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Feasibility of Developing a Protocol for Automated Protist Analysis

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16. Abstract (MAXIMUM 200 WORDS)

Ballast water treatment systems must be stringently tested relative to the Coast Guard's proposed ballast water discharge standard for live organisms prior to Coast Guard approval for routine shipboard use. Analysis for live organisms in sparsely populated samples is time limited and labor intensive. Skilled staff must use a microscope to observe, count, and verify the viability of live organisms greater than or equal to 10 µm and less than 50 µm in minimum dimension. This program built upon previous work performed by the Naval Research Laboratory to develop data management, data archive, and data analysis protocols for counting and determining the viability of organisms greater than or equal to 50 µm in minimum dimension. Results documented in this report clearly demonstrate the protocols are appropriate for organisms in the smaller size class, and they provide a high degree of confidence that automated methods can be further developed to process samples during production testing to determine the efficacy of ballast water management systems.

The findings in this report are the result of initial research into the potential to automate protist analyses and provide the most current information available at the conclusion of this initial research effort. Additional automation research efforts are ongoing and may affect details reported herein.

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EXECUTIVE SUMMARY

Ballast water is a known pathway for the introduction of non-indigenous species. In an effort to reduce the number of introductions of non-indigenous species into United States waters, the U. S. Coast Guard has proposed initial regulations requiring ships to meet stringent discharge standards. The proposed concentration for organisms ≥ 10 μm to < 50 μm in minimum dimension (nominally protists) is 10 living organisms per milliliter of ballast water. This concentration level may later be reduced (i.e., a more-stringent standard) if ballast water treatment systems can meet the initial standard. Before Coast Guard can approve treatment systems for routine use aboard ships, Coast Guard must verify they are capable of meeting the initial discharge standard.

A protocol for testing ballast water treatment equipment at full scale has been developed by Coast Guard and the Environmental Protection Agency's Environmental Technology Verification Program. Testing requires evaluating treated samples to determine the number of living organisms present. Current manual methods require skilled personnel using microscopes to observe, enumerate, and determine viability of organisms in concentrated samples within six hours of samples being taken. At present, this visual analysis is labor intensive, is subject to operator fatigue, and provides no detailed archive of results. The Coast Guard Research and Development Center, therefore, sought a method to automate analyses of organisms in the ≥ 10 μm to < 50 μm size class.

The Naval Research Laboratory in Key West, FL has previously shown the benefits of implementing detailed data management, data collection, and data analysis protocols for collecting and automating analyses of organisms ≥ 50 μm in minimum dimension (nominally zooplankton). This report focuses on recent activities to determine the feasibility of applying similar methodologies to organisms in the ≥ 10 μm to < 50 μm size class.

The results provided in this report provide a clear indication that automated analysis algorithms can be used to enumerate and determine the viability of organisms in the ≥ 10 μm to < 50 μm size class. This approach should significantly reduce analysis times and provide a more consistent means of analyzing and reporting the results from evaluations of ballast water management systems.

Recommendations for next steps include optimization of a multi-well sample tray to match the microscope's depth of focus such that organisms remain in focus throughout the observation time window. Concentrated natural samples should be used to optimize the system settings including the observation time window. The natural samples will also aid in determining the potential benefits of motion for assessing protist viability. Switches to monitor system settings should be developed such that settings are automatically recorded and archived with sample images. Improvement of light switching capabilities between brightfield and epifluorescence modes will allow more images to be collected in a shorter period of time and thus more samples could be analyzed within the six-hour timeframe of viability. Development of an operator friendly graphical user interface will provide ease of use for the analysis protocol.

The findings in this report are the result of initial research into the potential to automate protist analyses and provide the most current information available at the conclusion of this initial research effort. Additional automation research efforts are ongoing and may affect details reported herein.

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LIST OF ACRONYMS, ABBREVIATIONS, AND SYMBOLS

BWMS	Ballast water management system
CMFDA	5-Chloromethylfluroscein diacetate
CCD	Charged coupled device
DIA	Diascopic (brightfield)
FDA	Fluoroscein diacetate
GFP	Green fluorescent protein
GUI	Graphic user interface
m^3	Cubic meter
ml	Milliliter
mm	Millimeter
ms	Millisecond
ND	Neutral density
NRL	Naval Research Laboratory
PC	Personal computer
RGB	Red green blue
X	Magnification power
µL	Microliter
µm	Micrometer
µM	Micromol
<	Less than
>	More than
.nd2	Image file type
.xls	Excel® Spreadsheet file type

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1 BACKGROUND AND INTRODUCTION

Testing to evaluate the efficacy of ballast water management systems (BWMS) requires the characterization of samples to determine the number of live organisms following treatment. Standardized BWMS testing requires that organisms be characterized in three size classes based on maximum dimension of the smallest axis: organisms ≥ 50 micrometers (μm) (nominally zooplankton), organisms ≥ 10 μm to < 50 μm (nominally protists), and organisms < 10 μm (nominally bacteria).

The Naval Research Laboratory (NRL) recently published protocols for the collection of image sets, test documentation, image set analysis, and data archive for organisms in the ≥ 50 μm size class (Nelson, et al., 2010). That document additionally describes the equipment and software required to implement this "zooplankton" sample analysis protocol. The focus of this document is to identify the portions of the zooplankton protocol that are applicable to the analysis of organisms in the ≥ 10 μm to < 50 μm size class, or "protist" samples. It is additionally desired to identify specific elements of the zooplankton protocol that will need to be modified for working with ≥ 10 μm to < 50 μm size class organisms as well as new capabilities required for analysis of organisms in this smaller size class

With respect to organisms in the ≥ 10 μm to < 50 μm size class, Phase I of the U.S. Coast Guard proposed discharge standard requires that following treatment, there be less than 10 living organisms per milliliter (ml) (Federal Register, 2009). Statistical analyses suggest that to reliably characterize a sample for this concentration of viable organisms, 3-6 liters of sample should be concentrated to 1 liter. From this 1-liter concentrated sample, two to four 1-ml samples must then be analyzed (Lemieux et al., in review). To keep organisms slightly larger than 10 μm in focus in a sample, the typical sample volume will be approximately 20 microliters (μL), which necessitates that between 100 and 200 individual sample wells be analyzed to determine if a sample is compliant with discharge standards.

