in August 2021 by HW validation tests using the optimal medium (Table 1). Prior to starting these validation trials, source water was collected as described in section 3.2.1 to prepare growth media for the autotroph portion of the MPN method. To evaluate the precision and accuracy of the MPN method, samples were analyzed in triplicate using both the MPN (Section 3.3.2.1) and ETV (Section 3.3.2.2) methods.

3.2.3.1 *Sample Collection*

HW samples for validation testing were collected via a sump pump dockside at Montreal Pier Facility as described in section 3.2.1. Three 1-L HW samples were collected for biological analyses, as well as bottles for TSS, UVT, non-purgeable organic carbon (NPOC) and dissolved organic carbon (DOC) analyses. Water quality was measured at the time of collection as noted in section 3.3.1. Each HW sample was mixed well and divided into nine 50-mL Falcon tubes in order to do triplicate protist analysis via each of the three methods (autotroph, heterotroph, ETV). The schematic for these procedures follows that shown in Figure 1 with the addition of analysis of heterotrophs in each replicate HW sample used for autotroph analysis, as detailed in Appendix A.

3.2.4 Feasibility of MPN During Land-based Biological Efficacy Tests

A total of six biological efficacy (BE) trials (including a commissioning trial) were conducted in September and October 2021 during the *Land-based Evaluation of the Effectiveness of the Optimarin Ballast System in the Great Lakes* (Polkinghorne et al. 2022), model 334/340FX2 (hereafter OBS). The land-based testing of the OBS focused specifically on the BE consistent with the ETV Protocol (USEPA 2010) and examined the potential to reduce the environmental risk (i.e., number of propagules) associated with ballast water discharge within the Great Lakes. Ambient conditions of Duluth-Superior Harbor were employed as the physical, chemical and biological challenge water conditions, except that total suspended solids (TSS), particulate organic matter (POM), and mineral matter (MM) were augmented during BE testing to meet the minimum requirements specified by the ETV Protocol. When needed we also augmented protist densities to meet the ETV Protocol. Details of the OBS testing procedures and associated results not included in this report may be found in the associated technical report (Polkinghorne et al. 2022).

On the day of each trial, samples were collected according to LSRI/SOP/GWRC/28 – *Collecting Protist and Microbial Samples at the Montreal Pier Facility* (LSRI 2020a). MPN Samples were transported to the lab in coolers with no ice to maintain them at collection temperature. Each sample was mixed thoroughly and poured into six 50-mL Falcon tubes to be used as the three subsamples for analysis via the MPN method (autotroph and heterotroph procedures; section 3.3.2.1).

Each biological efficacy trial consisted of an uptake operation (ballasting simulation) with filtration and UV treatment, retention of control water and treated water, and a discharge operation (deballasting simulation) with further UV treatment. During uptake, ambient water was pumped from the Duluth-Superior Harbor through the Montreal Pier Facility at a flow rate of approximately 330 m^3/hr. Half of the water was directed through the control track and into a control retention tank while the other half passed through a treatment track (OBS) and into a treatment retention tank. Filling continued until a target retention volume of 225 m^3 was reached in each tank. Mechanical mixers provided a gentle agitation to keep non-neutrally buoyant organisms suspended in the water column for a minimum of 16 hours prior to discharge.

To minimize live organism contamination of the treated discharge water, prior to discharge the facility lines were flushed with municipal water and then steamed at 205 °C for a minimum of ten minutes as detailed in LSRI SOP GWRC/21 – *Cleaning and Performing Cleaning Validation of the Montreal Pier Facility* (LSRI 2021b). The sample collection tubs on the treatment track were also cleaned following LSRI/SOP/GWRC/24 – *Cleaning the Sample Collection Tubs and Zooplankton Collection Equipment* (LSRI 2021c). After the retention time had been reached, and line cleaning procedure was complete, the treatment retention tank was discharged and sampled first, followed by the control retention tank.

For the purposes of determining the feasibility of the MPN method during land-based testing, each BE trial included the collection of one additional control sample on uptake, one treatment sample on discharge, and one control sample during discharge of the control tank (Table 3). Control samples were analyzed in triplicate using both the ETV and MPN methods (sections 3.3.2.2 and 3.3.2.1, respectively). Due to the extended amount of time it takes to analyze the treated samples via the ETV method, treatment samples were not analyzed in triplicate via the ETV method but were for the MPN method.

3.3 Sample Analysis Methods

3.3.1 Water quality

Water quality parameters were measured during source water, uptake, and discharge events to determine experimental starting temperature and secondarily confirm that conditions were within normal ranges for the harbor. Water chemistry (temperature, pH, dissolved oxygen, conductivity, salinity, turbidity, and total chlorophyll) was measured using a YSI EXO2 Multiparameter Water Quality Sonde following LSRI/SOP/GWRC/27 – *Collecting Water Chemistry Samples and Water Quality Data at the Montreal Pier Facility* (LSRI 2021d) and LSRI/SOP/FS/41 – *Operation and Storage of YSI EXO2 Multiparameter Water Quality Sondes* (LSRI 2021a). The Sondes were calibrated prior to each test following LSRI/SOP/FS/39 – *Calibration of YSI EXO2 Multiparameter Water Quality Sondes* (LSRI 2017b). Total suspended solids (TSS), particulate organic matter (POM), and mineral matter (MM) were conducted according to LSRI/SOP/SA/66 – *Analyzing Total Suspended Solids, Particulate Organic Matter, and Mineral Matter* (LSRI 2017c). Sample analysis of percent transmittance (UVT) was conducted according to LSRI/SOP/SA/69 – *Determining Percent Transmittance of Light in Water at 254 nm* (LSRI

2018), and sample analysis for NPOC and DOC were conducted according to LSRI/SOP/SA/47 – *Measuring Organic Carbon in Aqueous Samples* (LSRI 2006).

3.3.2 Biological Enumeration Methods

3.3.2.1 *Most Probable Number (MPN) Method*

The MPN method (Appendix A) combines the number of viable autotrophs and living heterotrophs detected in a known volume of sample to provide an estimate of viable/live protists in the original sample. The day prior to each sampling event, source water was collected and filter-sterilized to prepare 50%BBM medium (or media described in section 3.2.2.1) for the autotroph portion of the procedure. (50%BBM was selected as the most promising medium based on the previous methods development testing.) Prepared media was then added into dilution tubes (9 mL) and autotroph tubes (4.9 mL) (Figure 1) and kept at source water collection temperature until test initiation or sample analysis.

3.3.2.1.1 Autotroph Procedure

Dilutions analyzed for the autotroph procedure were based on the source water or sample type (Table 3). A dilution series was prepared by adding 1 mL of sample into a 9 mL of media to create a 10^{-1} dilution (Figure 1). This was repeated with each dilution until the desired dilutions for the concentration of interest were met. From each dilution tube, 1 mL was pipetted into five replicates of the autotroph tubes, obtaining a final volume of 5.9 mL. Sterile caps were placed on the autotroph tubes, and they were vortexed to mix.

Autotroph tubes were analyzed for fluorescence using a Trilogy laboratory fluorometer equipped with a chlorophyll-*a in vivo* block (LSRI 2020b) to obtain the day 0 reading. Caps were loosened and tubes were incubated at collection temperature under continuous light (nominally 50 – 150 µmol photons m^{-2} s^{-1}) for 14 days and re-analyzed on the fluorometer on day 14 to assess growth. The increase in fluorescence for each autotroph tube was calculated:

> *Blank-corrected fluorescence increase = (Fluorescence Day 14 - Fluorescence Day 0) – (Average Blank Fluorescence Day 0 – Average Blank Fluorescence Day 14)*

As for HW testing, tubes were scored for growth and were determined to be positive if the fluorescence increase was greater than four times the standard deviation of the five initial measurements for the sample. In some cases, fluorescence exceeded the range of the fluorometer, so samples were diluted with sterile media to obtain a measurement. MPN was calculated by entering the number of positive tubes per dilution into an MPN calculator worksheet (Jarvis et al. 2010; see citation for direct link to calculation tool). Analysis replicates were averaged to determine the density (MPN/mL) of viable protists in the original sample. Calculations included 'rarity' which measures the probability of the actual outcome divided by the probability of the most likely outcome; i.e., indicating whether the outcome is likely. From the autotroph tubes that scored positive, 1 mL from each sample was composited, preserved and assessed for community composition (below).

3.3.2.1.2 Heterotroph Procedure

To have a complete MPN method it is recommended (First 2019) to supplement autotroph assessments with counts of heterotrophic protists, so this procedure was added for HW validation tests and land-based BWMS tests. To enumerate heterotrophic organisms in each sample, 1 mL aliquot of subsample was loaded into a Sedgwick Rafter chamber and placed on the stage of an epifluorescence microscope.

Motile organisms between 10 and 50 µm in minimum dimension that did not fluoresce red (due to a lack of chlorophyll) were counted in a known number of 1 X 1-mm squares until at least 100 organisms were counted. The concentration of each sample was calculated based on the number and volume counted (motile organisms/mL). Greater details of this method are provided in the SOP (Appendix A).

3.3.2.1.3 MPN Method Calculations

In each sample, the average number of autotrophs and the average number of heterotrophs in the 10 - 50 µm size category (strictly following IMO size criteria) were combined for the three replicate samples to determine the total number of viable/live organisms.

3.3.2.2 *Reference Method (ETV Method)*

3.3.2.2.1 Procedure for Enumerating Live Organisms in a Water Sample

Live analysis of samples occurred within six hours of sample collection, with samples stored in a refrigerator during the interim. Sample analysis was conducted according to LSRI/SOP/GWRC/30 – *Procedure for Protist Sample Analysis* (LSRI 2020c). Briefly, a 1.5-mL subsample was transferred to a 5-mL sample container, with 6 µL of fluorescein diacetate (FDA) and 15 µL of chloromethylfluorescein diacetate (CMFDA) viability stain stock solutions added. The subsample was allowed to incubate in the dark for 10 minutes. The incubated sample was mixed and immediately transferred to a 1.0-mL Sedgwick-Rafter counting cell, covered, and placed on the stage of an inverted microscope set for simultaneous observation using brightfield and epifluorescence at 200 X magnification. Prepared samples were counted for 20 minutes maximum and then counts were repeated two more times, preparing a new slide each time. Records of transect lengths and widths using the Sedgewick-Rafter grid were kept so that the total counted area and volume analyzed could be calculated. Cells between 10 and 50 µm in minimum dimension per IMO (2017) protocols were counted and totaled. Also, cells in protist entities (cells, colonies, filaments) with any dimension larger than 10 µm were also counted as a separate category. Remaining sample water up to 25 mL was archived using Lugol's solution for long-term storage.

3.3.2.2.2 Procedure for the Analysis of Preserved Samples to Determine Community Composition

To assess the amount and types of taxa that grew within each sample, community composition was compared to that in the day 0 water samples. Preserved sample analysis for taxonomic information was conducted according to LSRI/SOP/GWRC/30 – *Procedure for Protist Sample Analysis* (LSRI 2020c). Briefly, 1.0 mL of preserved well-mixed sample water was strewn in a Sedgwick-Rafter counting cell and assessed at 200 X magnification for at least one hour or until the entire cell was counted. At a minimum taxonomic resolution focused on taxonomic groups (diatoms, dinoflagellates, cyanophytes, microflagellates, greens, coccoid, etc.), though in many cases it was possible to identify taxa to the level of genus or species (e.g., *Tabellaria*, *Fragilaria crotonensis*). Though this method allows for a cell density calculation, the density from the associated live count was assumed to best represent actual cell densities, so densities from the preserved count were converted based on the total density from the live count.

4 RESULTS

For tests that relied on harbor water as a starting source, water quality measurements (Table 4) were within historical ranges for the Duluth-Superior Harbor. Because no anomalous conditions were observed in water quality we could assume that results should also reflect typical outcomes for the site.

4.1 MPN METHOD DEVELOPMENT AND OPTIMIZATION GROWTH EXPERIMENTS

During growth tests densities of protists in the harbor ranged from 87 – 1313 cells/mL within the strictly 10 – 50 µm category and 266 – 7833 cells/mL for total protists at sample collection temperatures between 6.6 °C and 19.8 °C (Appendix B). Given the dramatic increases in protist abundance after incubation – 10s and 100s of cells/mL in harbor water versus thousands and even millions of cells/mL in tubes – the various media were clearly able to support exponential algal growth. The most growth was observed in trials 3 and 4, corresponding to higher incubation temperatures in the summer season (Figure 2). ISO medium demonstrated the greatest growth at the coldest temperature (before 50%BBM was considered) though it was similar to BBM and MBF. Overall, 50%BBM supported the most growth based on the increase in fluorescence measurements in growth tubes after 14 days (Figures 2,3), though other media (ISO, WC) exhibited growth in a greater number of tubes, with WC consistently showing growth in all tubes.

Protist density assessments based on microscopic (ETV) assessment showed similar densities across media, with 10%BBM and 50%BBM having higher average total densities (Figure 3). A similar result was observed for protist counts adhering to IMO protocols (exclusion of < 10 µm cells), though MBF had a notably lower average protist density. The number of taxa identified following incubation was consistently lower for all media than the number of taxa observed in harbor water from day 0. 50%BBM generally supported more taxa under the strictly IMO size class, but when all protists were considered ISO, WC and 10%BBM were only slightly lower than 50%BBM. A medium of undiluted BBM was the poorest at maintaining taxa numbers overall. The full set of data used for Figures 2 and 3 is provided in Appendix B.

4.2 *SELENASTRUM* TESTS

Due to discontinuation of funding for the project, *Selenastrum* testing was truncated and is considered incomplete. Two tests were completed using 50%BBM (Table 5). Each test included two sets of incubated dilution tubes: 6 °C and 25 °C. No agitation was used during the first test and the second test included agitation at 100 rpm during the 14-day incubation period. No growth was observed for any tests conducted at 6 °C. For the warmer tests growth was observed, but unexpectedly during test 1 growth was only observed in the most dilute tubes. Test 2 showed growth in all dilutions except the highest, but even at the lowest dilution (10^{-0}) only 2 of 5 tubes had positive growth.

4.3 MPN METHOD VALIDATION USING AMBIENT HARBOR WATER

Based on growth experiments using initial samples from Duluth-Superior Harbor, no correlation between protist cell densities derived from MPN and ETV methods was observed (Figure 4). A paired *t*-test indicated that results from the two methods did not co-vary (N = 9 pairs, r = 0.41, t = 1.53, $P_{2\text{-tail}}$ = 0.163), confirming that protist densities based on day-14 MPN calculations did not change with higher initial concentrations derived from microscopic (ETV) counts of cell densities. A statistical comparison of

MPN and ETV (strictly 10 – 50 µm cells [IMO]) results using a one-way ANOVA further indicated no difference in means (N = 9 pairs, F = 1.74, P = 0.205; log_{10}-transformed F = 4.34, P = 0.053).

