Evaluation of Compliance Tools Using Variable Fluorescence Fluorometry to Detect Living Organisms in Ballast Water: A Test Protocol for Collecting Measurements

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A test protocol developed by the **Naval Research Laboratory (NRL)** and the **Alliance for Coastal Technologies (ACT)** for evaluation of variable fluorescence fluorometers for use as compliance tools

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Abbreviations and Definitions

ACT	Alliance for Coastal Technologies	IMO	International Maritime Organization
ADQ	Audit of Data Quality	ISO	International Organisation for Standardisation
ANS	Aquatic Nuisance Species	MERC	Maritime Environmental Resources Center
BWMS	Ballast Water Management System	MLML	Moss Landing Marine Laboratory
CMFDA	Chloromethylfluorescein Diacetate	NCMA	National Center for Marine Algae and Microbiota
CV	Coefficient of Variation	NRL	Naval Research Laboratory
DM	Document Manager	NRL-KW	Naval Research Laboratory in Key West, FL
DMSO	Dimethyl Sulfoxide	PAM	Pulse Amplitude Modulated
EFM	Epifluorescence Microscopy	PI	Principle Investigator
EPA	Environmental Protection Agency	QA	Quality Assurance
FDA	Fluorescein Diacetate	QC	Quality Control
FLW	Filtered Lake Water	TSA	Technical System Audit
FSW	Filtered Seawater	USCG	United States Coast Guard
GSI	Great Ships Initiative	VGP	Vessel General Permit
GUM	Guide to Uncertainty in Measurement		

1. Introduction

In an effort to mitigate the risk of transporting aquatic nuisance species, the United States Coast Guard (USCG) has finalized a rule limiting the concentrations of organisms in ships' ballast water discharged into US Ports (US Coast Guard 2012). The specified concentrations reflect those in the International Maritime Organization's (IMO) convention (IMO, 2004). Further, the limits are incorporated into the Vessel General Permit (VGP)—a set of guidelines on a suite of vessel operations (including the discharge of ballast water) regulated under the authority of the US Environmental Protection Agency (US EPA, 2013). In order to meet these limits, most ships will use a ballast water management system (BWMS). These systems incorporate a variety of technologies (including UV radiation, electrolytic chlorination, deoxygenation) to ensure that the discharge water meets the specifications.

Determining concentrations of sparsely populated living organisms requires extensive effort and sensitive equipment. For example, organisms ≥10 and <50 μm may be quantified using a set of vital stains to label living organisms and tally the organisms via epifluorescence microscopy (Steinberg et al. 2011). Direct counts of living organisms yield concentrations comparable to the numerical standard. While this rigorous, complex, and time-consuming analysis is appropriate for verification testing of BWMS (US EPA, 2010), it is not feasible to perform this analysis during routine shipboard inspections. Rather simple, hand-held, field instruments ("compliance tools") to rapidly assess the *likelihood* that the ballast water clearly exceeds the discharge limits will be of much greater value to the compliance officer. The tools for this approach must immediately produce measurements that are reliable indicators of the concentrations of living organisms within a regulated size class (Table 1).

Table 1. Ballast water discharge standards.

Organism	USCG Discharge Standard
Living Organisms ≥50 μm in minimum dimension [A]	<10 m^{-3}
Living Organisms ≥10 and <50 μm μm in minimum dimension [B]	<10 mL^{-1}
Toxigenic *Vibrio chloerae* [C]*	<1 cfu 100 mL^{-1}
Escherichia coli	<250 cfu 100 mL^{-1}
Intestinal enterococci	<100 cfu 100 mL^{-1}

[A] Nominally zooplankton, B Nominally protists, C Serotypes O1 and O139; *Note: the International Maritime Organization D2 Ballast Water Performance Standard also considers <1 cfu g^{-1} of wet weight of zooplankton as a standard for toxigenic *Vibrio chloerae*, USCG = US Coast Guard.

New or refined compliance tools require carefully considered test protocols for evaluating and verifying their performance. This test protocol describes the process of evaluating variable fluorescence as a compliance tool to estimate concentrations of living photosynthetic organisms in ballast water discharge. The core test elements will involve a series of laboratory and field experiments comparing individual instrument estimates of the concentration of living organisms

≥10 and <50 μm in size to a standard, validated approach (i.e., epifluorescence microscopy, EFM) accepted for use in verification testing of BWMS. The goal and objectives for this test protocol are described below.

2. Goals and Objectives

The overall goal is to evaluate the performance of a suite of individual variable fluorescence instruments as potential compliance tools for rapid assessment of ballast water discharge. The following objectives supervene from this specific goal:

- In a series of laboratory trials to be conducted at the Naval Research Laboratory in Key West, FL (NRL), determine **accuracy** and **precision** of the test instrument with samples containing both mixed assemblages of ambient organisms and algal monocultures over a range of concentrations, including concentrations below, equal to, and above the IMO and USCG discharge standard.
- Evaluate the impacts of interferences (dissolved and particulate materials) and disinfection byproducts (DBP) on the accuracy and precision of the test instrument.
- Evaluate the relationship between numerical concentrations of living organisms ≥10 and <50 μm and the accuracy and precision of the instrument using ambient organisms collected from natural waters at various locations (including Key West, Chesapeake Bay, and Lake Superior) and demonstrate how various assemblages of ambient organisms may affect this relationship.

3. Description of the Technology

This protocol evaluates variable fluorometers, and while the architecture of the instrument, the analytical routine, and the internal data processing algorithms will vary among manufacturers, all variable fluorometers measure chlorophyll *a* fluorescence at variable illumination intensities and intervals. These measurements are used to estimate concentrations of living organisms within an aliquot of water. Since the vast majority of organisms within the ≥10 and <50 μm size class are photosynthetic algae, the instruments may provide a reasonable estimate that a sample aliquot meets the discharge limit of 10 living organisms mL^{-1} in the ≥10 and <50 μm size class. Instruments will likely use both instantaneous, *in vivo* fluorescence measurements combined with measurements of variable fluorescence to detect the total concentration and the proportion of active microalgae in the sample, respectively. This data output may be adjusted based upon predetermined assumptions, such as the contribution of organisms in the sample but outside of the size class. Instruments will be *primarily* evaluated on whether they correctly identify exceedances, i.e., predict whether the organism concentration within the sample exceeds the discharge standard. When possible, secondary evaluation will consider the measurements collected and reported by the instrument. These may include initial fluorescence, variable fluorescence, or photochemical yield.

4. Summary of Basic Verification Approach

The verification of the instrument will be partitioned into laboratory and field environments. Experiments are designed to challenge the instruments by analyzing ranges of concentrations of both cultured microalgae and mixed assemblages of ambient organisms. Two microalgae monocultures will be used to sample with a range of cell concentrations encompassing the discharge standard. Since the discharge standard for living organisms ≥10 and <50 µm is <10 mL^{-1}, the set of target concentrations for both laboratory and field trials will be 0, 5, 10, 20, 50, and 100 mL^{-1}. Concentrations will be measured directly by fluorescently labeling organisms in a sample with a set of fluorochromes that are enzymatically activated and label only living cells (**Section 4.2; Appendix A**).