Because standardized tests also require that suspended solids and other water properties (e.g., mineral matter and dissolved organic carbon) fall within specified ranges, concentrating samples by 6:1 will also increase the amount of suspended and dissolved solids in samples in the same general size-range as the organisms being assessed. This process significantly increases the complexity of samples since organisms can be obscured from view by sample debris.

The requirement to analyze a large number of samples, combined with the complexity and diversity of organisms in the ≥ 10 μm to < 50 μm size class, along with the addition of suspended solids and other dissolved material in this same size-range make it challenging to accurately characterize protist samples using manual microscopy methods.

The motility of organisms in the ≥ 10 μm to < 50 μm class is an additional concern. Although many organisms in this size class are relatively non-motile, others are extremely motile and can move across an entire sample well in seconds. Further, suspended solids in the sample can allow organisms to "hide" or be obscured by debris during observation. Given the motility of some organisms, it is critical that the entire sample well is visible when conducting analyses. The alternative—magnifying selected regions of the field of view—provides information regarding only those organisms in the magnified region, which can result in organisms being under- or overcounted.

The requirement to observe the entire sample well can be addressed using a microscope and camera system with sufficient spatial resolution. When these systems provide adequate spatial resolution, it becomes feasible to "zoom in" on the individual organisms in the digital image set *after* the data has been collected from the entire sample well. If motility becomes important to determining the viability of protists, then time-resolved image sets can be collected, and motility algorithms can be directly applied to these image sets.

In an effort to find a method suitable for automation, NRL convened a workshop in January 2008 to compare multiple methods determining viability in the ≥ 10 μm to < 50 μm size class. Statistical analysis of the multiple experiments indicated that although there were significant differences between the methods, no one method could be considered superior to all others (Nelson et al, 2009). As a result of this workshop, NRL pursued the development of additional methods using manual microscopy.

NRL explored a variety of vital stains and mortal stains to determine the viability of protists in the ≥ 10 μm to < 50 μm class. During this effort, NRL stained ambient plankton samples as well as algal monocultures with numerous biological stains, both vital and mortal. Results of over 100 trials showed that no stain, when used alone or in combination, adequately stained all protists. Stains evaluated included Neutral Red, Aniline Blue, CellTracker™ Green (CMFDA), CellTracker™ Blue, SYTOX Green, SYTOX Orange, Calcein AM, and Fluorescein Diacetate (FDA). The work performed using stains has been summarized in a 2009 NRL Letter Report (Drake et al., 2009).

Although no single stain or combination of stains provided perfect results across all samples analyzed, one approach provided more consistent results than all of the others: a combination of two vital stains: Fluorescein Diacetate (FDA) and 5-chloromethylfluorescein diacetate (CMFDA, CellTracker™ Green) (both stains from Molecular Probes-Invitrogen; Carlsbad, CA; final concentration 5 micromol (μM) and 2.5 μM, respectively). After entering living cells, non-specific esterases in the cell cleave these stains, resulting in a molecule that fluoresces green when excited with blue light. Work performed at NRL indicates that by using a combination of these two stains, the viability of a greater number of organisms can be determined (compared to using these stains individually). This work also has demonstrated that many organisms' fluorescence signals can be observed even when an organism is obscured by debris.

Recent work has focused on performing measurements at a variety of geographic locations to demonstrate that these stains provide a location-independent means to identify viable protists in test samples (Steinberg et al., in prep.). This method, unfortunately, is limited because during experiments some heat-killed or freeze-killed protists fluoresced with these vital stains. Until a greater number of representative complex protist samples are analyzed, it is too early to determine if the staining of protists can be used exclusively to determine viability or whether staining will need to be used in conjunction with motility algorithms.

The major objective of this program was to determine if automated methods could be used to enumerate and determine the viability of organisms in the ≥ 10 μm to < 50 μm size class. Secondary objectives were to determine which portions of the zooplankton (≥ 50 μm size class) protocol are relevant to protist sample analysis and to identify how these protocols need to be modified to support data collection, analysis, and archive when working with organisms in the ≥ 10 μm to < 50 μm size class.

This report will first describe the equipment and software required to collect data, including images, that clearly demonstrate the applicability of this equipment and software for protist analysis. Description of data management and data collection protocols follow. Although originally developed to support work with

zooplankton, the same protocols are directly applicable to protist samples. Next, the methods used for data analysis are discussed along with an example using analysis of a protist sample. Finally, the results obtained to date are summarized, and the steps required to advance the technology to the level necessary to support production testing are provided.

2 APPROACH

2.1 Equipment

To characterize organisms in the ≥ 10 μm to < 50 μm size-range using an automated protocol similar to that developed for zooplankton, it is necessary to use two microscope modalities (i.e., brightfield [white light] and epifluorescence measurements). Combining these microscope modalities in conjunction with the NRL sample-staining procedure allows the user to assess the viability of a wide range of organisms in the complex samples associated with BWMS evaluations. Additionally, spatially correlated image data from both of these modalities is desired. Therefore, image sets from brightfield and epifluorescence must be collected in an interleaved fashion, not collected in series.

