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DEVELOPMENT OF AUTOMATED METHODS FOR THE ENUMERATION AND CLASSIFICATION OF ZOOPLANKTON AND PHYTOPLANKTON SAMPLES

FINAL REPORT
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N O T I C E

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16. Abstract (MAXIMUM 200 WORDS)

During standardized testing at the Ballast Water Treatment Test Facility located at the Naval Research Laboratory in Key West, significant time is spent characterizing zooplankton and phytoplankton samples to both ensure that the challenge water used to evaluate Ballast Water Treatment Equipment is consistent with the test standards and to determine the efficacy of treatment by the equipment. The objective of this effort was to develop and demonstrate methods for the automated assessment of ballast water including detection, classification and enumeration, and viability assessments of zooplankton and phytoplankton samples.

The methods that were demonstrated, developed, and described in this report have the potential to reduce the time required to analyze samples by at least one order of magnitude. These methods additionally produce digital image and video outputs that create a complete archive of the analyses that were performed to enumerate and determine the viability of zooplankton and phytoplankton samples. The systems explored and developed under this effort include an in-house developed microscope imaging system that uses image processing and classification algorithms to analyze zooplankton samples, and a commercial imaging flow cytometer system that has been modified and used in conjunction with cell impermeant DNA staining dyes for analyzing phytoplankton samples.

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Executive Summary

Ballast water is an inexpensive and efficient means for ships to maintain trim, counteract list, and adjust waterlines in response to cargo loading/unloading or in response to foul weather. Ballast water is taken aboard as needed, stored in tanks, moved about, and discharged as required. The ballast water volume for any given ship can be tremendous – hundreds to hundreds of thousands of metric tonnes. Frequently, ships' tanks will be ballasted in one port and deballasted in another. In addition to the water itself, ballast water contains organisms that were taken up along with the water. The entrained organisms are generally discharged along with the ballast water. This process has been identified as a vector for the translocation of non-indigenous species (NIS).

The United States Coast Guard (USCG) and the International Maritime Organization (IMO), which governs international maritime law, have promulgated regulations designed to reduce the transport and transfer of NIS. More stringent regulations for ballast water treatment are anticipated from IMO, USCG, and possibly other domestic regulators in the near future. These regulations will likely result in the need for onboard ballast water treatment to kill, remove or otherwise inactivate organisms in the ballast water.

In anticipation of the requirement for treating ballast water onboard ships, USCG has partnered with the Environmental Protection Agency's Environmental Technology Verification (ETV) Program to develop protocols for testing ballast water treatment equipment (BWTE). Likewise IMO has specified standardized protocols through their conventions. The protocols for shore-based tests call for characterization of basic water quality parameters and assessment of concentrations and viability of indigenous (ambient) and surrogate (challenge) phytoplankton and zooplankton. The evaluation of BWTE requires comparison of phytoplankton and zooplankton concentrations before and after treatment.

A ballast water treatment test facility has been established at the Naval Research Laboratory (NRL) in Key West and is used to evaluate the efficacy of BWTE according to standardized protocols, including those established in the ETV Program and the IMO's conventions. This unique facility's objective is to provide third party independent and objective testing of BWTE and to provide data from shore-based standardized tests to other entities for "certification."

During support of standardized testing at the NRL test facility, significant man-hours are expended determining the concentrations of phytoplankton and zooplankton in challenge and post-treatment samples and determining their viability. In the case of zooplankton, manual microscopic methods currently take in excess of 24 hours to fully characterize samples and assess the number of viable organisms. In the case of phytoplankton, most probable number (MPN) methods that are used to determine viability do not provide "real-time" results. Currently 4½ hours of microscope time is required to classify phytoplankton species and determine their concentrations. To reduce the man-hours required to perform these analyses, USCG contracted NRL to investigate the potential for automating methods for determining concentrations of phytoplankton and zooplankton and for determining the viability of organisms in samples obtained during shore-based tests at the NRL test facility.

For zooplankton, two potential analytical tools were tested. The commercially available LemnaTEC Scanalyzer system was evaluated in the laboratory. The system showed potential for

automating the counting of zooplankton and performing viability analysis. The system provided computer controlled lighting and camera settings, automatically archived data, and was capable of automatically characterizing multiple sample wells. The resolution of the archived photographs was limited by the camera's zoom capability whereas a second system capitalized on the resolution provided by a microscope. While the LemnaTEC Scanalyzer system had many advantages, the higher cost and lower resolution imagery compared to the second system tested resulted in it not being selected for further adaptation.

The second analytical tool was developed in-house and consisted of a microscope, a digital camera, and a suite of image processing algorithms. After initial tests and comparison to the LemnaTEC system, the in-house system was selected for further development and testing. Evaluations showed that the system could reliably count the surrogate *Artemia* in "real world" water samples obtained during BWTE tests. The system also performs live/dead evaluations on counted zooplankton and produces a digital archive consisting of both video and still images. The in-house system results compared favorably against manual observations. Lastly the system reduces the time required for sample observation from 3½ hours to 20 minutes to fully characterize 10 sample wells (at least 300 surrogate organisms).

For phytoplankton analyses, efforts centered on evaluation of a commercial imaging flow cytometer. The system, Fluid Imaging Technologies' FlowCAM®, was not adequate as purchased. After several modifications were made, the FlowCAM® unit has become suitable for characterization of phytoplankton samples. Methods were developed to optimally prepare samples for FlowCAM® analysis. DNA staining dyes were investigated, and Sytox® Green was found to work well with the modified FlowCAM® system. The system has significant potential to be incorporated into standardized protocols for phytoplankton analyses.

This study has identified two tools that show high potential for automating and improving analyses of zooplankton and phytoplankton during large-scale tests of BWTE. Each tool:

- significantly reduces the number of man-hours required to analyze samples;
- provides results that are comparable to the labor-intensive manual method;
- provides a hard copy of analysis results that can be reviewed after the fact;
- needs additional work to finalize the tool and protocol for use.

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List of Acronyms

BWTE = ballast water treatment equipment

BWTTF = ballast water treatment test facility

DNA = deoxyribonucleic acid

EPA = Environmental Protection Agency

ESD = equivalent spherical diameter

ETV = Environmental Technology Verification

GUI = graphical user interface

IMO = International Maritime Organization

L = liter

LED = light emitting diode

mL = milliliter

mm = millimeter

MPN = most probable number

NIS = non-indigenous species

NRL = Naval Research Laboratory

NRLKW = Naval Research Laboratory in Key West, Florida

PC = personal computer

pH = scale for degree of acidity and alkalinity

TIFF = tagged image file format

US = United States

USCG = United States Coast Guard

μL = microliter

= Number

® = Registered sign

1.0 Introduction

Nearly all commercial and military ships, and some leisure vessels, carry ballast water onboard. The ballast water is stored in tanks aboard the ship and serves a variety of purposes including management of ship trim and list as well as adjusting ship waterline in response to the on-loading and off-loading of cargo or as a function of ambient weather conditions. Ballast water volumes are, of necessity, very large (hundreds to hundreds of thousands of metric tonnes). Frequently, ships' tanks will be ballasted in one port and deballasted in another. This process has been identified as a vector for the translocation of non-indigenous species (NIS), and it is currently regulated to some degree by the United States Coast Guard (USCG). More stringent regulations are anticipated for ballast water treatment by the International Maritime Organization (IMO) (which governs international maritime law), USCG, and possibly other domestic regulators in the near future. These regulations will likely result in the need for on-board ballast water treatment to kill, remove or otherwise inactivate organisms within the ballast water.

With support from and in partnership with the USCG, the Naval Research Laboratory in Key West (NRLKW) has designed, constructed and operates a prototype ballast water treatment test facility (BWTTF). The BWTTF was created to provide a large-scale facility to simulate pumping, storage, treatment, and discharge of seawater in quantities and under conditions that can simulate shipboard ballasting and de-ballasting. The industry-provided ballast water treatment equipment (BWTE) can be inserted into the test facility piping configuration such that treatment on uptake, discharge, or both can be tested. This allows realistic tests of full-scale BWTE at a shore side facility where test conditions can be controlled. The BWTTF is used to evaluate the efficacy of BWTE according to standardized protocols including those established in the Environmental Protection Agency's Environmental Technology Verification (ETV) Program and International Maritime Organization's conventions. This is a unique facility with the objective of providing third party independent and objective testing of BWTE through the use of shore-based standardized tests and providing data from those standardized tests to other entities for "certification."

Standardized testing of BWTE necessitates the complete characterization of the water used to evaluate the efficacy of the equipment. Characterization of basic water quality parameters such as pH, total dissolved solids, salinity and other parameters will validate compliance with water chemistry parameters that are called out in the standardized test protocols. The local seawater in Key West is very clean compared with typical harbor water. To create ballasting conditions more typical of harbor environments, the Key West uptake water is augmented by adding large amounts of suspended sediments plus large numbers of zooplankton and phytoplankton (collectively called surrogates) to create what is called challenge water. The surrogates are selected according to several criteria, but two of the most important are that the surrogates are easy to culture and difficult to kill. To document biological treatment performance, concentrations and viability of both indigenous (ambient) and surrogate (challenge) phytoplankton and zooplankton must be determined before and after treatment. During such assessments of BWTE efficacy, significant man-hours are expended determining concentrations and viability in challenge and post-treatment samples. This primary objective of the current effort was the evaluation and development of automated techniques to count live and dead surrogates in challenge water that has been treated during shore-based tests of full-scale BWTE.

Current testing protocols require that for each zooplankton sample analyzed, analysts observe, count, and determine the viability of at least 300 organisms (or, with some treatment technologies, that some multiple of the volume of challenge water that generated 300 zooplankton counts be analyzed). Typically, these measurements are made using manual microscope observations and hand counting. Samples are placed in a tray with multiple small sample wells. For *Artemia* (one of the zooplankton surrogates), a typical well will have between 30 and 100 organisms present. The current protocol for viability requires that the microscope operator observe each *Artemia* for a period of 30 seconds. The *Artemia* is classified as living if it moves (including the movement of antennae and the flicking of an appendage) during this observation window.

