EVALUATION OF A METHOD FOR BALLAST WATER RISK-RELEASE ASSESSMENT USING A PROTIST SURROGATE

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Abstract

Aquatic invasive species cost the United States billions of dollars annually and are most often introduced via ships' ballast tanks. The International Maritime Organization (IMO), among other regulatory organizations, adopted a set of ballast water performance standards that sets limits on the number of viable organisms that can be discharged with ballast water. The IMO limit for the discharge of organisms 10 - 50 μm in minimum diameter (such as phytoplankton) is 10 viable organisms per mL. The National Research Council (NRC) hopes to ground the proposed IMO limits in scientific research by determining the risk-release relationship (the number of propagules needed to potentially lead to an invasion). To examine the risk-release relationship a surrogate invader, *Melosira varians* (a phytoplanktonic diatom), was used. Varying densities of the surrogate were added to 19-L mesocosms of water from the Duluth-Superior Harbor at Superior, Wisconsin. Each mesocosm was sampled weekly for four weeks and the response of the phytoplankton community was measured via assessments of cell densities, both of *M. varians* and of the background communities. Population responses varied by starting conditions (water quality and natural species assemblages) but in some cases *M. varians* was able to establish with starting densities higher than 20 cells/mL. Our findings suggest the mesocosm method has potential to inform the risk-release relationship. Additional surrogates and environmental conditions should be used in mesocosms to extend our understanding of organism discharge limits.

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INTRODUCTION

Invasive species cause economic and ecological harm. Some of these species outcompete native species, cause harm to human infrastructure, or are pathogenic or otherwise directly harmful to humans and other organisms (Bain et al., 2010, Pimentel et al., 2005, Rothlisberger et al., 2012, Strayer et al., 2006). Pimentel et al. (2005) estimated that invasive species cost the United States over $120 billion annually. Median damages estimated across multiple ecosystem services of invasive species introduced by ocean going vessels in the Laurentian Great Lakes in the United States were recently estimated at $138 million a year (Rothlisberger et al., 2012).

More than 70% of faunal nonindigenous species introductions since 1959 in the Great Lakes have been transported by ocean going vessels in ballast tanks (Holeck et al., 2004). Ships carrying cargo often adjust water mass in their ballast tanks for stability. An estimated 3-10 billion tons of water are transported annually in this way (Banerji et al. 2012). On unloading cargo a ship's ballast tanks are filled with ambient water (and the organisms therein), which may then be transported to another port where the ship deballasts to readjust buoyancy on loading new cargo. Freshwater species can be transported from one port to another that would normally be separated by inhospitable marine conditions or and/or significant distances (Gollasch et al., 2007). Through this vector a number of invasive species have been introduced to the Great Lakes, including zebra and quagga mussels, spiny water fleas, and a number of pathogenic bacteria and viruses (Gollasch et al., 2007).

The International Maritime Organization (IMO) is the United Nations body that regulates international shipping and has set up standards to minimize the spread of invasive species

via ballast water. The International Convention for the Control and Management of Ships' Ballast Water and Sediments was adopted in a diplomatic conference in 2004 (Gollasch et al., 2007). This Convention involves two different protection regimes: Regulation D-1, *Ballast Water Exchange Standard*, and Regulation D-2, *Ballast Water Performance Standard* (National Research Council, 2011). Regulation D-1 (the phase one standard) requires ships to exchange a minimum of 95% of ballast water volume at sea. Whenever possible this exchange should occur no closer than 200 nautical miles (or 50 nautical miles when this is impossible) to the nearest land and at depths of at least 200 meters. A ship is not required to deviate substantially from its required course to meet this standard and in many regions it cannot be met.

The idea of ballast water exchange (BWE) is to physically remove (dump) coastal organisms picked up from the original port and to kill the organisms remaining in the tank via osmotic shock when the ballast tanks are refilled with marine water. It appears that BWE has had some effect on reducing new invasive species in the Great Lakes (Bailey et al., 2011). Unfortunately ballast exchange is not wholly effective because some water and sediments remain at the bottom of tanks along with live organisms, and some ships are not capable of the pelagic exchange process. Furthermore, lakers (domestic vessels) within the Great Lakes have no requirements for BWE, so redistribution of species among ports is likely (Branstrator et al., 2015). For these reasons ballast exchange is viewed as an interim solution until ballast treatment systems are established (Gollasch et al., 2007).

Regulation D-2 (the phase two standard) sets limits for the density of organisms discharged with ballast water. The limits as summarized by Gollasch et al. (2007) are:

- fewer than 10 viable organisms per cubic meter greater than or equal to 50 μm in minimum dimension;
- fewer than 10 viable organisms per ml less than 50 μm in minimum dimension and greater than or equal to 10 μm in minimum dimension;

- ❖ less than the following concentrations of indicator microbes, as a human health standard:
  - Toxigenic *Vibrio cholerae* Pacini (serotypes O1 and O139) with less than 1 colony forming unit (cfu) per 100 ml or less than 1 cfu per 1 g (wet weight) of zooplankton samples,
  - *Escherichia coli* Migula less than 250 cfu per 100 ml, and
  - intestinal Enterococci less than 100 cfu per 100 ml.

These standards aim to minimize propagule pressure in natural systems and thus reduce establishment success (Simberloff, 2009). According to Briski et al. (2012) propagule pressure is the number of organisms released. However, Wonham et al. (2013) argue that propagule pressure is more than just numbers of introduced organisms, and encompasses the frequency, timing, viability, diversity, and abundance of released organisms.

A species needs sufficient propagule pressure to establish a population in a new environment in order to overcome demographic and environmental stochasticity. Simberloff (2009) defines demographic stochasticity as random fluctuations in a population of a finite size. Smaller populations are more likely than larger ones to die at any given time interval or be left with only individuals of a single sex. Simberloff defines environmental stochasticity as random abiotic events such as storms or fires that impact a population and may eliminate even large populations. In aquatic systems environmental stochasticity is especially important because conditions can vary temporally and spatially, thereby complicating our understanding of establishment dynamics.

Organism density standards (Regulation D-2) have been the source of continuing discussion and controversy. Though these numbers are heavily debated between various countries, few delegations have the necessary biological expertise to evaluate their ecological meaning (Gollasch et. al, 2007). The IMO largely initiated the development of regulatory criteria for maritime commerce, but the United States is not party to the

agreement and instead takes IMO recommendations into consideration when building its own regulations. Ballast water discharged in the United States is regulated at the federal level by the United States Coast Guard (USCG) and the United States Environmental Protection Agency (USEPA). The USCG regulates ballast water through the Nonindigenous Aquatic Nuisance Prevention and Control Act of 1990 (NANCPA) which was amended and reauthorized under the National Invasive Species Act of 1996 (NISA) (Albert et. al, 2013).

The USEPA acts under the Clean Water Act to regulate ballast discharge. Until 2009 all discharges incidental to the normal operation of a vessel, including ballast water, were not subject to the National Pollutant Discharge Elimination System (NPDES) permitting regime. In 2008 that exception was revised in the Vessel General Permit (VGP), which is similar to the standards set out by the USCG and focuses on ballast exchange (Albert et. al, 2013). The 2008 VGP expired in 2013 and a new VGP was released in 2013 that set numeric discharge limits identical to those in IMO Regulation D-2. Individual states are also free to enact their own standards that are stricter than federal standards. For example the Minnesota Pollution Control Agency (MPCA) requires a State Disposal System (SDS) permit for ballast water discharges into Minnesota Waters that is stricter than the federal standards.

The USEPA and USCG are interested in passing more stringent regulations (National Research Council, 2011), but to date little is known about whether active or proposed numeric standards for organism release are ecologically meaningful or, perhaps, too stringent. In 2011 the National Research Council (NRC) of the United States recommended a way to achieve a higher level of scientific knowledge on the relationship between the density of organisms released via ballast water and the associated risk (risk-release relationship). The NRC urged scientists to determine risk-release relationships of aquatic invasive species in diverse environments, and under varying seasonal conditions, in order to ground ballast water standards with evidence that they are protective. This

included an experimental component that would create scenarios for the release of surrogate invaders via ballast discharge.

Experimental studies of the risk-release relationship can be used to test propagule pressure as a variable, and the efficacy of the D-2 standard as a level of propagule pressure, by inoculating mesocosm tanks with different densities of study organisms (Roman & Darling, 2007). It is expected that higher propagule pressures will result in greater colonization success due to reductions in the effects of demographic and environmental stochasticity, although the effects of stochasticity may be evaluated by re-running experiments under varying environmental conditions. Experimental studies are valuable because they provide answers to questions regarding invasion risk without waiting for invasions to play out in real ecosystems. They are also replicable and it is possible to control and modify a variety of conditions, such as modifying water quality variables to create scenarios for invaders.

Lee et al. (2013) discuss the challenge of using mesocosms with aquatic organisms to set ballast water discharge standards because of artifacts associated with experimental methods (which may be avoidable in "natural" systems such as whole lakes) and because a limited number of taxa can be studied in this way. Mesocosms have a number of caveats associated with them including the occurrence of edge effects, and contrived biological, physical and chemical factors. However Lee et al. (2013) do conclude that experimental studies are critical for testing assumptions, generating insights into mechanisms of population establishment and propagule pressure, and may be able to aid in the development of population models.

While mesocosm work has been done to establish the effects of invasive species on community structure (Nyström et al. 1999, Johnson et al. 2008) and the impact of Allee effects on colonization success (Kramer, Sarnelle, & Knapp, 2008) mesocosm studies examining propagule pressure are relatively few. Britton & Gozlan (2013) performed a large-scale mesocosm experiment studying propagule pressure in freshwater systems.

They introduced pairs of the fish *Pseudorasbora parva* Temminck & Schlegels into 100-m^2 mesocosm ponds to characterize the likelihood of species establishment. They identified a propagule threshold above which there was a rapid attainment of high population densities. To date no comparable assessment has been performed for microorganisms.