To support the development of the zooplankton automation protocol, NRL made modifications to its microscope-camera system to collect interleaved time series of both brightfield and epifluorescence image sets. This task was accomplished using a mechanical shutter, under computer control, that switches between brightfield and epifluorescent modes. For the analysis of zooplankton samples, 62 images are collected (31 brightfield and 31 epifluorescent) over approximately 32 seconds.

These same microscope modifications are relevant to the analysis of protist samples because viable organisms that hydrolyze the vital stain can be detected in the epifluorescence images and then identified in the brightfield images. The number of images that should be collected and the optimum observation windows need to be defined for protist samples. Additionally, the potential benefits of applying motility algorithms similar to those used with zooplankton samples need to be determined. These parameters will be determined as more complex and representative protist samples are analyzed.

2.2 Data

The data management protocols that were developed to support zooplankton analyses are relevant—as written—for data management of protist samples. These protocols are detailed in Nelson et al., 2010 and are summarized here.

Data management and the documentation of system settings are critically important and assure the proper collection of image sets and archiving of test results. With the protocol developed by NRL for data management, all data associated with a given sample well analysis are stored in two files: a single, large file that contains all image data as well as certain key system settings (denoted as an *.nd2 file), and a test-specific Excel® spreadsheet (with embedded macros and forms). The microscope operator fills out specific fields in this spreadsheet to document all system settings. The spreadsheet and its embedded forms also provide a checklist to ensure that the operator has made all necessary system adjustments prior to data collection.

These files (and consequently the data associated with testing) are automatically stored in a directory with an intuitive structure using standardized file naming conventions. The file naming and directory structure provides a means to easily access data associated with a given test. Further, the file-naming conventions allow important information about the test to be discerned prior to analyzing the data. Also, as mentioned above, all data associated with a test are stored in only two files (both automatically generated during the data collection process) and these files archive all settings, other relevant test information, and the test data (image sets) itself associated with a given microscope measurement.

Data analysis for zooplankton is performed using a stand-alone application developed by NRL. When launched, this application provides a graphical user interface (GUI) that asks the operator to specify the *.nd2 file to be analyzed. The analysis application parses the *.nd2 file, performs all analyses, and stores its results into the same Excel® spreadsheet that was used to document test-specific settings.

In support of the protist effort, the data analysis application developed for zooplankton sample analyses was successfully used with protist samples. Indeed, the zooplankton algorithms needed no modification to analyze protist samples. This result clearly demonstrated the applicability of automated analysis algorithms in general and the transferability of the approach used for zooplankton analysis to protist analysis in particular. It is expected that a unique, stand-alone application for data analysis will be necessary to enumerate and determine the viability of protists in complex BWMS samples. This application will be refined and further developed as more complex and representative samples in the ≥ 10 µm to < 50 µm size class are collected and analyzed. It is expected that much of the stand-alone zooplankton analysis application will be used. Based on the work performed in support of this program and documented in this report, there is a high degree of confidence that with further development, automated analysis methods can be used to enumerate protists and determine their viability in the complex samples associated with BWMS testing.

3 EQUIPMENT AND SOFTWARE REQUIREMENTS

The imaging system, consisting of the microscope, camera, control hardware and software (e.g., a personal computer [PC] and applications), and the accessories described here are those selected and used at NRL. This same equipment and software was originally used to implement the zooplankton automation protocol and is applicable to the analysis of protist samples. To conduct reliable, robust protist analyses, the microscope-camera system must provide a resolution of at least 2 µm while imaging a full sample well approximately 3 - 4 millimeter (mm) in diameter. The sample well should accommodate a sample volume of between 20 and 25 µL. This latter requirement is to maintain a water column consistent with a microscope's depth of focus when imaging organisms in the ≥ 10 µm to < 50 µm size class. The microscope system must also be capable of switching automatically between brightfield and epifluorescence modalities under computer control. This switching needs to be accomplished quickly enough to afford a 1 second interval between successive images that make up the image set.

Other equipment with equivalent capabilities and resolution necessary to support this protocol can be used. However, many key system parameters that are currently monitored by system software on the NRL equipment may not be recorded as image file metadata with hardware and operating software different from that recommended in this document. Further, analysis routines will need to be modified to parse image data from a different source and may need to be additionally modified if camera resolution and dynamic range are not identical to those used in the NRL system.

3.1 Sample Wells

The requirements for the microscope and camera systems are driven largely by the sample well used to analyze samples and its dimensions. Images of the entire sample well must be collected while providing sufficient resolution to allow protist details to be observed in the same images by changing the digital zoom level (the NRL system provides < 2 μm spatial resolution when zoomed in and the entire well is visible).

Building upon work previously performed to optimize the sample wells for zooplankton, NRL performed a series of evaluations on sample well plates for protists. Accordingly, commercial, off-the-shelf SensoPlate™ sample-well plates with individual sample wells of approximately 3-4 mm in diameter were considered (Greiner Bio-One; Monroe, NC). These sample wells had a geometry that should result in the required 2 μm resolution when imaging the entire sample well.

Two SensoPlate™ wells were considered: the standard SensoPlate ™ 384 and SensoPlate Plus™ 384. Figure 1 provides images collected with the NRL microscope-camera system described in the next report section. Each of the sample wells was filled with 20 μL of Instant Ocean® (Spectrum Brands; Atlanta, GA) artificial seawater. Analysis of the images indicates that both of these well plates would provide sufficient resolution to support automated protist assessments.