Because fluorescence measurements used for MPN calculations account for all photosynthesizing organisms present within the sample, we considered whether total counts (including smaller cells than would be considered under strict IMO protocols) may better represent the quantity of photosynthetic pigments. Initial counts integrating all cell sizes provided much higher cell densities than that predicted by MPN. Instead, a comparison of MPN-derived densities and cell size-specific protist densities indicated that the strictly 10 – 50 µm cell size class (IMO) was relatively close to MPN estimates (i.e., overlapped the line indicating 1:1 relationship; Figure 4, upper panel). A comparison of IMO size class densities with MPN densities for all tests that employed 50%BBM (growth experiments, harbor water tests, land-based tests) also still did not show any correlation (Figure 4, lower panel), further indicating that MPN was not able to predict protist densities within the range observed in our starting conditions (194 – 3009 cells/mL).

The full set of data from harbor water experiments is provided in Appendix C.

4.4 MPN Method Feasibility During Land-based Biological Efficacy Tests

During commissioning and testing of the OBS, a total of six tests was completed, each providing protist density estimates for uptake, control discharge and treated discharge samples (full data in Appendix D). A summary of average protist densities (Table 6) indicates substantial reduction of protist densities by the BWMS, relative to control and uptake samples. For uptake and control samples, MPN tended to provide higher density estimates than ETV, except for test cycle 2 and the control discharge in test cycle 3. In contrast, MPN estimates of treatment discharge densities (average 0.7 cells/mL) were consistently much lower than ETV estimates (average 33.5 cells/mL).

The MPN calculation works irrespective of the order of dilutions and will generate estimated organism densities even if unlikely results are produced, such as an increase in the number of tubes with growth in more dilute sample sets. To evaluate such discrepancies a rarity index is also calculated (Jarvis et al. 2010). Across the 54 MPN analyses performed for the land-based tests the rarity index averaged 0.91 (Appendix D), indicating that results of the serial dilution tests represent concentrations that are likely close to estimated MPN values.

To generate a more robust set of data for the comparison of ETV and MPN methods, samples analyzed using the selected medium and methods were compiled (Figure 5). This included the two 50%BBM samples from growth tests 3 and 4, the nine HW samples from the HW validation trial, and all control uptake samples from the land-based trials of the OBS. As indicated by the factor of agreement (FOA), MPN-estimated densities were higher than protist densities derived from the ETV method using IMO size class criteria in 7 of 9 comparisons, except for harbor water tests, which had similar densities, and uptake from the second BWMS test. When all observed cell size classes were included in ETV calculations, MPN densities tended to be comparatively lower, though the third growth test (G-3) was a clear exception, and the OBS test cycles 1, 4 and 5 had similar results.

Growth tests G-3 and G-4 were very different in terms of comparisons between ETV and MPN results. An examination of community data indicates that G-4 had substantially more MPN growth of small-celled coccoid cyanophytes and filamentous green algae, neither of which were noted in the source water at the beginning of that experiment (Table 7). While that likely explains the much greater growth of ETV-determined cell densities in G-4, there are no taxonomic indicators that would explain a higher

MPN-inferred density via fluorescence in the growth tubes. However, tests OBS-4 and OBS-5 exhibited growth of large numbers of small-celled cyanophytes and coccoid green algae (Table 8), which may explain why MPN-inferred cell densities for those intake samples reflected much higher densities than ETV densities assessed using strictly IMO cell size criteria. More details of the data used to generate Figure 5 are provided in Appendix E.

4.5 Community Composition

Starting (day 0) and post-incubation taxonomic communities were assessed during growth experiments and land-based testing to identify whether taxonomic bias occurred during the growth phase (Tables 7 and 8). A total of 33 taxonomic categories (herein considered taxa) were identified across all tests. Across all analyses, heterotrophs comprised a very small portion of the protist assemblage, ranging from 0.4 – 1.6 % of protists in harbor water samples (Appendix C) and 0 – 6.9 % in samples collected during OBS tests (Appendix D).

Initial harbor samples from the harbor water tests yielded an average of 16 protist taxa. Autotroph tubes that exhibited growth over the incubation period, and were composited for community composition analysis, contained an average of eight taxa, indicating that MPN growth was possible for a smaller number of taxa. Certain taxa in certain tests only appeared on day 14, indicating that they were very rare on day 0 (below method detection) but enough initial viable cells were present to seed exponential growth of their populations during incubation in media. This phenomenon was most common with large-celled coccoid cyanophytes, *Scenedesmus*-like green algae and euglenoids. In most cases *Asterionella* (diatom), *Mallomonas*-type chrysophytes and several protozoan types were observed on day 0 but did not appear following incubation, indicating they were not favored to grow using the selected media. Many more cases of non-growability occurred, but those cases were distributed sporadically throughout the tests. Though little variation in taxonomic preference or inhibited growth was observed among media, undiluted BBM had the most ungrowable taxa, which contributed to that medium having the lowest number of taxa on day 14 (Figure 3). However, it is clear that a 50 % solution of BBM provided a growing environment for more taxa than other media.

At lower temperatures (6.6 ^{o}C, Table 7) diatoms were favored to grow in MPN tubes, whereas green algae and cryptophytes tended to do better at warmer temperatures. With the inclusion of smaller cells in the total protist category, there was more growth of small-celled cyanophytes, especially in warmer water temperatures.

During land-based BWMS testing, first-time appearance of taxa on day 14 was especially the case for *Microcystis*-like coccoid cyanophytes and fragilarioid diatoms. While those taxa were too small in at least one cell dimension to meet IMO size criteria, several cases of large-celled taxa (coccoid green algae, *Ankistrodesmus*-like green algae, filamentous diatoms) appeared in some uptake and control discharge samples on day 14 despite no observation on day 0. In contrast, some taxa had several cases where they were present on day 0 but did not appear in samples on day 14. For the large-celled (IMO size class) taxa this included *Anabaena*-like cyanophytes, coccoid green algae, euglenoids, cryptophytes, dinoflagellates, filamentous diatoms, naviculoid diatoms and ciliates. When considering smaller cells below IMO size criteria these non-growable taxa included filamentous cyanobacteria, *Scenedesmus*-like green algae, *Ankistrodesmus*-like green algae, *Asterionella* and fragilarioid diatoms.

5 DISCUSSION

Several experiments were performed to support ongoing discussion around the use of the MPN method in type approval testing of BWMSs. We believe these findings provide information to advance ballast water testing methodologies. The following discussion responds to multiple hypotheses, questions, and goals as posed in the Great Lakes Ballast Water R&D Plan.

5.1 WHAT IS THE MOST SUITABLE GROWTH MEDIUM?

A 50 % solution of Bold Basal Media supported the greatest growth and taxonomic diversity of freshwater protists throughout all trials, particularly those incubating at warmer temperatures reflecting summer in western Lake Superior. Similarly, a 50 % solution of growth medium was noted to maximize exponential growth of the chlorophyte *Chlorella vulgaris* (Blair et al. 2014, compared to 100 % and 25 % solutions), corroborating the need for a careful balance of nutrients to maximize MPN performance.

The prevailing literature on algal growth media pertains to solutions designed to generate exponential growth in low-diversity or axenic, non-siliceous algal cultures. Because diatom algae are dominant in the Great Lakes much of the year (Reavie et al. 2014b), we modified solutions to include silica to prevent silica limitation and support diatom growth. Though further study could optimize this addition, we recommend that future MPN assessments on ambient algal communities include silica addition. Other nuances in community composition may also dictate the best mix of nutrients and other chemicals in growth media. For instance, the balance of nitrogen-based nutrients can determine whether a chlorophyte or cyanophyte are successful in culture (Hyenstrand et al. 2000), a factor that likely played a role in determining growable and non-growable taxa in our tests.

5.2 WHAT IS THE EFFECT OF WATER CONDITION ON GROW-OUT PERFORMANCE?

We were able to complete tests using source water from early spring and summer conditions, so we captured a greater range of temperature conditions than those used in previous MPN studies. Diatoms displayed greater growth in cooler temperatures while cyanophytes and green algae displayed greater growth in warmer temperatures, corresponding with known temporal distributions of these taxa in the Great Lakes (Reavie et al. 2014b). Taxonomic diversity and overall growth were greater at warmer temperatures, in agreement with some other researchers (Singh & Singh 2014). Though our tests indicate that MPN may not be reliable across a range of water conditions, it is worth noting that substantial Great Lakes diatom blooms often occur during winter (Twiss et al. 2012), so in addition to temperature it is likely specific stoichiometry plays a role in successful growth. Rather, when a chemical nutrient is limiting, temperature effects on growth are probably of lesser importance.

5.3 HOW DO ESTIMATES OF PROTIST CELL DENSITY COMPARE BETWEEN MPN AND ETV METHODS?

The protist size criterion as defined by IMO (IMO 2017) reflects densities of cells that are strictly 10 – 50 µm in minimum visible dimension via microscopic methods, which we know excludes a large proportion of the Great Lakes protist community (Reavie & Cangelosi 2020). For instance, some of the most common phytoplankton from the Great Lakes are small-celled cyanobacteria and narrow-celled diatoms (Reavie et al. 2014b), which have at least one cell dimension that does not meet this strict size criterion. Regardless of the size class considered, there were no correlations between cell densities estimated

using the ETV and MPN methods, though it does appear that MPN estimates are more similar to cell densities calculated in the IMO size class, as was expected based on previous documentation of MPN applications (MacIntyre et al. 2018). This contrasts previous findings that both methods had similar precision at higher cell densities (~500 cells/mL, GWRC portion in First 2019). While our finding may highlight a weakness with MPN, it is especially important that MPN performs adequately at lower protist densities, such as near 10 cells/mL, as these densities determine whether ballast discharge meets the regulatory ballast discharge standard. The difference between ETV (average 33.5 cells/mL) and MPN (average 0.7 cells/mL) treatment discharge densities may reflect actual viable protist estimates as provided by the MPN method. Because we cannot verify whether the treatment discharge densities determined by ETV methods represented viable protists, it may be that the lower MPN estimates are an actual account of viable cells. It is plausible that some of the live cells recognized in treated samples during the ETV process were non-viable as a result of the UV treatment. This inactivation (without killing) of organisms by UV has been recognized by other researchers (Olsen et al. 2016). However, taxonomic specificity in MPN growth experiments – i.e., the inability to grow certain taxa – suggests our MPN-inferred protist densities are lower than actual viable densities following treatment.

Clearly there is variation in our results that is driven by the source water community. For instance, while MPN-inferred protist densities tended to be lower than ETV-inferred densities using all observed cells, there were a few exceptions owing to the taxa present in the source water. While small-celled cyanophytes and green algae were uncommon or undetected in the source water during OBS tests 4 and 5, these taxa thrived during 14 days of growth in media, likely resulting in a higher MPN-inferred protist density than could be provided by microscopic assessment of large-celled taxa that strictly meet the IMO size class.

A disadvantage of the ETV method is the possibility of false positives; i.e., observation of dead cells that appear alive because they fluoresce following staining with FDA and/or CMFDA. False positives can be determined by performing a validation step: ETV assessment on killed samples and determining the proportion of the community that provides a false live signal (Steinberg et al. 2011). After years of testing at the facility in Duluth-Superior harbor we know that false positives in local protists are rare (Reavie et al. 2017) and should not have had a significant affect on results presented herein, but elsewhere and for larger organisms such as zooplankton the phenomenon has been recognized as a greater problem (Adams et al. 2014).

5.4 Is MPN an acceptable approach for mixed-species viability measurements? Are certain protist taxa favored or disfavored under MPN procedures?

Under our testing conditions taxa that exhibited strong growth were green algae, cyanophytes and diatoms. We also observed several non-growable freshwater autotroph taxa. For instance, chrysophytes, cryptophytes, and dinoflagellates were frequently important components of the harbor (day 0) microalgae community. However, after the incubation period these taxa were rare or not present. This finding is a likely result of inter-taxa competition under a scenario of rich nutrient availability (Tilman 1977, Titman 1976). Further, the MPN approach assumes that each dilution is thoroughly mixed and clumps or aggregates of cells do not occur or, if these issues do occur, that they are not disrupted during further dilution stages – an assumption that is likely incorrect. Certain opportunistic taxa with environmental preferences for the condition during incubation may rapidly bloom, thereby outcompeting other viable taxa due to increasing physical and/or nutrient constraints

during the growth period. Phytoplankton taxa from the Great Lakes have a wide range of environmental optima for many water quality variables (Reavie et al. 2014a), so attempts to grow cultures of protists from ambient collections are challenged by the specific environmental optima and tolerances of each species. Hence it is not surprising that several taxa confirmed as alive at the beginning of experiments were extirpated.

That some taxa were not favored to grow could result in an underestimation bias of the MPN method. The number of non-growable autotrophs is also likely impacted by seasonality within the Laurentian Great Lakes, potentially because of their tolerance to specific ranges of surface water temperature or illumination. This underestimation may have played a role in the notable lower MPN-inferred cell densities relative to ETV counts that included all cell sizes.

It was suggested by First (2019) that quantifying the effect of non-cultivable organisms could be supported by having data on natural protist communities at land-based test locations. This would involve surveyed comparisons of taxa present in collections and grow-out cultures so that there is a pre-understanding of which taxa are likely to grow. Using Duluth-Superior Harbor at the foot of the St. Louis River Estuary as an example, seasonal variation and stochastic changes resulting from short-term weather events make for a diverse and highly temporally variable protist community, so efforts to adequately characterize these parameters would be complex. Costs and effort associated with such characterization and culturability of protist communities across multiple Great Lakes test locations would need to be carefully considered if it is decided that MPN will become part of type approval testing of BWMSs in the Great Lakes. During the comment period on the R&D Plan (January 2021, R&D Plan Data Working Group) it was further suggested that a location-specific target organism that is abundant at a water source under consideration may serve as a proxy for the entire community. This too is a challenging proposition because at our location and in the Great Lakes in general, substantial seasonal and short-term variation dictates several dominant taxa throughout a given year.

Our *Selenastrum* growth experiment results suggest that temperature is an important factor in MPN incubation – i.e., colder temperatures are less likely to provide valid results – and that growth in response to dilution can provide unexpected outcomes. However, because of a need for additional data we hesitate to report on the significance of these results and hope that future work may revisit additional assessments of MPN and cultured protists.

Using the single-celled green alga *Tetraselmis* as a challenge organism for validation of a UV-based BWMS, Sun & Blatchley (2017) determined that MPN assays successfully estimated viable protist endpoints following treatment. This suggests that single-celled organisms may be better suited to MPN applications than the diverse community present in Great Lakes samples. In most cases throughout the Great Lakes most of the protist biomass comprises colonial taxa that form multi-celled filaments and geometrically organized or amorphous globular colonies Reavie & Cangelosi (2020). This likely poses a problem for the dilution process required for MPN, namely the uncertainty of the desired dilution that is close to 1 cell/mL which provides the critical density for viability estimates assessed by fluorescence following incubation. We suspect the lack of correlation between ETV and MPN approaches may be at least partly caused by the patchiness inherent in Great Lakes protist communities. For instance, a single colonial entity containing 10 viable cells may be captured in a “1 cell/mL” tube, thereby creating an erroneous, 10-fold increase in growth potential. In contrast it is likely many more tubes with purported lower starting concentrations will receive no living organisms due to the substantial patchiness of diluted samples containing mixed communities of colonial entities.