To determine the number and viability of 30 barely viable (gross movements are easy to see but hard to keep track of) *Artemia* in a sample well requires 15 minutes of continuous concentrated microscope observation time. It is difficult for a microscope operator to maintain full concentration during this 15-minute observation window. The operator additionally is required to move the sample well and zoom in on each *Artemia* in the sample well to make these observations. This conservatively adds 6 minutes to the time required to make these microscope observations. Consequently, it takes at least 21 minutes to count and classify 30 *Artemia* in a single sample well. Counting 300 *Artemia* takes over 3½ hours of intensive microscope observations. This time estimate is quite conservative in that using the current protocol implemented at the BWTTF for characterizing zooplankton takes over 24 hours to complete because a two-step process is required to perform these assessments.

The conservative estimate assumes that the biologist takes no breaks during the 3½ hour elapsed time interval required to make these observations. Additionally, there is evidence that over this length of time the sample may degrade (e.g., *Artemia* decompose). In general, challenge water characterization or ballast water treatment technology evaluations involve the analysis of multiple samples collected from multiple locations which further increases the length of time required for microscope observations. During testing, one or more biologists/microscope operators will need to perform these types of observation functions on a nearly full-time basis. It is also important to note that microscope observations of these types do not generate a "hard copy" of results in the event that viability assessments show that a ballast water treatment system fails to meet required efficacy levels.

2.0 Objective

The primary goal of this research was to investigate and evaluate potential reliable and economic methods for automating enumeration and viability characterization of zooplankton and phytoplankton samples during standardized tests of ballast water treatment equipment. The man-hour costs using human-on-microscope analysis techniques are prohibitive for routine use during shore-based approval tests of full-scale ballast water treatment systems. Two different equipment selections and associated automated analysis techniques were investigated for zooplankton. One system was evaluated for phytoplankton.

3.0 Technical Discussion

In an effort to develop automated methods of analysis for counting zooplankton and determining their viability for full-scale tests of BWTE two systems were explored. A commercial system, the LemnaTEC Scanalyzer system, was evaluated in the laboratory to determine its applicability towards characterizing zooplankton samples. Second, an in-house developed microscope system was used in conjunction with NRL-developed advanced image processing and classification algorithms to evaluate zooplankton samples. Both methods showed the potential for automating methods of counting zooplankton and performing viability classification. Although the LemnaTEC Scanalyzer system was capable of automated analyses and had several advantages, it was not selected for in-depth evaluation because it was not deemed to be as accurate as the NRL system and was more expensive. Results of the LemnaTEC Scanalyzer system tests are presented first in this section. They are followed by an in-depth discussion of the NRL-developed system.

The second aspect of this research was evaluating methods for automating analyses of phytoplankton. A commercial off-the-shelf system was selected for testing in Phase 1. A description of efforts to modify this commercially available particle counter for use during phytoplankton analyses follows the two-part zooplankton discussion mentioned above. The phytoplankton analysis effort is not yet complete and further refinement of automated analysis is required before a final protocol can be established.

3.1 Zooplankton

For the purposes of this research effort, zooplankton are divided into two artificial size classes based on the mesh size of the nets used to collect samples. The size classes are in accordance with proposed USCG and IMO discharge standards. Organisms greater than 50 microns are represented by *Artemia*. Rotifers represent zooplankton greater than 10 microns but less than 50 microns.

3.1.1 Zooplankton Sample Preparation

In BWTTF evaluations, nets are used to collect the zooplankton samples from a 3-m^3 collection tank. The material collected in the net is rinsed into the net's cod end to capture a concentrated sample from the tank being characterized. The sample is flushed from the cod end into a flask. This results in approximately 3 liters of concentrated sample that is evaluated to determine the concentration and viability of zooplankton in the tank.

The flask is homogenized and twenty 1-mL samples are transferred to 7-mL sample wells. (The 7-mL sample well allows the sample to spread into a shallow layer thus allowing better viewing of organisms.) Twelve sample wells are housed on a single plate and each of these wells is independently characterized using the methods described below or the automated methods described elsewhere in this report. There are typically between 200 to 300 organisms observed in each sample well (*Artemia* and rotifer surrogates and indigenous zooplankton). The procedure

currently in use takes in excess of 24 hours to enumerate the number of zooplankton in each sample well and to determine viability. A two-step counting process is used in which the dead zooplankton are first counted, then the sample is fixed (organisms are killed) and the zooplankton recounted. When manually characterizing the samples, there may be some advantage in diluting the samples in each sample well, characterizing more sample wells and having a lower density of organisms in the individual sample wells.

In support of this program's work with *Artemia*, 1-mL samples containing *Artemia* were also analyzed in 7-mL sample wells. These samples were created by diluting stock solutions of the zooplankton. The stocks were diluted to levels that were typical of what was expected when performing BWTTF water sample characterizations. In many of the tests conducted with the *Artemia*, there were between 100 and 200 *Artemia* present in the sample well. The automated methods conducted during this program analyzed relevant size samples of approximately the same organism density (same or higher *Artemia* densities) as the BWTTF zooplankton samples.

During this program's work with rotifers, 50-µL, 100-µL and 1-mL samples were analyzed in 3-mL sample wells and 1-mL samples were further analyzed in 7-ml sample wells. Again the samples were created by diluting stock solutions of the zooplankton. Many of the tests conducted had between 50 – 150 rotifers (and ciliates) present in the test solutions. The imaging methods worked better with the rotifers in the 7-mL sample wells because these wells were shallower than the 3-mL sample wells.

Future work with the in-house developed systems will be conducted with mixed samples of *Artemia*, rotifers and indigenous zooplankton using samples that are prepared in the same manner as those prepared for manual microscopic analysis.

3.1.2 LemnaTec Scanalyzer System

The LemnaTec Scanalyzer system was evaluated as a potential means for characterizing BWTTF water samples. Advantages of this system include complete and repeatable computer control of camera settings and lighting parameters and data automatically being archived to a database. (Data archive includes dealing appropriately with data custody issues). A very attractive feature is the ability to automatically characterize multiple sample wells on a tray (in the case of the Scanalyzer system) and multiple trays (in the case of the Scanalyzer High Throughput System) without manual operation. The major disadvantages of this system included high cost (~$100,000 - $200,000 per system) for performance obtained and lower resolution imagery, since a camera and its optical zoom is used to capture images of the sample wells, not a microscope system. It also requires the user to "build" and validate individual application-dependent image processing and classification algorithms as a result of this system being designed for a broad range of counting, classification, and characterization applications and not for BWTTF water sample evaluations specifically. Because these systems were not selected for further development/evaluation, the results obtained during the initial feasibility demonstration are also provided here.

The two LemnaTEC Scanalyzer Systems are shown in Figure 1. Shown on the left is the standard Scanalyzer System, and on the right is the Scanalyzer High Throughput System. Not shown in either of the photographs are the servers and personal computers (PCs) that are used to control the systems and archive the collected data. The Scanalyzer System shown on the left

consists of a high-resolution digital camera mounted to a translation stage. Not apparent in the photograph but quite impressive are the variable sample lighting options that are provided with this system. Both the camera parameters as well as the lighting parameters are completely controlled by a system PC. This allows experimental setups (sample well configuration, lighting, camera setup parameters, etc.) to be first prototyped and demonstrated and then fully repeated under computer control. This has the advantage of providing greater repeatability of these parameters than that provided using the in-house developed system. The high throughput system shown at the right allows multiple trays each containing multiple sample wells to be sequentially evaluated completely under computer control without requirements for manual operation. This could provide some benefits to BWTTF water sample characterizations.

Figure 1. LemnaTEC Scanalyzer Systems.

A LemnaTec Scanalyzer System was evaluated at NRLKW using a mixed sample of live and dead *Artemia*. A LemnaTec representative was present for these evaluations and was involved with both the optimization of the camera and lighting settings as well as for using their image processing "building block" functions to develop some basic algorithms for counting the total number of *Artemia* and identifying specific *Artemia* that moved during the observation interval.

Figure 2 provides two images that were generated during the evaluation of the LemnaTec system. The image on the left is of a single sample well on a sample well tray. It shows a number of *Artemia* and air bubbles in the sample well. The particles that are colored in red were detected by the rudimentary enumeration routine that was configured. Note that the majority of the *Artemia* in the well are all colored red, indicating that they were counted by the routine. Note also that the air bubbles and some of the debris (and maybe one or two *Artemia*) are not colored red and consequently are not counted by the routine. The assessment of this sample well was that it had a total of 26 *Artemia* present. The image on the right was generated by a routine that was used to detect movement of the *Artemia* over an observation window. This routine identified 19 motile *Artemia* in the sample well. This result (26 total *Artemia*/19 motile *Artemia*) was consistent with the results that were obtained using manual microscopy and biologist observations.

The results presented in Figure 2 were very encouraging. However, the high cost and lower resolution compared to the in-house developed system resulted in the in-house developed system being selected for additional development under the support of this program.