The NOAA Great Lakes Aquatic Nonindigenous Information System (http://www.glerl.noaa.gov/res/Programs/glansis, 2015) lists more than 20 nonindigenous microalgae present in the Great Lakes. This list includes organisms associated with potentially harmful blue-green algal blooms such as *Actinocyclus normanii* fo. *subsalsa* (Juhl.-Dannf.) Hust., biofouling organisms such as *Bangia fuscopurpurea* (Dillwyn) Lyngbye, and taste and odor-causing organisms such as introduced via ballast water (http://www.glerl.noaa.gov/res/Programs/glansis, 2015). Some organisms such as *Thalassiosira baltica* (Grunow) Ostenfeld even have such a wide salinity tolerance that they are able to survive BWE (Edlund et al., 2000).

To better inform a growing body of research on risk-release dynamics, we evaluated a method for experimental determination of the risk-release relationship for organisms in the 10 - 50 μm size range.

METHODS

TEST ORGANISM

Melosira varians Agardh was selected as a surrogate invader for our study because it is native to the harbor, and so there was no risk of an accidental, non-native introduction at the test facility. Also, *M. varians* is a large-celled diatom that fits the IMO size criterion of 10 – 50 μm in minimum dimension, and it is easily identified using inverted light microscopy, thus simplifying analysis of the many samples collected for these experiments. While this taxon is native to the Great Lakes it still served the purpose of assessing risk-release relationships by artificially augmenting its occurrence in samples

collected from the Duluth-Superior Harbor.

According to Passy (2007) *M. varians* is an r-strategist or ruderal species. According to Grime (1977) ruderal species are adapted to high disturbance, low stress situations. They produce a large number of offspring and are early colonizers of an area, making them prime invaders. We hypothesized that this adaptation would favor growth of *M. varians* in our mesocosms.

Many diatoms such as *M. varians* are capable of reproducing both sexually and asexually. However deleterious effects of inbreeding have been reported in various diatoms (Chepurnov et al. 2011). In one study the viability of F1 clones of *Melosira moniliformis* (Müller) Agardh was compared to the viability of *M. moniliformis* that had been outcrossed. This study found a significant reduction of viability and fertility in the clones. Chepurnov (2011) concludes that the incidence of selfing is probably low in natural populations. This is an important factor to consider when examining the risk-release relationship because the number of available mates is highly subject to demographic stochasticity with low propagule numbers.

M. varians has its best development in warm waters with high light intensities. According to Patrick (1977) *M. varians* is often dominant in Eastern United States lakes and streams that are nutrient rich in the late summer. It can be dominant year round if light and nutrient levels are high but it ismost common when the days are long. With increasing global temperatures species like *M. varians* and other warm adapted species may progress north.

PRELIMINARY EVALUATION OF PROTIST DIVERSITY IN THE DULUTH-SUPERIOR HARBOR (2012)

In the initial phase of the study, we evaluated the density and diversity of the natural assemblage of protists in water from Duluth-Superior Harbor during July-September,

2012. This work was done in coordination with the Great Ships Initiative (http://www.greatshipsinitiative.net, 2015) team which was undertaking a series of experiments to develop and validate methods for mesocosm studies on the crustacean zooplankton *Bythotrephes longimanus* Leydig. To simulate stochasticity among trials it was desirable to have different biological and water quality conditions each time. Duluth-Superior Harbor water quality (both biotic and abiotic factors) can vary dramatically over the course of a year, from day to day and even over the course of one day (Matt TenEyck, personal communication), making the harbor a sufficiently variable source of water for experiments. For a single trial, 22 1-m^3 ("large") mesocosm tanks (Figure 1) were filled simultaneously to minimize the heterogeneity in the ambient water between replicate mesocosm tanks for a given trial. A preliminary trial was conducted to confirm that abiotic factors such as filling, lighting, and pumping could be consistent and that heterogeneity was minimized. Three complete trials (July 10 - July 13 2012, August 16 - September 13, 2012, and September 17, 2012 - October 17, 2012) were conducted to confirm that biotic heterogeneity was also minimized. Tanks were filled on day 1 of these periods.

All tanks were filled simultaneously by pumping water from the nearby harbor through specially designed manifolds that divided the water uniformly 22 times (Appendix A1). After filling, temperature, dissolved oxygen, pH, specific conductivity, and total chlorophyll were measured weekly with a calibrated, handheld Yellow Springs Instruments (YSI) Multiparameter Water Sonde.

Protist densities were measured (see methods below) from two of the mesocosms immediately after filling and from the remaining 20 mesocosms when they were drained after four weeks. Protist density and assemblage composition was assessed across the mesocosm replicates within each trial and across trials to identify the range of variations in the background community of protists, and whether a bias existed in any of the mesocosms.

PRIMARY TESTS (2013)

Surrogate Culture

In September 2012, specimens of the diatom *M. varians* were collected from Duluth-Superior Harbor (lat. 46.781, long. -92.096) using a 53-μm mesh plankton net. Subsampling and culturing (Appendix B2) of *M. varians* was performed by Matthew L. Julius and his research team at the St. Cloud State University (SCSU) Department of Biological Sciences, and SCSU currently maintains this culture.

Mesocosm Tank Setup

Each trial initiation in 2013 (July 9 - August 6, September 6 - October 4, and October 11 - November 8) paralleled the filling of the large mesocosm tanks for study of *B. longimanus*. As above, the large mesocosms were filled simultaneously with ambient Duluth-Superior Harbor water. At the end of filling, 20 of the large mesocosms were subsampled in random order to fill the small mesocosms (20-L food-grade buckets) to a calibrated 19-L level (see Figure 1 for depictions of the mesocosm arrangement). This was achieved by stirring the large mesocosms with a paddle and immediately submerging the 19-L mesocosms in the homogeneous water. Immediately after subsampling, all 20 of the 19-L mesocosms were characterized for water quality parameters (temperature, conductivity, salinity, pH, turbidity, dissolved oxygen (DO), and total chlorophyll) using a calibrated YSI sonde. Mesocosms were exposed to indoor climate control (air temperature = 15.7 °C; light:dark 16:8 hrs, gentle aeration) during the experiment. The complete apparatus was lit by 32-watt fluorescent lights, ensuring light intensities did not vary among mesocosms.

Spiking and Sampling

A sufficient volume of *M. varians* culture was obtained for amending 20 19-L mesocosms. *M. varians* density in the culture was determined as follows. *M. varians*

culture was shaken to homogenize and minimize clumping. A 1.1-mL subsample was pipetted on a Sedgewick-Rafter slide. One transect of the slide was counted for *M. varians* cells that visibly contained chloroplasts. This process was repeated twice, counting one transect per slide. The counts from three transect lengths were used to calculate cell density. A calculation table (Appendix A2) was used to determine appropriate amendments based on cell density in the culture and desired densities in mesocosms. Mesocosms (4 for each desired cell density) were inoculated with enough *M. varians* to obtain final densities of 0 (control), 1, 10, 30 or 100 cells/ml.

Each mesocosm was stirred with a long-handled, stainless steel ladle to homogenize the protist assemblage (Figure 1). An airstone bubbler was placed on the bottom of each mesocosm to maintain constant circulation. A 500 mL sample was immediately collected from each mesocosm in the same random order they were filled. This was achieved by stirring with the ladle, scooping and adding the 500-mL sample to a clean, 1-L Nalgene bottle (Figure 1). Each 500-mL sample was immediately preserved with 5 mL of Lugols solution and inverted five times to mix the sample. Subsequent subsampling of the mesocosms occurred weekly for four weeks, for a total of 100 preserved samples for each experiment. On a sampling day the same subsampling procedure as above was followed to acquire a new set of 20 subsamples. On each sampling day all mesocosms were re-assessed for water quality parameters.

Sample Processing

Each preserved sample was concentrated prior to analysis. We homogenized the sample by inverting at least six times, then measured 100 mL of sample water in a graduated cylinder. This water was filtered using standard methods for chlorophyll *a* filtration (Reavie et al. 2010), substituting a 7 X 7-cm sheet of 7 μm plankton netting in place of a filter (see Appendix A3). To prevent cell damage, vacuum pressure did not exceed approximately 200 mm Hg (~2 psi or ~0.13 atm). If filtration occurred rapidly, we measured and added an additional 50 or 100 mL of sample and filtered this volume as

well. We continued to add sample water in increments until filtration slowed. For trials 1 and 2 approximately 200 mL and 400 mL was concentrated, respectively, for each sample. Trial 3 had sufficiently dense protists that samples did not need to be concentrated. The filter was rinsed with 15 mL of filtrate from the vacuum chamber using a washing tube (Appendix A4). To aid backwashing of the algae into the collection dish a rubber pipette bulb was placed neck-down on the washing tube and gently pressed from the top to force water through the filter and backwash the concentrated protist assemblage. Concentrated samples were stored in 25 mL bottles.