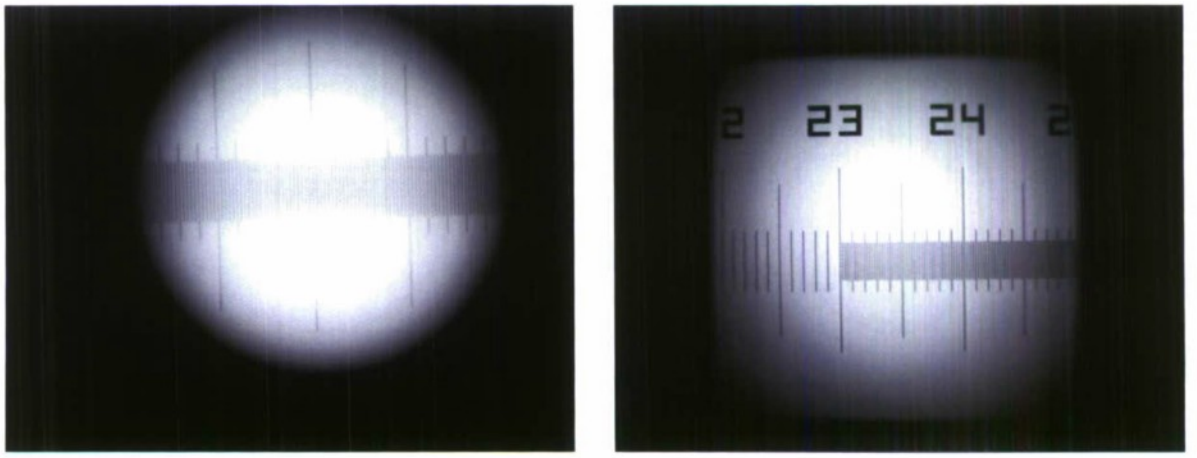

Figure 1. Images of samples wells from SensoPlate ™ 384 (left) and SensoPlate Plus™ 384 (right) plates, each with 20 μL of artificial seawater added.

Figure 2 demonstrates additional suitability of these plates for protist analysis. Shown in the figure are the brightfield and epifluorescence images of the flagellate *Tetraselmis* sp. (strain PLY 429; cultures were purchased from Reed Mariculture; Campbell, CA) in a well of a SensoPlate ™ 384. The sample consisted of artificial seawater with added *Tetraselmis* sp. (from a monoculture); the sample was stained with FDA and CMFDA according to the NRL protocol. Because *Tetraselmis* sp. are 6 to 12 μm in minimum dimension, they are at the small end of the ≥ 10 μm to < 50 μm size class. The images shown in Figure 2 demonstrate that organisms of this size can be identified in both the bright field and the epifluorescence images.

Although the initial results with commercial sample well trays are promising and demonstrate the potential for their use in support of automated protist analyses, a few elements complicate their use. First, even with a very small sample volume of 20 μL, the water column is sufficiently high to allow organisms to swim into and out of the microscope's depth of focus. Second, well edge effects make it difficult to visualize

organisms at the perimeter of the well. These effects are obviated to some extent with SensoPlate Plus™ 384 plates and their geometry, which is superior to the standard SensoPlate™ 384 . However, some distortion is still encountered with these wells because water surface tension forms a meniscus, resulting in a variable height of the water column across a sample well.

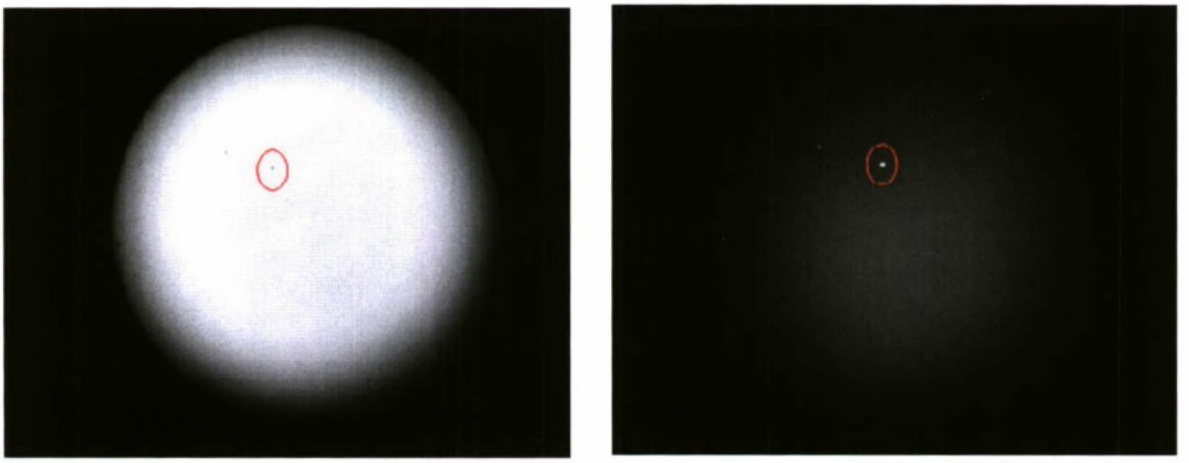

Figure 2. Brightfield (left) and epifluorescence (right) images of the flagellate *Tetraselmis* sp. in a well of a SensoPlate ™ 384.

As a result of these potential deficiencies in commercial sample well trays, the use of custom sample well trays was explored. Figure 3 provides photographs of initial work that NRL performed to explore the feasibility of developing a custom well-plate. A Sedgwick-Rafter slide was modified by inserting a machined plate containing several "sized" holes into the slide's sample well. There are several advantages of this approach. First, sample well size can be optimized and fully matched to the microscope's field of view. This feature should result in improved spatial resolution over commercial well plates. Second, the well depth can be optimized for a 20 µL sample to better match the microscope's depth of focus to the sample well depth. The wells could additionally be designed to use a cover glass to reduce the impacts of distortion due to surface tension. Lastly, this approach can be easily scaled to produce an improved multi-well tray with sample wells fully optimized for protist analyses.