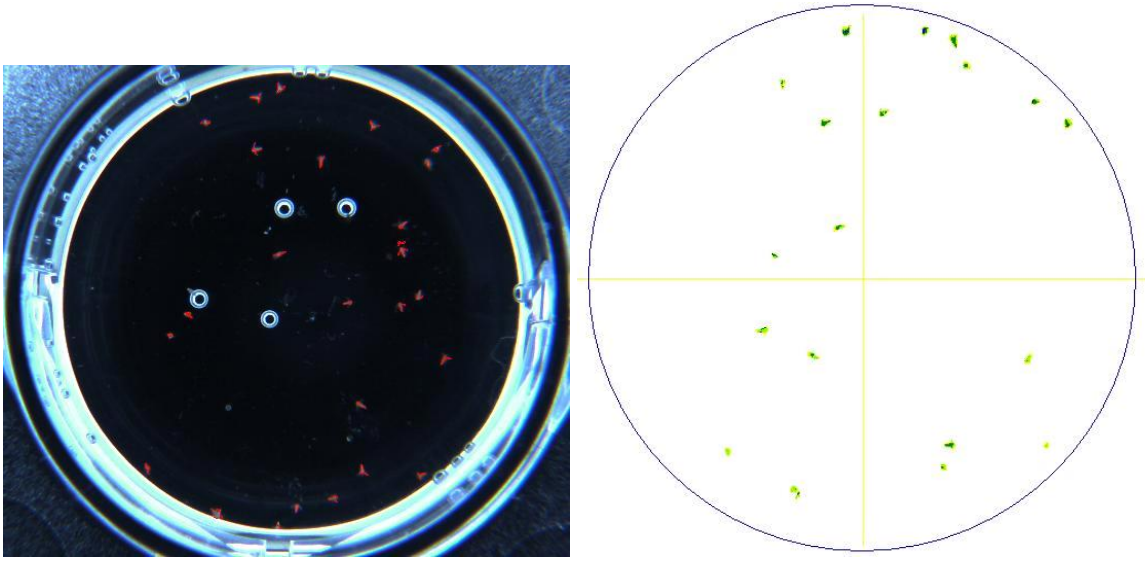

Figure 2. *Artemia* enumerating by the LemnaTec Scanalyzer and determined viable using a basic image processing routine.

3.1.3 NRLKW Developed System

NRLKW developed a system for enumerating and classifying zooplankton in BWTTF water samples. Based on initial evaluations of the system's performance, it was decided to further the development of the system. The hardware, image processing and classification algorithms, and the outputs that are produced when these algorithms are applied to the collected data using a software application Graphical Users Interface (GUI) are described below. Experimental results obtained using this system to analyze zooplankton samples are provided in Section 4.1 of this report.

3.1.3.1 Hardware

The setup for capturing time-resolved high resolution image sets uses a standard stereo-microscope (Olympus SZH10 Stereoscope – with approximately 10X to 15X magnification) interfaced to a high resolution monochrome progressive firewire (IEEE 1394) camera (Q-Imaging's Retiga 1300i Fast 1394 Camera). A personal computer is used to capture time-tagged multi-framed TIFF (tagged image file format) images, with each frame having a 1300x1022 pixel resolution. The time between frames is user adjustable, but our work to date has shown ½ second between frames is adequate for the detection of viability. The system just described is used to collect a 150-frame TIFF image. The header for each frame in the multi-framed TIFF image provides the time that the specific frame was captured. In this manner a time-tagged high resolution multi-framed TIFF image set is collected that fully documents 75 seconds of total observation time. This image set will be referred to as "multi-frame TIFF" set or series in the

remainder of this report. The single image series is optimized for both counting and isolating the *Artemia* present in the sample well (using the zoom-adjusted image) and for producing a time resolved sub-image series optimized for the detection of both gross and small *Artemia* movements.

A sample is first placed under the stereo-microscope and a guide system is used to roughly position the sample well in the microscope's field of view. The sample well is centered horizontally in the image. The microscope's zoom factor is next adjusted until the top and bottom section of the sample well are barely clipping the edge of the field of view (i.e., further zooming would delete part of the sample well's top and bottom image). A typical image of a sample well is provided in Figure 3.

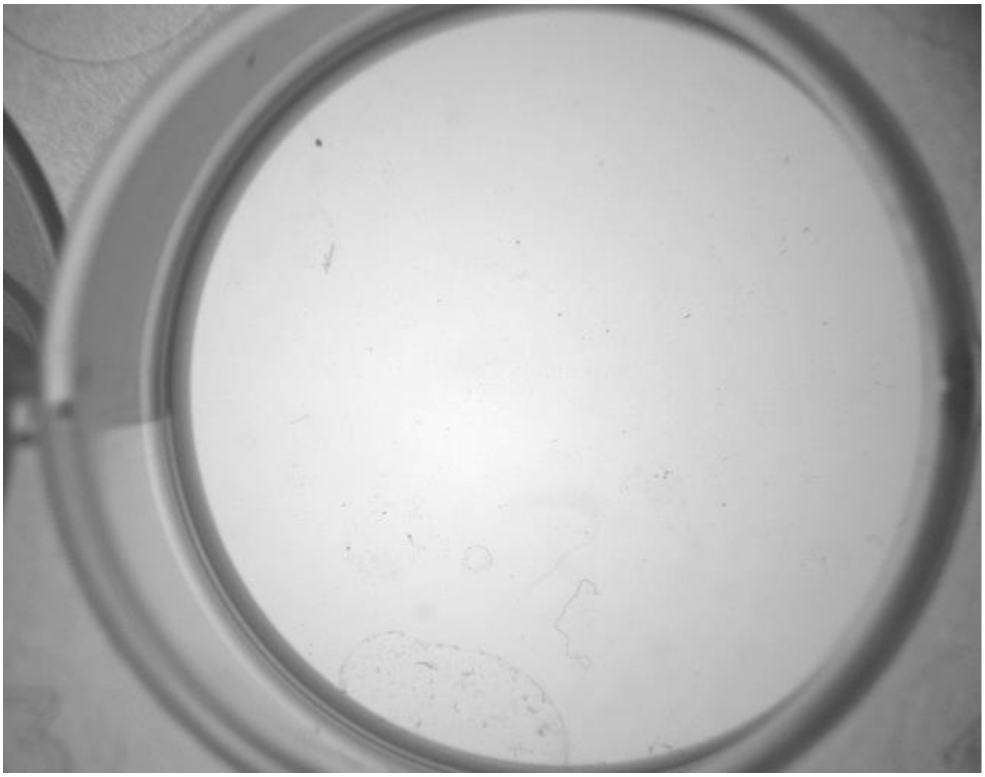

Figure 3. Image of sample well following zoom factor adjustment.

Figure 4 provides an image of a sample well with *Artemia* present. Image processing algorithms (described in the next section of this paper) are applied to this image to segregate individual *Artemia* from the background. Once the individual *Artemia* are segregated, they are counted. The image processing algorithms additionally define a bounding box that fully encompasses the segregated *Artemia*. Figure 5 provides a sub-image of one of the *Artemia* that was extracted from the image shown in Figure 4. This sub-image provides sufficient spatial resolution for the detection of small visceral movements of the *Artemia* antennae or intermittent or occasional flicking of an *Artemia* appendage (the actual images worked with are of significantly better quality than those shown in this figure). These movements (as well as gross movements) are detected by analyzing a time series of the sub-images similar to that shown in Figure 3. The sub-images are extracted from the overall multi-frame Tiff image series generated by the camera and captured using the PC.

The time required to position the sample well and to collect the required imagery for enumerating the *Artemia* in the well and determining their viability is less than 2 minutes or almost a 20-minute savings over manual microscopy techniques for performing these functions. Further, the TIFF image set serves as an archive of the microscope observations. To perform a complete characterization of a BWTTF water sample (analysis of 10 sample wells at typical sample densities) takes approximately 20 minutes compared with the 3½ hours of continuous intensive manual microscope observations that are required for a microbiologist to characterize the same sample.

The equipment discussed in this section consists primarily of equipment that was available in the NRL Key West Microscopy laboratory. The most unique aspect of this system is the high resolution progressive firewire camera. This component specifically affords the ability to obtain the high resolution image of the individual particles as shown in Figure 5.

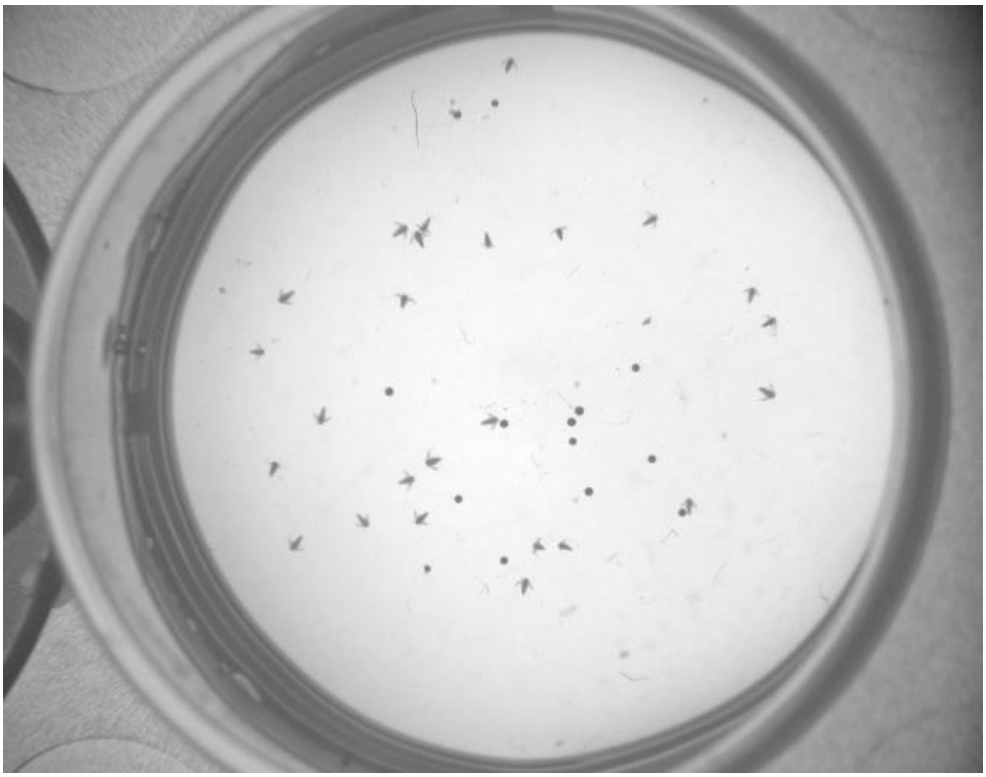

Figure 4. Image showing *Artemia* in a sample well. Automated image processing algorithms to isolate and enumerate are applied to the full sized zoom back image.

Figure 5. Sub-image of an individual *Artemia* extracted from the image shown in Figure 4. Small movements or gross motions of the *Artemia* are detected in a time-resolved series of sub-images similar to that shown in this figure.