Cell Counts

Concentrated samples were analyzed in a Sedgwick-Rafter cell under brightfield using an Olympus CKX41 inverted microscope at 200 X magnification. We aimed to count at least 100 entities and two transects. Depending on whether samples were dominated by more filamentous or globular entities the number of cells counted varied from hundreds to thousands. Single-celled entities and cells comprising colonial and filamentous entities were characterized as alive if cells contained chloroplasts and other visible organelles. "Dead" cells that were significantly degraded or had empty cell walls (usually diatoms) were not counted. Per standard analytical protocols for ballast water (Great Ships Initiative, 2010), entities that were less than 10 μm in all visible dimensions or greater than 50 μm in minimum dimension (e.g., zooplankton) were not counted. Transect lengths and widths were recorded so that the total counted area could be calculated later. Taxonomy was based upon Smith (1950) and our taxonomic resolution followed that shown in the count sheet used during microscopic assessment (Appendix A5). Cell or entity densities were calculated as in equation 1:

$$x = \frac{counted\ total\ (number)}{\left(\left.\begin{matrix}water\ settled \\ in\ chamber\ (mL)\end{matrix}\right/ \begin{matrix}concentrated\ sample \\ volume\ (mL)\end{matrix}\right) \times \left(\begin{matrix}water\ volume \\ filtered\ (mL)\end{matrix}\right) \times \left(\begin{matrix}proportion\ of \\ chamber\ counted \\ (fraction)\end{matrix}\right)} \quad (1)$$

a. **Counted total (number)** = Total number of cells counted (for cells/mL endpoint) or total number of entities counted (for entities/mL endpoint)
    - The total number of cells is the sum of all recorded protist numbers on the datasheet.
    - The total number of entities is the number of data entries recorded on the datasheet (i.e. single-celled protists, filaments and colonies).
b. **Water settled in chamber (mL)** = 1.1 mL (Sedgewick-Rafter volume)
c. **Concentrated sample volume (mL)** = Volume of concentrated sample rinsed with filtrate (usually 15 mL)
d. **Volume of water filtered (mL)** = Volume of raw water filtered using plankton netting
e. **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 Sedgewick-Rafter chamber (10^9 μm^2)

The remaining concentrated sample was archived.

DATA ANALYSIS

Data were recorded on countsheets (Appendix A5) and entered into a spreadsheet database and analyzed using R (R Core Team, 2014). For unknown reasons one mesocosm tank in Trial 2 had an abnormally large protist bloom, and so was considered an outlier and not part of further analyses. The R package ggplot2 (Wickham and Chang, 2014) was used to plot *M. varians* and other cell densities over time. Growth rates were also plotted against actual initial *M. varians* cell density to account for differences

between intended and actual starting densities. A risk-release curve was generated to compare *M. varians* inoculation densities with the number of *M. varians* tanks that exhibited growth. *M. varians* densities were also plotted against water quality parameters to examine possible confounding effects and interactions. Paired t-tests comparing *M. varians* density at different days in different trials were done to generate p-values to measure the significance of slopes between each time period.

RESULTS

PRELIMINARY TESTS (2012)

Results from the preliminary tests indicated that different test conditions (including different initial ambient phytoplankton communities) occurred among trials (Figure 2). Both seasonal and inter-tank variability were observed. Trials 1and 3 indicated growth while trial 2 indicated population decline after four weeks. Although it is ideal to minimize inter-tank variability to better detect the effects of propagule pressure, these preliminary experiments provided information on background variability we may expect to see in the system. None of the experiments suggested bias in any tanks across trials (such as preferential high or low growth or taxonomic specificity). In general the standard deviation in discharge assemblages was relatively larger (as much as 77% of the mean density) for cell densities compared to entity densities (as much as 30%). Data from these preliminary assessments verified that we could expect significant variations in test conditions across trials, and that we were unlikely to experience bias in the system that might hinder interpretations of results from simulated invasions by a surrogate organism.

PRIMARY TESTS (2013)

Each of the three trials had very different initial cell densities and the added *M. varians* growth responses varied. During Trial 1 *M. varians* growth mainly occurred early at the

100 cells/mL inoculation density (Figure 3). According to paired comparisons of *M. varians* densities among sample times, significant growth occurred from days 1 through 21 at the 100 cells/mL inoculation and significant death occurred between days 21 and 28 at the 30 cells/mL inoculation (Table 1). Aside from a decline in abundance with time (Figure 3) there was no clear response pattern among other protists based on *M. varians* inoculation. This trial had a relatively low intake density of ambient phytoplankton (between 600 and 1,000 cells/mL) compared to the subsequent trial.

A very different response occurred in Trial 2: *M. varians* density in the highest inoculation rapidly declined while the abundance of other protists increased. Significant *M. varians* death occurred between days 14 and 21, and days 21 and 28 (Table 1). Growth of other protists may have occurred due to decaying organic matter (i.e. dead *M. varians*) providing a nutrient supply for other phytoplankton. Initial densities of ambient phytoplankton in this trial ranged from 7,000 - 13,000 cells/mL, and by the end of the trial all of the mesocosms inoculated with *M. varians* had established equilibrium populations lower than 100 cells/mL.

In Trial 3 *M. varians* density increased up to day 14 in the 100 cells/mL inoculum and up to day 7 in the 30 cells/mL inoculum, followed by a decline in abundance. However, none of these changes were significant based on paired t-tests (Table 1). Other protists increased in abundance, reaching a maximum at day 21. Again, slightly higher abundances of other protists occurred in treatments with more *M. varians* added, suggesting a recycled nutrient supply from dead *M. varians*. In all trials, no apparent growth of *M. varians* occurred with inoculation densities of 10 cells/mL or lower. In this trial, ambient phytoplankton levels were lower than the previous trial, ranging from about 2,000 to approximately 6,000 cells/mL.

Although we attempted to provide *M. varians* inoculum densities based on set amounts of 0, 1, 10, 30 and 100 cells/mL, initial *M. varians* densities tended to be higher than intended (x-axis in Figure 4). This is likely due to occurrence of *M. varians* in the ambient water, and more so due to error associated with the spiking method. *M. varians*

is a filamentous diatom and the filaments were prone to clumping in the culture, so it is probable this made accurate quantification difficult. Nonetheless this provided a continuum of initial *M. varians* densities based on actual counts on day 0.

M. varians growth rates exhibited a clear, heteroscedastic distribution indicating increasing variation in growth rates at higher inoculation densities (Figure 4). While variable within and between trials, growth of *M. varians* appeared to occur at inoculation densities higher than 20 cells/mL. Some growth was observed in all trials, especially in trials 1 and 3, and the highest growth rates occurred within the first week, especially in Trial 1 (Table 1). Growth rates below 0 (prominent in Trial 2) indicate die-off of *M. varians*. Standardizing the growth rates by initial cell density (right-hand panels in Figure 4) was performed to correct for death rates that might simply be greater because there were more *M. varians* cells to begin with. Standardizing this way removed the heteroscedastic trend, indicating that larger death rates were a result of higher starting densities of *M. varians*.

In order to demonstrate results comparable to other risk-release experiments (Wonham et al., 2013) we generated a risk-release curve (Figure 5) that plots risk (% establishment) against release (inoculation density). Establishment is defined as a growth slope greater than 0. Trial three showed a mostly linear risk-release curve, with the risk of establishment increasing at higher inoculation densities. Trial 2 revealed the highest risk at 10 cells/mL inoculation with some risk associated with higher and lower inoculations. Trial 1 had maximum risk at 100 cells/mL inoculation but no risk at the adjacent 30 cells/mL inoculation. The risk-release curve that integrated all three trials indicates maximum risk at the highest inoculation but overall there was no notable overall trend. We acknowledge that these results are suitable to illustrate a method for risk-release analysis, but that confirmation of a risk-release relationship at a critical threshold of inoculation is not possible from our data without further testing.

We examined variations in water quality parameters among *M. varians* inoculum treatments and over time (Figure 6) to identify possible confounding variables in our

growth rate observations. For instance, higher conductivity in mesocosms with higher inoculum densities might indicate higher phosphate content which could contribute to algal growth. Overall, water quality did not vary predictably with *M. varians* inoculation density, indicating little or no confounding of our results by variations in water quality. Starting water quality conditions (at day 0) were comparable across inoculation treatments. There may have been a slight bias in temperature among treatments (e.g. temperature tended to be highest in the 10 cells/mL treatment and lowest in the 1 cells/mL treatment), but it was not related to inoculum density and only reflected a range of about 1 °C. Conductivity, salinity, and pH all increased over time while turbidity and DO decreased. Conductivity and salinity may have increased due to evaporation in the tanks. Turbidity may have decreased due to increased particle settling over time. DO probably decreased over time as it was respired by organisms. Chlorophyll tended to follow algal abundance. Further, no significant relationship was observed between *M. varians* abundance and water quality parameters (Figure 7), with the exception of chlorophyll, indicating no confounding effect of water quality on our observations of protist growth rates.

DISCUSSION

This study is the first to evaluate a method for experimental determination of the risk-release relationship in microorganisms. With *M. varians* as a surrogate invader we successfully indicated the potential for mesocosm assessments in evaluating this relationship. Preliminary assessments with no surrogate added indicated the variation that can be expected in mesocosm systems resulting from initial conditions and between replicate tests conducted at this site with water from the Duluth-Superior Harbor. Our assessment then demonstrated establishment and variability in the risk-release relationship for our surrogate protist.

M. varians establishment and growth was observed at starting densities generally 20 cells/mL or greater, although the risk-release curve indicated some evidence of at least temporary establishment at all inoculation densities. The success of *M. varians*

colonization varied by the time of year. The first trial, which began in early July, had a relatively low intake density of ambient phytoplankton and had the greatest rates of *M. varians* establishment with all four of the tanks with 100 cells/mL inoculum exhibiting *M. varians* growth from day 0 to day 28. This is also the time of year when *M. varians* traditionally thrives, in mid-summer when days are long and warm (Patrick, 1977). The second trial (early September) started during the autumn diatom bloom in Duluth-Superior Harbor, and by the end of the trial all of the inoculated mesocosms had low *M. varians* densities. This may be because the ambient water was already close to carrying capacity of phytoplankton at the beginning of the trial. In trial 3, which began in October, ambient phytoplankton levels were down. Growth of *M. varians* was highly variable but there were higher rates of establishment at the 30 and also at the 100 cells/mL inoculation densities, suggesting again that lower ambient phytoplankton densities may have allowed for establishment of inoculated diatoms.