5.5 Is the combination of autotroph and heterotroph analyses valid?

Implementing the combined assessment of autotrophs and heterotrophs was not a major challenge, however in the Great Lakes (and likely most freshwater systems) non-phototrophic protists are relatively rare most of the time, though instances of high abundance occasionally occur (Carrick & Fahnenstiel 1990, Fahnenstiel et al. 1998). In our tests heterotroph abundance was consistently a very low proportion of the total protist community, and they contributed little to overall calculations. Because of their low abundance, heterotrophs contributed little to the overall variability in MPN results, and so are not considered a confounding issue. In water containing higher densities of heterotrophic protists some growth supplement may be helpful to support growth of these organisms during MPN incubation.

6 Conclusions

A comparison of microscopic (ETV) and grow-out (MPN) methods was performed in a freshwater location in western Lake Superior to compare the potential of these methods in the evaluation of type approval testing of BWMSs. Though we did not evaluate all possibilities for growth media, of our selection of commonly used media, a 50 % solution of Bold Modified Basal Media provided the best MPN results in terms of taxonomic diversity and autotroph growth and reproduction. Our growth experiments indicated that warmer temperatures maximized growth in MPN assays. Though it may be possible to maximize total growth in assays, one can always assume that some viable taxa in a mixed community will not grow in the specific microenvironment provided during incubation. If MPN is adopted as a method to support the protist assessments in type approval of BWMSs, not accounting for the non-growable phototrophic species present in source waters may be problematic for meeting regulatory limits. Though challenging to develop initially, alternative considerations like having clear lists of MPN-suitable (growable) taxa occurring at ballast uptake locations may increase reliability of results.

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8 APPENDICES

STANDARD OPERATING PROCEDURE

Assessing the Viability of Freshwater Protists

SOP Written by Desi Niewinski **Signature:**

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Date:

DISTRIBUTION LIST:
All Lake Superior Research Institute (LSRI) and Great Water Research Collaborative (GWRC) staff and student assistants, including subcontractors, involved in analysis of sample for determining viability of organisms in the 10 to 50 µm size class.

RECORD OF REVISIONS:

No.	Date	Type	No.	Date	Type
1			7		
2			8		
3			9		
4			10		
5			11		
6			12		

Analysis method for determining organism viability in the ≥10 to 50 µm size class

INTRODUCTION

This Great Waters Research Collaborative (GWRC) Standard Operating Procedure (SOP) describes the culture-based analysis method for determining the viability of organisms in the ≥10 µm to <50 µm size category for biological efficacy or ballast water management system type-approval tests. Based on International Maritime Organization (IMO, 2004) and United States Environmental Protection Agency's Environmental Technology Verification Program Criteria (US EPA, 2010), this size category refers to organisms that range from ≥10 µm to <50 µm in minimum dimension and is typically dominated by phytoplanktonic algae but usually includes some protozoans and suspended benthic algae. This viability assessment method uses an integrative approach of enumerating viable autotrophs and living heterotrophs in a water sample. The autotroph method uses chlorophyll-based dilution-culture techniques in order to determine organisms that are living and able to reproduce based on fluorescence measurements. The number of heterotrophic organisms is determined by the count of organisms that are motile, but do not contain chlorophyll, therefore do not exhibit fluorescence from photosynthetic pigments. The densities counted within these methods are combined to calculate a total concentration of viable organisms, which is used to calculate biological efficacy of a ballast water management system (BWMS).

Samples are collected according to SOP GWRC/28 – *Collecting Protist and Microbial Samples at the Montreal Pier Facility*. Samples analyzed in triplicate are serially diluted and placed into a most probable number (MPN) culture tube array with 5 replicates of each dilution plus 5 replicates of a media blank. Autotroph MPN tubes are analyzed for chlorophyll via fluorometer following SOP SA/72 – *Measuring In Vivo Chlorophyll A Using the Trilogy Laboratory Fluorometer.* 1-mL subsamples are collected for heterotroph analysis, where water is placed onto a Sedgwick-Rafter cell and assessed for non-fluorescent, motile organisms. Replicate MPN tubes are incubated at sample collection temperature ±5 °C under continuous light for 14 days and measured again by fluorometer and protist analysis to determine cell growth. After the 14-day incubation samples are assessed for positive growth and tubes that exhibit growth are composited and analyzed for community composition.

DEFINITIONS IN THE CONTEXT OF THE PROTIST VIABILITY STUDY

Autotroph – an organism that is able to produce its own food (e.g., via photosynthesis), In this method an autotroph is specifically an organism that contains chlorophyll.

Autotroph method – the portion of the MPN method involving quantifying photosynthesizing organisms (i.e., not including heterotroph assessment)

Discharge control sample – a sample of water from a BWMS test, collected at the end of the testing period during the discharge of a tank that held uptake water that had not been treated by the BWMS

Discharge treated sample – a sample of water from a BWMS test, collected at the end of the testing period during the discharge of a tank that held uptake water that had been treated by the BWMS

Heterotroph – an organism that is not able to photosynthesize, specifically an organism that does not contain chlorophyll

Viable autotroph – an organism with chlorophyll that is able to reproduce

Living heterotroph – an organism without chlorophyll that exhibits motility

MPN – most probable number, a mathematical determination based on presence/absence data

Uptake sample – a sample of water from a BWMS test, collected at the start of the testing period from a location prior to treatment

Viability – the ability to reproduce. In the context of ballast water treatment and preventing successful invasions, it is equally protective to render organisms either non-viable or dead. Organisms that are non-viable are rendered harmless in the context of preventing successful invasions. Thus, treatment standards based on enumeration of living organisms are equivalently met by enumerating viable organisms.

SCOPE AND APPLICATION

This method provides instruction for carrying out a two-part method to measure the number of viable organisms within the 10-50 µm size range for water samples. These samples may be collected from any water source or during uptake and discharge processes during testing the of a BWMS.

The first part of the method, the autotroph method, includes a chlorophyll-based most probable number (MPN) dilution-culture technique to determine the concentration of viable organisms. In the context of ballast water treatment, non-reproductive organisms are considered to be rendered harmless and non-viable. In this method, viable autotrophs are defined as those capable of reproduction and are measured by fluorescence increase (*in-vivo* chlorophyll-a). In theory this method cannot be biased by organism bodies that are dead or alive and nonviable, so it should be applicable to BWMS that use any treatment technologies.

The second part of the method, the heterotroph method, is an adaptation of the microscopic method described in the ETV Protocol for assessing the number of live organisms in the >50-µm size class, in which living organisms are defined as those organisms that exhibit motility. In this method, an epifluorescent microscope is used to detect, identify, and eliminate organisms with chlorophyll (as those are assessed by the autotroph method). Heterotrophs are defined as organisms without any chlorophyll; as such they are organisms that do not show any red auto-fluorescence. Thus, the number of living heterotrophs in the size range 10-50 µm is determined as the count of organisms in the size range 10-50 µm, that do not exhibit red auto-fluorescence, and that do exhibit motility. This portion of the method does not evaluate the ability of heterotrophs to reproduce and is therefore not providing a true assessment of damage caused by technologies that impact organism reproduction (e.g., UV treatment). As there is no method available at this time to evaluate the ability for heterotrophs to reproduce, this is recognized as a limitation.

The living organism concentrations measured by the two techniques (autotroph method and heterotroph method) are summed to provide a total concentration of living organisms in the original sample volume which is used to assess the performance of the BWMS relative to the discharge standard in type-approval testing or biological efficacy testing.

PERSONNEL QUALIFICATIONS

All analysts read, understand and commit to strictly follow this SOP and must document that commitment in a signed *LSRI SOP Compliance Form*. An analyst may not perform this procedure until the analyst has passed an initial demonstration of capability (IDC). After hands on training by a supervisor has been completed, the IDC will consist of the analyst conducting the procedure as written in this SOP, properly making media, pipetting samples, and understanding the use of incubators required by the SOP to a supervisor. Results of the IDC must be documented on the *LSRI Training Completion/Competency Testing Form*.

EQUIPMENT LIST

- Adjustable-volume pipettes and sterile pipette tips
- Autoclave
- Autoclave bags
- Biohazard trash receptable
- Biosafety cabinet (BSC)
- Bunsen burner
- Disposable sterile filter unit (e.g., Stericup™) -0.22 µm (for sterilizing test water and media)

- Deionized (DI) water
- Epifluorescence microscope with blue excitation and red emission filter cube
- Falcon tubes (15 mL and 50 mL)
- Fume hood
- Forceps
- Germicide (1% bleach solution, 70% ethanol, or equivalent)
- Glass test tubes (6 mL and 15 mL)
- 1 L HPDE bottles
- Incubator with grow light (nominally 50-150 µmol photons m^{-2} s^{-1})
- Labels
- Laboratory glassware (beakers, graduated cylinder, volumetric flasks, etc)
- Microcentrifuge tubes (2 mL) for stain
- Min/Max thermometer (accuracy must be verified annually)
- Personal protective equipment (PPE; eye protection, gloves, lab coat, etc)
- Sedgwick Rafter counting chamber
- Sterile test tube caps
- Test tube racks
- Turner Trilogy Laboratory Fluorometer with *in-vivo* chlorophyll module and 12-mm test tube adapter
- Vacuum pump
- Vortex tube mixer

STANDARDS AND REAGENTS

Personal protective equipment (PPE) including lab coat, gloves, safety glasses, and adequate ventilation are required when preparing stain stock solutions. Note: expired stain solutions must be discarded in compliance with University of Wisconsin-Superior or University of Minnesota Duluth guidelines depending on where the stain is disposed.

1. Lugol's solution. In a fume hood, dissolve 100 g of potassium iodide (KI) and 50 g iodine (I_2) in approximately 800 mL of deionized (DI) water in a 1-L volumetric flask. Mix until the chemicals are completely dissolved. Add 100 mL of glacial acetic acid and bring volume to 1 L with DI water. Store solution in a dark bottle at room temperature.
2. With the exception of ISO 8692, stock concentrates were pre-purchased from vendors as listed in Table 1 of the main report. ISO 8692 stock (ISO 1989) was made using in-house ingredients. Concentrates may be stored in the fridge in the dark until needed.
3. Silica stock 28.46 g/L – Dissolve 28.46 g of sodium metasilicate nonahydrate ($NaSiO_3$ $9H_2O$) into approximately 800 mL of DI water then adjust final volume to 1000 mL. Sterilize stock solution by filtering through disposable 0.22 µm sterile filter unit. Cap tightly in sterile bottle and store for up to one year at <10 °C and above freezing in the dark.

SAMPLE HANDLING REQUIREMENTS

1. Collect samples, and source water for media preparation in clean containers according to the relevant project planning documentation (e.g., Quality Assurance Project Plan (QAPP)) or according to SOP *GWRC/28 – Collecting Protist and Microbial Samples at the Montreal Pier Facility*. Label and track samples according to the LSRI SOP *REC/15 – Sample Custody, Tracking, and Acceptance*.
2. Transport collected samples to the analyst immediately after collection. Store samples in a cooler as close to sample collection temperature as possible (±5°C).
3. Samples should be analyzed as soon as possible after collection to avoid unpredictable changes in the organism community. If samples are not processed immediately, samples should be stored in an incubator set at sample collection temperature (±5°C) without a light and can be held for up to 6 hours.

PROCEDURE

Personal Protective Equipment (PPE) must be worn during this procedure including laboratory coat, gloves, and eye protection. Follow aseptic technique throughout procedure, working in the BSC when preparing dilutions and MPN tubes. For more on aseptic technique, see LSRI *General Microbiology Laboratory Procedures Handbook* (LSRI, 2019).

Media Preparation

1. Check and document that the accuracy of pipettes has been verified within the last three months according to LSRI SOP *GLM/27 – Procedure for Verifying the Accuracy of Laboratory Pipettes.*
2. Prepare filter sterilized source water (FSW) for media preparation. If feasible, this may be done up to two days prior to a sampling event, if water can be collected from the uptake location, and inclement weather is not expected to change water quality.
   2.1. Filter water through a disposable 0.22 µm sterile filter unit to sterilize water. If done prior to the start of the test date, water should be collected without inclement weather between collection and start date. Store FSW in the refrigerator and bring to source water temperature prior to sample analysis.
3. Each growth medium has similar preparation methods, and 50% Bold Basal Media (50%BBM) is presented here as an example. Add stock solutions for BBM medium (5 mL, with added vitamins) and silica stock (1 mL) to pre-sterilized test water (993.5 mL), and then add K_2HPO_4 stock (0.5 mL; considered part of BBM stock, but added during dilution to prevent precipitation). Mix thoroughly and adjust pH to 6.8 ± 0.2. Once pH is adjusted, filter media through a disposable 0.22 µm sterile filter unit to ensure sterility. Cap tightly in sterile bottle and store for up to one year at <10 °C and above freezing in the dark.

Table 1: Media components for 50% BBM.

Media	Stock Component	Amount (mL)
50% BBM	BBM Concentrate with Vitamins	5
	K_2HPO_4 Stock	0.5
	Silica Stock	1
	Filter Sterilized Test Water	993.5
	Total Volume	1000

4. Sterilize all glass test tubes and other glassware by autoclaving for a minimum of 15 min at 121°C following guidelines specified in LSRI SOP *GLM/33 – Autoclave Use and Maintenance*.
5. Prepare dilution tubes by pipetting 9 mL sterile media into sterile tubes (e.g., 15 mL Falcon tubes) and cover with caps. Label tubes with test ID, test date, sample ID and dilution. Can be stored at <6 °C for up to two days.
6. Prepare autotroph MPN tubes by pipetting 4.9 mL sterile media into sterile 6-mL, 12-mm glass tubes and cover with caps or aluminum foil. Label tubes with test ID, test date, sample ID, dilution and replicate number. Can be stored at <6 °C for up to two days.

Autotroph Method

7. Mix sample thoroughly by inverting 25-30 times, pour sample into three 50 mL Falcon tubes labeled with test ID, and sample ID (e.g., 21-VA-G-1 AUT-1 for the first sample on the first growth test date).
8. For each medium and each sample create a set of serial dilution tubes, selecting the appropriate dilutions based on Table 2. Create dilutions by pipetting 1 mL of sample into the first dilution tube filled with 9 mL sterile media, gently vortex the tube to mix. Pipette 1 mL of the first dilution tube into the second dilution tube and continue until all desired dilutions are created.

Table 2: Dilution selections according to protist concentration.