In work that has been performed on samples containing rotifers (which are less than 50 microns in size), the hardware configuration is the same as that described previously. In this case, however, a smaller volume sample well is used to position the sample under the stereomicroscope. The same procedure as described earlier is used to "frame" the sample well, thereby insuring that high-resolution imagery is available for each detected rotifer.

3.1.3.2 Classification Algorithms

This section details the automated analysis of time-series photography of each of the 20 prepared samples. The general steps (*in italics*) provide a basic outline of the procedures. The remainder of the section is a detailed, step-by-step discussion of these general steps.

- *Using digital image processing techniques {developed and modified by NRLKW}, identify, count, and determine viability of zooplankton in each sample using the following general sequence of steps:*
  - *Develop a method to eliminate areas within the view that are not of interest. This amounts to a mask that allows operation only on the sample well and not on the full image area.*
  - *Create a pixel-counting scheme to identify individual Artemia. This is done by creating a new entropy filtered image by applying a special function (see Equation 1) to 5x5 square blocks of pixels from the original masked image. The function operates on the density information from all 25 pixels in the block but the function value (a summation) is assigned only to the center pixel of the block. This operation is repeatedly applied to all pixels inside the original mask. The result is the new entropy filtered image*
  - *Normalize the intensity levels associated with the entropy-filtered image to create a grayscale entropy filtered image.*
  - *Process the pixels by applying a threshold to the grayscale image to create a binary image. The binary image is pure black and pure white.*
  - *Create a time series of binary images that covers the 75 seconds of observation using the above steps for each of the 150 images taken during that period.*
  - *Analyze the series of binary images by using a succession of logical **ON/OFF** operations. The logical **AND / OR** operations are used to count and determine the viability of each detected zooplankton by sensing image-to-image movement as follows: Note: each identified zooplankton is isolated as a sub- image.*

- *Using time-adjacent sub-images, perform a binary sub-image to sub-image subtraction of corresponding pixel values (1s or 0s). Corresponding pixels of the time-adjacent sub-images that have no motion remain unchanged. Black is a 0. White is a 1. Thus, black minus black = black, and white minus white = black.*
- *Using rules for forming sums and ratios of white and black pixel counts called Parameters 1, 2, and 3 in the text, determine viability of the zooplankton in each sub-image. Label each of the organisms according to a viability scheme.*

The first image processing step is to develop a mask that allows the image processing algorithms to operate only on the sample well and not on the whole rectangular image area. Applying the mask to the image being analyzed eliminates well image artifacts including the sample tray area between sample wells, reflections, and the edges of the sample well itself. Thus the subsequent processing algorithms operate only on the sample images. Applying a mask to the image also allows the contrast of the objects in the well to be enhanced.

In order to accommodate variations in sample well placement, the mask is developed interactively with input from the operator. A software graphical user's interface (GUI) brings up an image from the camera system and prompts the operator to click on the left and right sides of a sample well using a mouse (the vertical positioning of the well is fixed adequately by the microscope zoom adjustment process). The coordinates associated with the mouse clicks are analyzed and a mask is developed for the specific multi-frame TIFF image series. Figure 6 shows the results of applying a mask that was developed using the above process to the image previously shown in Figure 4. As a result of the masking operation, image processing operations are not applied to areas in the image outside of the sample well.

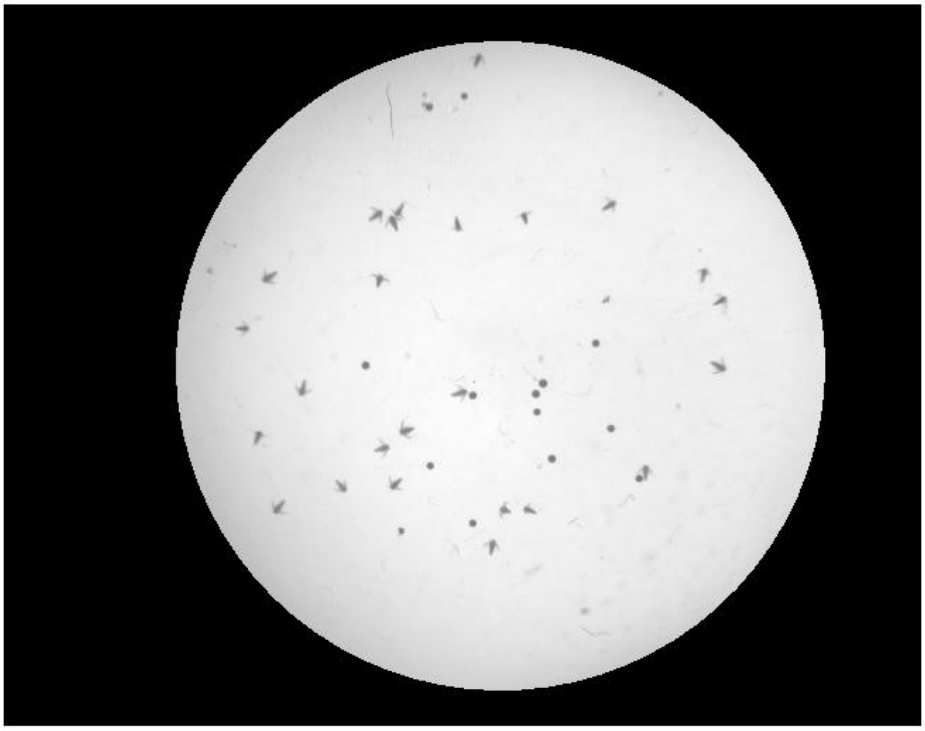

Figure 6. Masked image of the sample well previously shown in Figure 4.

The next step in the image processing algorithm is to segregate each of the individual *Artemia* in the image being analyzed. This is accomplished by applying an entropy filter with a 5x5 square block to an image such as that shown in Figure 6. The image entropy is a measure of the information content or randomness (noise) of the intensity levels that make up an image. The image entropy's units are bits/pixel, and it is calculated by generating a normalized histogram and performing the following operation:

$$IEntropy = -\sum_{I=1}^{I=n} countsbinI * \log 2(countsbinI) \qquad \text{(Equation 1)}$$

where:

IEntropy is the image entropy and

countsbinI are the normalized counts in bin I.

The summation is performed on all bins (1 through n) of the normalized histogram.

Entropy filtering is a block-processing operation that in this case uses a 5-pixel by 5-pixel block (the size of the block is a variable that impacts the processing results). A 5x5-pixel neighborhood is defined for each pixel of the pixels that make up the image being filtered. (Neighborhoods for pixels on the perimeter of an image use a padding process to define an

appropriate 5x5-pixel area). The pixel being worked with is the pixel in the center of the 5x5-pixel neighborhood. The image entropy is calculated for the neighborhood, and the value of the image entropy replaces the intensity value for the pixel being worked with. This process is performed on all of the pixels in the image creating an entropy filter image. The intensity levels associated with the entropy-filtered image are then normalized to create a grayscale entropy-filtered image. A threshold is next applied to the grayscale image to create a binary image. The value of the image entropy is in general high for blocks in which there are edges in an image (such as the transition from background to an *Artemia*), so this process tends to define the edges associated with each of the *Artemia* or other particulates in the image being analyzed. These processes create a binary image from an entropy-filtered image. The result of performing these operations on the images previously shown in Figure 4 and 6 is the binary image shown in Figure 7. The binary image clearly delineates each of the individual *Artemia* (as well as cysts and other items) in the sample well being analyzed. Additional size and shape filters are used to isolate *Artemia* in lieu of other items that might be in the sample well. As a result of this process each of the images that make up the time-resolved high resolution TIFF image series is entropy filtered to create a time-resolved binary image representation of the isolated objects in the TIFF image series.

The next procedure is isolating mobile particles from immobile particles. The software GUI prompts the operator to define a specific image in the TIFF image series to work with. The binary image representation of the objects in the specified image (Figure 7 for example) is then labeled (or labeled and size and shape filtered). The labeling operation directly provides a count of the total number of *Artemia* (or particles) in the sample well since the number of independent objects or "blobs" in an image is an output of the image-labeling process. The labeling operation also allows definition of a bounding box, which fully encompasses each of the isolated *Artemia* or other particles in the binary image. The coordinates of the bounding box allow sub-images of each of the isolated *Artemia* to be extracted from the image being analyzed. To perform motion analyses, sub-images of each isolated *Artemia* can also be extracted from any of the images in the multi-frame TIFF image series or from any of the images in the time-resolved binary image representation of the objects in the TIFF image series (generated through the entropy-filtering process).

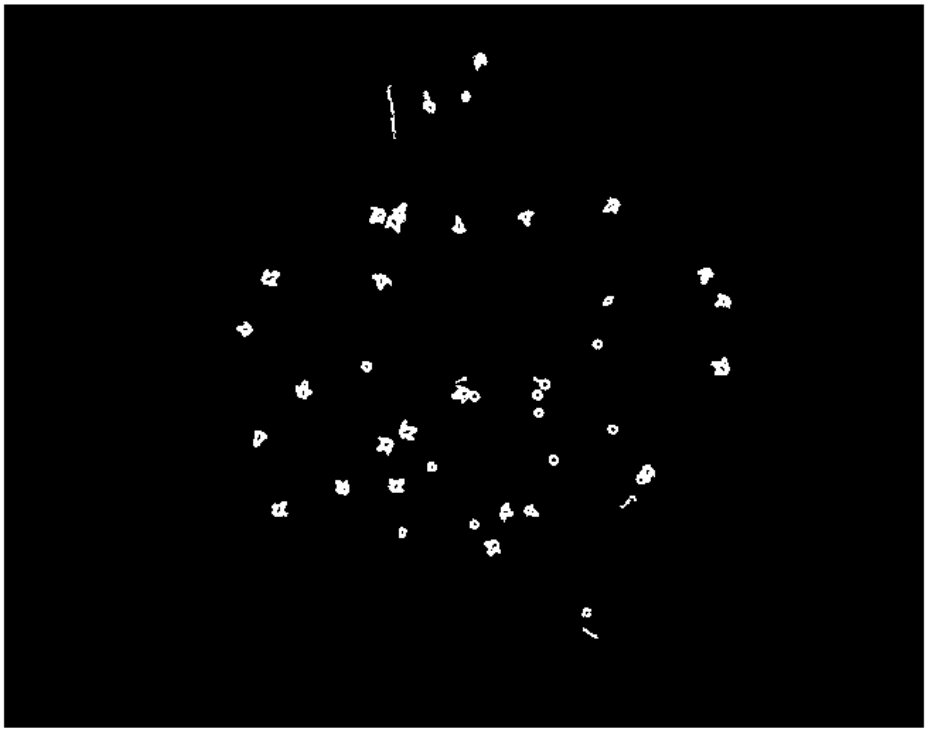

Figure 7. Binary image representation of the entropy-filtered image generated from the image previously shown in Figures 4 and 6.