For laboratory experiments, samples will be prepared with the addition of dissolved organic matter (DOM), particulate organic matter (POM), and mineral matter (MM), which are used to achieve 'challenge water' conditions (US EPA, 2010). Additional samples will be prepared by mixing living organisms with natural water treated with hypochlorite and held for 5 d prior to the experiment. For field experiments, characteristics of the ambient water, including temperature, salinity, DOM, POM, and MM, will be measured. The concentrations of organisms <10 and ≥50 µm will be measured in the whole-water samples collected for field experiments. Organisms in field samples will be classified to general taxonomic groups (e.g., flagellates, copepod nauplii, diatoms, dinoflagellates, etc.). Organisms outside the ≥10 and <50 µm size spectra will also be identified and counted in preserved samples collected from the whole-water sample. These measurements may yield information on how organisms outside of the ≥10 and <50 µm size class affect measurements of variable fluorescence. Characterization of the assemblage of organisms (including those outside the ≥10 and <50 µm size range) allows for analysis that is more detailed and may provide insight into instances where the direct counts are unaligned with the output from the compliance monitoring device.

The critical evaluation of the instrument is to determine whether it provides accurate and consistent estimates of the abundance or living organisms via measurements of chlorophyll *a* fluorescence that can be directly compared to concentrations of living organisms ≥10 and <50 µm. The test instrument will be evaluated at a range of concentrations below and above the discharge standard to measure its performance in assessing whether the sample *meets* or *exceeds* the discharge standard. The test instruments may not report quantitative measurements; therefore, it may not be possible to measure the linear response of the instrument across the dynamic range. However, where quantitative measurements are provided or available, these analyses will be performed[1]. In addition to the accuracy of the instrument in predicting concentrations of living organisms are greater or less than 10 mL^{-1}, the precision of the instrument will be evaluated by repeated analysis, including the analysis of replicate subsamples.

[1] At the Workshop held on 8-9 April, vendors agreed to provide all available measurements. Vendors will provide a list and description of the parameters measured, and a list of these available measurements will be compiled before the start of the field experiments.

The response of the test instrument will be determined using both and algal monocultures in controlled laboratory experiments (**Section 5**) and mixed assemblages of ambient organisms (**Section 6)**. A thorough description of these test elements is available in these sections. The standard method for directly counting organisms ≥10 and <50 μm using epifluorescence microscopy (EFM) is described in detail in **Appendix A**. **Appendix B** contains descriptions of some additional analytical methods—including a description of the gravimetric approach for calculating sample volume—and a discussion of some approaches for additional data analysis. The following sections provide a brief and general overview of variable fluorometry and direct counts by EFM.

4.1. Measuring Chlorophyll *a* fluorescence and Photochemical Yield

The instruments evaluated in this study do not require reagents or sophisticated sample preparation and processing. Thus, all instruments are designed to read a whole water sample with minimal manipulation. Apart from this shared criteria, the instruments may employ unique approaches to assess the sample. This diversity can include a variety of illumination routines to excite the algal photopigments, various configurations of light emitting diodes (LED) and photodiodes, and differences in signal processing and data analysis. The specific approaches and characteristics of each of the instruments are not relevant for the testing; however, this information will be addressed in the final report for each of the instruments. Whatever the approach, at a minimum the instruments report a single characterization of living biomass in disposition of the sample water (e.g., ***meets*** discharge standard or ***exceeds*** discharge standard). The specific protocols for operating the test instruments (including a description of all the data reported by the instrument) will be provided by the instrument manufacturers. For completeness, all preliminary measurements and metadata reported by (or available from) the instrument will be collected and recorded.

4.2. Determining Concentrations of Microalgae by Epifluorescence Microscopy (EFM)

The procedure for direct counts via EFM will vary among locations. Critically, each of the participants in the field experiments should use a standard protocol that has been validated for the evaluation of their water samples. The procedure described in this protocol which will be used for the laboratory experiments and field experiments at Key West, was developed at the Naval Research Larboratory in Key West, FL (NRL-KW). The detailed procedures are available in **Appendix A**. In general, the approach employs a set of two fluorochromes, which are molecules capable of fluorescence following a reaction with cellular enzymes. The fluorochromes recommended for use in verification used in ballast water (US EPA, 2010) and validated for ambient microbiota (including determining rates of false positives and negatives; Steinberg et al., 2011) are chloromethyl fluorescein diaceteate (CMFDA) and fluorescein diacetate (FDA). These fluorochromes are reconstituted from lyophilized stock by dissolving the powder in dimethyl sulfoxide (DMSO) to yield stock solutions of 250 μM CMFDA and 1 mM FDA, and 10 μL and 5 μL of the stock solutions, respectively, are added to 0.985 mL of sample.

The labeled sample is then incubated in the dark at room temperature for 10 minutes, during which time the non-fluorescent molecules diffuse or are transported across cell membranes, react with non-specific enzymes in the cytoplasm, and fluoresce.

The labeled sample water is then transferred into a 1-mL gridded chamber (i.e., a Sedgewick-Rafter counting chamber). When covered with a glass coverslip, the dimensions of the chamber (50 x 20 x 1 mm) accept exactly 1 mL. Horizontal and vertical grid lines are spaced every 1 mm, so each grid represents 1 µL (i.e., 1 mm^3). Because of this, a portion of the chamber can be counted and that area counted corresponds to a known volume of water. For example, every 50 mm long x 1 mm wide row contains 50 µL of sample water.

The Sedgewick-Rafter counting chamber is transferred to an epifluorescence microscope equipped with the light filter cubes appropriate for fluorescein fluorescence. In general, the excitation and emission maxima of fluorescein occur at 485 and 530 nm, respectively. A dichroic mirror at 500 nm will prevent the detection of backscattered excitation light. Typically, organisms in the ≥10 and <50 µm size class are detectable at 100 – 200x magnification. Higher magnification may be necessary for ambient samples to size organisms near the extremes of the size class.

Counting should be completed within 20 minutes, as after 20 minutes the background fluorescence becomes significant and results in difficulty detecting and identifying living organisms. The volume counted during this time will vary based upon the concentrations of organisms within the sample: for sparse concentrations (<50 mL^{-1}), it is possible to scan the entire 1-mL chamber. For complex samples with high concentrations (>100 mL^{-1}) only a portion (30-50%) can be scanned. In this case, it is necessary to select individual rows randomly and to scan the entire row; a random number generator is used to supply a counting order (**Appendix A**). Fluorescent organisms are tallied as the grid is scanned; for ambient samples, it is necessary to classify the organisms into general taxonomic groups. A pre-generated data sheet with entries for common organisms (and picture references) should be used during counting. The concentration of total organism (or organisms in each of the general groups) is calculated as the count per volume counted. If necessary, the final output is adjusted to account for the addition of the fluorochromes and concentration or dilution, if used.

5. Methods for Laboratory Experiments at the Naval Research Laboratory in Key West, FL (NRL)

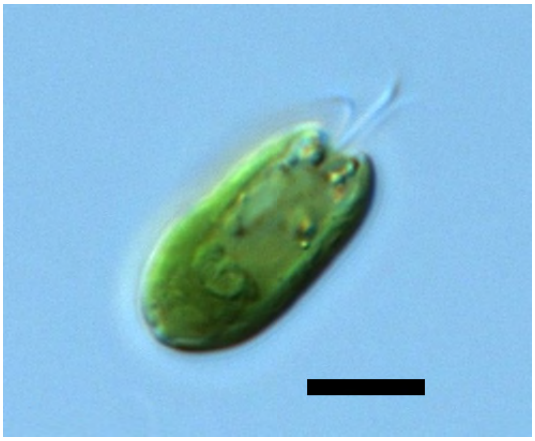
Laboratory tests will use a range of concentrations of cultured microalgae. These trials represent the most straightforward samples for the instruments, as samples will consist of only a single alga cultured in filtered, oligtrophic seawater. Thus, complications arising from a mixed assemblage of organisms (including organisms outside the ≥10 and <50 µm size class) are avoided, and interferences from dissolved and particulate matter are minimized. The laboratory experiments will show the baseline performance of the instrument, which may be useful in

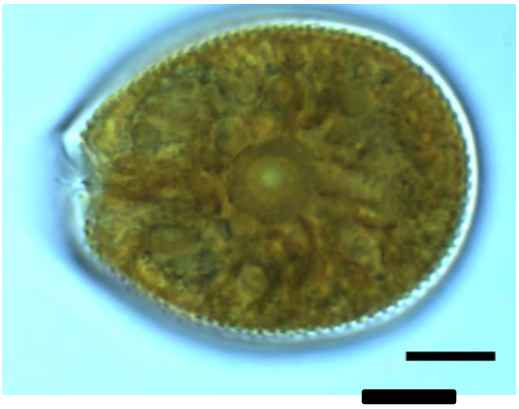
interpreting results from field tests designed to examine complex, natural samples. Additionally, the laboratory tests will incorporate samples with dissolved and particulate materials or samples containing dead cells and, potentially, disinfection byproducts. These test elements are described in the following sections.