M. varians abundance and establishment did not relate to water quality (temperature, conductivity, salinity, pH, turbidity, or DO) and instead establishment success appeared to vary more due to competing phytoplankton, as described above. This may have been due to competition for resources such as light or nutrients. As a ruderal species *M. varians* is traditionally an early colonizer and is not suited to thrive with existing competition (Passy, 2007; Grime, 1977). Nutrient levels were not measured in this study, and in the future it may be useful to understand which nutrients may have been limiting in order to understand more fully how competition impacts establishment success.

The cycling of nutrients in mesocosm systems may not relate well to natural systems. In natural systems nutrient cycles include interactions with surrounding soil and water conditions, whereas contrived biological, chemical, and physical factors in mesocosms are not able to fully participate in the cycles they constantly undergo in natural systems. Such non-analogue circumstances are probably magnified with a longer trial as nutrient resources become more stressed or limiting. In the future it may be helpful to include replenishment with filtered harbor water to minimize nutrient limitation. Despite limitations of mesocosm tests it is probable that this method is suited to additional

surrogate protist species. Larger organisms such as zooplankton are more affected by tank effects (requiring far more water and resources to mimic their natural environments) and thus require additional considerations.

For future studies, non-native protists and protists from additional groups (e.g. protozoans, cyanobacteria, dinoflagellates, etc.) should be considered. Non-native species with different life history traits and strategies (e.g. K-strategists) should also be considered. For example, an organism that is better adapted to high stress or is more competitive in low disturbance regimes could provide valuable information on useful release limits since variable release conditions may favor these life histories.

In future investigations it will be important to not only consider inoculation densities but also increasing propagule pressure by increasing the number of releases or inoculations over the course of the study, as happens with ship-borne introductions (Simberloff, 2009). Hedge et al. (2012) performed a similar study on the release and establishment of the oyster *Crassostrea gigas* Thunberg*,* finding that their organism was more effective at establishment under a scenario of several small releases compared to more sporadic, large releases. Repeated introductions contribute to increased genetic diversity which allows organisms to adapt to a wide variety of conditions (Roman & Darling, 2007). This becomes particularly important as climate change alters environmental conditions allowing organisms to live in places that were previously inhospitable and gives organisms that would have necessarily been separated increased opportunities to hybridize (Chown et. al., 2014).

Until this work is expanded our investigation should be considered preliminary and limited to our surrogate “invader” and the environmental conditions we observed at Duluth-Superior Harbor. In this case we found that the threshold for growth in *M. varians* was greater than the 10 cells/mL IMO discharge standard. It is not known whether other taxa will exhibit similar responses. Until further work is performed and consistencies in the risk-release relationship under varying conditions are identified we cannot determine the extent of future work that will be needed to establish ecologically relevant bounds

(critical densities of discharge, environmental conditions in receiving environments) on the risk-release relationship.

It should be noted that observation of a critical inoculation density represents what would be observed in a receiving environment such as the water body at a port, and not the discharge density from a ship. Site-specific considerations, largely dilution, need to be considered to further enhance understanding of risk associated with discharges of non-native protists. To achieve our inoculation densities in a harbor receiving ballast water, obviously concentrations in the ballast tanks would need to be much higher.

Although we observed relatively clear trends in biological dynamics related to surrogate inoculum density, quantitative accuracy was difficult given the colonial nature of the test organism. Many algae can contain hundreds of propagules in a single colonial entity, and characterizing these propagules is critical in assessments of potential establishment. Such a problem may be unavoidable, but is less likely to be a problem with larger, single-celled surrogate entities (e.g. most dinoflagellates).

Given the small mesocosm volumes associated with tests in the 10 - 50 μm size range, budgeting resources (time, personnel, materials) are feasible for future work on additional surrogates. The larger volumes needed for organisms in larger size categories (e.g. zooplankton) will necessitate greater logistic considerations (Lee et al., 2013).

This is the first experimental study to examine the risk-release relationship of protists and one of the first to examine the risk-release relationship overall. Through careful examination of the risk-release relationship at the experimental and field levels the USEPA and USCG will be able to ground ballast water standards with evidence they are appropriately protective (National Research Council, 2011). Standards that are too stringent have a high cost for shipping companies without significant gains for the ecosystem. Standards that are too lenient can lack necessary environmental protection. Ballast discharge standards have been set in order to minimize the spread of invasive species (Gollasch et al., 2007) and here we successfully tested the practicality of such

standards using a specific organism. Halting the spread of invasive species in the Laurentian Great Lakes is a critical issue due to the well-known loss of ecosystem services (Rothlisberger et al., 2012), so further development of such assessments is needed to support realistic discharge standards.

FIGURES

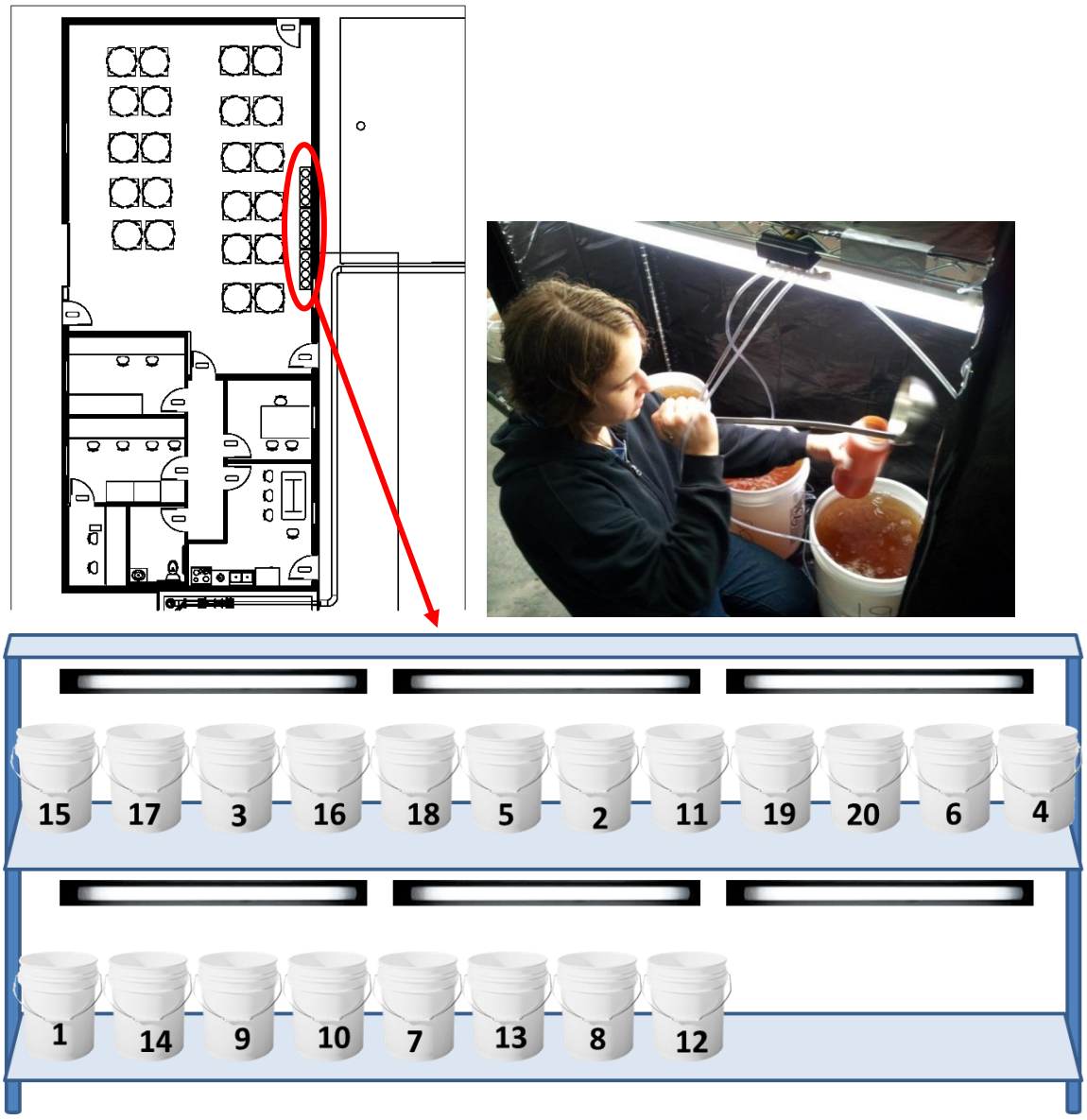

Figure 1: Top Left: Mesocosm building top-down floor plan. The large (3.8-m^3) mesocosm tanks are shown in the upper portion of the diagram while the small (19 L) tanks are arranged to their right. Below: more detailed small mesocosm tank arrangement. Mesocosms (20-L buckets) were lit by overhead fluorescent lights and marked with randomly generated numbers that matched corresponding large mesocosms. A black curtain over all of the tanks protected the system from outside influences and ensured even lighting. Tanks were sampled weekly with a 500 mL ladle (photo, top right).