Dynamic analyses are first performed to determine whether the *Artemia* or other isolated particle is mobile or immobile. These analyses are performed on both the time-resolved TIFF image series and on the binary representation of the objects in the TIFF image series. The analysis works with the images 20 images before and 20 images following the image that is specified by the operator (image 21). The analysis thus works with a sequence of 41 images.

The next step is the separation of the very mobile (swimming) *Artemia* from the immobile *Artemia*. Sub-images are extracted from each of the 41 binary image representations of the objects in the TIFF image series using the bounding box coordinates of the isolated *Artemia*. The binary sub-image series is analyzed in eight independent groups of five images. In binary images, the pixels that make up white areas have a numerical value of 1 and pixels in the dark areas have numerical value of 0. Furthermore, logical operations can be used to process these images. These operations include the logical AND and logical OR operations. Both of these operations are performed on a pixel-by-pixel basis for all pixels in the image.

When two binary images are AND'd, pixels in both of the images being processed need to be ON (e.g., white or having a numerical value of 1) for the pixel to be ON in the output binary image. When two binary images are OR'd, a pixel in either image needs to be ON for the pixel to be ON in the output binary image. These operations can be used effectively to detect changes between binary images and consequently are effective in determining movement of the *Artemia* between frames.

Each of these groups of five images that make up the set are first logically OR'd. This creates a new binary image where the ON pixels from any of the five independent images in the set are turned ON in the output binary image. This operation has an averaging effect that helps to accommodate variations in camera lighting levels and camera response. This new binary image is next logically AND'd with the binary sub-image representation of the *Artemia* being worked with (extracted from frame 21 in the series). If the *Artemia* has not moved, the ANDing operation results in a large number of ON pixels in the AND output image. If the *Artemia* is mobile, then the ANDing operation results in very few pixels being ON in the AND image (in many cases the *Artemia* are so mobile that no pixels are turned on).

This process is repeated for each of the eight groups of five images. The average number of ON pixels in the eight images that resulted from the ANDing operation is computed. The number of ON pixels in the binary sub-image representation of the *Artemia* being worked with (extracted from image 21 in the series) is next computed. The average number of ON pixels from the eight independent analyses is next divided by the number of ON pixels in the sub-image representation of the *Artemia* being worked with. If the result of this division is less than 0.85 (indicating that far fewer pixels are turned ON in at least one of the eight independent analyses), the *Artemia* or other particle is classified and counted as very mobile and viable.

If the value is greater than 0.85 (which indicates a relatively stationary particle), additional analyses are performed using the time-resolved TIFF image series to classify the *Artemia* or other particulate as dead (immobile) or as viable based on detected small movements of antennae or other *Artemia* appendages. A grayscale image differencing approach is used in these analyses. The sub-images of the particle of interest extracted from the entire 41-image time resolved TIFF image series are used for these analyses. The analysis starts with the first sub-image in the series, an example of which is shown on the left in Figure 8. The next sub-image in the series, shown in the center of Figure 8, is first subtracted from the previous sub-image in the series. Thresholds (a positive and negative threshold) are next applied to this "difference" image to create a binary image that highlights any movements that occurred between the successive frames. An example of this difference image is shown on the right in Figure 8. This binary image clearly depicts the small motion that occurred between these two frames.

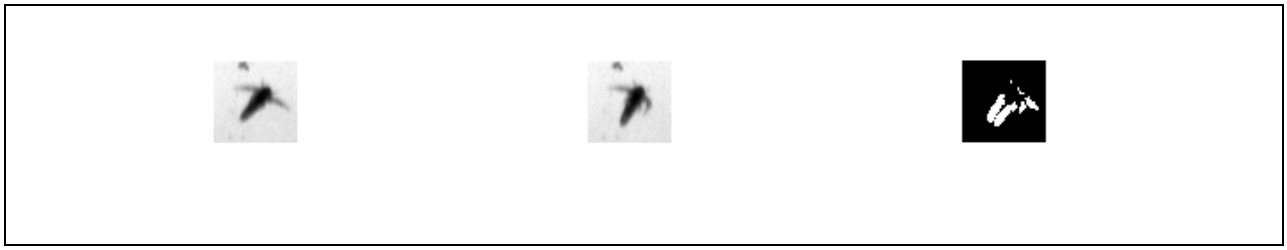

Figure 8. Two successive images indicating movement and a binary difference image.

Figure 9 provides a similar image for the same particle shown in Figure 8, from later in the time resolved sub-image set. These two successive images do not indicate any real motion of the *Artemia* being analyzed. The sub-image shown on the left was extracted from frame 5 of the time-resolved image set. The sub-image shown in the center was extracted from frame 6 of the time-resolved image set. Neither the successive images nor the binary image indicate movement of the *Artemia* between these two frames.

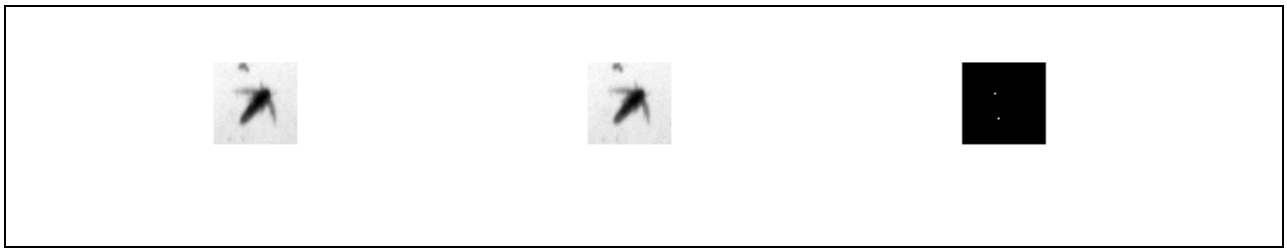

Figure 9. Two successive images indicating little or no movement and a binary difference image.

Three new binary images are generated using the binary difference images. Information extracted from these three binary images are used to classify the particles as mobile (minimally) or immobile. To obtain these three images, the binary difference is generated for each pair of successive images in the time-resolved sub-image series (starting with image 1 and 2, then image 2 and 3, continuing to image 40 and 41). The 40 binary difference images are next added. Thresholds are applied to the summed binary difference image to create the binary images that are used for classification. The first of these binary images is generated by applying a threshold of 1 to the summed binary image. This creates a binary image in which any of the ON pixels from any of the difference images is ON. The second of these binary images is generated by applying a threshold of 2 to the summed binary image. This operation results in ON pixels when there are ON pixels in at least 2 of the 40 difference images. The third of these binary images is created by removing objects that are of a size of less than 10 pixels from the "first" binary image (generated by applying a threshold of 1 to the summed binary difference image).

An example of the three binary images generated using the above operations for a viable *Artemia* is provided in Figure 10. The image on the left in the figure is the binary image created by applying a threshold of 1 to the summed binary image. The image in the center is the image created by applying a threshold of 2 to the summed binary image. Note that the "blob" is smaller in center image than in the image on the left. The image created by size filtering the image on the left is shown on the right. Note the elimination of the single (or few) ON pixels at the top of the image that resulted from this operation. The sub-image of the *Artemia* being analyzed (extracted from the 21st frame) is provided at the bottom of Figure 10.

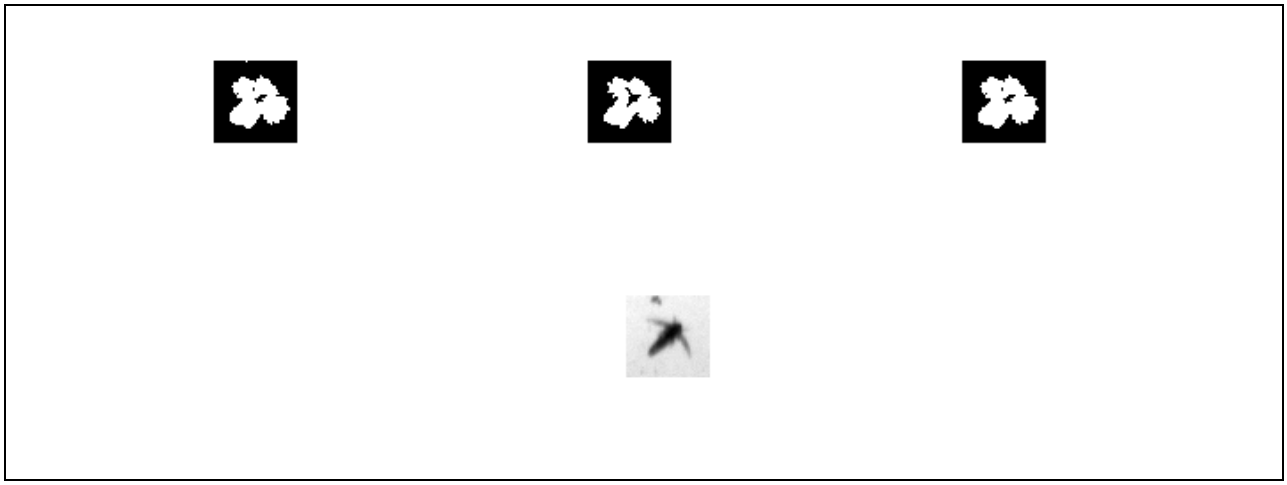

Figure 10. Three binary images used for viability classification and the *Artemia* sub-image for a viable *Artemia* (particle 41).