5.1. Test Organisms

For laboratory trials, two organisms will be used to create a series of samples that will be analyzed by the suite of variable fluorometers (Table 2). The organisms represent cells at the extremes of the ≥10 and <50 µm size class. While the minimum size range for the width of *Tetraselmis marina* is reported to be 9 µm, cells are typically >10 µm in cell width. For the laboratory experiments with cultured algae, all living cells will be counted during the microscope analyses (even though some individuals may have dimensions at the outside of the size threshold). A thorough evaluation of the size of the test organisms will be performed on the source culture immediately prior to sample preparation using photomicrographs and image analysis to determine the size distribution of the test organisms.

Table 2. Marine algae used in laboratory experiments. Cultures were obtained from the National Center for Marine Algae and Microbiota (NCMA, Bigelow Laboratory for Ocean Sciences; East Boothbay, ME). Cell size ranges and images of the strain (or a closely related strain for *T. marina*) were obtained from the web page referenced below.





Alga	*Tetraselmis marina*	*Procentrum micans*
Culture	CCMP898	CCMP683
Size	9 – 15 µm	25 – 50 µm
Link	https://ncma.bigelow.org/ccmp898	https://ncma.bigelow.org/ccmp683

5.2. Preparing Samples with a Range of Cell Concentrations

Stock cultures acquired from the NCMA have been maintained at the Naval Research Laboratory in a temperature-controlled culture chamber (Model I-66LL; Percival; Perry, Iowa). Both *T.*

marina and *P. micans* have been held at 20°C under a 16:8 light:dark cycle. To maintain actively growing populations (i.e., as observed in the logarithmic growth phase), cultures were transferred weekly or bi-weekly into new nutrient media prepared by adding f/2 nutrients (Guillard and Ryther 1962) to 0.22-µm filtered seawater. The nutrient media was autoclaved and cooled to 20°C prior to seeding it with a mature culture. Typically, 50 to 100 mL is added to 500 mL of media. These culturing conditions are in place to ensure that variations among independent trials (each with a series of samples) are minimized. Nevertheless, algal populations will undergo periods of rapid growth, stagnation, and autogenous mortality. These factors will lead to variations in the per capita chlorophyll *a* content within cells and may cause variation in measurements amid stability in cell concentrations.

Immediately prior to preparing the dilution series, the stock culture is counted using the direct count method (**Appendix A**). This count is not performed in triplicate, as it is only a rapid estimate of cell concentration in the stock culture, but this concentration is used to calculate the volume of stock culture needed to produce a sample with a target concentration. Because the variable fluorometers are optimized to measure concentrations near the discharge standard (<10 living organisms mL^{-1}), the target values include concentrations below, at, and above the discharge standard. These concentrations are listed in Table 3. When the concentration of organisms in the culture stock (C_{Stock}) has been determined, this value is used to calculate the volumes of culture stock (V_{Stock}) and filtered seawater (FSW; V_{FSW}) needed to prepare the samples using the following equations:

Eq. 1 $$V_{Stock} = \frac{V_{Final} \cdot C_{Target}}{C_{Stock}}$$

Eq. 2 $$V_{FSW} = V_{Final} - V_{Stock}$$

Table 3. Target cell concentrations and an example of the values used for sample preparation. The volumes of stock culture and filtered seawater (FSW) used as a diluent are calculated based upon an assumption that there are 500 cells mL^{-1} in the stock culture and that the final sample volume is 2,000 mL. Because the stock concentration will vary, these values should be recalculated using Eq. 1 and Eq. 2 (**Data sheet XXX**).

Target Concentration (cells mL^{-1}; C_{Target})	Final Sample Volume (mL; V_{Final})	Stock Concentration (cells mL^{-1}; C_{Stock})	Stock Volume Needed (mL; V_{Stock})	Volume of FSW Needed (mL; V_{FSW})
100	2,000	500	400	1,600
50	2,000	500	200	1,800
20	2,000	500	80	1,920
10	2,000	500	40	1,960
5	2,000	500	20	1,980
0	2,000	500	0	2,000
		Sum Volume:	740	11,260

Samples of *T. marina* and *P. micans* are prepared separately. Therefore, a single, independent trial will generate 12 unique samples (6 concentrations x 2 organisms).

5.3. Preparing Samples with Interfering Compounds

Natural waters will also contain dissolved and suspended materials that can interfere with the measurements of light intensity, primarily by absorbing or reflecting light. The quantity of interfering compounds will vary among sample locations. In order to mimic water conditions with dissolved and particulate materials representative of ports in coastal regions, a sample will be prepared with 'challenge water' additives (US EPA, 2010). The additives will be introduced into samples of *T. marina* and *P. micans* with target concentrations of 10 cells mL^{-1}. These samples are prepared separately from the samples in **Section 5.2**.

A 100x-concentrated stock solution of the additives is prepared using quantities displayed in Table 4 (100x stock in 100 mL of FSW). The sample with either *T. marina* or *P. micans* is created following the procedure in Section 5.3 (target concentration: 10 mL^{-1}), but the volume of FSW added to the sample is decreased by 20 mL. The sample is then filled to capacity (2,000 mL) by adding 20 mL of the 100x-concentrated additive mixture, which has been well mixed by inverting the sample container 5 times prior to transferring the 20-mL volume. In addition to the 12 unique samples generated in **Section 5.2**, these samples with the additive mixture will generate an additional 2 unique samples (1 additive mixture x 2 organisms at 10 cells mL^{-1}).

Table 4. Additives for challenge water. The final concentration is the quantity *added* to the sample; it does not include dissolved or particulate material already in the sample. Concentrations of DOM and POM are specified as dry mass of total organic matter.

Challenge Water Component	Additive	Additive Source	Final Concentration	100x Stock (100 mL)
Dissolved organic matter (DOM)	*Camellia sinesis* extract	Lipton® decaffeinated, instant iced-tea mix (Unilever; Glasgow, Scotland)	6 mg L^{-1}	60 mg
Particulate organic matter (POM)	Humic matter	Micromate humates (Mesa Verde Resources, Placitas, NM)	4 mg L^{-1}	40 mg
Mineral Matter (MM)	Ultrafine Arizona test dust (ISO 12103-1)	Powder Technology, Inc. (Burnsville, MN)	20 mg L^{-1}	200 mg

5.4. Preparing Samples with Disinfection Byproducts

A shipboard sample may contain byproducts from the BWMS (i.e., disinfection byproducts [DBP]), which can interfere with measurements of variable fluorescence. Additionally, a post-treatment sample may contain killed organisms that contribute a fluorescence signal. Electrolytic chlorination is one of the common approaches used to treat ballast water (Lloyd's Register, 2011). Real world, post-treatment ballast water samples are not well characterized (and will likely vary among ships, seasons, water types, and treatment regimens). Therefore, *a priori* knowledge of interfering compounds and DBP is not feasible at this time. A sample simulating chlorination, however, will be created from ambient seawater and may provide an initial test of some of the characteristics of a water sample collected following treatment.