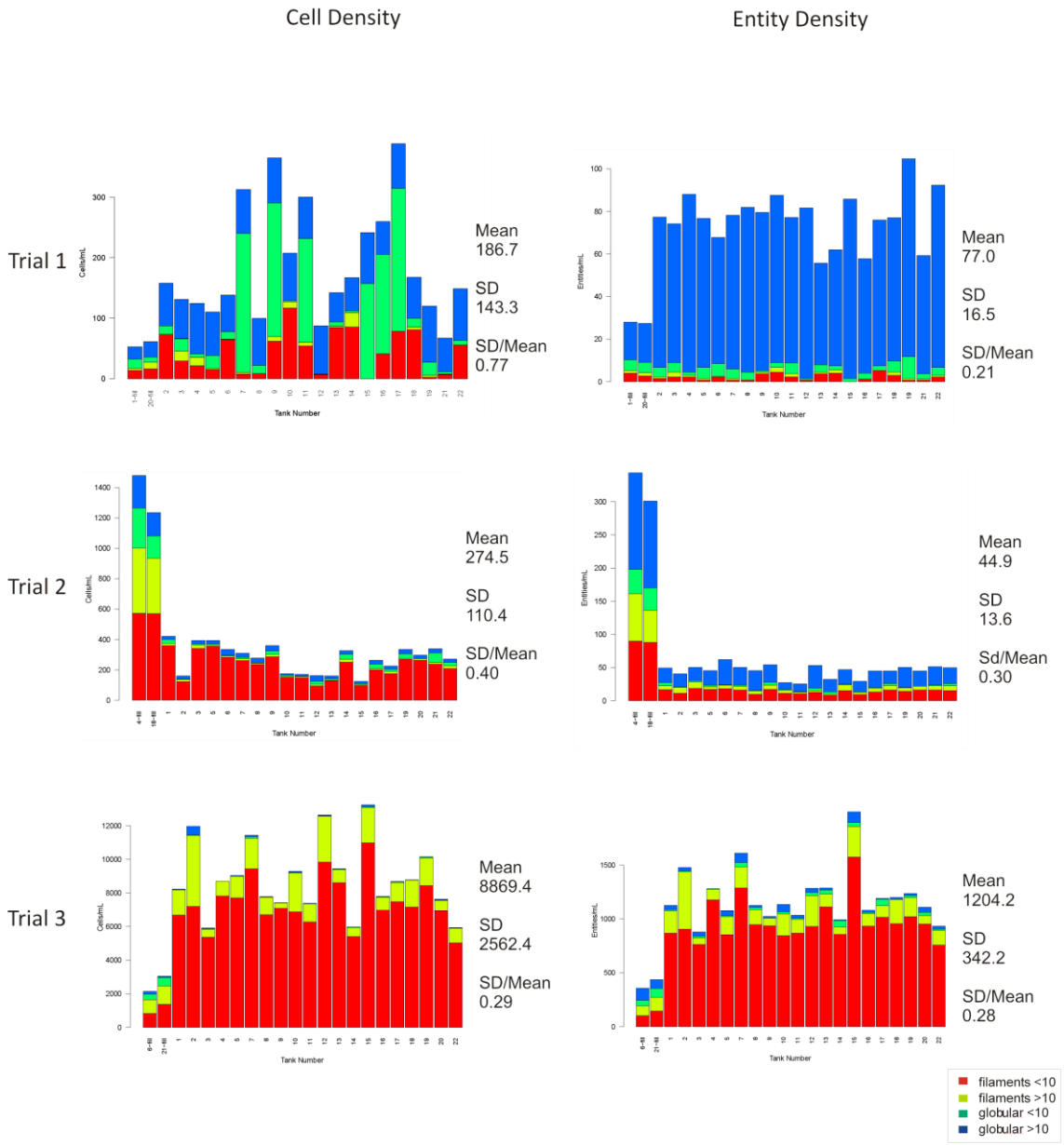

Figure 2: Protist cells/mL in fill (first two stacked bars in each plot) and post-discharge samples from 1-m^3 mesocosm tanks, collected from three trials in 2012. Mean, standard deviation (SD) and the proportion of the mean represented by the SD for total cell and entity values from discharge samples are presented. Colors of stacked bars represent cell sizes (i.e. minimum dimension less than or more than 10 μm) and whether entities were filamentous or globular (non-filamentous). Note the variability in y–axis scales.

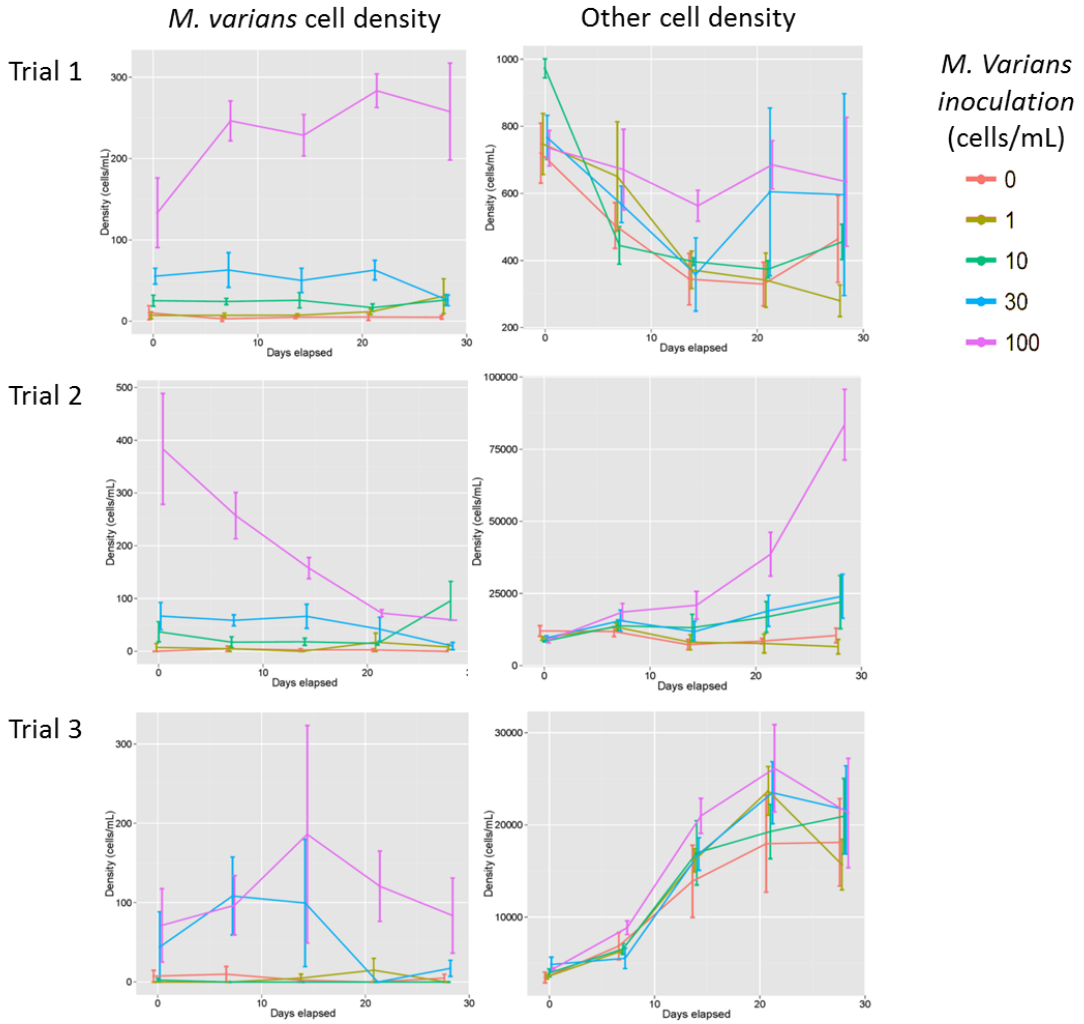

Figure 3. Effects of time and initial *M. varians* inoculum on cell densities over four weeks. Note that density scales (y-axis) vary for each plot.

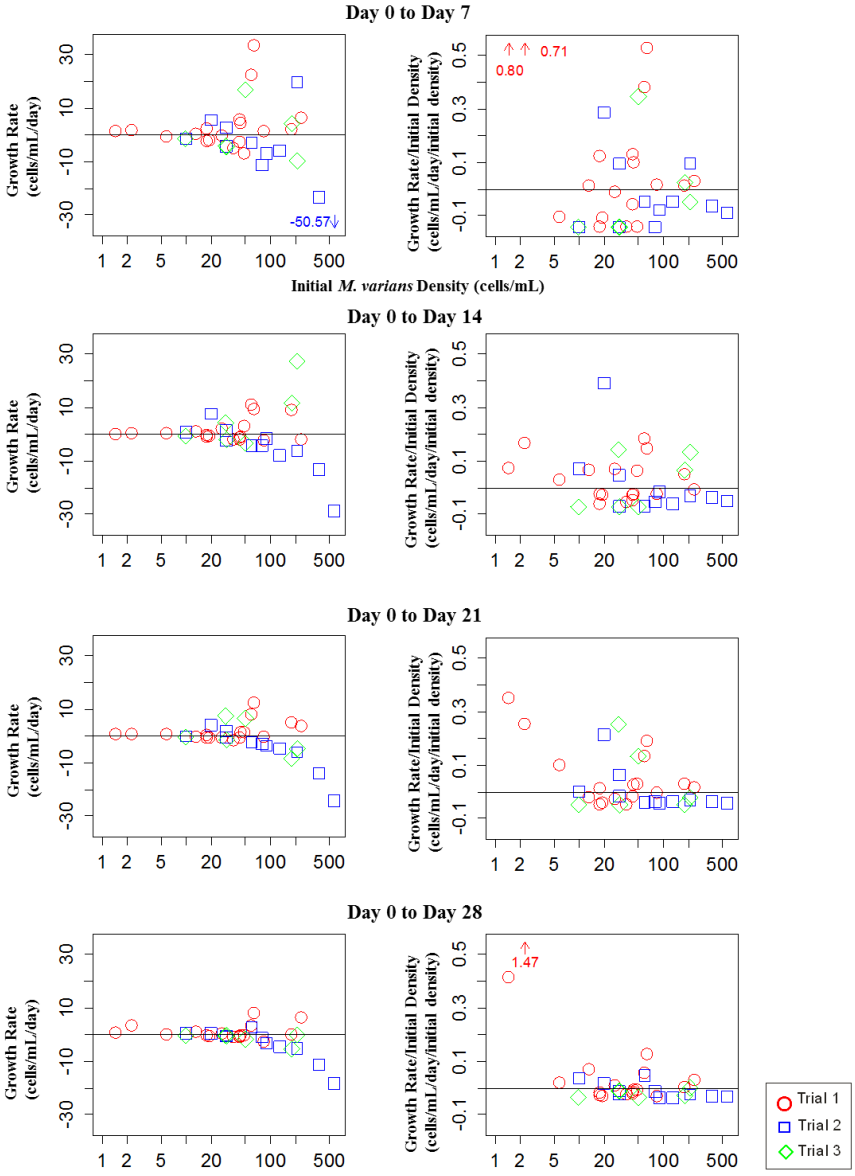

Figure 4. *M. varians* growth rates as they relate to inoculum density. Panels represent rates calculated over four time periods. Each point represents a single mesocosm and the slope of the line connecting the cell density at the beginning and end of the time period. Left panels indicate growth rates and right panels are standardized (divided) by inoculation density. Negative values in standardized plots indicate net cell death over the time period.