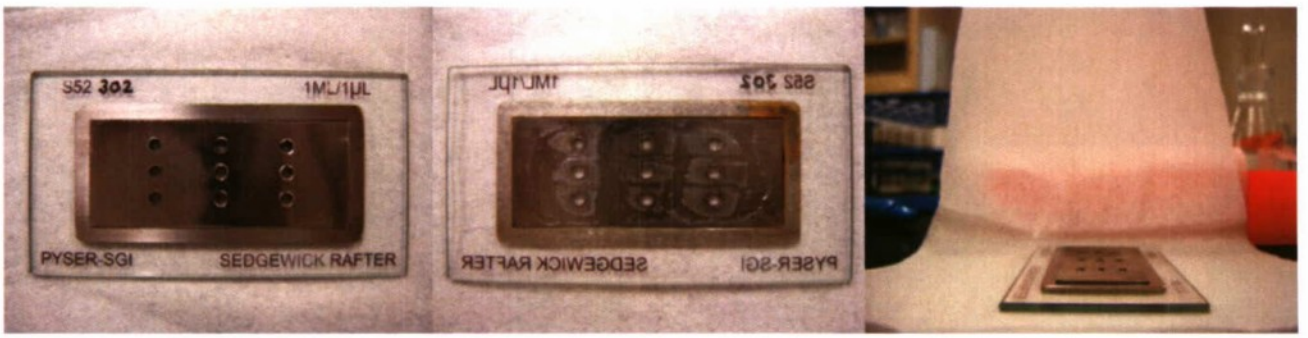

Figure 3. Photographs of a "custom" sample well tray designed by NRL. This approach may provide the ability to match well geometry to the microscope-camera system's imaging capabilities.

3.2 Microscope System

NRL worked with Nikon to design a microscope with the desired capabilities when used in conjunction with an existing Q-Imaging 1300 Retiga IEEE 1394 camera system. NRL has determined that modifications implemented in its system are also applicable to the analysis of protist samples. The capabilities in the microscope and camera system include:

- Ability to image a 3 mm – 4 mm diameter field of view with < 2 μm spatial resolution.
- Capability to collect images using brightfield illumination and epifluorescence.
- Ability to rapidly switch between brightfield and epifluorescent modes under computer control.
- Ability to move the sample well tray under computer control to allow all of the sample wells on a given tray to be imaged under computer control.
- Ability to focus the microscope under computer control.
- Ability to monitor the majority of key system settings under computer control.

The data previously shown in Figure 2 demonstrate that this microscope-camera system provides sufficient resolution for detecting small protists such as *Tetraselmis* sp. cells while imaging a complete sample well.

3.2.1 Microscope

The microscope used at NRL is the Nikon Multizoom AZ-100 Multi-Purpose Zoom Microscope. As purchased, this microscope provides basic magnification of 10X, 20X and 50X with an 8:1 variable zoom available at each magnification. Switching magnification is accomplished using a triple nosepiece that allows each of the three objectives to be rotated into position. This microscope was originally purchased with the Nikon AZ-FL epifluorescence attachment, which allows the microscope to operate in both brightfield and epifluorescence modes.

For work with protists, it is anticipated that this microscope will be operated with a 20X magnification with some variable zoom or with 50X magnification with a variable zoom level of approximately 1.

3.2.2 Light Sources

In July 2009, new light sources that allowed the switching between brightfield and epifluorescence under computer control were integrated into the NRL microscope. These light sources are additionally applicable to the optimum analysis of protist samples.

3.2.3 Sample Stage

In October 2009, a computer controllable motorized X-Y-Z stage was integrated to the NRL microscope. This stage allows each sample well on the sample well plates to be moved into position (and image sets collected) under computer control. The stage also provides the capability to focus the microscope under computer control.

This same stage control can also be used to move to a specific well to be analyzed during protist analyses. The number of wells and their locations are programmed into the image acquisition software for the automated collection of data on multiple sample wells on a multi-well tray.

3.3 Imaging and Image Acquisition Software

Nikon NIS-Elements Advanced Research Imaging System Software (Elements) is used to control the microscope system during automated data collection. This software controls the entire image acquisition process, the storing of image data into a single *.nd2 file, the switching of the light sources during image acquisition, and the movement of the motorized stage when multiple sample wells are evaluated in sequence on a single multi-well plate. Finally, the software also controls the camera settings and provides image display and image processing capabilities.

It is expected that an application macro different from the one used for zooplankton analysis will be used to control the system's imaging and image acquisition of protists. This task is easily accomplished using the Nikon Elements Software once the specific requirements for protist sample data collection and analysis are better defined.

3.4 Camera

As mentioned above, NRL uses a Q-Imaging 1300 Retiga IEEE 1394 camera. It provides a maximum resolution of 1300 x 1030 pixels using a large area CCD (charge-coupled device) detector that provides high light sensitivity (compared with consumer CCD-based imaging systems, such as video cameras). The camera is fully controllable using the NIS-Elements software. Because the camera's detector is no longer manufactured, this camera is no longer available from Q-Imaging and has been replaced by the Q-Imaging 2000 R Retiga IEEE 1394 camera, which provides 1600 x 1200 pixel resolution. As such, it will result in a higher resolution imaging system than the system that is available at NRL. For this application, the monochrome, un-cooled version of the camera is recommended.

3.5 Computer

To complete the system, a PC with at least two RS-232 ports, four USB-2 ports, and an IEEE 1394 (Firewire) interface is required.