The laboratory in Key West has the capacity to produce hypochlorite through the electrolysis of seawater (the process used in shipboard BWMS), and this approach will be used instead of adding diluted commercial bleach to the sample, as commercial bleach contains additional components (e.g., sodium polyacrylate) that would not be produced by electrolytic chlorination. Hypochlorite will be produced through the electrolysis of ambient seawater (35 psu [practical salinity units]) collected from Key West and filtered through a 0.22-µm membrane filter. Electrolysis is performed by placing platinized wire electrodes, controlled by a benchtop potentiostat (HA-151; Hokuto Denko; Tokyo, Japan), into 1 L of FSW. The seawater is exposed to 10 V for ~1 h; after, it is filtered again to remove precipitates. For all measurements, the concentration of hypochlorite (measured as the concentration of free chlorine) will be determined using a colorimetic test (Pocket Colorimeter Kit®; Hach, Inc.; Loveland, CO; APHA, 1998). The colorimeter is calibrated with gel standards (Hach, Inc.), and readings should be within the margin of uncertainty of the standards. The solution will be kept in an opaque glass bottle, and the concentration of hypochlorite will be measured again immediately prior to creating stock solutions.

Simulated treated water will be made by adding the hypochlorite to ambient water (collected as described above) to yield an *applied* dose of 10 mg L^{-1} (~10 ppm) solution. At least 2 L of water will be stored at 20°C for 5 d prior to the experiment. During this incubation, the chlorinated solution will be kept in an opaque bottle at 20°C. The total residual oxidant (TRO) is expected to be ≤0.01 mg L^{-1} after 5 d, but if the TRO is greater than this value, the solution can be neutralized using sodium thiosulfate. This treated and neutralized water is used as the diluent to make samples for measuring variable fluorescence and cell concentrations, and samples will be produced for both *T. marina* and *P. micans* at target concentrations of 10 cells mL^{-1}, which will generate an additional 2 unique samples (1 treated sample x 2 organisms at 10 cells mL^{-1}).

5.5. Sample Analysis and Workflow

Sample processing efforts will produce 16 unique samples. Each sample will require the analysis using each of the methods or instruments in Table 5. Samples are prepared concurrently

at the start of the day of the experiment. Because organisms are acclimated to laboratory temperatures, the prepared samples will be held in opaque bottles at 20°C until analysis. The order of analysis is random, so the sample selected for analysis may contain either *T. marina* or *P. micans* at any of the target concentrations or the treatments with additives or DBP. Once the sample has been prepared, the identity and composition of the sample will be masked by labeling the sample with a random code. This process is critical, so a quality officer will oversee this step, assuring that the master list linking the random code to the sample identification is correct. Throughout the day, samples will be selected, mixed by slowly inverting the sample five times, and removing an aliquot with a volume sufficient for direct microscope counts and all four variable fluorometers. This process will be repeated to remove a second aliquot. The third aliquot is only used for microscope counts.

Table 5. Analyses required per each of the samples.

Method or Instrument	Maximum Subsample Volume and Number	Maximum Volume Required (mL)
Direct microscope counts via EFM	500 mL x 3	1,500
Chelsea FastBallast	500 mL x 2	1,000
Turner Designs Ballast-Check2	100 mL x 2	200
BBE Moldaenke 10Cells	100 mL x 2	200
YSI Xylem	150 mL x 2	300

5.5.1. Analysis Procedure for Direct Microscope Counts

The most time-consuming analysis will be the direct microscope counts. There will be 14 samples to analyze per day (the two blank samples will not be analyzed by microscopy). Three subsamples will be analyzed per sample, yielding 42 counts to be performed each day. Even with multiple microscopists and two microscopes that can be continuously used, this still presents a major effort. To accomplish this task in a single day (without over taxing microscopists), the ***average*** time of the each microscope count should be ***15 minutes*** *and a single microscope count should not exceed* ***20 minutes***. Four microscopists will rotate between counting and preparing samples. The sample preparation will include concentrating a large

volume (up to 500 mL) into a volume of 10 to 50 mL. The final volume is measured gravimetrically as described in **Appendix B.** This concentrated sample will be mixed, subsampled, and labeled using the procedure described above (**Section 4.2; Appendix A**). A successful count will survey a portion of the Sedgewick-Rafter chamber (ranging from >0.2 mL, or 20% of the gridded area to the entire area, which is equivalent to 1 mL) *and* will have an organism count greater than 30 individuals. The final concentration of organisms in the population (*P*) is calculated by the following equation:

Eq. 3 $$P = \frac{I \cdot C \cdot D}{A \cdot S}$$

Which considers the number of individuals counted (*I*), the volume of concentrated sample (*C*, mL), the dilution due to the addition of fluorescent labels (*D* = 1.015x), the volume of the aliquot counted (*A*, the area of the Sedgewick Rafter x 1 mL), and the sample volume (*S*, mL), which will be ≤500 mL.

5.5.2. Analysis Procedures for the Variable Fluorometers

The procedures used to conduct a single measurement for each of the four fluorometers are specified by their respective manufacturers. These procedures should be understood and performed ***exactly*** as written. If the procedure includes steps for basic troubleshooting, performing a blank measurement, or executing a set of simple steps, these will be followed. However, the troubleshooting steps should not require excess sample volume (in addition to the sample volume reserved). The specific protocols will be available prior to the start of the test, and vendors should make certain that analysts are properly trained in the procedure.

Four analysts will work concurrently, and each analyst will be responsible for analyzing samples using a specific instrument. Since all analysts will be trained and familiar with all of the variable fluorometers, analysts will periodically rotate to work with different instruments. At a minimum, all instruments should report a metric indicating whether the sample meets or exceeds the discharge standard. Other data and metadata that is displayed or available through interfacing with a computer, however, should be recorded. Analysts can record relevant notes and observations as the analyses are in process.

6. Methods for Field Tests at Various Test Locations

6.1. Overview

Instrument performance will also be tested in field experiments using ambient water samples collected from three separate locations representing a range of water temperatures, salinities, and community compositions. As the ambient concentrations of organisms vary among locations (and at a location over time), the ambient concentrations should be first measured by direct counts using the method describe above. Because of the site-to-site variation in concentrations of ambient organisms, it will be necessary to sample and process water differently among

locations. These differences, however, should not affect the integrity of the comparison between concentration measurements by direct counts and variable fluorescence.

For locations with high concentrations of ambient organisms (>>100 mL^{-1}; e.g., Chesapeake Bay), a discrete volume of water (10 – 15 L) will be collected by pooling multiple grabs of water from 1 m depth. Within 3 h of this sample collection, water will be sampled at the same location using the same sampling technique. This water will be designated for filter sterilization and will be used to dilute the sample water to yield water with organism concentrations in the target ranges:

- 0 mL^{-1}, the filter-sterilized water to be used as a baseline or blank for fluorescence,
- 5 – 20 mL^{-1}, representing concentrations near the discharge standard [DS],
- 30 – 50 mL^{-1}, representing concentrations above the DS, and
- ≥100 mL^{-1}, representing concentrations well above the DS.

Because all of the organisms for analysis will be contained within a single sample vessel, which is well mixed prior to subsampling, there is only a need to collect one aliquot from the vessel for auxiliary measurements (e.g., dissolved organic matter, concentrations of organisms <10 μm).