Sample Type	Dilutions	Range of Protist Concentration (Cells/mL)
Uptake – land-based	10^{-2}, 10^{-3}, 10^{-4}	18 – 16,000
Uptake – shipboard	10^{-1}, 10^{-2}, 10^{-3}	1.8 – 1,600
Discharge control – land-based	10^{-1}, 10^{-2}, 10^{-3}	1.8 – 1,600
Discharge treated – land-based or shipboard	10^{-0}, 10^{-1}, 10^{-2}	0.18 – 160
Method/media blank	10^{-0}	NA

9. From each dilution tube pipette 1 mL of sample into each of the 5-replicate autotroph MPN tubes containing 4.9 mL of sterile media. Place sterile caps onto each tube using forceps and following aseptic techniques and gently vortex or invert sample to mix.
10. For each media blank transfer 50 mL of sterile media into a falcon tube, then pipette 1 mL from the falcon tube into five replicate 4.9 mL autotroph MPN tubes to use as media blanks for the duration of the incubation. Place sterile caps onto each tube using forceps and following aseptic techniques and gently vortex or invert sample to mix.
11. Measure the fluorescence of each of the autotroph tubes on the Trilogy Fluorometer following SOP *SA/72 – Measuring In Vivo Chlorophyll A Using the Trilogy Laboratory Fluorometer.* Record the date, time in and out of the incubator, temperature in and out of the incubator, analyst, sample ID and raw fluorescence unit (RFU) onto the fluorometer datasheet.
12. Once sample fluorescence has been read, lightly place the cap on top of each MPN tube, allowing for exchange of CO_2 and O_2 and place the MPN tubes under the grow light in an incubator set at sample collection temperature. Incubate samples for 14 days in continuous light.
13. Light intensity within the incubator will be measured using a light meter read in foot candles on Day 0 and Day 14 of incubation.
14. After the 14-day incubation period, read samples again on the fluorometer to measure for positive growth. Vortex samples prior to reading fluorescence to ensure homogenization.

Autotroph Quantification

15. Calculate the average fluorescence of the media blank tubes at Day 0 and Day 14.

$$average\ blank\ fluorescence\ day\ X = \frac{\sum fluorescence\ of\ day\ X\ in\ n\ replicate\ tubes}{n\ replicate\ tubes}$$

16. Calculate the blank-corrected fluorescence increase in each autotroph tube.

Blank-corrected fluorescence increase = (tube fluorescence day 14 - tube fluorescence day 0) – (average blank fluorescence day 0 – average blank fluorescence day 14)

17. Calculate the standard deviation of the fluorescence of the day 0 initial media blank tubes on Day 0. (In Excel: = STDEV.S ($x_1, x_2, x_{3,...}$)

$$\sqrt{\frac{\sum(x-\overline{x})^2}{(n-1)}}$$

18. Score each autotroph tube for positive growth. Tubes are considered positive if the blank- corrected fluorescence increase is greater than four times the standard deviation of average fluorescence measurements of the blanks on Day 0.

19. Calculate the MPN result by entering the number of positive tubes per dilution into an MPN calculator worksheet (Wilrich, 2018). (e.g., we used MPN_ver6.xls)

Heterotroph Method

20. From each subsample and media blank collect a 50 mL sample in a falcon tube for heterotroph analysis.
21. Invert or vortex sample to mix and immediately pipette a 1.0 mL sample onto the Sedgwick-Rafter cell.
22. Flood the counting cell with the subsample and cover to seal with a coverslip. Place the counting cell on the stage of the microscope and set the microscope for simultaneous observation using brightfield and epifluorescence. Let the sample settle for at least 30 seconds before counting.
23. Count and identify specimens along horizontal transect that are motile (disregard organisms which exhibit a red fluorescence) using 200 X magnification. Keep track of the number of squares counted on the gridded Sedgwick-Rafter cell and record the total number of squares counted (1000 maximum possible per counting cell; 20 transects X 50 squares/transect).
24. Entities that are characterized as alive or ambiguous are enumerated on the datasheet, entities characterized as dead are not included. Cells are counted per the following categories:
    - 24.1. Multiple cell entities with cells that are less than 10 µm in all dimensions.
    - 24.2. Multiple cell or single cell entities with cells that are less than 10 µm in minimum dimension, but greater than 10 µm in maximum dimension.
    - 24.3. Multiple cell or single cell entities with cells that are greater than 10 µm in all dimensions, but less than 50 µm in minimum dimension.
    - 24.4. Multiple cell or single cell entities with cells that are greater than 50 µm in minimum dimension or organisms (e.g., zooplankton) greater than 50 µm are noted.
25. Densities are calculated using the following equation:

$$x = \frac{counted\ total\ (number)}{(1.0\ mL\ sample\ counted) \times (proportion\ of\ chamber\ counted\ (fraction))}$$

Where:

- Counted total (number) = total number of cells is the sum of all recorded heterotrophic protist numbers on the datasheet
- 1.0 mL sample counted = portion of sample placed onto the Sedgwick-Rafter cell.
- Proportion of chamber counted (fraction) = total transect length (µm) multiplied by the field of view width (typically 980 µm for 200 X on inverted microscope), then divided by the total area of the Sedgwick-Rafter chamber (10^9 μm^2).

Species Diversity

26. Tubes that test positive will be composited and 1 mL per 100 mL sample of Lugol's will be pipetted into the composited sample for later analysis in order to determine species diversity and composition.
27. Invert or vortex sample to mix and immediately collect the exact volume to fill the counting chamber using a pipette. The Sedgwick-Rafter cell will accept 1.0 mL

- Count and identify species according to steps 25 - 28.

INTERFERENCES, BIAS, AND LIMITATION

We acknowledge the following factors that may affect results, and the likelihood of impacts are described in Table 3.

1. Inclusion of organisms >50 µm – Whole water samples are not screened through a 50 µm, filter prior to start to analysis to remove >50 µm autotrophs.
2. Inclusion of organisms <10 µm – Whole water samples are filtered on a 10 µm filter and resuspended to remove <10 µm autotrophs.
3. Removal of organisms <10 µm – The 10 µm filtration and resuspension step can result in some autotrophs >10 µm squeezing through the 10 µm filter.
4. Filtration mortality – The 10 µm filtration and resuspension step can result in some autotrophs being killed during the filtration step.
5. Non-growing autotrophs – Some species of autotrophs may not be able to reproduce or grow in the culture conditions (media, light, temperature).
6. Slow-growing autotrophs – Some species of autotrophs may not be able to reproduce or grow fast enough to result in the detection of growth from the most dilute autotroph tube, within the incubation period.
7. Chain-forming autotrophs – Some species of autotrophs can form chains of individual cells.
8. Grazing by heterotrophs – The presence of heterotrophs within the autotroph tube can lead to the grazing of autotrophs.
9. Assessment of live-dead status – Motility is used to distinguish between live and dead heterotrophs. This also used in the ETV protocol and validates the use for stains for determining the count in the ETV method for organisms in the 10-50 µm size class.

Table 3. Summary of possible biases for the autotroph and heterotroph methods.

	Bias on number of living 10-50 µm organisms	**Probable magnitude**
Autotrophs		
Inclusion of >50 µm autotrophs	Overestimate	Small, <3%
Inclusion of <10 µm autotrophs	Overestimate	Effect is described in detail in primary manuscript
Removal of >10 µm autotrophs	Underestimate	Can be minimized
Filtration mortality of >10 µm autotrophs	Underestimate	Can be minimized
Non-growing autotrophs	Underestimate	Small, near 0% by abundance
Slow-growing autotrophs	Underestimate	Small
Chain-forming autotrophs	Under- & overestimate	Small
Grazing by heterotrophs	Underestimate	Small
Heterotrophs		
Inclusion of >50 µm heterotrophs	Overestimate	Can be minimized
Inclusion of <10 µm heterotrophs	Overestimate	Can be minimized
Removal of >10 µm heterotrophs	Underestimate	Can be minimized
Filtration mortality of >10 µm heterotrophs	Underestimate	Can be minimized
Assessment of live-dead status	Overestimate	Large for UV treatment

QUALITY ASSURANCE AND QUALITY CONTROL

1. For each sample type 3 replicate subsamples will be collected. The autotroph method will consist of 3 dilutions per subsample with 5 replicate tubes per dilution.
2. A method blank is processed for both the autotroph and heterotroph method from a 50 mL subsample taken from the sterilized media and processed as a regular sample. No growth should be exhibited in the tubes.

3. Calibrations
   3.1. Fluorometer - the fluorometer is used to track growth in autotroph tubes and is measured as Raw Fluorescence Units (RFUs), due to the raw fluorescence units the fluorometer does not need to be calibrated prior to use. The secondary solid standard will be read before and after each use and the readings must be within 5 % of each other per SOP *SA/72 – Measuring In Vivo Chlorophyll A Using the Trilogy Laboratory Fluorometer.*
   3.2. Incubator – Temperature readings of the incubator are to be recorded twice daily when the lab is staffed, with a minimum of 4 hours in between readings to ensure stability of operation.
   3.3. Light Intensity – Light intensity of the grow light within the incubator will be read at Day 0 and Day 14 of the incubation period to ensure growth conditions are stable within the incubation duration.
   3.4. Thermometer – Verify the thermometers use have been calibrated yearly as per SOP LSRI/
   3.5. Pipettes – Verify the accuracy of the pipettes have been verified quarterly as per SOP/ LSRI
4. Heterotroph samples will be counted in triplicate.

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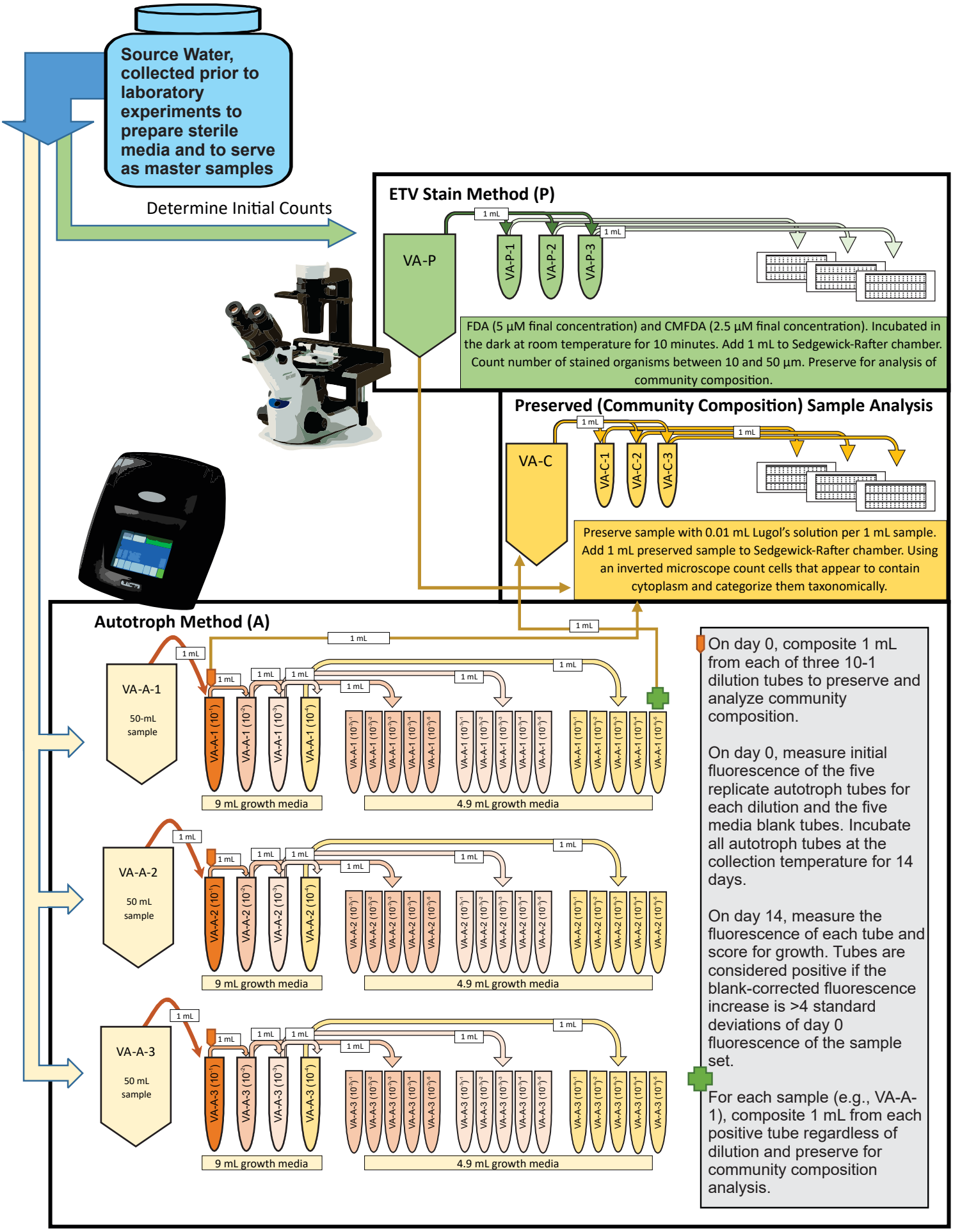

Figure 1. Sample analysis schematic for the ETV and MPN methods, as performed on water samples containing protist communities.

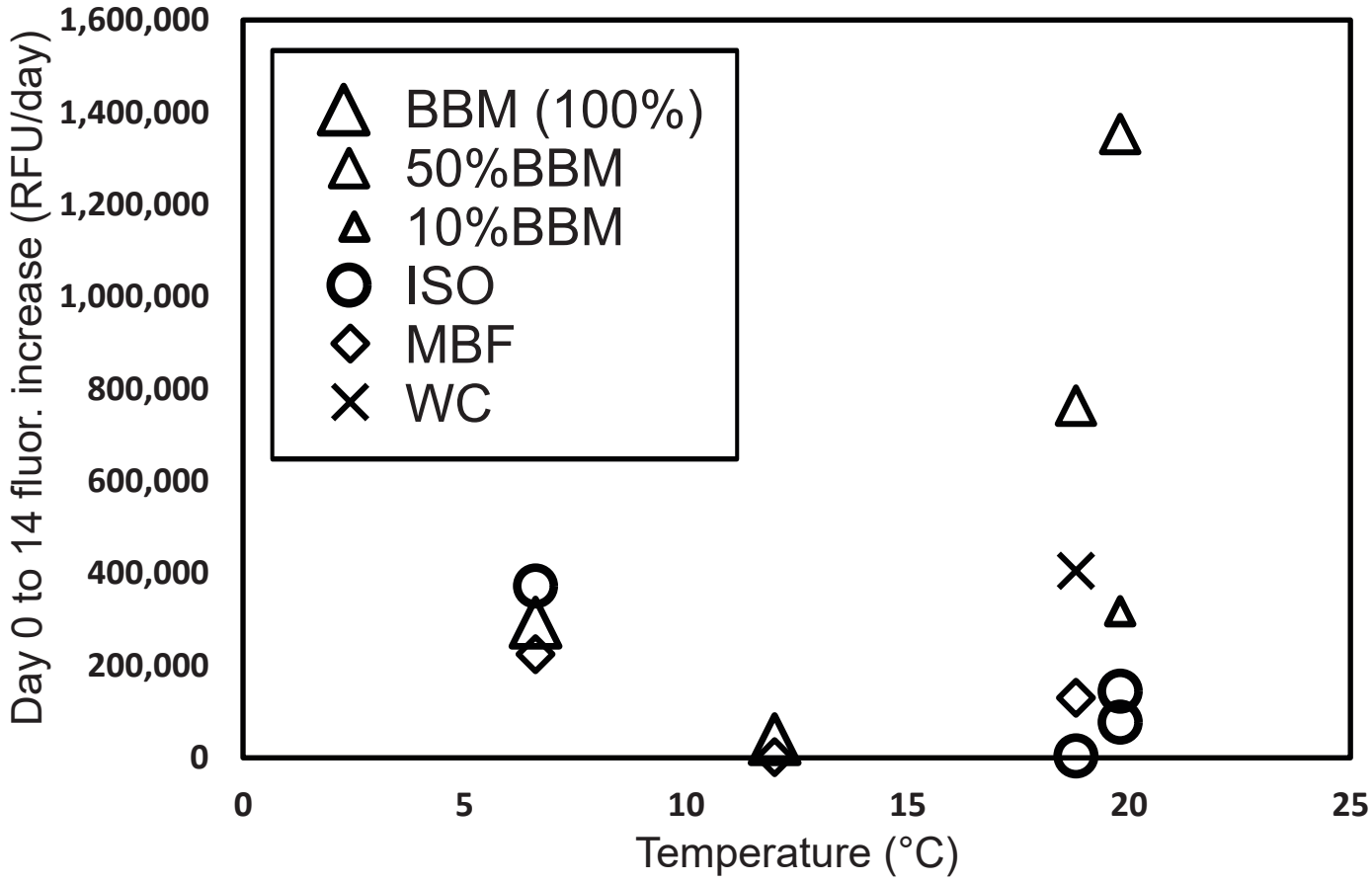

Figure 2. Autotroph growth as indicated by fluorescence increase over a 14-day period in MPN tubes, measured across various temperatures and growth media types.