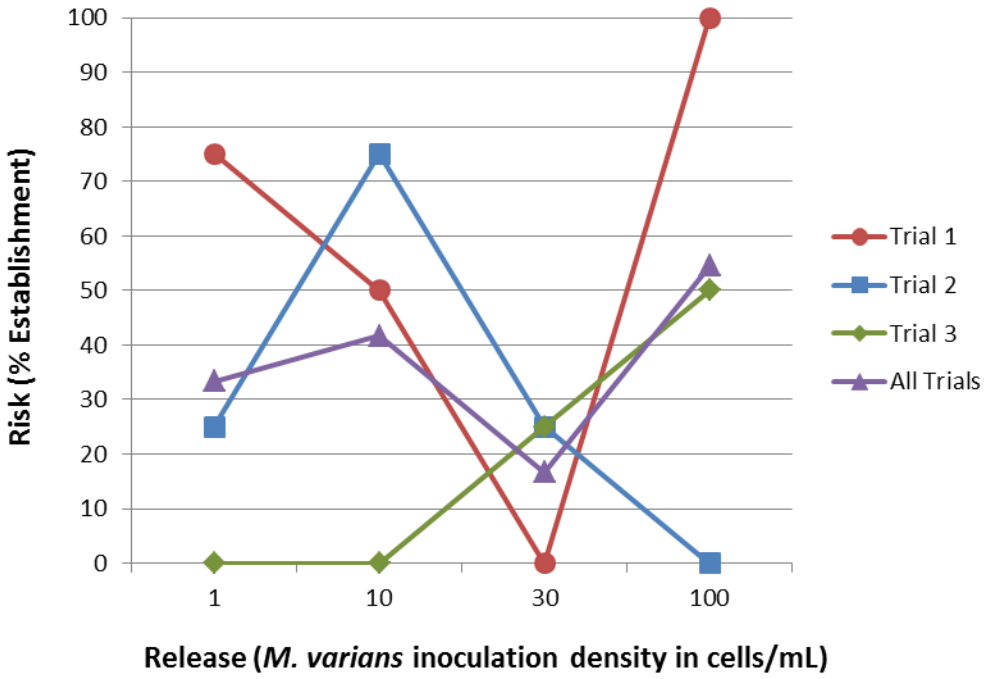

Figure 5. Risk-release curves representing *M. varians* establishment success compared to inoculation density. A tank was considered to have *M. varians* establishment if there was positive growth from day 0 to day 28. Percent establishment represents the proportion of tanks that had positive growth (n = 4 for each inoculation density in trials 1 - 3 and n = 12 for pooled results at each inoculation).

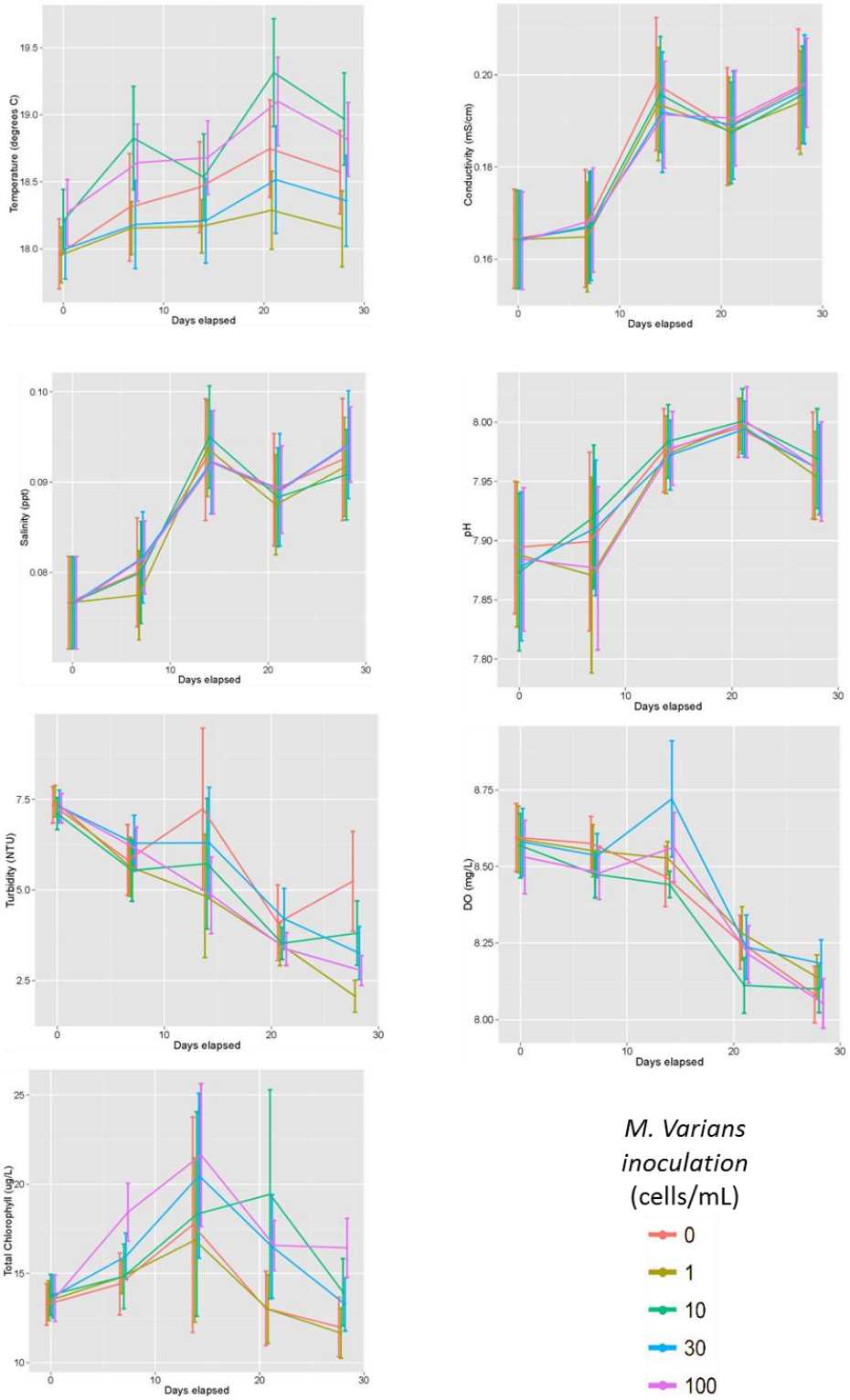

Figure 6. Effects of time and initial *M. varians* inoculum on water quality variables. Trials and replicates were pooled.

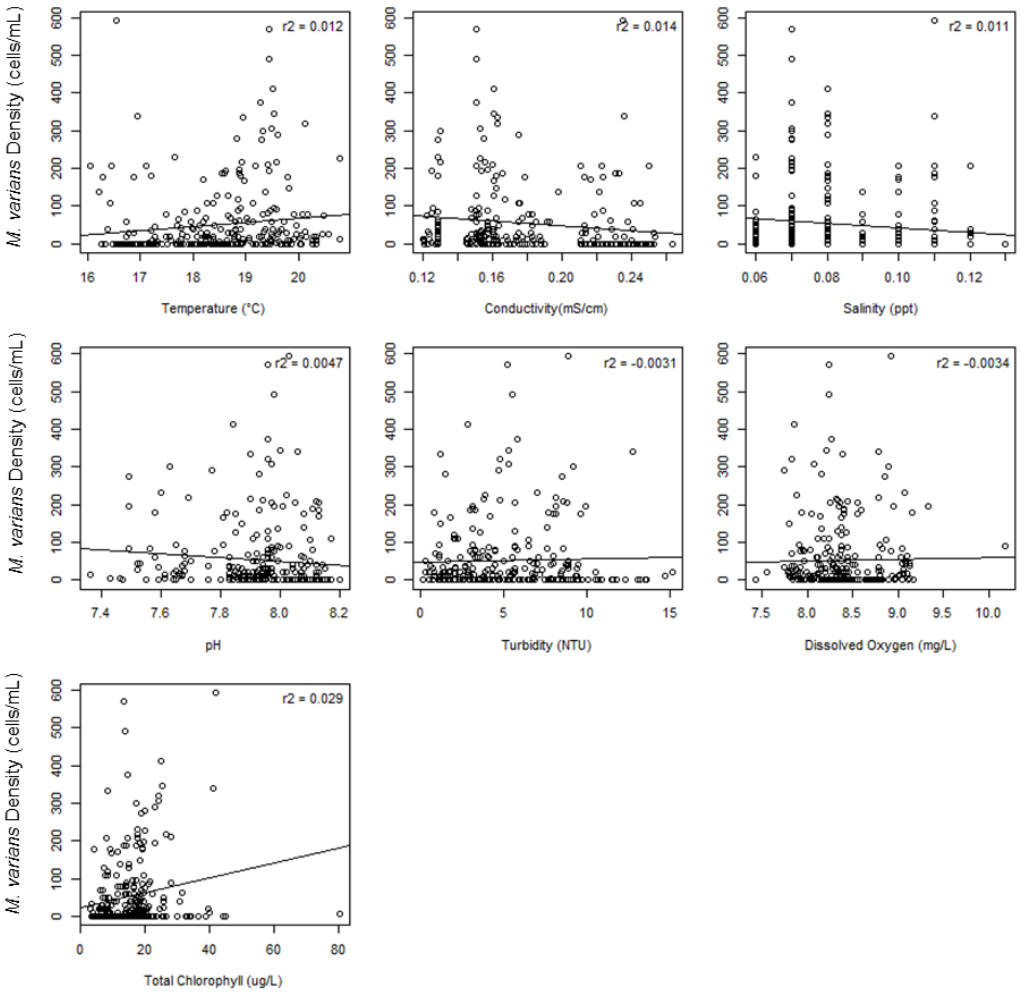

Figure 7. Relationship between *M. varians* density and water quality in 19-L mesocosms. The squared correlation coefficient is shown for the linear fit. The only significant correlation (P < 0.05) was between *M. varians* density and chlorophyll.