Figure 11 provides the same three binary images for an immobile *Artemia*. A sub-image of the *Artemia* being analyzed (extracted from the 21st frame) is also shown at the bottom of the figure. It is important to note that the most common result of performing these operations on an immobile or dead *Artemia* would be three binary images without a single ON pixel. In the case shown in this figure, the large number of disparate ON pixels in the middle of the image shown on the left is a result of image noise. This is the case because in virtually no cases were there ON pixels in two or more binary difference images in the middle/upper sections of the image. Further, all of these ON pixels were turned off by the size filter used to create the binary image shown on the right. The large blob at the bottom of the images shown in Figure 11 is a result of another *Artemia* swimming past the *Artemia* being analyzed in the time series. It is also important to note that there are no ON pixels in the binary images shown in Figure 11's middle and right images, in the vicinity of the *Artemia* shown in the sub-image. If the *Artemia* shown in the sub-image were mobile, then the binary image should show ON pixels (white) in the same locations as the *Artemia* in the sub-image.

To generate information for classification, the three binary images (such as those shown in Figures 10 and 11) are next labeled. The number of discreet objects or "blobs" in the binary image is a direct output of the image labeling process. Three parameters that are used in the final viability classification are next generated from the labeling operation.

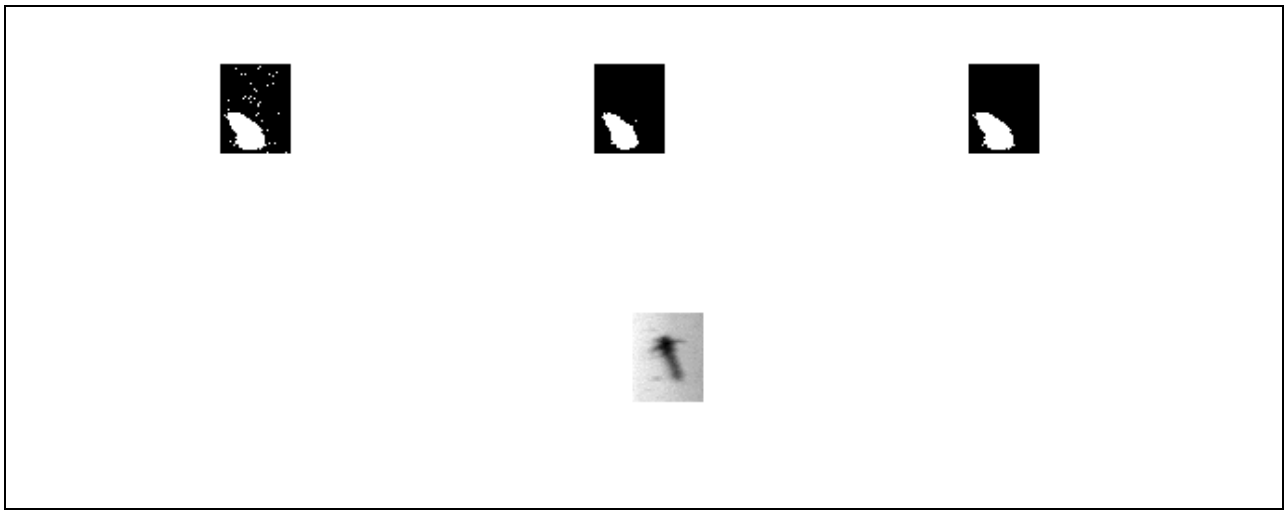

Figure 11. Three binary images for viability classification and the *Artemia* sub-image for a dead *Artemia* (particle 71).

The first parameter, Parameter 1, is the number of objects in the binary image shown on the left in Figures 10 and 11. It has been observed that image noise (which can result in ON pixels) typically produces many ON pixels in this binary image and consequently many discrete objects. It has also been noted that image noise is significantly reduced in sub-images that have a well-defined and well-focused particle present.

The second parameter, Parameter 2, is the number of objects in the binary image shown on the right in Figures 10 and 11. It has been observed that when a particle is mobile (small movements) that the number of objects in the image shown on the right is always greater than one and generally not more than three. This is consistent with the binary operations used to

create this image, which in general produces "blobs" that surround the detected particle that result from small appendage motions, tail motions and other small visceral motions (such as antennae movement).

The third parameter, Parameter 3, is the ratio of the number of objects in binary image shown in the left to the number of objects in the binary image shown in the middle. A low ratio indicates motions that correlate across two or more binary difference images while a high ratio indicates that detected ON-pixels may be a result of image noise and not movement.

A rule base is used for the final viability classification. If Parameter 1 is greater than 20 objects, the particle is considered immobile or dead. If Parameter 2, is greater than or equal to 1 and less than or equal to 3, the particle is considered mobile, or viable. If neither of the above conditions are met, and if Parameter 3 is less than or equal to 4, the particle is classified as viable. If none of these conditions are met, than the particle is considered immobile or dead.

The above rules have done an excellent job in classifying the viability of many *Artemia* (1000s) in many different system evaluations. These same rules have additionally provided excellent results with other zooplankton including rotifers and ciliate protists.

Additional robustness in the classification routines could most likely be obtained by performing a more detailed analysis of the locations of each of the ON pixels in the binary images shown in Figures 10 and 11. Additionally more advanced classification methods that utilize information fusion methods instead of a logical rule set may provide additional robustness.

However, the routine as currently written is providing results that are consistent with the observed number of zooplankton and their viability in the multi-framed TIFF sets. Further, as described in the next section, the software GUI produces a number of outputs that can be reviewed by biologists for final classification and confirmation of automated results.

3.1.3.3 Software GUI Outputs

The software GUI generates a number of outputs that fully document the results of analyses performed by the image processing and classification routines. The tools described in this paper are meant to augment and not replace microbiologists that still oversee microscope image data acquisition and operate the GUI-initiated analyses of the collected data. The tools described significantly reduce the time required to analyze the significant amount of samples that need to be analyzed in the testing of ballast water treatment technology efficacy at NRL's BWTTF. The system outputs provide an archive of the analyses results for microbiologist review and final results tabulation. As described in this section, the results archive additionally provides a "hard record" of the conclusions drawn regarding the state of the challenge water prior to treatment and the efficacy of a ballast water treatment technology.

The software GUI produces six outputs associated with each analysis that it performs. Two output images, a text file, and three output videos (.avi files) are generated and archived by the algorithm.

The first archived algorithm output is an image of the sample well associated with the image being analyzed. Each detected *Artemia* or other particle is labeled with a number in this image. The number is written in green for very mobile *Artemia*, yellow for slightly mobile *Artemia*, or red for a dead *Artemia*. A title included in the image provides the total number of particles counted, the number of very mobile *Artemia* detected, and the number of slightly mobile *Artemia* detected (classified as "Flinchers"). The title used on this image effectively tabulates the important results of the sample well analysis. As will become apparent in data provided later in this section, a microbiologist or technician can review the data in this image, and adjust results up or down to accommodate the few particulates that were classified as "dead" *Artemia* or the few *Artemia* that were not counted as a result of masking operations.

The second archived output is a binary image representation of the objects detected in the sample well being analyzed. Each detected *Artemia* in this image is labeled with the same number as in the previously described image and with the same color code. The title is also the same on this archived image output as it was in the first archived image output. Again, this title effectively summarizes the results of the analyses. The major purpose of this output image is as a diagnostic tool for evaluating the efficacy of the image processing algorithms and as an aid in algorithm diagnostics and forensics in the event that the algorithm produces a "questionable result."

The text file output provides an archive of user information that was entered into the GUI. Specifically, it provides the coordinates of the mouse clicks used to develop the mask, the frame number of the image being analyzed, and a complete record of input and output files and their locations. Again, this output is produced primarily to aid in algorithm diagnostics and forensics. Information provided in this file allows the algorithm results to be re-tabulated without any additional user inputs (e.g., to develop the mask and to specify the image to be analyzed). Information in this file has been used to show that a mask was not properly defined (too large) by the system operator.

The first video output produced is a time series (video) of the complete sample well for the 41 images that were used in the viability analyses. This video clearly shows all of the *Artemia* in the well. Very mobile *Artemia* are additionally quite visible in this video output.

The second video output produced is a time series (video) of the binary representation of the complete sample well for the 41 images that were used for the viability analyses. Again, this output is produced mostly as a diagnostic aid for evaluating the efficacy of the image processing algorithms. Very mobile *Artemia* are clearly delineated in this video segment. This output has also been used to alert the operator that other smaller zooplankton or mobile protists have been detected in the sample.

The final and most important video output is a movie of each of the individual *Artemia* detected in the sample generated using the 41 sub-images that were used for the viability analyses. Prior to showing an image of a detected particle, the number that was used to designate a detected particle in the algorithm output is displayed. This allows the operator to rapidly hone in on a short video segment of any of the detected *Artemia*. Review of these video segments clearly shows the large movements or small movements that were used to designate a detected particle as viable. The operator can also review this video to fine tune analysis results. In more dense samples a non-viable *Artemia* can be physically moved by other *Artemia* in the sample, which can result in a dead *Artemia* being declared very mobile or slightly mobile. Additionally, a live

Artemia swimming over a dead *Artemia* being evaluated can be mistaken as a small movement of the dead *Artemia* being observed. The live/dead counts generated automatically by the image processing algorithms described in this paper can be validated and/or adjusted through an operator's review of this video segment, if required.

The time-resolved high-resolution TIFF image series itself additionally provides a complete archive of the 75 seconds of microscope observations conducted on each sample well. If enhanced versions of the algorithms described in this paper are developed, previously archived image series can be re-examined to generate improved results or additional information. Further, the complete high resolution time-resolved TIFF image series can be "stepped through" and worked with interactively using standard software (e.g., Windows Picture and Fax Viewer) allowing a microbiologist to draw his or her own conclusions on the data.