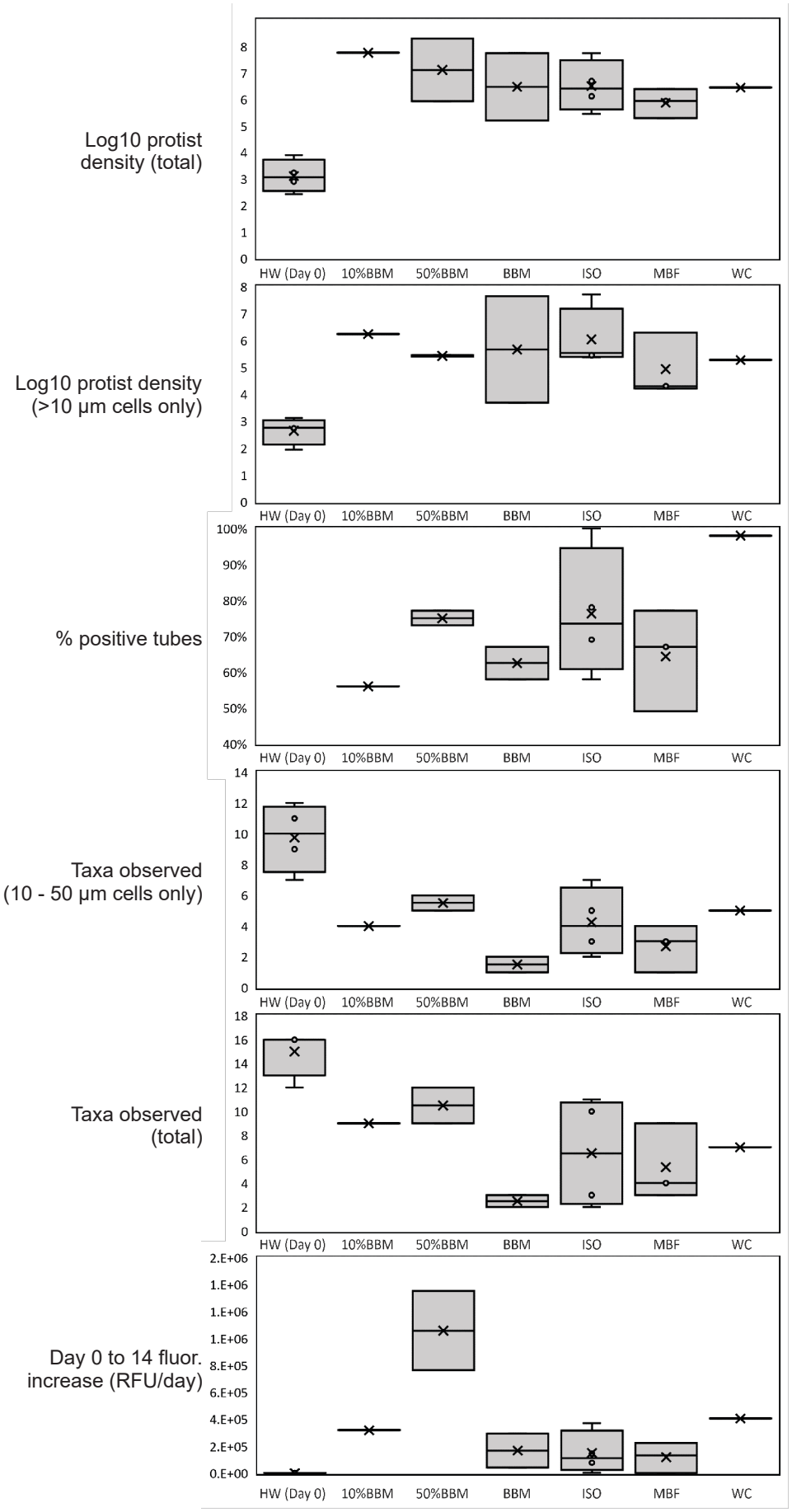

Figure 3. Comparison of protist community parameters in starting samples (HW) and in MPN tubes following 14 days of incubation in six types of growth media (10%BBM, 50%BBM, BBM, ISO, MBF, WC).

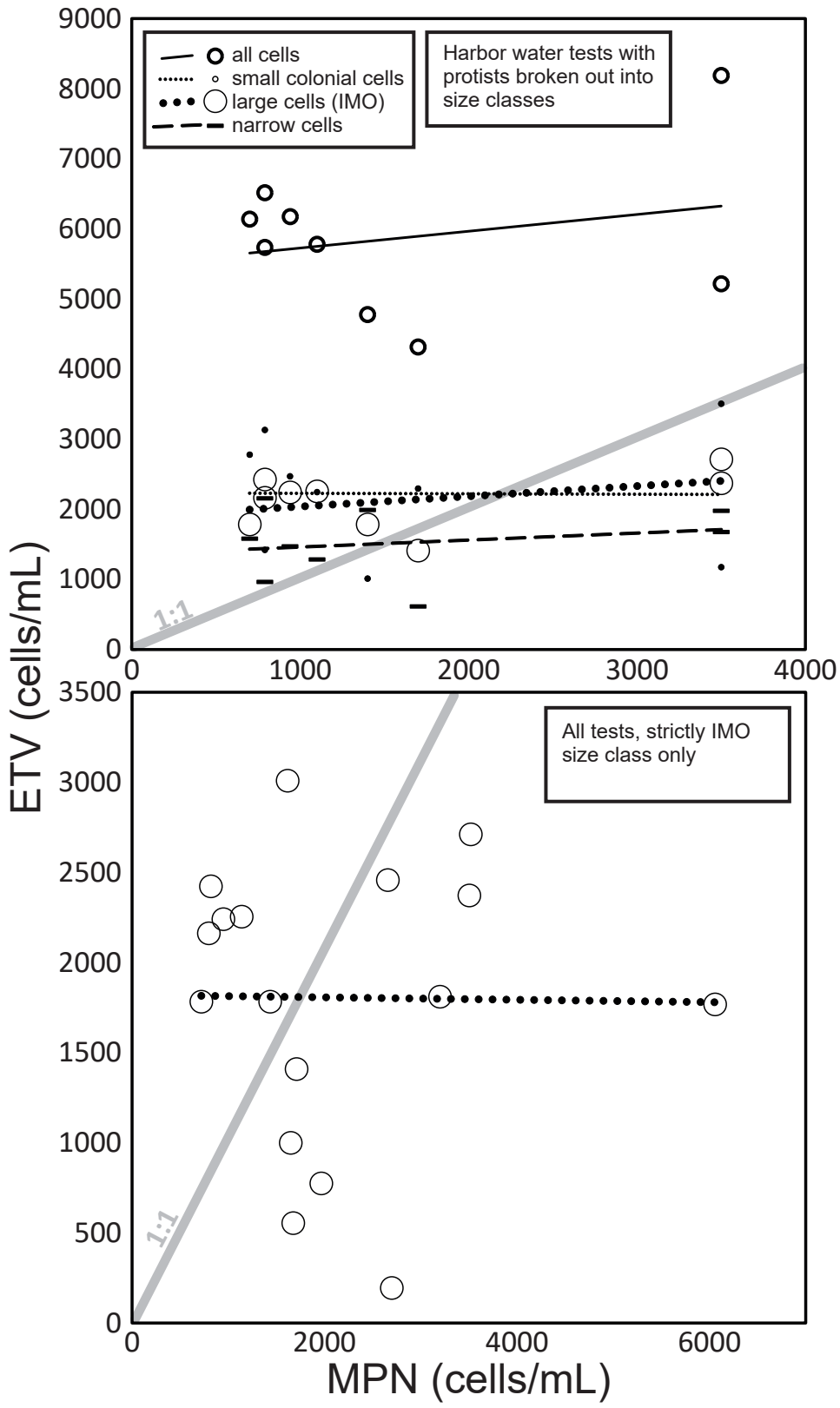

Figure 4. Comparison of cell density estimates derived from ETV and MPN methods. Lines represent regression lines based on four different ways of counting cell size classes in ETV analysis (no significant trends observed; *t*-test, P > 0.05). The 1:1 line is provided for reference as the perfect relationship between the analyses.

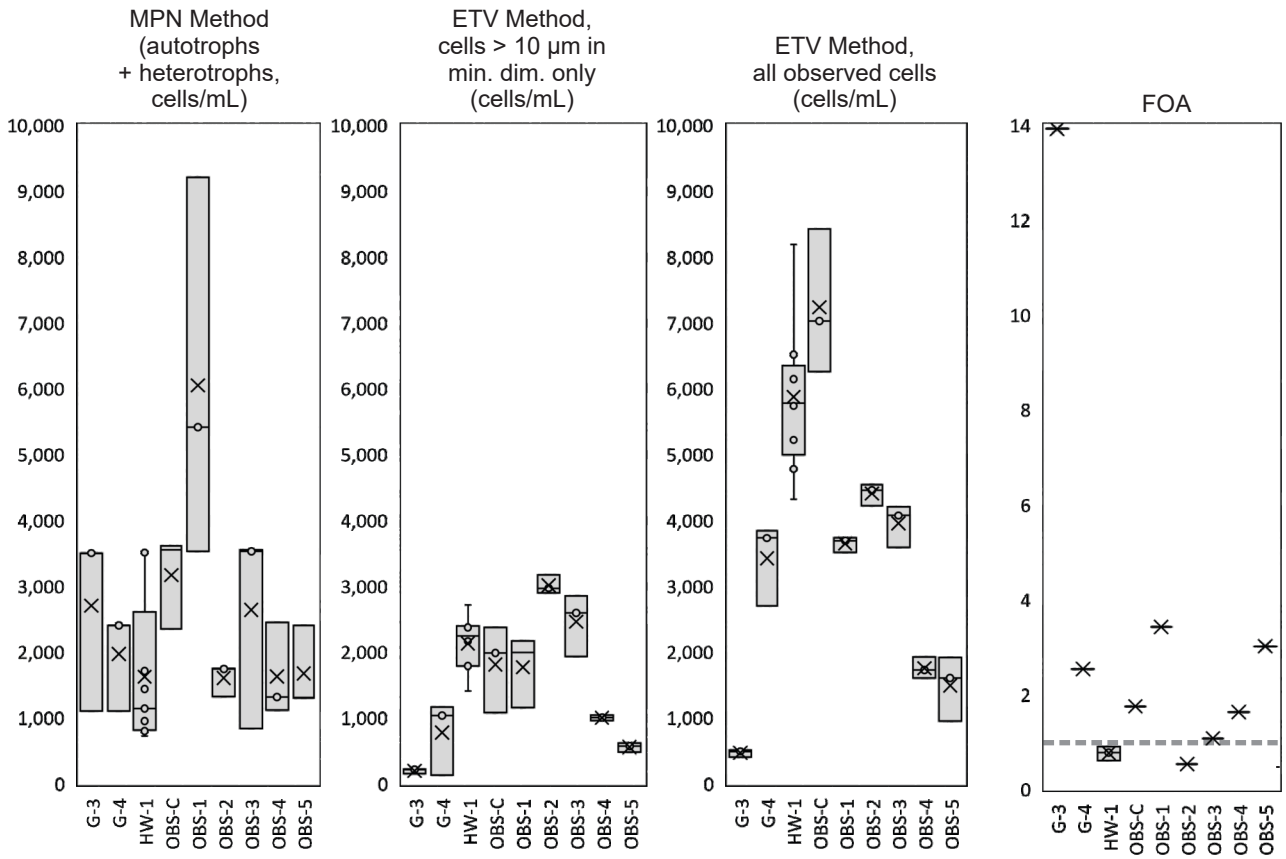

Figure 5. Comparison of cell density estimates in source-water samples derived from ETV and MPN methods. Boxplots represent results from two growth experiments (G), harbor water tests (HW) and the six tests run during testing of the treatment system (OBS). The factor of agreement (FOA, MPN:ETV) is the ratio of results between methods, with 1 (dashed gray line) representing perfect correspondence.

Table 1. Summary of the various growth tests evaluated in subprojects presented herein. The last column reflects days passed for measurements of potential growth. Starting protist densities are based on ETV microscopic methods (cells strictly 10 - 50 µm in minimum visible dimension), with the exception of the *Selenastrum* tests which were based on hemacytometer assessment of the cultured cells.