TABLES

Table 1. Slopes (change in cells/mL/day) of *M. varians* density in all possible time periods for all three trials. Significance of changes was evaluated by paired *t*-tests comparing *M. varians* densities. Significant slopes are indicated in green and near-significant slopes (0.05 < P < 0.10) are in yellow.

Inoculation		Trial 1				Trial 2				Trial 3			
0 cells/ mL	Day	7	14	21	28	7	14	21	28	7	14	21	28
	0	-1.5	-0.6	-0.4	-0.3	0.7	0.2	0.1	0.0	0.3	-0.4	-0.4	-0.1
	7		0.4	0.2	0.1		-0.4	-0.2	-0.2		-1.1	-0.7	-0.2
	14			0.0	0.0			0.0	-0.2			-0.4	0.2
	21				0.0				-0.4				0.7
1 cell/mL	0	0.0	0.0	0.2	0.8	0.3	-0.5	0.5	0.0	0.0	0.4	0.7	0.0
	7		0.0	0.3	1.1		-0.7	0.9	0.2		0.7	1.1	0.0
	14			0.6	1.7			2.5	0.6			1.4	-0.4
	21				2.7				-1.3				-2.1
10 cells/mL	0	-0.1	0.0	-0.4	0.0	-2.8	-1.4	-1.1	2.1	-0.4	-0.2	-0.1	-0.1
	7		0.2	-0.5	0.1		0.1	-0.2	3.7		0.0	0.0	0.0
	14			-1.3	0.0			-0.5	5.6			0.0	0.0
	21				1.3				11.6				0.0
30 cells/mL	0	1.1	-0.4	0.4	-1.1	-1.1	0.0	-1.2	-2.0	9.2	4.0	-2.1	-1.0
	7		-1.8	0.0	-1.8		1.1	-1.2	-2.3		-1.3	-7.7	-4.3
	14			1.8	-1.7			-3.5	-4.0			-14.2	-5.9
	21				-5.3				-4.6				2.5
100 cells/mL	0	16.1	6.8	7.1	4.4	-18.1	-16.1	-14.8	-11.6	3.6	8.2	2.3	0.4
	7		-2.5	2.6	0.5		-14.2	-13.2	-9.4		12.8	1.7	-0.6
	14			7.8	2.1			-12.2	-7.0			-9.4	-7.3
	21				-3.7				-1.9				-5.3

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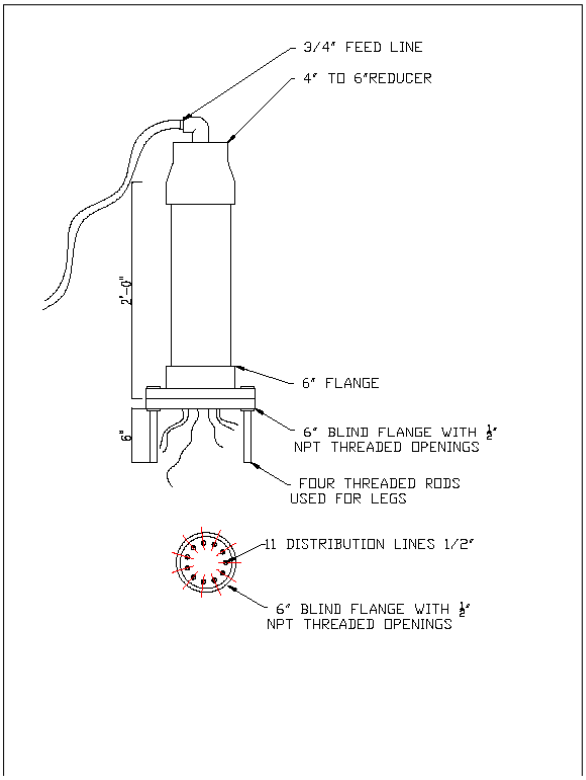
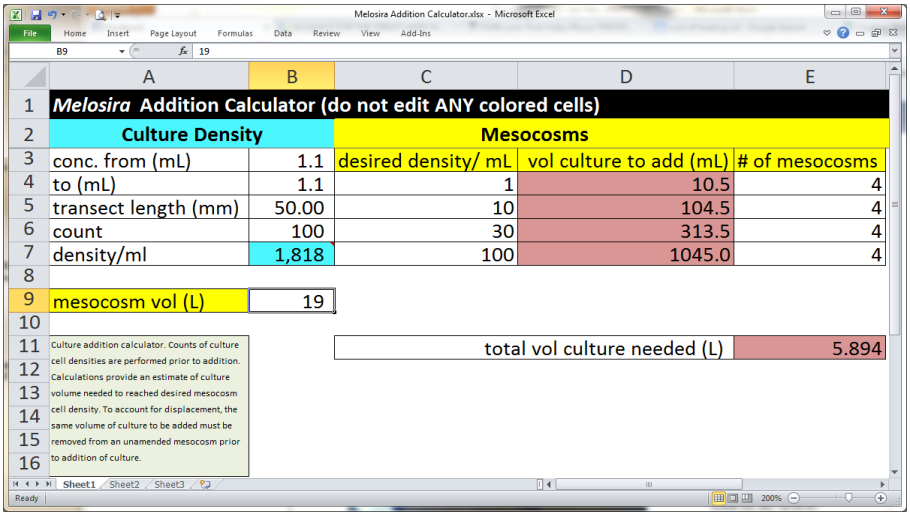
APPENDIX A

A1: Manifold that divided intake harbor water 22 times to make sure it was appropriately mixed for tank filling.



A2: *M. varians* spiking calculations worksheet



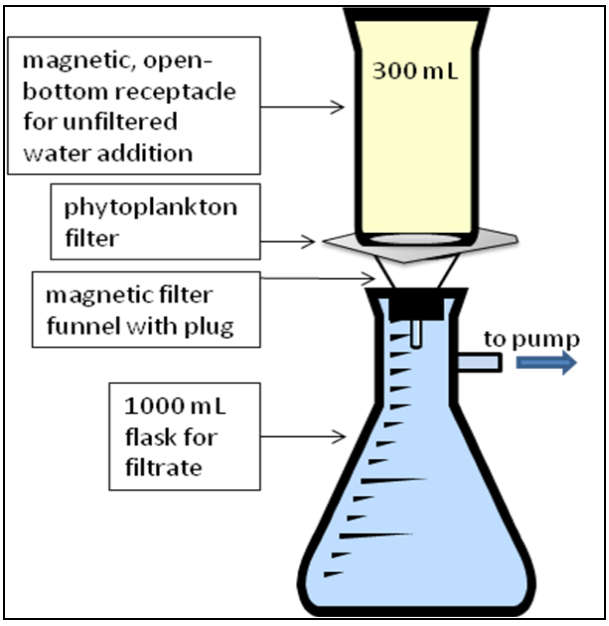
Melosira Addition Calculator.xlsx - Microsoft Excel

	A	B	C	D	E
1	***Melosira*** **Addition Calculator (do not edit ANY colored cells)**				
2	**Culture Density**		**Mesocosms**		
3	conc. from (mL)	1.1	desired density/ mL	vol culture to add (mL)	# of mesocosms
4	to (mL)	1.1	1	10.5	4
5	transect length (mm)	50.00	10	104.5	4
6	count	100	30	313.5	4
7	density/ml	1,818	100	1045.0	4
8					
9	mesocosm vol (L)	19			
10					
11	Culture addition calculator. Counts of culture			total vol culture needed (L)	5.894
12	cell densities are performed prior to addition.				
13	Calculations provide an estimate of culture				
14	volume needed to reached desired mesocosm				
15	cell density. To account for displacement, the				
16	same volume of culture to be added must be removed from an unamended mesocosm prior to addition of culture.				

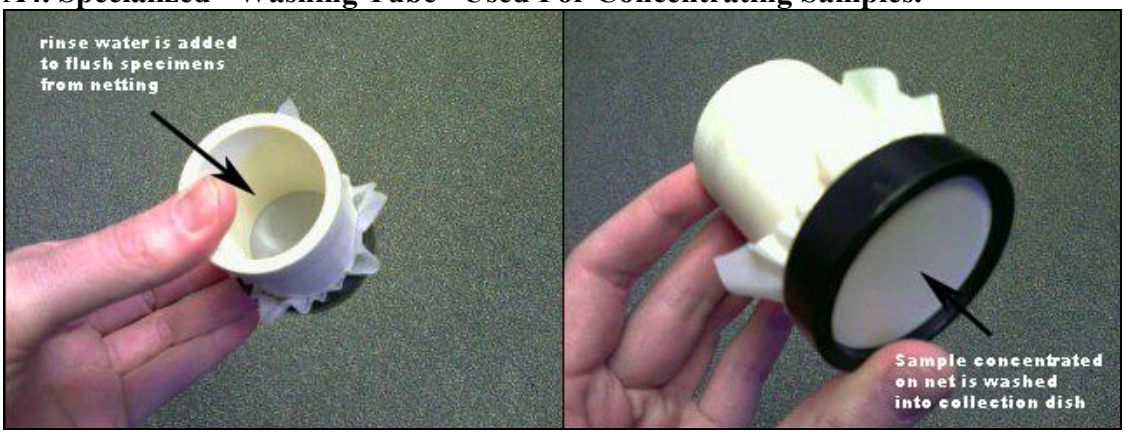
Sheet1 Sheet2 Sheet3

Ready

A3: Filtration setup used for concentrating phytoplankton samples



A4. Specialized "Washing Tube" Used For Concentrating Samples.