3.6 Settings

Following the October 2009 updates to the microscope system, the majority of system settings are either directly monitored by the computer or entered by the microscope operator into the spreadsheet (as discussed in the next report section). Many of the parameters entered into the spreadsheet described in the next report section can be corroborated, as these same data are recorded in the *.nd2 files.

There are currently four parameters that are adjusted manually by the microscope operator that cannot be corroborated in the *.nd2 files. First, the operator manually adjusts (or ensures) that the variable zoom level is set to 1 for implementation of the data collection protocol. The operator is asked by the spreadsheet to confirm this zoom level prior to the start of data acquisition. Second, the filter cube position is manually set to position 2 – for the Green Fluorescent Protein (GFP) filter cube. Again, the operator is asked by the spreadsheet to confirm the filter cube position prior to the start of data acquisition. Third, the microscope operator needs to ensure that the 5 X objective (50X system magnification with the ocular lenses) is used. The operator is also asked to corroborate this point prior to data acquisition. Lastly, the microscope operator needs to ensure that the neutral density filters are in the proper configuration: the ND2 filter should be not

engaged and the ND8 and ND16 filters should be engaged. The microscope operator is asked to corroborate this configuration prior to data collection.

The total cost of a new system for implementing the zooplankton protocols as well as to implement the protist protocols is approximately $66,000 (Table 1). With the upgrades that have been made in its microscope system, the imaging system available at NRL is now configured to optimize, finalize, and implement protocols for protist analyses.

Table 1. Equipment summary and costs.

Item	Model Used	Approximate Cost
Microscope	Nikon Multizoom AZ-100 Multi-Purpose Zoom	$30,000
Light Source	Computer Controlled Light Switcher	$7,500
Sample Stage	Motorized XYZ Stage	$15,000
Imaging Software	Nikon NIS-Elements Advanced Research Imaging System Software	$3,600
Camera	Q-Imaging 2000 Retiga IEEE 1394 camera	$7,000
Computer	PC with 2 RS-232, 4 USB-2 Ports & IEEE 1394 (Firewire)	$3000
Analytical Software	Government developed	$0
TOTAL		**~$66,000**

4 DATA MANAGEMENT AND PROTIST DATA COLLECTION PROTOCOLS

Based on the recent automation work with zooplankton (≥ 50 µm), two protocols have been developed by NRL to satisfy the requirements of standardized data collection: the Data Management Protocol and the Data Collection Protocol. They are intended to be used in concert and ensure that system settings are properly set, documented, and recorded. As described below, in many cases, redundant information is recorded in the two major outputs: an Excel® Spreadsheet, which contains comments by the operator and lists the settings used, and an *.nd2 file, which contains the diascopic (DIA, transmitted light [brightfield]) and epifluorescent image sets (GFP). NRL has been successfully using both of these protocols to support its data collections and analyses for zooplankton since August 2009. Protist data have been collected according to this protocol since October 2009.

It is anticipated that as more complex protist data are collected and analyzed that some details associated with the protocols will change. However, the basic approach described for data management and collection outlined in the following report sub-sections are likely to be very similar to the final protocols that will be used in support of protist samples.

4.1 Data Management Protocol

The Data Management Protocol serves two purposes. First, it provides a standardized means of storing data with a file naming convention into a directory structure that allows specific test data to be easily identified. Second, it produces a test-specific spreadsheet that documents key system settings and allows the microscope operator to enter test-specific comments following a review of the image set after it is collected. The Data Management Protocol is the same for zooplankton and protist samples.

4.2 Data Collection Protocol

The purpose of the Data Collection Protocol is to ensure that standardized image data sets are collected. It is used to set the microscope and camera settings prior to data collection and to collect the standardized image sets in an *.nd2 file format following system setup. Once finalized, most parameters such as lamp brightness, camera gain, and exposure can be automatically set using configuration files (not editable) accessed by Nikon Elements. These meta-data are additionally stored in the *.nd2 file.

It is anticipated that all parameters associated with protist image collection, including the number of images collected and specific microscope settings will be finalized as more complex sample data are generated and analyzed in support of ongoing programs.

4.3 Using the Protocols – Summary Steps

Brief descriptions for using the Data Management Protocol and Protist Data Collection Protocols follow. The protocols assume that the microscope operator has basic familiarity with the AZ-100 microscope and the Nikon Elements Software.

For a more detailed description of the specific protocols used by NRL, please refer to Appendix A of "Protocol for Automated Zooplankton Analysis", January 2010 (Nelson et al., 2010). This provides a step by step procedure for performing the steps outlined in the following report sub-sections.

Prior to running the management and collection protocols, the sample should have been concentrated and stained. A 20-µL subsample should have been dispensed into the sample well and the sample plate placed onto the microscope stage. Note that previous NRL work has shown the stains fade 30 minutes after their addition to a sample.

4.3.1 Data Management – File naming

The observer starts by creating a new experiment folder for each new *.nd2 file. A strict file/folder naming convention allows all sub-sample data to be associated with the corresponding .xls spreadsheet. The folder should be labeled with the date and a sequential number. For example, a folder Desktop > ANS > 2009 > Month > Day > Treatment > Run Folder could be Desktop > ANS > 2009 > 07_July > 09 > rotifer_300ms > 20090709_rotifer_300ms_001. The file itself should be named to match the run folder.

Once the folder name is established, an Excel® data sheet template (.xls) is saved to the folder with the .xls file having the same name as the folder. If necessary, the operator may need to change the Excel® security setting to medium to allow Excel® to open embedded macros. The operator then opens the Excel® worksheet and enters appropriate data for the run. All entries, including redundant data, must be filled before saving. Comments can be added to the worksheet after the .nd2 images are reviewed.