Trial #	Live Protist Starting Concentration (cells/mL, strict IMO size class)	Test Start Date	Conditions	Growth media	Days RFU measured
Growth tests using ambient harbor water					
G1	778	20-Apr-21	6.1 °C, continuous light	BBM, MBF, ISO	0,3,6,8,10,14,20
G2	1475	10-May-21	12.0 °C, continuous light	BBM, MBF, ISO	0, 14, 18
G3	465	11-Jun-21	18.8 °C, continuous light	50%BBM, ISO, MBF, WC	0,14
G4	357.8	8-Jul-21	19.8 °C, continuous light	50%BBM, 10%BBM, ISO	0,14
***Selenastrum* Tests**					
S1	1000	13-Apr-22	6.0 °C, 25.0 °C, continuous light	50%BBM, EPA	0,7,14
S2	1000	14-Jun-22	25.0 °C, continuous light, 100 rpm	50%BBM, EPA	0,14
S3	1000	30-Jun-22	6.0 °C, 25.0 °C, continuous light, 100 rpm	50%BBM, EPA	0,7,14
Validation Test (harbor water)					
V1	2029	11-Aug-21	21°C, continuous light	50%BBM	0,14
V2	2092	11-Aug-21	21°C, continuous light	50%BBM	0,14
V3	2258	11-Aug-21	21°C, continuous light	50%BBM	0,14
Land-based Test					
Commissioning	1810	14-Sep-21	18.8°C, continuous light	50%BBM	0,14
1	1768	21-Sep-21	18.8°C, continuous light	50%BBM	0,14
2	3009	28-Sep-21	16.1°C, continuous light	50%BBM	0,14
3	2457	30-Sep-21	16.1°C, continuous light	50%BBM	0,14
4	999	6-Oct-21	16.8°C, continuous light	50%BBM	0,14
5	554	13-Oct-21	16.8°C, continuous light	50%BBM	0,14

Table 2. Media and components used in Great Lakes VA method optimization growth tests. Asterisks mark the siliceous compound not included in the original recipe, which was added to accommodate the abundant diatom community in Great Lakes waters.

Components	Bold Modified Basal Media with f2 Vitamins (BBM)	ISO 8692 Growth Media (ISO)	Bold Medium (MBF)	Wright's Chu #10 (WC)
Purchased from	Canadian Phycological Culture Collection, University of Waterloo, Waterloo, ON, Canada	Stocks prepared in accordance with ISO 8692 using LSRI chemical stock	Sigma Aldrich (B5282)	Canadian Phycological Culture Collection, University of Waterloo, Waterloo, ON, Canada
pH	6.8	8.1	6.6 - 6.8	8.0 - 8.5
Calcium	Calcium Chloride Dihydrate	Calcium Chloride Dihydrate	Calcium Chloride Dihydrate	Calcium Nitrate Tetrahydrate
Magnesium	Magnesium Sulfate Heptahydrate	Magnesium Sulfate Heptahydrate	Magnesium Sulfate Heptahydrate	Magnesium Sulfate Heptahydrate
Nitrogen	Sodium Nitrate	Ammonium Chloride	Sodium Nitrate	Ammonium Nitrate
Phosphorus	Potassium Phosphate Monobasic and Dibasic	Potassium Phosphate Monobasic	Potassium Phosphate Monobasic and Dibasic	Potassium Phosphate Dibasic
Sodium	Sodium Chloride	Sodium Bicarbonate	Sodium Chloride	Sodium Bicarbonate
Potassium	Potassium Hydroxide		Potassium Hydroxide	
Iron	Iron (II) Sulfate	Iron (III) Chloride Hexahydrate	Iron (II) Sulfate	Iron (III) Chloride Hexahydrate
Cobalt	Cobalt (II) Nitrate	Cobalt (II) Chloride Hexahydrate	Cobalt (II) Nitrate	Cobalt (II) Chloride Hexahydrate
Copper	Copper (II) Sulfate	Copper (II) Chloride Dihydrate	Copper (II) Sulfate	Copper (II) Sulfate
Manganese	Manganese (II) Chloride Tetrahydrate	Manganese (II) Chloride Tetrahydrate	Manganese (II) Chloride Tetrahydrate	Manganese (II) Chloride Tetrahydrate
Zinc	Zinc Sulfate	Zinc Chloride	Zinc Sulfate	Zinc Sulfate
Molybdenum	Sodium Molybdate	Sodium Molybdate	Molybdenum (VI) Trioxide	Sodium Molybdate
Vitamins	B12, Biotin, Thiamin			B12, Biotin, Thiamin
Silica	Sodium Silicate	Sodium Silicate	Sodium Silicate	Sodium Silicate
Other components	Boric Acid, EDTA	Boric Acid, EDTA	Boric Acid, EDTA	Boric Acid, EDTA
Trace Metals			Sodium Selenite, Tin (IV) Chloride, Vanadium (II) Sulfate, Nickel (II) Chloride, Potassium Iodide	

Table 3. Preparation of dilutions during MPN testing was based on source water type (modified from First 2019).

Sample Type	Concentration of interest (Cells/mL)	Dilutions	Range of protist concentration (cells/mL)
Uptake – land-based and harbor water	1,000	10^{-2}, 10^{-3}, 10^{-4}	18 – 16,000
Discharge control – land-based	100	10^{-1}, 10^{-2}, 10^{-3}	1.8 – 1,600
Discharge treated – land-based or Shipboard	10	10^{0}, 10^{-1}, 10^{-2}	0.18 – 160
Method/media Blank	0	none (10^{0})	NA

Table 4. Water quality measured at sample collection for growth trials. High turbidity numbers (*) may be erroneous due to turbulence within the sonde flow cell.

Trial	Sample type	Temp. (°C)	Salinity (PSU)	Dissolved Oxygen (mg/L)	Spec. Cond. (µS/cm)	pH	Turb. (FNU)	Chl. *a* (µg/L)	TSS (mg/L)	POM (mg/L)	MM (mg/L)	% UVT filtered	NPOC (mg/L)	DOC (mg/L)
Growth tests using ambient harbor water														
G1	Harbor	6.6	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
G2	Harbor	12	0.08	10.42	161.2	7.7	8.65	1.75	6.0	1.4	4.6	17.1	20.2	19.4
G3	Harbor	18.8	0.09	8.57	190.4	7.79	16.15	3.68	NA	NA	NA	NA	NA	NA
G4	Harbor	20.5	0.08	8.71	173	7.96	9.81	7.09	6.3	1.8	4.5	54.5	7.0	7.2
Validation Test (harbor water)														
V1-V3	Harbor	21	0.08	8.38	172	8.02	11.05	7.95	5.1	1.8	3.3	68.2	5.6	5.4
Land-based Test														
COM	Harbor uptake	18.79	0.08	8.76	171.6	7.85	109.60	3.99	29.0	6.4	22.6	71.2	5.8	4.2
COM	D Control	18.83	0.08	7.95	172.2	7.73	10.86	2.23	6.6	2.0	4.6	71.7	5.1	4.1
COM	D Treatment	18.89	0.08	8.38	171.9	7.82	12.23	1.07	8.1	2.2	5.9	72.0	5.0	4.0
1	Harbor uptake	17.86	0.08	9.26	171.5	7.72	22.05	6.26	35.7	7.1	28.5	72.8	5.6	3.9
1	D Control	17.44	0.08	8.99	166.3	7.73	13.88	4.96	13.6	3.0	10.6	73.6	5.2	3.8
1	D Treatment	17.57	0.08	9.37	166.4	7.77	14.67	1.85	14.0	2.9	11.1	73.7	5.3	4.0
2	Harbor uptake	16.23	0.08	9.58	169.7	7.85	807*	7.54	30.4	6.1	24.3	70.6	5.3	4.2
2	D Control	16.33	0.08	9.12	169.1	7.82	13.12	6.25	12.7	2.7	9.9	70.4	4.5	4.1
2	D Treatment	16.57	0.08	9.51	169.0	7.90	13.07	2.22	12.3	2.3	10.0	71.7	5.1	4.0
3	Harbor uptake	16.44	0.08	10.12	176.3	7.91	105.90	4.62	25.3	5.1	20.2	70.8	4.8	4.1
3	D Control	16.45	0.08	9.30	177.3	7.85	11.03	6.52	10.3	2.4	7.8	71.9	4.2	3.7
3	D Treatment	16.75	0.08	9.56	177.4	7.93	10.91	1.81	9.7	2.0	9.4	72.4	5.1	4.0
4	Harbor uptake	16.75	0.09	8.48	181.4	7.66	3440*	3.69	28.4	5.7	22.7	69.9	4.6	3.9
4	D Control	16.41	0.09	8.24	181.7	7.67	12.97	2.56	10.9	2.5	8.4	70.2	4.3	3.8
4	D Treatment	16.78	0.09	8.60	181.8	7.70	13.29	1.16	10.9	2.4	8.5	70.8	4.7	3.7
5	Harbor uptake	16.47	0.09	8.39	198.1	7.50	298.10	2.28	31.5	6.6	24.9	65.5	5.2	4.2
5	D Control	15.90	0.09	7.86	198.4	7.56	17.15	1.94	11.4	2.6	8.7	63.9	5.1	4.5
5	D Treatment	16.30	0.09	8.39	198.5	7.60	16.56	1.14	11.2	2.2	9.0	65.8	5.4	4.5

Table 5. Results of MPN growth experiments on cultured *Selenastrum capricornutum* . Two tests were completed using 50%BBM. Empty cells indicate that calculation of a value was not possible.

Test	Shaking	Temp.	Dilution	Positive Tubes	MPN/mL	Log MPN	SD of Log	95% Lower	95% Upper
22-VA-SC-1 (test 1)	No	6°C	10^{-0}	0	0			0	0.74
			10^{-1}	0	0			0	7.4
			10^{-2}	0	0			0	74
			10^{-3}	0	0			0	740
			10^{-4}	0	0			0	180
		25°C	10^{-0}	0	0			0	0.74
			10^{-1}	0	0			0	7.4
			10^{-2}	0	0			0	74
			10^{-3}	3	920	3	0.26	280	3000
			10^{-4}	1	2200	3.3	0.44	300	17000
22-VA-SC-2 (test 2)	100 rpm	6°C	10^{-0}	0	0			0	0.74
			10^{-1}	0	0			0	7.4
			10^{-2}	0	0			0	74
			10^{-3}	0	0			0	740
			10^{-4}	0	0			0	180
		25°C	10^{-0}	2	0.51	-0.29	0.31	0.12	2.1
			10^{-1}	2	5.1	0.71	0.31	1.2	21
			10^{-2}	3	92	2	0.26	28	300
			10^{-3}	1	220	2.3	0.44	30	1700
			10^{-4}	0	0			0	7400

Table 6. Average number of protist cells/mL and standard error of the mean (negative numbers below density values) in samples analyzed on uptake and discharge during biological efficacy tests completed with UV-based Optimarin BWMS. Results indicate protist densities based on ETV (microscopy, strictly cells in the 10 - 50 µm size class) and MPN methods. An asterisk Indicates the value is less than ETV uptake requirement.

	Uptake		Treatment Discharge		Control Discharge	
Test Cycle	ETV	MPN	$ETV^{(n=1)}$	MPN	ETV	MPN
COM	1,810	3,202	12	2.8	974	1,578
	-382	-392		-0.53	-94	-473
1	1,768	6,060	52	0.7	2,109	8,342
	-313	-1667		-0.37	-146	-3,890
2	3,009	1,618	51	0.3	2,188	1,699
	-82	-149		-0.33	-123	-914
3	2,457	2,658	41	0	1,848	1,353
	-275	-907			-74	-263
4	999*	1651	21	0.2	977	3,400
	-24	-433		-0.13	-136	-1,028
5	554*	1677	24	0.2	350	647
	-42	-366		-0.01	-38	-155

Table 7. Representation of protist taxa during growth experiments. Numbers are cell densities (cells/mL) after at least 14 days of incubation. Colors reflect growth dynamics: present only on day 0 (black), present on both day 0 and last day (yellow), and present only on last day (blue).

		Cyanophytes					Greens								Cryptophytes			Diatoms									Chryso-phytes		Protozoans and Animals			
Sample or medium	Temperature	*Anabaena, Dolichospermum* + similar	large-celled coccoid	*Microcystis* -like coccoid	non-coccoid filamentous	filamentous with poor cell definition (cells = length/5 µm)	*Scenedesmus* + similar	*Pediastrum*	coccoid	singular spindles (e.g., *Ankistrodesmus*)	filamentous	colonial with spindle-shaped cells	euglenoid	Desmidiales	*Cryptomonas* , *Chroomonas* + similar	round microflagellates	irregular-shaped microflagellates	filamentous (e.g., *Aulacoseira*, *Melosira*)	*Asterionella*	*Tabellaria*	*Diatoma*	centric non-filamentous (e.g., cyclotelloid)	fragilarioid (ribbon-shaped colonies)	singular fragilarioid (includes nitzschioid)	naviculoid (+ other single pennates)	*Rhizosolenia* + similar	*Mallomonas* + similar	*Dinobryon* , *Kephyrion* + similar	ciliates	round or oval protozoan	irregular-shaped protozoan	heterotroph egg or resting stage
Taxa strictly 10 - 50 µm in minimum dimension																															
Harbor Water	6.6°C								4						38	34	2	400				38			6		8		2	4	2	
	12.0°C														290	130		120				40					20		10		10	
	18.8°C								1		2		2		6	9		12				21			4		2		25			1
	19.8°C													7	67	13	7	1,113				87			7		7		7			
BBM	6.6°C																	348,857				44,362,931										
	12.0°C												4,859																			
10%BBM	19.8°C									1,336,442			78,614			157,229						157,229										
50%BBM	18.8°C								182,750				6,527			79,953		9,790				1,632							4,895			
	19.8°C														62,438	83,198		62,438				20,761			20,761							
ISO	6.6°C																	304,894				50,460,025										
	12.0°C															35,623		47,200				202,158										
	18.8°C								3,332							9,997	3,332				16,662	73,311			126,629				3,332			
	19.8°C		135,901										5,663		118,913	147,226													11,325			
MBF	6.6°C						10,036		20,072							66,907						1,880,093										
	12.0°C												20,229																			
	18.8°C								3,262							3,262		9,785														
WC	18.8°C							112,462	56,231						3,124	6,248											9,372					
All observed taxa																																
Harbor Water	6.6 °C						8		4						42	34	2	472	14	88		42	4	32	28		8		2	6	2	
	12 °C								60	10					880	160		360	90			40			10		20	80	10		10	
	18.8 °C					25			1	2	2		2		11	10		78	32			22	35		11	2	2		25			1
	19.8 °C	67		2,900		1,173	47		187	13				7	94	20	7	3,200				87			14		7		7		7	
BBM	6.6 °C																	348,857				55,468,200			116,286							
	12 °C									157,565			4,859																			
10%BBM	19.8 °C		52,828,781				314,457			3,459,027			78,614		157,229	157,229		393,071				157,229			314,457							
50%BBM	18.8 °C	24,475			11,422		45,687		252,913	146,853			6,527			79,953		16,317				1,632	236,596		16,317				4,895			
	19.8 °C		15,047,466	2,018,764							894,887				62,438	83,198		811,689				20,761		20,761	41,521							
ISO	6.6 °C																	304,894				55,567,006										
	12 °C															35,623		47,200				202,158										
	18.8 °C			199,940			353,227		23,326	469,859		49,985				9,997	3,332				16,662	73,311			126,629				3,332			
	19.8 °C		3,663,663	651,192		169,876				124,576		50,963	5,663		124,576	152,889									5,663				11,325			
MBF	6.6 °C						10,036		20,072							66,907						2,321,680										
	12 °C									171,943			20,229						6,743													
	18.8 °C	13,047				16,308	215,267		3,262	319,639						3,262		19,570					231,576		55,448							
WC	18.8 °C						318,643	137,454	103,090	2,218,002					3,124	6,248											9,372					

Table 8. Taxa detected in preserved community composition samples collected on uptake and discharge during treatment system evaluation. Numbers are cell densities (cells/mL) at day 14 following incubation. Colors reflect growth dynamics: present only on day 0 (black), present on both day 0 and day 14 (yellow), and present only on day 14 (blue).