3.2 Phytoplankton

Phytoplankton are defined in this effort as being greater than 10 microns and less than 50 microns in size. This size range results from the nets used to fractionate the plankton classes in the ballast test water. Note that zooplankton smaller than 50 microns (i.e., rotifers) are also part of this size classification and they are analyzed differently from the phytoplankton.

3.2.1 Phytoplankton Sample Preparation

In BWTTF evaluations, 3-m^3 sample tanks are used to collect the phytoplankton samples. The tanks are homogenized and three 1-Liter (L) samples are taken. For each of these three 1-L samples, the following sequence is repeated three times. The samples are homogenized and a 10-mL sample is removed. From each 10-mL sample, one 1-mL sub-sample is extracted for analysis. This results in three sub-samples from the 10-mL samples taken from the three 1-L samples for a total of 9 samples for analysis. One 3-m^3 sample tank is filled with the challenge water and a second is filled with post-treatment water. The above sampling process is conducted on both tanks.

It currently takes approximately 4½ hours of microscope observations to enumerate, classify and determine the viability the nine 1-mL phytoplankton samples taken from one 3-m^3 sample tank. Observations are all made manually. These samples are currently being analyzed using the florescent microscope and the Sytox® dye system described in this report. In implementing this approach the efficacy of this dye system for the detection of non-viable phytoplankton has been further demonstrated.

For the current effort to find automated means of analysis, a commercially available flow cytometer, the FlowCAM®, was investigated and some of the manual sample preparation had to be changed to accommodate requirements or limitations of the instrument.

When using the FlowCAM® it is essential that the sample be diluted and the pump speed be adjusted to ensure that only one particle is in the instrument's field of view when the unit triggers. This is important since the same chlorophyll florescent, 520 nm florescent, and forward

scatter intensity level is assigned to each particle that is in the instrument's field of view when the unit triggers. If five particles are in the field of view, each of these five particles are assigned the same florescent values. This means that these parameters are not valid for four of the five detected particles. Further, it is impossible to determine which of the five particles should be assigned the florescent and scatter values. Consequently, when multiple particles are in the instrument's field of view it is not possible to use this important information for the classification of the particles. As an example, when multiple particles are in the field of view, it is not possible to use the chlorophyll florescent values to discriminate phytoplankton from sediment. It also precludes using the 520 nm florescence value for cell viability assessment.

To prepare samples for analysis, the stock *Tetraselmis* or *Thalassioira weissflogi* solutions were diluted to a level sufficient to prevent multiple particles being in the FlowCAM®'s field of view simultaneously. It is anticipated that the 1-mL samples that are currently used in conjunction with florescent microscope analyses are at a concentration level that is optimal for direct analysis by the FlowCAM®. After further demonstration, the FlowCAM® technology promises to reduce the time required for BWTTF phytoplankton characterization from 4 ½ hours to approximately ½ hour including all preparation time.

3.2.2 FlowCAM® System

For the automated detection and classification of phytoplankton, this effort concentrated on the use of a commercially available and unique imaging flow cytometer. This system, a modified version of the Fluid Imaging Technologies' FlowCAM® system, has shown its suitability to determining the concentration of phytoplankton in solution and, when used in conjunction with deoxyribonucleic acid (DNA) staining dyes, offers the potential of determining the live/dead ratio of detected phytoplankton. The sensitivity of the forward scattering detector of the system was significantly increased so that the unit now reliably triggers on all objects in the size range of BWTTF surrogate phytoplankton. The system produces high-resolution imagery as well as useful florescence intensity information (at the chlorophyll florescence wavelength and at a wavelength optimized to the DNA staining dye). The florescence intensity levels, in conjunction with image features generated by the FlowCAM® and additional features extracted from the high-resolution imagery using in-house developed algorithms, offer the potential to classify the two surrogate phytoplankton used in support of the BWTTF as well as indigenous phytoplankton that are introduced during testing.

For analyzing phytoplankton samples, most probable number (MPN) methods are generally used to determine viability. This method does not provide "real-time" results. As part of this study, DNA staining membrane impermeant dyes were identified that have been shown to reliably stain non-viable cells. These dyes were originally identified for use with a commercial imaging flow cytometer system, specifically the Fluid Imaging Technologies FlowCAM® system. These dyes are currently also being used in conjunction with manual microscopy methods to classify phytoplankton species and determine their concentrations and viability in "real-time." This provides a significant improvement to MPN methods, but it still takes approximately 4½ hours of extensive microscope observation time to complete these analyses using manual methods. In seeking an automated method for determining the concentration and viability of phytoplankton, the current effort concentrated on the evaluation of the FlowCAM® system. Although this system as purchased was not adequate for performing these functions, modifications were made

during this study which greatly improved its capabilities for phytoplankton sample characterization. This system also promises to significantly reduce the overall time required to characterize BWTTF samples for phytoplankton concentration and viability.
Much of the effort associated with this program was directed at obtaining the required modifications in the FlowCAM® system to make it suitable for automated characterization of BWTTF phytoplankton water samples. These changes were implemented throughout this program's period of performance and only as the end date of the program was approached have they been fully completed. With the additional testing recommended in Section 4.5 of this report, the FlowCAM®'s applicability to characterizing BWTTF phytoplankton samples will be fully demonstrated. It should also be noted that many of the changes that have been implemented on the FlowCAM® over the course of this program are now available in Fluid Imaging Technologies Systems.

3.2.2.1 Hardware and Hardware Modifications

The FlowCAM® system that is produced by Fluid Imaging Technologies is shown in Figure 12. System components are depicted in the two photographs shown on the left of the figure. The system consists of a PC that is used to control the system and to archive the collected data and a separately housed opto/mechanical breadboard. The breadboard itself is shown in the photograph on the right in Figure 12. Housed on the breadboard are all of the mechanical components required to bring the sample being analyzed through a flow cell (pumps, controls, flow cell holder etc.). Also on the breadboard are all of the optical components used to induce and measure florescence from particles passing through the flow cell and a detector that monitors particle-induced forward scatter from this same laser. When a particle is detected by either of the florescence channels or the forward scatter value, it is imaged as it passes through the flow cell. Imaging is accomplished using a unique optical configuration that uses a "flash" LED (light emitting diode) to illuminate the particle and a unique microscope objective/camera/custom optical element configuration that extends the microscope objective's depth of focus. As a result, this system produces very high quality imagery of all detected particles.

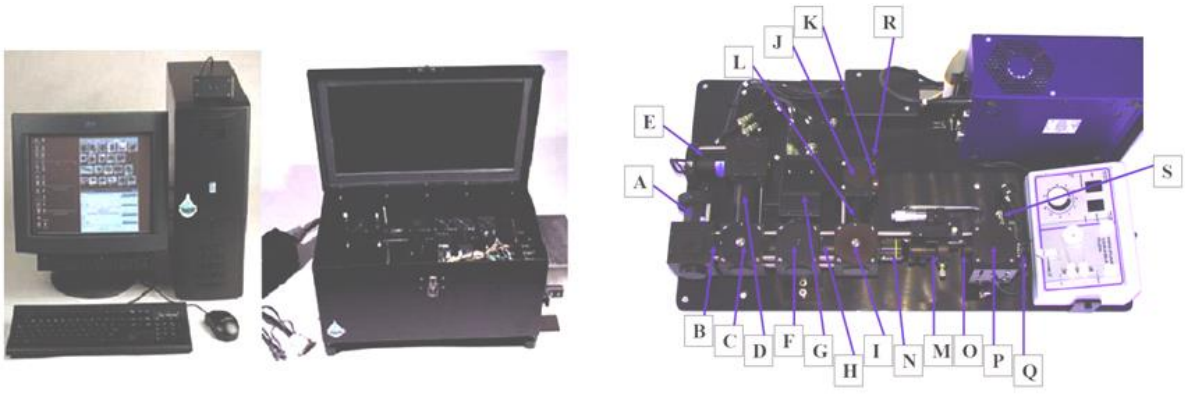

Figure 12. Fluid Imaging Technologies FlowCAM® Imaging Flow Cytometer.

Over the course of this program, significant modifications were implemented in the FlowCAM® system originally purchased in 2004 to allow automatic characterization of phytoplankton.

These modifications and the improvement in automated phytoplankton water sample analysis that resulted from these modifications are described below.

Upgrade to a 4-channel system

The FlowCAM® unit originally purchased was a 2-channel system. As such it could only be configured to simultaneously monitor two florescence channels or a florescence and forward scatter channel. The system was upgraded to a 4-channel system such that the unit could simultaneously monitor both the florescence channels and the forward scatter channel. The unit additionally triggers whenever any of the intensity values measured in these channels exceeds a user-selected threshold value. The 4-channel configuration is required for reliably determining the live/dead ratio of detected phytoplankton and helps in performing discrimination between phytoplankton and sediment in the same size range.

Upgrade to a high resolution digital camera system

The originally purchased FlowCAM® unit used an interleaved (two interleaved frames are used to generate an image) analog video camera and image capture board to capture imagery of the detected particles. The analog camera was replaced with a progressive (one frame is used to generate an image) high resolution digital camera. The camera upgrade significantly increased the resolution and size of the images collected using the FlowCAM®. Figure 13 provides images of *Artemia* (a zooplankton surrogate) collected using the analog camera (on the left) and the digital camera (on the right).

The image data provided in Figure 13 clearly shows the size and resolution improvements that are obtained using the new camera system. In addition, reviews of these images show that there is enhanced dynamic range in imagery that is collected with the new camera system. This improves the image features that can be extracted from the collected imagery which, in general, results in improved discrimination capabilities.

Figure 14 displays FlowCAM® generated imagery of *Tetraselmis* (a phytoplankton surrogate) collected using the analog camera (on the left) and the digital camera (on the right). These images show why it was important to upgrade the camera if the FlowCAM® system is to be used to detect and classify phytoplankton. The imagery collected using the analog camera was pixel limited. As such there was not sufficient information to discriminate between *Tetraselmis* and other phytoplankton species. The imagery generated by the digital camera provides significantly improved resolution. Furthermore, the improved dynamic range of the digital camera results in the images that provide information on the cell structure that will likely be useful for the discrimination of various phytoplankton species.