4.3.2 Data Collection

With the camera and both lights (DIA and epifluorescence) on, the Elements software is turned on. After the operator focuses the microscope and the optical pathway is sent to the camera, the operator opens a live preview in Elements. Clicking the DIA optical configuration on the toolbar allows the operator to focus the camera based on the Live Preview window. The operator then checks and records the physical microscope settings. The operator then changes to the GFP optical configuration on the toolbar and verifies the GFP settings.

With all settings verified and recorded, the operator selects which folder to save the images in and enters the first filename as "filename_001". The Elements software will automatically increment the number at the end of each run. For zooplankton, the operator sets the interval to 1 second and the Duration to 31 seconds. The specific interval and duration still need to be determined for the analysis of protist samples. Finally the operator checks the Lambda tab to verify that the first Lambda is GFP and the second is DIA.

The operator then clicks "Run now" to start automatically collecting images of the sample under the ocular.

5 DATA ANALYSIS AND BASIC ALGORITHM DESCRIPTION

The Data Analysis algorithms are expected to be based on similar methodologies as those that are used to perform automated analyses of zooplankton samples. Preliminarily, it is expected that for protists, the automated analysis algorithms will emphasize staining over motility. However, until more and representative data of complex protist samples are acquired and analyzed, substantive details of the algorithm approach that will ultimately be used for automated protist analyses cannot be provided. That said, the algorithms currently used for assessing the viability of zooplankton have already been successfully applied to the analysis of protist samples, in this case a homogeneous culture of *Tetraselmis* sp. in artificial seawater.

Figure 4 provides two successive epifluorescence images that were collected of a 20 µL sample of stained *Tetraselmis* sp. in a SensoPlate ™ 384 well. The same algorithm used to detect motility in zooplankton samples was applied to these two images. The results of performing this operation are provided in Figure 5.

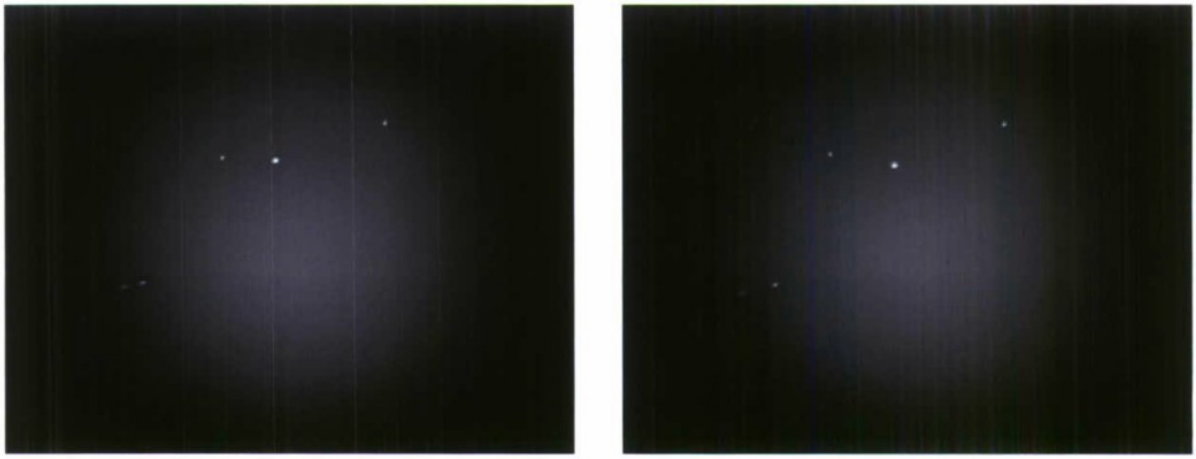

Figure 4. Two successive images collected on a 20 µL sample of stained *Tetraselmis* sp. in a SensoPlate ™ 384 well.

The data shown in Figure 5 were generated by thresholding each of the images shown in Figure 4 to create two binary images. These images were next subtracted and this result squared to generate the image shown in Figure 5. Non-zero pixels (white) in the image shown in Figure 5 are indicative of protists that were motile between the collection of successive images. A review of the data shown in Figure 5 clearly shows that this approach successfully identified four motile Tetraselmis sp. in these image pairs. Applying this approach to the successive brightfield images of Figures 4 and 5 yielded similar results. This data set shows the algorithms developed for automated zooplankton analyses are also applicable to the analysis of protist samples.

Figure 5. The results of applying the motility algorithm developed for zooplankton analyses to the successive images shown in Figure 4. The motility algorithm identified four *Tetraselmis* sp. that were motile between the collection of the two images shown in Figure 4.

For zooplankton, the Data Analysis algorithms are executed as a stand-alone, Windows-based application. When run, the application asks the operator to specify a *.nd2 file to be analyzed. The *.nd2 file associated with a given test is parsed and the motility algorithms operate on the parsed image sets. The algorithm provides a display of the DIA and GFP image sets and provides a screen output of the number of living organisms detected in the analyzed data. Additionally, the data analysis routine outputs its final (as well as intermediate) counting results directly into the spreadsheet described in the Data Management Protocol.

It is expected that the stand-alone protist analysis software application will be used in a similar fashion to the zooplankton routines and provide much of the same functionality.

6 SUMMARY AND NEXT STEPS

The work performed in support of this program and documented in this report clearly demonstrates the applicability of the protocols, equipment, and analysis routines that were developed for automated zooplankton analyses (organisms in the $\geq$ 50 µm size class) to protist samples (organisms in the $\geq$ 10 µm to $<$ 50 µm size class). Based on the results described above and the high probability for success, we think additional work will result in a tool that can support BWMS production testing.