		Cyanophytes					Greens						Single-celled flagellates				Diatoms								Chryso-phytes		Protozoans and Animals		
Sample	Test ID	*Anabaena, Dolichospermum* + similar	large-celled coccoid	*Microcystis*-like coccoid	non-coccoid filamentous	filamentous with poor cell definition (cells = length/5 µm)	*Scenedesmus* + similar	*Pediastrum*	coccoid	singular spindles (e.g., *Ankistrodesmus*)	colonial with spindle-shaped cells	euglenoid	*Cryptomonas*, *Chroomonas* + similar	round microflagellates	irregular-shaped microflagellates	dinoflagellates	filamentous (e.g., *Aulacoseira*, *Melosira*)	*Asterionella*	*Diatoma*	centric non-filamentous (e.g., cyclotelloid)	fragilarioid (ribbon-shaped colonies)	singular fragilarioid (includes nitzschioid)	naviculoid (+ other single pennates)	*Rhizosolenia* + similar	*Mallomonas* + similar	*Dinobryon*, *Kephyrion* + similar	ciliates	irregular-shaped protozoan	*Keratella*
Taxa strictly 10 - 50 µm in minimum dimension																													
Uptake Control	COM							3,769,147					5,182,577	942,287													471,143		
	1								159,951					2,799,137			719,778			879,729									
	2								1,057,856	32,717				796,118						250,832									
	3								600,041					2,644,626			5,311,475			1,400,096						22,224			
	4								3,015,556				327,778																
	5												118,000	53,636			21,455			986,909									
Discharge Treatment	COM								64				128	815		16			80										
	1											30	15	44		7				30									
	2																												
	3													1,062						118									
	4													741						114									
	5								45,265					236						94									
Discharge Control	COM								838,068			251,420		2,095,170		167,614				670,455									
	1								29,819	29,819				656,029			59,639			357,834									
	2													9,060			27,179			498,273									
	3								712,453			89,057	712,453	11,488,302			890,566			979,623									
	4									11,974			2,035,609	311,328			23,948			778,321									
	5													17,811			17,811			765,887									
All observed taxa																													
Uptake Control	COM	1,413,430			413,663,884			7,067,151	1,884,574				6,124,864	942,287													471,143		
	1				159,951		639,803		4,078,743	159,951			159,951	3,039,063			9,437,092			879,729			79,975						
	2								3,195,379	1,461,368				796,118			109,057			294,455	556,192		54,529						
	3				533,370				2,489,059					2,644,626			5,444,817			1,422,320						22,224			
	4			11,275,556			262,222		16,978,889				327,778				131,111			131,111			524,444						
	5			1,137,091	4,666,364				85,818	53,636			150,182	53,636			53,636	332,545		1,029,818			10,727						
Discharge Treatment	COM		640						5,628				128	815		16			80										
	1								30			30	15	52		7				30									
	2																												
	3			75,815										1,062						118									
	4		912	31,324									29	741						114									
	5								86,801					236						94									
Discharge Control	COM								6,034,091	1,592,330		251,420		2,514,205		167,614	1,005,682			670,455			1,005,682						
	1	238,556	715,668				119,278		1,610,253	5,665,704				715,668			178,917			417,473	1,162,960		1,252,419						
	2						36,238							9,060			688,522	45,298		534,511	45,298		1,023,724						
	3				356,226		356,226		15,050,566			89,057	712,453	11,488,302			890,566			979,623									
	4				407,122				742,399	359,225			2,047,583	323,303			455,018			790,295									
	5			329,509	1,255,698		605,585			8,906	62,340			17,811			17,811			872,755									

Appendix B. Cell densities and proportion of media tubes showing growth (positive fluorescence) by the end of the experiment. Average chlorophyll-a fluorescence (RFU) growth (/day) measured from positive growth tubes.

Trial	Media	Total protist counts (cells/mL)	Strictly 10-50 µm protist counts (cells/mL)	% positive tubes	Total Taxa (strictly 10 - 50 µm)	Total Taxa (all observed)	Day 0 to 14 fluorescence increase (RFU/day)
Trial 1 (6.6°C)	HW (Day 0)	7.90E+02	5.40E+02	--	12	16	--
	BBM	5.59E+07	4.47E+07	58%	2	3	293229
	ISO	5.59E+07	5.08E+07	58%	2	2	144331
	MBF	2.42E+06	1.98E+06	49%	4	4	224455
Trial 2 (12.0°C)	HW (Day 0)	1.73E+03	6.20E+02	--	7	12	--
	BBM	1.62E+05	4.86E+03	67%	1	2	40219
	ISO	2.85E+05	2.85E+05	100%	3	3	3546
	MBF	1.99E+05	2.02E+04	67%	1	3	1412
Trial 3 (18.8°C)	HW (Day 0)	2.66E+02	8.70E+01	--	11	16	--
	50%BBM	8.44E+05	2.86E+05	77%	6	12	763901
	ISO	1.33E+06	2.37E+05	78%	7	11	77145
	MBF	8.77E+05	1.63E+04	77%	3	9	129958
	WC	2.80E+06	1.87E+05	98%	5	7	405445
Trial 4 (19.8°C)	HW (Day 0)	7.83E+03	1.31E+03	--	9	16	--
	50%BBM	1.99E+08	2.50E+05	73%	5	9	1352719
	10%BBM	5.79E+07	1.73E+06	56%	4	9	317830
	ISO	4.96E+06	4.19E+05	69%	5	10	372224

Appendix C. Harbor water validation testing of MPN versus ETV microscopy method. Triplicate analysis of (1) number of viable organisms (MPN/mL) present in ambient harbor water samples, (2) live heterotrophs present in ambient harbor water, (3) live protist densities counted via the ETV method. Growth applied 50% BBM solution. During microscopic analysis of initial harbor samples cell and entity sizing was characterized in detail, allowing for seven size classes to be presented (2 X heterotrophic only, 5 X all protists).

		1							2		3				
Sample ID	Analysis Rep	MPN/ mL	Log_{10} MPN	SD of Log_{10} MPN	lower 95% conf limit	upper 95% conf limit	Rarity index	MPN Category	Total Heterotrophic Organisms per mL	Strictly 10-50 µm Heterotrophic Organisms per mL	Small-celled colonies (colony >10 µm, but cells <10 µm in all dimensions)	Narrow cells (cells ≥10 µm in any dimension but <10 µm in minimum dimension)	Large cells matching strict IMO requirements (cells ≥10 and <50 µm in all visible dimensions)	All cells counted ("allowed cells" - excludes entities <10 µm in all dimensions)	Very large cells (>50 µm in minimum cell dimension)
HW-1	Rep 1	790	2.9	0.25	250	2500	1.000	1	48	30	3131	962	2423	6515	0
HW-1	Rep 2	1100	3.0	0.22	390	3100	0.574	1	94	41	2243	1282	2254	5780	6
HW-1	Rep 3	1700	3.2	0.21	640	4600	0.941	1	72	11	2293	612	1409	4315	4
HW-1	Average	1197	3.0	0.23	427	3400	0.838	1	72	28	2556	952	2029	5537	3
HW-2	Rep 1	1400	3.1	0.20	550	3600	0.148	1	67	36	1009	1987	1783	4778	0
HW-2	Rep 2	700	2.8	0.24	230	2200	0.358	1	88	20	2776	1580	1783	6140	0
HW-2	Rep 3	3500	3.5	0.25	1100	11000	1.000	1	88	20	3504	1975	2711	8190	0
HW-2	Average	1867	3.1	0.23	627	5600	0.502	1	81	25	2430	1848	2092	6369	0
HW-3	Rep 1	790	2.9	0.25	250	2500	1.000	1	25	9	1419	2154	2162	5735	0
HW-3	Rep 2	3500	3.5	0.25	1100	11000	1.000	1	34	7	1173	1673	2372	5218	0
HW-3	Rep 3	940	3.0	0.22	340	2600	0.062	1	28	9	2468	1468	2241	6176	0
HW-3	Average	1743	3.1	0.24	563	5367	0.687	1	29	8	1687	1765	2258	5710	0

Appendix D. Analytical results from ETV and MPN assessments during testing of the Optimarin BWMS.

Collection Date	Test ID	Sample ID	Analysis Rep	"strictly" Live Cell Density: ≥10 and <50 µm in all visible cell dimensions	Total Cell Density: ≥ 10 and < 50 µm cells	"strictly" Heterotrophic Organisms per mL	Total Heterotrophic Organisms per mL	MPN	Log_{10} MPN	SD of Log_{10} MPN	lower 95% conf. limit	upper 95% conf. limit	Rarity index	Category	Total Viable Organisms Viability Assessment Method	Total # Species Day 0	Total # Species Day 14	# Species Present on Day 0 and Day 14
9/14/2021	21-VA-OBS-C	C-SP2c	Rep 1	1079.27	6256.10	107.01	129.15	3500.00	3.54	0.25	1100.00	11000.00	1.00	1.00	3607.01			
9/14/2021	21-VA-OBS-C	C-SP2c	Rep 2	1980.58	8427.18	145.00	220.00	2200.00	3.34	0.20	880.00	5600.00	0.30	1.00	2345.00			
9/14/2021	21-VA-OBS-C	C-SP2c	Rep 3	2369.57	7021.74	46.67	56.67	3500.00	3.54	0.25	1100.00	11000.00	1.00	1.00	3546.67			
9/14/2021	21-VA-OBS-C	Uptake Control	Average	1809.81	7235.01	99.56	135.27	3066.67	3.48	0.23	1026.67	9200.00	0.77	1.00	3166.23	14	7	6
9/16/2021	21-VA-OBS-C	T_SP10b	Rep 1	12.20	30.20	0.00	0.00	1.69	0.23	0.21	0.63	4.50	0.92	1.00	1.69			
9/16/2021	21-VA-OBS-C	T_SP10b	Rep 2			0.00	0.00	3.29	0.52	0.24	1.07	10.13	1.00	1.00	3.29			
9/16/2021	21-VA-OBS-C	T_SP10b	Rep 3			0.00	0.00	3.29	0.52	0.24	1.07	10.13	1.00	1.00	3.29			
9/16/2021	21-VA-OBS-C	Treatment Discharge	Average	12.20	30.20	0.00	0.00	2.76	0.42	0.23	0.92	8.25	0.97	1.00	2.76	12	6	5
9/16/2021	21-VA-OBS-C	C_SP10c	Rep 1	1037.19	1789.26	27.25	70.84	2400.00	3.40	0.24	780.00	7400.00	0.74	1.00	2427.25			
9/16/2021	21-VA-OBS-C	C_SP10c	Rep 2	786.41	1598.71	39.06	74.22	1300.00	3.11	0.23	440.00	3800.00	1.00	1.00	1339.06			
9/16/2021	21-VA-OBS-C	C_SP10c	Rep 3	1091.35	1908.65	42.86	80.00	790.00	2.90	0.25	250.00	2500.00	1.00	1.00	832.86			
9/16/2021	21-VA-OBS-C	Control Discharge	Average	971.65	1765.54	36.39	75.02	1496.67	3.14	0.24	490.00	4566.67	0.91	1.00	1533.06	16	8	7
9/21/2021	21-VA-OBS-1	C-SP2c	Rep 1	1150.86	3732.76	13.76	16.82	5400.00	3.73	0.27	1600.00	19000.00	1.00	1.00	5413.76			
9/21/2021	21-VA-OBS-1	C-SP2c	Rep 2	2165.52	3510.34	22.86	48.57	3500.00	3.54	0.25	1100.00	11000.00	1.00	1.00	3522.86			
9/21/2021	21-VA-OBS-1	C-SP2c	Rep 3	1987.58	3689.44	6.67	13.33	9200.00	3.96	0.26	2800.00	30000.00	1.00	1.00	9206.67			
9/21/2021	21-VA-OBS-1	Uptake Control	Average	1767.99	3644.18	14.43	26.24	6033.33	3.75	0.26	1833.33	20000.00	1.00	1.00	6047.76	12	9	8
9/22/2021	21-VA-OBS-1	T_SP10a	Rep 1	51.73	79.23	0.00	1.00	0.00			0.00	0.66	1.00	1.00	0.00			
9/22/2021	21-VA-OBS-1	T_SP10a	Rep 2			1.00	1.00	0.20	-0.70	0.44	0.03	1.47	1.00	1.00	1.20			
9/22/2021	21-VA-OBS-1	T_SP10a	Rep 3			0.00	0.00	0.00			0.00	0.66	1.00	1.00	0.00			
9/22/2021	21-VA-OBS-1	Treatment Discharge	Average	51.73	79.23	0.33	0.67	0.07	-0.70	0.44	0.01	0.93	1.00	1.00	0.40	13	6	5
9/22/2021	21-VA-OBS-1	C_SP10c	Rep 1	1868.29	3297.56	24.92	43.61	16000.00	4.21	0.24	5300.00	49000.00	1.00	1.00	16024.92			
9/22/2021	21-VA-OBS-1	C_SP10c	Rep 2	2372.78	4094.67	38.58	41.54	3500.00	3.54	0.25	1100.00	11000.00	1.00	1.00	3538.58			
9/22/2021	21-VA-OBS-1	C_SP10c	Rep 3	2085.00	3360.00	24.62	40.00	5400.00	3.73	0.27	1600.00	19000.00	1.00	1.00	5424.62			
9/22/2021	21-VA-OBS-1	Control Discharge	Average	2108.69	3584.08	29.37	41.72	8300.00	3.83	0.25	2666.67	26333.33	1.00	1.00	8329.37	15	10	8
9/28/2021	21-VA-OBS-2	C-SP2a	Rep 1	3166.67	4456.79	37.59	55.14	1700.00	3.24	0.21	640.00	4600.00	0.94	1.00	1737.59			
9/28/2021	21-VA-OBS-2	C-SP2a	Rep 2	2966.85	4220.99	47.77	70.06	1700.00	3.24	0.21	640.00	4600.00	0.94	1.00	1747.77			
9/28/2021	21-VA-OBS-2	C-SP2a	Rep 3	2892.22	4538.92	20.51	28.21	1300.00	3.11	0.23	440.00	3800.00	1.00	1.00	1320.51			
9/28/2021	21-VA-OBS-2	Uptake Control	Average	3008.58	4405.57	35.29	51.14	1566.67	3.20	0.22	573.33	4333.33	0.96	1.00	1601.96	11	7	6
9/29/2021	21-VA-OBS-2	T_SP10c	Rep 1	51.20	73.80	0.00	0.00	0.00			0.00	0.66	1.00	1.00	0.00			
9/29/2021	21-VA-OBS-2	T_SP10c	Rep 2			0.00	0.00	0.00			0.00	0.66	1.00	1.00	0.00			
9/29/2021	21-VA-OBS-2	T_SP10c	Rep 3			0.00	1.00	0.00			0.00	0.66	1.00	1.00	0.00			
9/29/2021	21-VA-OBS-2	Treatment Discharge	Average	51.20	73.80	0.00	0.33	0.00			0.00	0.66	1.00	1.00	0.00	8	0	0
9/29/2021	21-VA-OBS-2	C_SP10b	Rep 1	2023.15	2736.11	25.71	34.29	700.00	2.84	0.24	230.00	2200.00	0.36	1.00	725.71			
9/29/2021	21-VA-OBS-2	C_SP10b	Rep 2	2428.57	3368.13	35.00	46.67	790.00	2.90	0.25	250.00	2500.00	1.00	1.00	825.00			
9/29/2021	21-VA-OBS-2	C_SP10b	Rep 3	2112.50	3508.33	22.22	26.67	3500.00	3.54	0.25	1100.00	11000.00	1.00	1.00	3522.22			
9/29/2021	21-VA-OBS-2	Control Discharge	Average	2188.07	3204.19	27.65	35.87	1663.33	3.09	0.25	526.67	5233.33	0.79	1.00	1690.98	13	7	6
9/30/2021	21-VA-OBS-3	C-SP2a	Rep 1	1928.20	3585.60	51.43	100.00	3500.00	3.54	0.25	1100.00	11000.00	1.00	1.00	3551.43			
9/30/2021	21-VA-OBS-3	C-SP2a	Rep 2	2852.27	4204.55	48.13	53.48	790.00	2.90	0.25	250.00	2500.00	1.00	1.00	838.13			
9/30/2021	21-VA-OBS-3	C-SP2a	Rep 3	2590.16	4071.04	26.51	28.92	3500.00	3.54	0.25	1100.00	11000.00	1.00	1.00	3526.51			
9/30/2021	21-VA-OBS-3	Uptake Control	Average	2456.88	3953.73	42.02	60.80	2596.67	3.33	0.25	816.67	8166.67	1.00	1.00	2638.69	12	6	5
10/1/2021	21-VA-OBS-3	T_SP10b	Rep 1	41.00	57.20	0.00	0.00	0.00			0.00	0.66	1.00	1.00	0.00			
10/1/2021	21-VA-OBS-3	T_SP10b	Rep 2			0.00	0.00	0.00			0.00	0.66	1.00	1.00	0.00			
10/1/2021	21-VA-OBS-3	T_SP10b	Rep 3			0.00	0.00	0.00			0.00	0.66	1.00	1.00	0.00			
10/1/2021	21-VA-OBS-3	Treatment Discharge	Average	41.00	57.20	0.00	0.00	0.00			0.00	0.66	1.00	1.00	0.00	10	3	2
10/1/2021	21-VA-OBS-3	C_SP10b	Rep 1	1811.06	3152.07	55.56	99.03	1700.00	3.24	0.21	640.00	4600.00	0.94	1.00	1755.56			
10/1/2021	21-VA-OBS-3	C_SP10b	Rep 2	1743.17	3218.58	31.38	73.22	1300.00	3.11	0.23	440.00	3800.00	1.00	1.00	1331.38			
10/1/2021	21-VA-OBS-3	C_SP10b	Rep 3	1990.00	3705.00	37.21	97.67	790.00	2.90	0.25	250.00	2500.00	1.00	1.00	827.21			
10/1/2021	21-VA-OBS-3	Control Discharge	Average	1848.08	3358.55	41.38	89.98	1263.33	3.08	0.23	443.33	3633.33	0.98	1.00	1304.72	14	8	7
10/6/2021	21-VA-OBS-4	C-SP2a	Rep 1	1000.00	1922.48	13.33	26.67	1100.00	3.04	0.22	390.00	3100.00	0.57	1.00	1113.33			
10/6/2021	21-VA-OBS-4	C-SP2a	Rep 2	956.52	1601.45	48.54	109.22	2400.00	3.38	0.24	780.00	7400.00	0.74	1.00	2448.54			
10/6/2021	21-VA-OBS-4	C-SP2a	Rep 3	1040.00	1726.67	12.00	16.00	1300.00	3.11	0.23	440.00	3800.00	1.00	1.00	1312.00			
10/6/2021	21-VA-OBS-4	Uptake Control	Average	998.84	1750.20	24.63	50.63	1600.00	3.18	0.23	536.67	4766.67	0.77	1.00	1624.63	13	7	5
10/7/2021	21-VA-OBS-4	T_SP10a	Rep 1	21.30	28.80	0.00	0.00	0.00			0.00	0.66	1.00	1.00	0.00			
10/7/2021	21-VA-OBS-4	T_SP10a	Rep 2			0.00	0.00	0.45	-0.35	0.31	0.11	1.86	1.00	1.00	0.45			
10/7/2021	21-VA-OBS-4	T_SP10a	Rep 3			0.00	0.00	0.20	-0.70	0.44	0.03	1.47	1.00	1.00	0.20			
10/7/2021	21-VA-OBS-4	Treatment Discharge	Average	21.30	28.80	0.00	0.00	0.22	-0.53	0.37	0.04	1.33	1.00	1.00	0.22	7	5	3
10/7/2021	21-VA-OBS-4	C_SP10a	Rep 1	956.73	1629.81	50.14	105.85	2400.00	3.38	0.24	780.00	7400.00	0.74	1.00	2450.14			
10/7/2021	21-VA-OBS-4	C_SP10a	Rep 2	753.21	1439.10	22.22	51.11	5400.00	3.73	0.27	1600.00	19000.00	1.00	1.00	5422.22			
10/7/2021	21-VA-OBS-4	C_SP10a	Rep 3	1221.59	2011.36	18.00	44.00	2200.00	3.34	0.20	880.00	5600.00	0.30	1.00	2218.00			
10/7/2021	21-VA-OBS-4	Control Discharge	Average	977.18	1693.42	30.12	66.99	3333.33	3.49	0.24	1086.67	10666.67	0.68	1.00	3363.45	12	7	7