For locations with low concentrations of ambient organisms (<10 mL^{-1} e.g., Key West), a discrete volume of water (<50 L) will be collected by pooling multiple grabs of water from 1 m depth. Within 3 h of this sample collection, water will be sampled at the same location using the same sampling technique, and this water will only be used to create a blank (0 mL^{-1}) sample and for rinsing. The larger volumes of water will be sieved to concentrate organisms ≥10 μm to yield water with organism concentrations in the target ranges:

- 0 mL^{-1}, the filter-sterilized water to be used as a baseline or blank for fluorescence,
- 5 – 20 mL^{-1}, representing concentrations near the discharge standard [DS],
- 30 – 50 mL^{-1}, representing concentrations above the DS, and
- ≥50 mL^{-1}, representing concentrations well above the DS.

All of the organisms for analysis will not be contained within a single sample vessel, rather, each vessel will contain a concentrated sample that is representative of one of several sampling and pooling events. Because of this, it is necessary to collect and aliquot from each of the three preparations (excluding the 0 mL^{-1} blank) for auxiliary measurements (described in more detail below).

6.2. Auxiliary Measurements

For each pooled water sample, the temperature and salinity of the initial sample will be measured, and the water will be subsampled for analysis via direct counts to determine concentrations of organisms ≥10 and <50 μm. Additional subsamples will be aliquoted after mixing from this source sample for analysis of organisms <10 μm and >50 μm (preserved to be

measured within 2 weeks), dissolved and particulate organic matter (analyzed within 1 week). Four samples prepared from this collected water will contain concentrations of living organisms mL^{-1} in the ≥10 and <50 µm size class. To achieve this, the water collected may require filtered water from the site (referred to here as either filtered [<0.22µm] lake water or seawater, FLW or FSW, respectively). At sites with low concentrations of organisms ≥10 and <50 µm, the sample may require concentration using a monofilament mesh sieve. The analyses procedure will mirror that as described in the laboratory experiments: each sample that was prepared to have a specified, target concentration and is subsampled three times for analysis. Each subsample contains sufficient volume to perform one direct count via microscopy and two discrete analyses by each of the four variable fluorometers. All four concentrations will be prepared and analyzed in a single day. Specific details of these procedures are described in the following sections.

6.3. Preparing Filtered Water from the Site for Dilution

Filtered water from the site is required to dilute samples to achieve the target concentrations and to rinse concentrated water from the surface of monofilament mesh sieved. Water for dilution should be collected from the sampling location and prepared prior to, but not more than 3 h before, sample collection. During this time interval, it is necessary to keep the FLW or FSW isothermal to the collected water. The rapid temperature changes induced by mixing , e.g., 0.1 L of water at 8°C into 1.9 L of water at 15°C, should be avoided. This may be performed by keeping sealed bottles or carboys with FLW or FSW submerged in a flow-through trough with ambient water or in a temperature-controlled incubator. However, after samples are diluted, it is not necessary to keep the samples at the temperature of ambient water. If samples are analyzed within 6 h of collection, and if samples are kept out of direct sunlight, the gradual temperature changes due to the acclimation to laboratory conditions are not necessary to avoid, as they should affect all samples equally, regardless of their starting concentration of organisms.

6.4. Sampling Procedure

A single, independent sampling event consists of deploying a Van Dorn sampling bottle (or equivalent device) one or more times into the source water and collecting a final composited sample within a clean vessel for further subsampling. The Van Dorn sampling bottle and the compositing vessel will be carefully cleaned prior to each sampling event following the institution's cleaning protocol. Typically, cleaning consists of thorough rinsing with municipal water three times followed by three rinses with deionized water and air-drying. If detergents are used, the rinsing should be sufficient to remove residuals. An initial sample will be collected and discarded to remove any residual materials from the Van Dorn bottle. The water should be gently poured into the carboy. A large funnel with a flexible tube leading to the bottom of the carboy will prevent splashing and bubbling of the water. The volume collected should be >10 L. After each use, the Van Dorn bottle (and all other materials in contact with the sample) should be cleaned.

6.5. Concentrating Organisms ≥10 µm

Concentrations of organisms ≥10 and <50 µm will vary among field locations: in some cases, concentrations may exceed the 'challenge water' threshold of 1,000 mL^{-1} (U.S. EPA, 2010). At NRL-KW, concentrations of organisms ≥10 and <50 µm are typically <10 mL^{-1} (unpub. data), and therefore, organisms must be concentrated prior to detection by either microscopy or variable fluorescence fluorometry. In this case, organisms are concentrated by sieving sample water through a 7-µm mesh netting in a sieve. Sample water is poured gently and continuously through the sieve, but the filtrate is not allowed to completely drain, which would expose the organisms retained on the mesh to air. It is recognized that this process will effectively concentrate *all* organisms ≥10 µm (including organisms ≥50 µm), but organisms <10 µm will be at concentrations similar to their ambient concentrations. Because the whole water sample will be analyzed by direct counts of preserved samples, concentrations of organisms <10 and ≥50 µm will be measured. This may provide insight into the situations where the variable fluorescence reading, which may include a signal from organisms outside the ≥10 and <50 µm size class (depending on the pre-processing protocol), deviates from direct microscope counts of organisms within the size class.

6.6. Sample Analysis and Workflow

In general, the sample analysis and workflow will follow that described for the laboratory experiments. Only three concentrations will be performed each day: on the first day, the collected water will be used to prepare samples with 100, 50, and 20 organisms mL^{-1}; on the second day, the collected water will be used to prepare samples with 10 and 5 organisms mL^{-1}. Negative controls (i.e., FLW or FSW with 0 organisms mL^{-1}) will be analyzed using the variable fluorometers on both days, but it is *not* necessary to perform microscope counts of these blank samples.

7. Data Recording and Archiving

The procedures outlined here are similar to those used in other test protocols published by the Alliance for Coastal Technology (ACT; www.act-us.info). An example protocol describes the data recording procedures for water quality analyses (MERC, 2013). Data sheets for direct microscope counts are filled out as the sample analysis is underway. The datasheets are signed upon completion and stored until the data are manually logged into a digital file. The Quality Officer (QO) verifies that each datasheet has been completed and correctly logged into a digital format. Data reported by the instrument will be manually transcribed on formatted data sheets, which will be tailored to each instrument's data output. Additionally, data from other analyses will be recorded in standard formats such as data collection forms, bound and paginated laboratory and field notebooks, spreadsheets, and electronic data files.

Hand-written data logs and records are submitted to a team member familiar with the parameter for review, and the originator and the reviewer both sign, date, and initial the form. The

originator creates a digital copy of the document, and both the digital document and the hand-written form are turned into the data manager (DM) at the end of the day. The DM files the hard copies in a binder specific to the trial and uploads the digital scans to a secured website that is backed up daily to an offsite location. All the documents (both hand-written and digital) must include the test cycle number, the sample date, and other relevant metadata. Data on hand-written forms are also transcribed into electronic data files. Each of the fields is included in a raw data table in a spreadsheet compatible with Microsoft Excel. Data on data sheets are manually entered into the spreadsheet and the entry is verified by a second analyst. Datasheets will have signature lines for the analyst recording the data, entering the data, and reviewing the data entry; all analysts will sign the data sheet analysis collecting the data upon the completion and verification of their task. Data sheets are assigned a unique identification code so that the data in electronic data tables can be quickly traced back to the original data sheet.

If off-site analysis becomes necessary, chain-of-custody procedures are strictly followed for all samples that are prepared or collected and sent to a contracted analytical laboratory. The possession of the samples from its time of collection until the time of analysis must be well documented and traceable. The chain of custody form will track the sample release from the sampling location to the analysis laboratory. Each sample will contain a unique identification number, the sample date and time, the sample type (e.g., ambient seawater, lake water), a description of its contents (e.g., "ambient organisms" or "*T. marina* (culture CCMP898): marine microalgae," and the analyses required. The original chain-of-custody form will remain with the samples at all times, and each form is signed by the person relinquishing the sample once that person has verified its accuracy. Chain-of-custody forms are archived in the same manner as the data sheets (described above).