Feasibility of Developing a Protocol for Automated Protist Analysis

The automated protist protocol will clearly leverage significant work previously performed to support the development of an automated zooplankton analysis protocol. For example, the directory structures and file naming conventions developed for the zooplankton data management protocol are directly applicable to a protist data management protocol without modification. The major benefit of this protocol is that all test data and results are stored in only two files in a directory structure that allows specific tests to be easily identified. Further, saving analysis results in the test-specific Excel® spreadsheet makes the archiving of test data and results simpler.

The equipment used to support zooplankton sample data collection is also directly applicable to support protist sample data collection. By modifying the sample wells used for data collection and operating the microscope at higher magnifications, optimum imagery with sufficient spatial resolution (< 2 μm) was determined using this equipment. Work performed has shown that the major microscope modifications made to support zooplankton data collection–including the incorporation of light sources that allow for interleaved collection of brightfield and epifluorescence image data, the automated stage for sample well positioning, and the monitoring of many key system parameters—are directly applicable to protist data collection.

The analysis methods developed for zooplankton samples were additionally found to be directly applicable to protist samples. First, and most importantly, protists can be clearly observed in both the brightfield and epifluorescence image data that have been collected, and these protists can be directly detected in this imagery using the same algorithms as those used for the detection of zooplankton. The motility algorithms used to determine the viability of zooplankton have been successfully applied to determine the viability of motile protists. The ability to use the epifluorescence image data to detect organisms in the presence of debris is directly applicable to the enumeration of viable protists in the presence of sample debris.

In order to mature this technology to the level required to support production analysis of protist samples during BWMS evaluations, additional work is needed. First, optimization of a multi-well sample tray for protist analyses is necessary. Additional work should be performed with more complex samples in the commercial sample well trays that were investigated in support of this program. It is believed that a significant payoff will be realized by fully matching the sample well geometry to the imaging capabilities of the microscope-camera system. Further development of the custom multi-well plate described in this report is thought to be the best way to achieve that task.

Second, complex and representative protist samples must be generated and evaluated using the systems and approaches described in this document. It is essential to collect and analyze these data to fully optimize the system settings for protist analyses and to finalize data collection protocol details. Initial emphases need to be directed at defining an optimum observation temporal window and determining the potential benefits that motility algorithms will provide for assessing the viability of protists in complex BWMS test samples. Other automated analysis algorithm modifications appropriate for protists, such as the emphasis of epifluorescence image data over brightfield image data and staining over motility can be further developed and demonstrated using this more complex sample data. Once sufficient data are generated and analyzed, other important parameters can be finalized and incorporated into a more formal protocol for "Automated Protist Analyses."

Third, as with the Automated Zooplankton Protocol, a software application should be developed with a user-friendly GUI that calls Excel®, the Nikon Elements software, and the analysis algorithms. This would make it easier to implement the protocol, as it would no longer be necessary to run separate independent software applications during its implementation.

Fourth, system settings (the zoom level, the filter cube position, the objective used, and the neutral density filter arrangement) that are currently not independently monitored by the computer system should be monitored using appropriate switches that allow the computer system to monitor these key system settings. This information would then be written into the Excel® spreadsheet and into the *.nd2 metadata.

Fifth, because the camera used in the existing configuration is no longer available from Q-Imaging, a new camera should be purchased to collect image set data. In discussions with the camera manufacturer, the camera that replaced the existing camera will allow improved image quality and spatial resolution in its image sets. Further, if this protocol is implemented by other organizations that perform standardized testing, it will be important that NRL researchers have the same equipment as that used by these outside organizations in order to respond to questions from these organizations.

Lastly, Nikon representatives believe they can provide a modified light source arrangement that would allow significantly faster (2 to 10 times faster) switching between the epifluorescent and brightfield microscope modes. The current setup, which was implemented to provide results within programmatic time milestones, is limited to approximately a 1 second interval between the collection of successive epifluorescent images (the brightfield image is captured within this 1 second interval). Faster switching would potentially allow the individual sample well observation time to be reduced by at least a factor of two to four. If faster switching were available, an equal number of images could be collected in less time than it currently takes. This would translate to more individual sample-wells being analyzed within the six hour time window for viability of organisms. This in turn would be advantageous in production testing since a large number of sample wells (~ 200) must be analyzed to ensure that BWMS efficacy is compliant with discharge standards.

7 CONCLUSIONS AND RECOMMENDATION

The standardized evaluation of the efficacy of BWMS requires a large number of samples be evaluated to determine the number of viable organisms in the $\geq$ 10 µm to < 50 µm size class. The addition of suspended solids (to meet testing requirements), the complex aggregates of ambient organisms in this size class, and the natural debris present in seawater make these samples extremely complex. This task is further complicated by the requirement to concentrate samples by between three and six times prior to analysis. Additionally, recent work performed indicates that it may be difficult or impossible for even a trained expert to be able to observe and count the number of viable organisms in this size class using manual microscopy methods in these complex concentrated samples.

Based on the results described in this report and the high probability for success, it is highly recommended that this work be advanced to the level that is required to support BWMS production testing. The work performed thus far has clearly demonstrated that a suitable protocol can be developed to support the automated collection and analysis of protist samples. When finalized, the equipment, protocols and analysis methods explored under this program are expected to provide advantages to the data management and

archive of protist sample data and to provide a more efficient, less labor intensive, and more uniform means of enumerating and determining the viability of protists in complex BWMS samples.

It is highly recommended that this work be continued to formalize and finalize the details of a protocol for analyzing samples with organisms in the ≥ 10 μm to < 50 μm size class. This recommendation includes the additional work to develop a fully optimized multi-well tray for protist analyses. If the recommended changes are implemented, it is anticipated that the equipment and protocols described in this report will result in efficient means for working with samples, for managing and collecting data, and for enumerating and determining the viability of protists in complex BWMS test samples.

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