Collection Date	Test ID	Sample ID	Analysis Rep	"strictly" Live Cell Density: ≥10 and <50 µm in all visible cell dimensions	Total Cell Density: ≥ 10 and < 50 µm cells	"strictly" Heterotrophic Organisms per mL	Total Heterotrophic Organisms per mL	MPN	Log_{10} MPN	SD of Log_{10} MPN	lower 95% conf. limit	upper 95% conf. limit	Rarity index	Category	Total Viable Organisms Viability Assessment Method	Total # Species Day 0	Total # Species Day 14	# Species Present on Day 0 and Day 14
10/13/2021	21-VA-OBS-5	C-SP2b	Rep 1	615.59	1604.84	5.00	10.00	1300.00	3.11	0.23	440.00	3800.00	1.00	1.00	1305.00			
10/13/2021	21-VA-OBS-5	C-SP2b	Rep 2	571.43	1914.29	1.69	11.86	1300.00	3.11	0.23	440.00	3800.00	1.00	1.00	1301.69			
10/13/2021	21-VA-OBS-5	C-SP2b	Rep 3	474.84	949.67	3.80	9.49	2400.00	3.38	0.24	780.00	7400.00	0.74	1.00	2403.80			
10/13/2021	21-VA-OBS-5	Uptake Control	Average	553.95	1489.60	3.50	10.45	1666.67	3.20	0.24	553.33	5000.00	0.91	1.00	1670.16	13	10	8
10/14/2021	21-VA-OBS-5	T_SP10c	Rep 1	24.00	67.00	0.00	0.00	0.20	-0.70	0.44	0.03	1.47	1.00	1.00	0.20			
10/14/2021	21-VA-OBS-5	T_SP10c	Rep 2			0.00	0.00	0.18	-0.74	0.43	0.02	1.34	0.09	1.00	0.18			
10/14/2021	21-VA-OBS-5	T_SP10c	Rep 3			0.00	0.00	0.20	-0.70	0.44	0.03	1.47	1.00	1.00	0.20			
10/14/2021	21-VA-OBS-5	Treatment Discharge	Average	24.00	67.00	0.00	0.00	0.19	-0.71	0.43	0.03	1.43	0.70	1.00	0.19	8	3	3
10/14/2021	21-VA-OBS-5	C_SP10b	Rep 1	414.55	650.91	3.64	7.27	330.00	2.52	0.24	110.00	1000.00	1.00	1.00	333.64			
10/14/2021	21-VA-OBS-5	C_SP10b	Rep 2	351.43	628.57	3.44	12.05	790.00	2.90	0.25	250.00	2500.00	1.00	1.00	793.44			
10/14/2021	21-VA-OBS-5	C_SP10b	Rep 3	284.38	495.31	3.33	11.67	790.00	2.90	0.25	250.00	2500.00	1.00	1.00	793.33			
10/14/2021	21-VA-OBS-5	Control Discharge	Average	350.12	591.60	3.47	10.33	636.67	2.77	0.25	203.33	2000.00	1.00	1.00	640.14	11	8	6

Appendix E. Summary of agreement for all tests that included both alternative (growth + fluorescence + heterotroph counts) and ETV (fluorescence microscopy) assessments of ambient harbor water. All growth assessments were performed with 50%BBM medium. Factor of agreement (FOA) reflects the ratio of estimated protist densities via the MPN and ETV methods. E.g., a FOA greater than 1 indicates an overestimation of protist densities by the MPN method relative to ETV assessment. Results are provided for ETV assessments using strictly cells >10 µm in minimum dimension and all observed protists (the Great Lakes-adapted approach, right).

								Cells strictly > 10 µm in minimum dimension												All observed cells										
Test ID	Test Rep.	Analytical Rep.	Autotroph Method (MPN Viable/mL)					Heterotroph Method (live organisms/mL)			MPN Method (autotrophs + heterotrophs, cells/mL)			ETV Method (cells/mL)					FOA	Heterotroph Method (live organisms/mL)			MPN Method (autotrophs + heterotrophs,			ETV Method (cells/mL)					FOA
			MPN	L CI	U CI	Mean	SE	Count	Mean	SE	Tcount	Mean	SE	Count	Mean	SE	L CI	U CI		Count	Mean	SE	Tcount	Mean	SE	Count	Mean	SE	L CI	U CI	
G-3	**1**	**1**	3500	1100	11000	2700	800	0	0	$NC^{n=1}$	3500	2700	800	148	194	23	149	239	13.92	3	3	$NC^{n=1}$	3503	2703	800	489	466	35	398	533	5.80043
G-3	**1**	**2**	3500	1100	11000			0			3500			220									3503			510					
G-3	**1**	**3**	1100	390	3100			0			1100			213									1103			398					
G-4	**1**	**1**	1100	390	3100	1967	433	0	0	$NC^{n=1}$	1100	1967	433	1164	775	327	134	1416	2.538	1	1	$NC^{n=1}$	1101	1968	433	3729	3422	363	2710	4133	0.5751
G-4	**1**	**2**	2400	780	7400			1			2400			125									2401			2699					
G-4	**1**	**3**	2400	780	7400			0			2400			1035									2401			3837					
HW-1	**1**	**1**	790	250	2500	1197	267	30	28	9	820	1224	261	2423	2029	314	1414	2643	0.603	48.3	72	13	838	1268	272	6515	5537	647	4269	6804	0.229
HW-1	**1**	**2**	1100	390	3100			41.2			1141			2254						94.1			1194			5780					
HW-1	**1**	**3**	1700	640	4600			11.4			1711			1409						72.2			1772			4315					
HW-1	**2**	**1**	1400	550	3600	1867	841	35.6	25	5	1436	1892	840	1783	2092	309	1486	2698	0.904	48.3	72	13	1448	1938	839	4778	6369	992	4426	8313	0.30429
HW-1	**2**	**2**	700	230	2200			20			720			1783						94.1			794			6140					
HW-1	**2**	**3**	3500	1100	11000			20			3520			2711						72.2			3572			8190					
HW-1	**3**	**1**	790	250	2500	1743	879	9.1	8	1	799	1752	879	2162	2258	61	2138	2378	0.776	25.5	29	3	815	1773	882	5735	5710	277	5167	6252	0.31051
HW-1	**3**	**2**	3500	1100	11000			6.8			3507			2372						34			3534			5218					
HW-1	**3**	**3**	940	340	2600			9.4			949			2241						28.2			968			6176					
OBS-C	**1**	**1**	3500	1100	11000	3067	433	107	100	29	3607	3166	411	1079	1810	382	1061	2559	1.749	129.2	135	47	3629	3202	392	6256	7235	636	5989	8481	0.44257
OBS-C	**1**	**2**	2200	880	5600			145			2345			1981						220			2420			8427					
OBS-C	**1**	**3**	3500	1100	11000			46.7			3547			2370						56.7			3557			7022					
OBS-1	**1**	**1**	5400	1600	19000	6033	1676	13.8	14	5	5414	6048	1671	1151	1768	313	1155	2381	3.421	16.8	26	11	5417	6060	1667	3733	3644	68	3511	3778	1.66301
OBS-1	**1**	**2**	3500	1100	11000			22.9			3523			2166						48.6			3549			3510					
OBS-1	**1**	**3**	9200	2800	30000			6.7			9207			1988						13.3			9213			3689					
OBS-2	**1**	**1**	1700	640	4600	1567	133	37.6	35	8	1738	1602	141	3167	3009	82	2848	3169	0.532	55.1	51	12	1755	1618	145	4457	4406	95	4219	4592	0.36723
OBS-2	**1**	**2**	1700	640	4600			47.8			1748			2967						70.1			1770			4221					
OBS-2	**1**	**3**	1300	440	3800			20.5			1321			2892						28.2			1328			4539					
OBS-3	**1**	**1**	3500	1100	11000	2597	903	51.4	42	8	3551	2639	900	1928	2457	275	1918	2996	1.074	100	61	21	3600	2657	907	3586	3954	188	3585	4322	0.67198
OBS-3	**1**	**2**	790	250	2500			48.1			838			2852						53.5			843			4205					
OBS-3	**1**	**3**	3500	1100	11000			26.5			3527			2590						28.9			3529			4071					
OBS-4	**1**	**1**	1100	390	3100	1600	404	13.3	25	12	1113	1625	416	1000	999	24	952	1046	1.627	26.7	51	29	1127	1651	433	1922	1750	93	1567	1933	0.94343
OBS-4	**1**	**2**	2400	780	7400			48.5			2449			957						109.2			2509			1601					
OBS-4	**1**	**3**	1300	440	3800			12			1312			1040						16			1316			1727					
OBS-5	**1**	**1**	1300	440	3800	1667	367	5	3	1	1305	1670	367	616	554	42	472	635	3.014	10	10	1	1310	1677	366	1605	1490	284	932	2047	1.1255
OBS-5	**1**	**2**	1300	440	3800			1.7			1302			571						11.9			1312			1914					
OBS-5	**1**	**3**	2400	780	7400			3.8			2404			475						9.5			2409			950					
Overall Mean ± SE					**2364 ± 386**			**26 ± 8**			**2389 ± 386**			**1631 ± 253**					**2.742**	**47 ± 11**			**2410 ± 385**			**3998 ± 616**					**1.13028**