8. Data Analysis

Accuracy is measured as the portion of tests that correctly assess whether a sample meets or exceeds the discharge standard, but samples with concentrations near the discharge standard should be weighed higher in the final calculation. Precision is measured as the variation among replicate readings and subsamples. Additional analyses (such as establishing the dynamic range and determining linearity by plotting fluorescence or calculated cell concentrations versus actual cell concentrations) may also be performed (see **Appendix B** for a further discussion of data analyses). The critical data point is the metric that indicates whether a sample meets or exceeds the discharge standard. The instrument should clearly indicate this "meets or exceeds" metric or display a value that is simple to interpret (e.g., "if the output is greater than a certain value, the sample exceeds the discharge standard"). The disposition specified by the instrument will be compared to the mean concentration of organisms as determined by direct counts. For example, if the mean cell concentration is 15 organisms mL^{-1}, and if the instrument specifies, "exceeds," the data point is considered to be correct. Thus, the main metric has a binary outcome—the instrument can only be correct or incorrect. A simple logistical regression analysis is commonly

used for this type of scenario, where a binary outcome is compared to a continuous variable (e.g., organism concentration).

9. Quality Management

Work performed for this project will be conducted following the quality management system (QMS) developed by the Alliance for Coastal Technologies (ACT). The QMS is a comprehensive set of policies, processes, and procedures that ensure that the quality of data, products, and services consistently meet or exceed meeting the clients stated quality requirements and comply with all applicable quality standards. The QMS also ensures that data collection and processing activities are carried out in a consistent manner, to produce data of known and documented quality that can be used with a high degree of certainty by the intended user to support specific decisions or actions regarding technology performance. The QMS provides the framework for quality assurance (QA) functions, which cover planning, implementation, and review of data collection activities and the use of data in decision making, and quality control (QC), which is a technical function that includes all the scientific precautions that are needed to acquire data of known and adequate quality.

Preventive actions will be made throughout this evaluation to anticipate and resolve potential problems before the quality of performance is compromised. The QA/QC procedures for this evaluation will follow the requirements described in this protocol, any vendor specified requirements, and the general principles and specific QA/QC from technical documents for measuring fluorescence in aquatic systems. Technical staff has the responsibility to identify problems that could affect data quality or the ability to use the data. Any problems that are identified will be reported to the Principle Investigator (PI), who will work with the Quality Assurance (QA) Manager and Technical Advisory Committee (TAC) to resolve any issues. Action will be taken to control the problem, identify a solution to the problem, and minimize losses and correct data, where possible.

9.1. Quality Control Requirements

Quality control measures are implemented by technical staff and monitored by the PI. These provide information on data quality on a day-to-day basis to ensure the integrity, correctness, and completeness of the collected data and include:

- Duplicate sampling to ensure sample representativeness with respect to sampling and handling procedures. The acceptable range of relative percent difference between a sample and its duplicate is 10%.
- Replicate analysis to ensure sample representativeness with respect to sample processing and analysis. Triplicate measurements will be done on every field sample. The acceptable range of relative standard deviation among replicate analyzes is 10%.

- Calibration and maintenance procedures, schedules, and standards (certified reference materials) for all equipment used in the tests.

The responsibility for interpreting the results of QC checks and resolving any potential problems resides with the PI.

9.2. Quality Assessment

Quality assessments include technical audits and data quality assessments. Fundamental principles of the assessment process include:

- Assessments are performed by the QA Manager, who is independent of direct responsibility for performance of the Verification.
- Each assessment is fully documented.
- Each assessment must be responded to by the appropriate level of the testing team. Quality assessment reports require a written response by the person performing the inspected activity, and acknowledgment of the assessment by the PI.
- Corrective action must be documented and approved on the original assessment report, with detailed narrative in response to the assessor's finding. Initials and date are required for each corrective action response. Acknowledgment of the response will be provided by the PI.

9.3. Technical Audits

Technical audits are systematic and objective examinations of the verification test implementation to determine whether data collection activities and related results comply with the test protocol, are implemented effectively, and are suitable to achieve its data quality goals. Audits for the evaluation will include: 1. Technical system audits and 2. Audits of data quality. The PI is responsible for ensuring that audits are conducted as part of this verification.

9.3.1. Technical System Audit

A Technical System Audit (TSA) is a thorough, systematic, and qualitative evaluation of the sampling and measurement systems associated with a Verification test. The objective of the TSA is to assess and document the conformance of on-site testing procedures with the requirements of the test protocol, published reference methods, and associated procedures. The TSA assesses test facilities, equipment maintenance and calibration procedures, reporting requirements, sample collection, analytical activities, and QC procedures. For this project, assessing compliance tools based upon the variable fluorescence of chlorophyll *a*, both laboratory and field TSAs are performed.

The QA Manager will conduct a TSA of the laboratory component and at least one field test during the verification. The TSA is performed following the Environmental Protection Agency (EPA) document Guidance on Technical Audits and Related Assessments for Environmental

Data Operations (EPA QA/G-7, January; 2000). A TSA checklist based on the test protocol is prepared by the QA Manager prior to the TSA and is reviewed by the PI. At the close of the TSA, an immediate informal debriefing will be conducted. Non-conformances are addressed through corrective action. The QA Manager will document the results of TSAs and any corrective actions in a formal audit report.

9.3.2. Audit of Data Quality

An Audit of Data Quality (ADQ) is a quantitative evaluation of the verification test data. The objective of the ADQ is to determine if the test data were collected according to the requirements of the test protocol and associated procedures and to verify whether the data were accumulated, transferred, reduced, calculated, summarized, and reported correctly. The ADQ assesses data accuracy, completeness, quality, and traceability.

The ADQ is conducted after data have been 100% verified by the technical staff. The ACT QA Manager conducts the ADQ. The ADQ entails tracing data through their processing steps and duplicating intermediate calculations. A representative set of the data (10%) is traced in detail from raw data and instrument readouts through data transcription or transference through data manipulation through data reduction to summary data, data calculations, and final reported data. The focus is on identifying a clear, logical connection between the steps.

Problems that could impact data quality are immediately communicated to the PI. The results of the ADQ are documented in a formal audit report with conclusions about the quality of the data from the verification and their fitness for their intended use.

9.4. Audit Reporting

The QA Manager is responsible for all audit reports. These written reports:

- identify and document problems that affect quality and the achievement of objectives required by the test protocol and any associated procedures;
- propose recommendations (if requested) for resolving problems that affect quality;
- independently confirm implementation and effectiveness of solutions;
- identify and cite noteworthy practices that may be shared with others to improve the quality of their operations and products;
- provide documented assurance that when problems are identified, further work performed is monitored carefully until the problems are suitably resolved.

9.5. Data Quality Assessment

The QA manager reviews all data so that only sound data that are of known and documented quality and meet technology testing quality objectives are used in making decisions about technology performance. Data assessment is conducted in two phases. The first phase, data

verification and validation, consists of reviewing and determining the validity of the analytical data. The second phase, usability assessment, consists of interpreting the data to determine its applicability for its intended use.

9.5.1. Data Verification

Data verification is the process of evaluating the completeness, correctness, and consistency of the test data sets against the requirements specified in the test protocol. Data verification is conducted by the QA Manager. The process includes verifying that:

- the raw data records are complete, understandable, well-labeled, and traceable;
- all data identified in the test protocol has been collected;
- instrument calibration and QC criteria were achieved;
- data calculations are accurate.