A5: Simplified countsheet used for the first of three trials. The second and third trials were taxonomically assessed using the more taxonomically detailed countsheets for algae/small protozoan sample analysis.

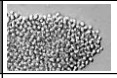
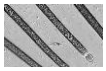
Great Ships Initiative	ANALYST NAME:			Issue date: May 29, 2013	
	SIMPLIFIED DATASHEET FOR ALGAE/SMALL PROTOZOAN SAMPLE ANALYSIS (GSI/FORM/XX/X/X)				
SAMPLE NAME:		WATER FILTERED:	mL	FIELD OF VIEW WIDTH (µm):	980
EXPERIMENT:		CONC. SAMPLE VOL.:	15 mL	TRANSECTS (start>finish):	
DATE SAMPLED/ANALYZED:	/	WATER SETTLED:	1.1 mL		
		CHAMBER AREA:	10 cm^2		
	min. dim. < 10µm		min. dim. > 10 µm		
Filamentous					
Globular					
Melosira varians					
ADD ANY ADDITIONAL NOTES IN THIS AREA (e.g. dominant taxa, etc.):					

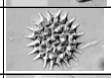
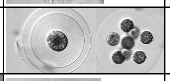
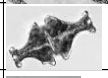
Great Ships Initiative

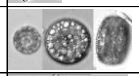
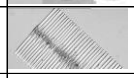
Issue date: July 13, 2011

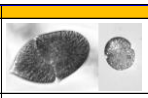
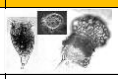
DATASHEET FOR ALGAE/SMALL PROTOZOAN SAMPLE ANALYSIS (GSI/FORM/LB/A/3)

SAMPLE NAME:		WATER FILTERED:	mL	FIELD OF VIEW WIDTH:	980 µm
EXPERIMENT:		CONC. SAMPLE VOL.:	mL	TRANSECTS (start>finish):	
TIME/DATE:		WATER SETTLED:	1.1 mL		
OTHER NOTES:		CHAMBER AREA:	10 cm^2		

	ALIVE		PICTURES / NOTES	
Taxon	**min. dim. < 10µm**	**min. dim. > 10 µm**	**sample**	
BLUE GREENS				
Anabaena **-like**				
Oscillatoria **-type**				
coccoid				
Microcystis **-like coccoid (cells <10 µm)**				
other filamentous-cells				
filamentous-nocells (length)				
Merismopedia				

GREENS				
Scenedesmus **- type desmid**				
Pediastrum				
coccoid				
single spindle (could be blue-green)				
filamentous - cells				
other colonial (non-coccoid)				
other colonial (spindle)				
euglenoid				
CRYPTOPHYTES (and other small flagellates)				
Cryptomonas/ Chroomonas **- types**				
round micro-flagellates				
irregular micro-flagellates				

DIATOMS				
Chain (*Aulacoseira, Melosira, S. binderanus*)				
Asterionella				
Tabellaria				
Diatoma				
Centric solitary (*Cyclotella, Stephanodiscus*)				
Fragilarioid (ribbon colony)				
Synedra **-like** (includes nitzschioid)				
naviculoid (or other single pennate)				
Rhizosolenia				
CHRYSOPHYTES				
Mallomonas				
Synura				
Dinobryon (and Kephyrion)				

DINOFLAGELLATES				
round/ teardrop/ pointy Dino.				
Ceratium (Eiffel Tower)				
PROTOZOANS and ANIMALS				
ciliate				
round or oval protozoan				
irregular protozoan				
Keratella				
Polyarthra				
Egg				
UNKNOWN ENTITIES/CELLS				
round/oval "could be anything"				
irregular "could be anything"				

Great Ships Initiative

ANALYST NAME:

Issue date: May 29, 2013

ADDITIONAL DATASHEET FOR ALGAE/SMALL PROTOZOAN SAMPLE ANALYSIS (GSI/FORM/XX/X/X)

SAMPLE NAME:		WATER FILTERED:	mL	FIELD OF VIEW WIDTH (µm):	980
EXPERIMENT:		CONC. SAMPLE VOL.:	15 mL	TRANSECTS (start-finish):	
DATE SAMPLED/ANALYZED:	/	WATER SETTLED:	1.1 mL		
		CHAMBER AREA:	10 cm^2		

	min. dim. < 10µm	min. dim. > 10 µm
Aulacoseira		
Fungi from culture		
Melosira varians		

ADD ANY ADDITIONAL NOTES IN THIS AREA (e.g. dominant taxa, etc.):

APPENDIX B

M. varians culturing SOP

M. varians varians was isolated into axenic culture December 5, 2012 from a Lake Superior collection obtained from Duluth Harbor, MN. A single *M. varians* cell was extracted from the sample for culture initiation using a borosilica micropipette. This cell was transferred in series through droplets of sterile WC media (Guillard, 1975) until the medium surrounding the cells was free of contaminant organisms, ensuring that the initial culture was unialgal and homozygous. Isolation from this preliminary culture continued, until a bacteria-free stock culture was established. A 20-L stock culture was maintained in WC media (see below).

Stock Culture Conditions and Duration

Cultures were grown in 23-L glass carboys with a 20-L culture volume. Additionally, cultures were constantly agitated via a bubbling system using sterile air. The cultures were maintained on a light cart. Temperature was held at 20 °C with and light cycles were 12:12. Light intensity was >400 μE/m^2/sec on both carts. *In vivo* chlorophyll *a* was recorded on alternate days at the 6th hour of light to ensure growth was occurring. Cultures were diluted with fresh WC media once cell densities exceeded 50,000 cells/ml. Cell density was determined by direct enumeration of cultures via clove oil preparations.

Table 1. WC Media.

Component	Stock Solution (g/L)	Quantity Used/L Media
$CaCl_2{*}2H_2O$	36.76	3 ml
$MgSO_4{*}7H_2O$	36.97	3 ml
$NaHCO_3$	12.6	3 ml
K_2HPO_4	8.71	3 ml
$NaNO_3$	85.01	3 ml
$Na_2SiO_3 \cdot 9H_2O$	28.42	3 ml
Trace Metals Solution	See Following Recipe	3 ml
Vitamins Solutions	See Following Recipe	1.5 ml
Trace Metal Solution		
FeCl3 * 6H2O	N/A	3.15 g
Na2EDTA * 2H2O	N/A	4.36 g
MnCl2 * 4H2O	180	1 ml
ZnSO4 * 7H2O	22	1 ml
CoCl2 * 6H2O	10	1 ml
CuSO4 * 5H2O	9.8	1 ml
Na2MoO4 * 2H2O	6.3	1 ml
Vitamin Solutions		
Thiamine * HCI	N/A	2000 mg
Biotin	1	1 ml
Cyanocobalamin	1	1 ml

Guillard, R. R. L., 1975. Culture of phytoplankton for feeding marine invertebrates. In W. L. Smith & M. H. Chantey (eds), Culture of Marine Invertebrate Animals. Plenum Publishers, New York: 29–60.

APPENDIX C

Correlation matrix of *M. varians* density with other organism densities in mesocosms samples. This appendix is not referred-to in the body text of this manuscript, but is provided to the reader in case additional data on relationships among protist types are desired.

	Melosira Cells	Melosira Entities
Anabaena Small Cells	0.158933454	0.143642416
Scenesdesmus Big Entities	0.125535366	0.151499733
Colonial Spindle Small Cells	0.114301551	0.140244924
Colonial Spindle Small Entities	0.139123052	0.152868525
Cryptomonas Small Cells	0.197176555	0.171351704
Cryptomonas Small Entities	0.200803995	0.182725489
Cryptomonas Big Cells	0.189788738	0.191991984
Cryptomonas Big Entities	0.21517603	0.210891693
Round Microflagellates Small Entities	0.306194301	0.342172268
Round Microflagellates Big Cells	0.199617681	0.146264165
Round Microflagellates Big Entities	0.199465656	0.148038399
Chain Diatoms Small Cells	0.30421451	0.314056663
Chain Diatoms Small Entities	0.373738836	0.332128262
Centric Diatoms Big Cells	0.382912316	0.393002409
Centric Diatoms Big Entities	0.388163089	0.405976302
Naviculoid Small Cells	0.23287316	0.24974729
Naviculoid Small Entities	0.41629506	0.463456851
Rhizosolenia Small Cells	0.198129521	0.178070473
Rhizosolenia Small Entities	0.167610362	0.157872445
Mallomonas Small Entities	0.128845899	0.147575532
Round Protozoan Small Cells	0.286737221	0.280412426
Round Protozoan Small Entities	0.265081396	0.252859259
Irregular Protozoan Small Cells	0.171672949	0.054346094
Irregular Protozoan Small Entities	0.209377018	0.156422992
Irregular Protozoan Big Entities	0.129586162	0.183652879
Aulacoseira Small Entities	0.191120705	0.204241364
Fungi Small Entities	0.182490781	0.193793484
Melosira Cells	1	0.949419528
Melosira Entities		1

n=195

$p < 0.05$
$p < 0.01$
$p < 0.0005$