Corrective action procedures are implemented if data verification identifies any non-compliance issues.

9.5.2. Data Validation

Data validation evaluates data quality in terms of accomplishment of measurement quality objectives, such as precision, bias, representativeness, completeness, comparability, and sensitivity. Data validation:

- establishes that required sampling methods were used and that any deviations were noted;
- ensures that the sampling procedures and field measurements met performance criteria and that any deviations were noted;
- establishes that required analytical methods were used and that any deviations were noted;
- verifies that QC measures were obtained and criteria were achieved; and that any deviations were noted.

Data validation is performed by the QA Manager. Any limitations on the data and recommendations for limitations on data usability are documented.

9.5.3. Data Usability

Data usability assessments determine the adequacy of the verified and validated data as related to the data quality objectives defined in the test protocol. All types of data and associated information (e.g., sampling design, sampling technique, analytical methodologies) are evaluated to determine if the data appear to be appropriate and sufficient to support decisions on technology performance.

A data usability assessment has an analytical and a field component. An analytical data usability assessment is used to evaluate whether analytical data points are scientifically valid and of a sufficient level of precision, accuracy, and sensitivity. The field data usability assessment evaluates whether the sampling procedure (e.g., sampling method, sample preservation and hold times) ensures that the sample that is collected for analysis is representative.

9.6. Corrective Action

Corrective action is implemented in response to any situation that compromises the quality of testing or data generated in the execution of this project. The need for corrective action can be identified by any project personnel and implemented with the prior approval of the PI, in consultation with the QA Manager. The PI is responsible for determining appropriate corrective action to address an issue. Any findings that have a direct impact on the conduct of the verification test will be corrected immediately following notification of the finding. Implementation of corrective actions must be verified by the QA Manager to ensure that corrective actions are adequate and have been completed. This will be done in real-time if corrective actions can be immediately performed. All corrective actions are documented. Any impact that an adverse finding had on the quality of the verification test data is addressed in the test report.

10. References

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Appendix A: Direct Counts of Organisms ≥10 and <50 µm

A1. Preparation of fluorochrome stock solutions

Fluorescein diacetate (FDA) stock preparation
Weigh approximately 100 mg of dry FDA powder on a lab scale. Using the ratio of 0.048 mL DMSO to 1 mg FDA, add DMSO to yield a 50 mM solution (the dried powder can be reconstituted in the plastic weight boat or within a 1.5-mL centrifuge tube). The volume of DMSO will depend upon the final weight of material. For example, exactly 100 mg of FDA will require 4.8 mL of DMSO to yield a 50 mM solution. Add 20 µL of the 50 mM FDA solution to 980 µL of DMSO to create a 1 mL volume of working stock solution (1 mM). Divide the 1 mL volume (1 mM working stock) into 100 µL aliquots and dispense into individual centrifuge tubes (1.5 mL capacity). Label each tube as FDA and indicate the date of reconstitution and the concentration. Store stock solutions at -20°C.

Chloromethyl fluorescein diacetate (CMFDA) stock preparation
Completely thaw a 50-µg vial of CMFDA (special packaging option from the manufacturer). Add 430 µL of DMSO to the vial and vortex to mix. The concentration of the stock solution is 250 µM. Divide the working stock into 100 µL aliquots and dispense into individual centrifuge tubes (1.5 mL capacity). Label each tube as CMFDA and indicate the date of reconstitution and the concentration. Store stock solutions at - 20°C.

A2. Fluorescently labeling protists

Add 10 µL of CMFDA (250 µM stock solution) and 5 µL of FDA (1 mM stock solution) to an empty centrifuge tube (1.5 mL capacity). Then, add 985 µL of sample to the centrifuge tube. Mix by pulsing with a pipettor and close the tube. Incubate the centrifuge tube in the dark for 10 min. Use a timer set for 10 min with an alarm to notify when the incubation period has completed.

A small amount (~10 µL of concentrated micro-bead suspensions) of 50 µm and 10 µm fluorescent microbeads should be added to the counting chamber before addition of labeled sample. The microbeads are used as a reference allowing the observer to count only the organisms in the defined size class (≥ 10 µm and < 50 µm). Upon completion of the incubation, the sample should be loaded into a gridded SR chamber. The cover glass is slid across the chamber as sample is added to prevent the loss of sample due to overflow.

The sample dilution due to fluorochrome addition should be incorporated into the final concentration calculation by multiplying the volume analyzed by the dilution factor. For example, the sample volume of 1 mL consists of 0.985 mL of sample and 0.015 µL of fluorochrome solutions; if 0.5 mL of sample is analyzed, the cell count should be divided by 0.4925 mL to estimate concentrations. Analysis should be completed within 20 min after the start of the incubation. CMFDA and FDA stock solutions should be refrozen at -20°C after use.

A3. Epifluorescence microscopy

Turn on E600 epifluorescence (or AZ100 if E600 is not available) mercury bulb to allow bulb to heat up (~5 min) and run at optimal level. Place the gridded counting chamber with the protist sample on the stage of microscope. The rows assigned to be counted are determined prior to analysis via a random number generator (MS Office Excel; See below). Lists of randomly assigned rows are only used once. Move the stage horizontally to the row of the randomly assigned row. The microscope can be configured to allow both brightfield and epifluorescence illumination simultaneously. In this configuration, the green fluorescence filter set is in place (causing a slight discoloration of the sample when viewed using brightfield illumination).

The observer should move horizontally across the row of the SR chamber examining each square until a protist is identified. If protist exhibits a green fluorescence (or is moving), the observer should mark the protist as "live" and categorized into one of the following categories: ***Crustacean Nauplii, Flagellates, Rotifers, Dinoflagellates, Ciliates, Annelids, Smooth Worms, Flat Worms, Trochophors /Veligers, Diatoms, and Other (not otherwise specified).***

If a mobile protist moves into or out of the square while in the field of view of the microscope, it should still be counted. That is, cells do not need to remain in the field of view throughout the analysis period. After the entire square is assessed for protist viability, the illumination should be switch back to brightfield and the observer should move horizontally to the next square. When the entire row has been assessed, the observer should move vertically on the SR chamber to the next randomly selected row and repeat the steps above. The observer should continue quantifying viable protists for 20 min after the completion of the incubation. Typically, between 12 and 35 µL are counted per min.

A4. Generating Random Numbers

Random row order assignments are created using spreadsheet software with a random number generator (Microsoft Excel 2007, Microsoft, Redfield, WA). The examples in this section are specific to Excel. However, other programs capable of generating random numbers and ranking number lists can also be used to generate random row counting assignments.

A table of random numbers is generated by using the Excel function, *rand()*. The number of columns (*n*) can be determined by the number of sample wells (each column will yield row assignments for 1 plate). There should be exactly 20 rows in the table and all of the cells should have the following: = rand(). A secondary table is created with n columns and 20 rows (Table A1, random numbers).

Once a series of random numbers is generated, a ranking function is used to determine the counting order. The Microsoft Excel spreadsheet includes the ranking function, rank (r1c1,range), where r1c1 is the row number and column number and range is the data range (Table A2, Ranked Row Order). In the table below, the data range is r1:r20 in column 1.

The entire SR counting chamber is rarely counted due to the limits of sample analysis time (20 min). It is possible that protists would be sparse enough to allow for the analysis of an entire chamber. However, typically 5 – 10 rows are counted in the analysis time. Each set of random row assignments should only be used once. The row should be marked or crossed out to indicate the counting order has been used. New counting tables can be rapidly generated in Excel by refreshing the random numbers (Table C1). This is performed by hitting the "delete" key in an empty spreadsheet cell. Once the new list is generated, the table should be printed and the printout stored near the microscope. Typically, 10 unique tables (each with assignments for 8 slides) are generated at a time.

Table B6. Example table generated in Microsoft Excel demonstrating the routine for generating random row counting orders. The first two rows in the first column (Slide 1) show the Excel formula.

Random numbers (each slide = 20)			**Ranked Row Order**			
Slide 1	**Slide 2**	**Slide 3**	**Counting Order**	**Row No. Slide 1**	**Row No. Slide 2**	**Row No. Slide 3**
=rand()	0.76	0.15	1st	***=rank(r1c1, range)***	3	14
=rand()	0.96	0.24	2nd	***=rank(r2c1, range)***	1	11
0.45	0.22	0.39	3rd	10	17	9
0.90	0.75	0.09	4th	4	4	18
0.66	0.71	0.13	5th	7	6	16
0.19	0.21	0.18	6th	17	18	13
0.96	0.28	0.44	6th	2	13	6
0.97	0.43	0.27	8th	1	10	10
0.26	0.61	0.14	9th	15	8	15
0.26	0.22	0.39	10th	14	16	8
0.61	0.30	0.18	11th	8	12	12
0.40	0.24	0.80	12th	12	15	1
0.09	0.74	0.68	13th	19	5	2
0.74	0.27	0.60	14th	5	14	3
0.14	0.01	0.09	15th	18	20	17
0.68	0.50	0.48	16th	6	9	5
0.43	0.62	0.03	17th	11	7	19
0.22	0.42	0.00	18th	16	11	20
0.94	0.81	0.40	19th	3	2	7
0.36	0.16	0.60	20th	13	19	4

Appendix B: Tangential Measurements and Data Analyses

B1. Gravimetric Measurements of Sample Volume

Transferring volumes of liquid ranging from 1 µL to 5 mL is best performed using calibrated (and calibration-verified) volumetric pipettors. In many cases, the quantity of water is best measured gravimetrically on a calibrated and calibration-verified balance. In particular, gravimetric measurements of volume are useful for measuring the concentrated water after sieving. For example, a known volume of sample water (e.g., 200 mL) is concentrated and the concentrated water is rinsed into a 50-mL centrifuge tube. In this case, the volume may be estimated from the gradations on the tube, but gravimetric measurements will indicate the mass added (after subtracting the tare weight of the tube) with much greater precision. The volume (V) is calculated from the sample mass (M) by estimating the density of the sample (D). The density can be estimated using standard equations that incorporate the temperature and salinity of a sample (Fofonoff 1985):

Eq. 4 $$V = \frac{M}{D}$$

For the measurements of large volumes (e.g., sample carboys which will contain approximately 10 L of sample water), the pre-weighed container is weighed on a balance with at least 0.5 g resolution. Smaller sample volumes can be measured on balances of higher precision. For samples <100 mL, measurements with precision of 0.1 mg (~0.1 mL) are achievable on a calibrated bench-top balance.

B2. Measurements of Particulate Organic Matter and Mineral Matter

A 0.5-L volume of water for water quality analysis should be removed from the collected water immediately after collection and mixing by inversion. The water quality analyses are performed following standard procedures (US EPA, 1983). Briefly, total suspended solids are measured by filtering 100-500 mL of the sample water through a pre-combusted, glass fiber filter (GF/F, effectively retaining particles >0.7 µm). The filtrate is collected for analysis of dissolved organic matter (described below). After the filtrate is removed, the filter was rinsed three times with Type I water, which has been deionized and purified by reverse osmosis. Three rinses of 50 mL are used to remove residual dissolved salts. After the rinsing, the filter is dried at 104°C for 3 h. In previous experiments, this time was sufficient to achieve a constant mass. The difference between the dry filter mass and the mass of the filter prior to filtration is the mass of total suspended solid (TSS).

This filter is then combusted at 550°C in a combustion oven for at least 20 min. The filter is cooled to room temperature and reweighed. The mass remaining is the non-combustible material or mineral matter (MM). The difference between the mass of TSS and MM is the particulate organic matter (POM). All these mass quantities are converted to concentration by adjusting the mass to the volume filtered.

B3. Data Analyses

Determining Linearity

The linear response of an instrument is a measure of the proportionality of a signal produced along a range of quantities of a measurand (ISO 2008). In this case, the measurand (i.e., the quantity measured) is the concentration of cells in the sample. All samples yielding a valid measurement (i.e., samples within the dynamic range) will be used for linear regression analysis. Additionally, seawater samples will be prepared to measure the linear response of the instrument when analyzing a mixed assemblage of ambient organisms.

Determining Accuracy and Precision

Accuracy is a measure of the difference between a measurement and the actual or expected value, whereas precision is a measure of the variation among repeated analyses. Chemical or biological standards are not available for measurements of variable fluorescence as the complex and dynamic reaction of chlorophyll *a* to excitation is difficult to reproduce consistently. Therefore, accuracy is measured as the standard error of the estimate, which is calculated as part of linear regression routine. However, since the instruments may not actually produce a numerical output on a linear scale, accuracy will be primarily measured as whether the instrument correctly determines if a sample meets the discharge standard, with adjustments to consider the difference between the actual concentration and the numerical standard. Precision is measured as the coefficient of variation among subsamples (separate aliquots of the same source sample) and repeated readings of a single subsample. In both cases, repeated analyses are not independent, but represent the range of variation within the sample among readings. Precision is measured as the coefficient of variation (*CV*), which is calculated from the mean ($\overline{x}$) and standard deviation (σ) of the measurements:

Eq. 5
$$CV = 100 \cdot \frac{\overline{x}}{\sigma}$$

Accuracy

Accuracy is measured as the proportion of samples that correctly assess whether a sample meets the discharge standard. Because samples near the discharge standard will be more difficult to correctly assess, these samples will be weighed higher in the final assessment. For all samples, a positive result (in which the instrument correctly predicted whether the concentration of organisms was above or below 10 mL^{-1}) is set equal to 1. A negative result, where the instrument incorrectly predicted the outcome, is set equal to 0. In this manner, incorrect results will not contribute to the accuracy of the instrument. Correct readings will be adjusted to consider the differences between the concentration and the discharge standard using the following adjustment factor (*A*):

Eq. 6 $$A = \frac{\log(P) - \log(DS)}{\log(DS)}$$

Where *P* is the population concentration, *DS* is the discharge standard (10 mL^{-1}). In theory, when *P* = *DS*, then *A* = 1, which is the highest adjustment factor possible. When P = 5 mL^{-1}, A =0.7. The adjustment factor, however, may be changed so that the factors are normally distributed around the mean of
10 mL^{-1}.

Precision

From each sample, ten subsamples are aliquoted by first mixing the sample vessel three times, opening the vessel, and aspirating 3 mL from the center of the water. The sample is aspirated with a volumetric pipette, which should be verified for accuracy prior to use. The five subsamples each are deposited into separate cuvettes specific for the instrument, and each of the five subsamples is analyzed following the protocol described by the manufacturer**.**

B4. References

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Appendix C: Tentative Testing Schedule

Table C7. Tentative testing schedule. NRL: Naval Research Laboratory; GSI: Great Ships Initiative; SERC: Smithsonian Environmental Research Center

Testing Event	**Testing Location**	**Tentative Dates**
Field Test 1	NRL; Key West, FL	June 1 – 4, 2015
Field Test 2	GSI; Superior, WI	July 12 – 17, 2015
Field Test 3	SERC; Edgewater, MD	August 8 – 11, 2015
Laboratory Tests	NRL; Key West, FL	September 7 – 11, 2015