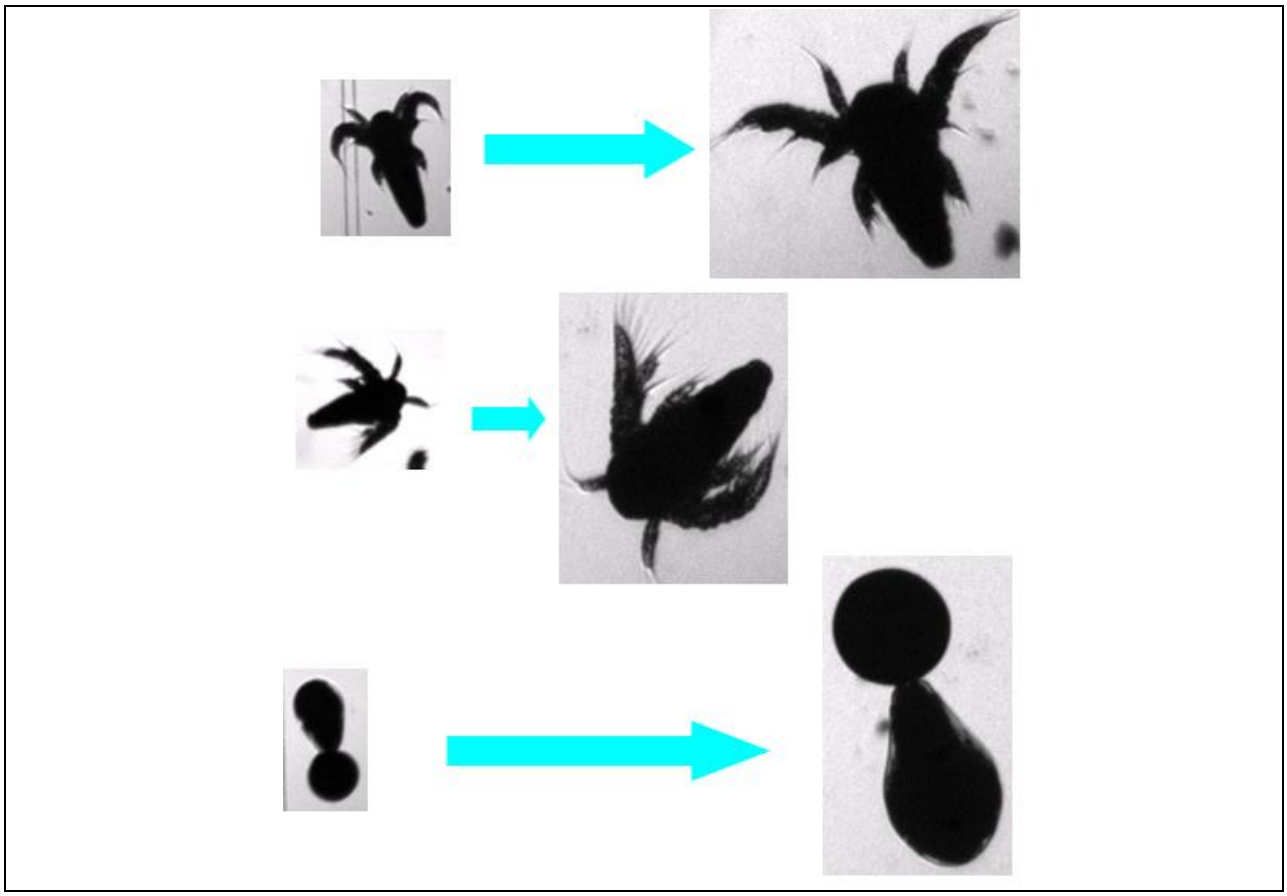

Figure 13. FlowCAM® *Artemia* Images - Analog camera images on left and digital camera images on right.

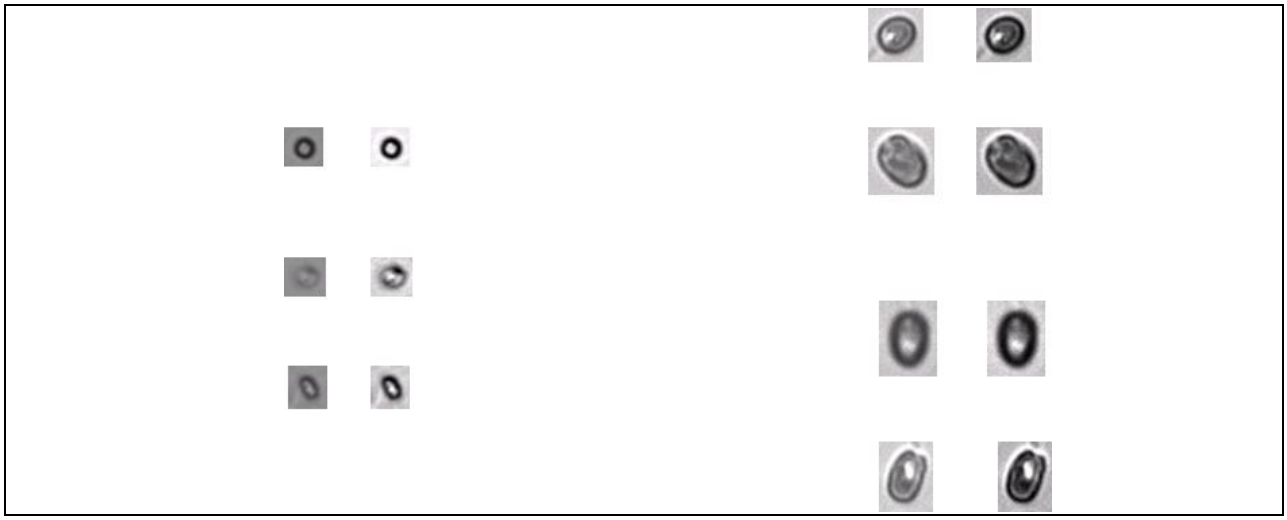

Figure 14. FlowCAM® *Tetraselmis* images - Analog camera images on left and digital camera images on right.

Upgrading to a blue laser (from a green laser)

The original FlowCAM® unit was provided with a green laser. The laser's wavelength was too long to efficiently induce fluorescence in either metabolic dyes or DNA staining dyes that were explored for cell viability determination. The unit was upgraded to a blue laser to explore the use of dyes. The blue wavelength also increased the measured chlorophyll intensity levels and resulted in requirements to change the detector used to monitor forward scatter.

Modifying the bandpass of the second florescence channel to 520 nm

The bandpass of the second florescence channel was modified to provide a wavelength bandpass at 520 nm to obtain compatibility with the Sytox® Green DNA staining dye that consistently identifies cells with compromised cell membranes (non-viable cells).

Increasing the sensitivity of the forward scatter detector

The forward scatter detector in the original FlowCAM® system was not sufficiently sensitive and did not have appropriate circuitry to reliably trigger on particles in the size range of the BWTTF phytoplankton surrogates. The sensitivity was increased and the circuitry was modified so that the forward scatter detector now reliably detects all particles in the size range of the BWTTF phytoplankton. This capability is required for the reliable enumeration of phytoplankton concentrations and the discrimination between these cells and sediments in the same size range.

3.2.2.2 Dyes for Viability Classification

Two types of dyes were explored as potential means for automatically identifying viable cells: metabolic dyes and cell membrane permeant DNA staining dyes. It is important to note that the selection of optimal fluorescent stains to assist in determining viability is intimately tied to analysis equipment settings and automated counting techniques.

The metabolic dye Calcein AM was explored as one potential method for the detection of viable cells. This dye's florescent properties are altered when acted upon by enzymes (esterase) associated with cell metabolic activity [Brussaard, C. P. D., Marie, D., Thyrhaug, R., Bratbak, G. (2001)]. Work performed using florescence microscopes with this dye showed that the variability in cell metabolism that results from diurnal and other external conditions results in the variability in the dye's florescent intensity levels. This variability makes it difficult to automate viable cell identification using Calcein AM.

The cell permeant dye Syto® 9 was also explored as a means of detecting viable cells. This dye permeates the cell membrane and its florescence becomes active when the dye contacts genetic materials (DNA) inside the cell [Brussaard et al (2001); Veldhuis, M. J. W., Kraay, G.W., Timmermans, K. R. (2001)]. Results with this dye were inconsistent and depended significantly on the process (time, temperature, etc.) used to "stain" the cells being evaluated. Further, as a cell permeant dye, it represents a health hazard during the cell staining process when the dye is worked with.

In addition to the dyes for detecting viable cells, one dye system was explored for the detection of non-viable cells. The cell impermeant dye Sytox® was explored as a potential means of detecting non-viable cells [Brussaard et al (2001); Veldhuis et al (2001)]. This dye cannot travel through the cell membrane of a healthy cell. A break in cell membrane is a strong indication of a non-viable cell, and in these cases the dye can interact and "stain" the genetic material within the cell. Work performed using the florescent microscope provided a strong indication that this dye system consistently stained non-viable phytoplankton cells. The literature suggests that different cell staining procedures might be required for different types of phytoplankton, but the work performed in support of this program has initially indicated that this dye effectively stains cells of both BWTTF surrogates (*Tetraselmis* and *Thalassioira weissflogi*i) as well indigenous phytoplankton. As a cell impermeant dye, it also provides less of a health hazard than the cell permeant dyes that were explored as a potential means of detecting viable cells. Based on work performed using the florescent microscope, it was decided to pursue phytoplankton viability classification using the cell impermeant DNA staining dye, Sytox® under this program.

An initial experiment was performed that has demonstrated the applicability of this dye to cell viability classification using the FlowCAM®. As mentioned in the previous section, two modifications were made in the FlowCAM® system to make it compatible with this dye system. These changes involved switching to a blue laser to excite florescence and the modification of the wavelength passband to be compatible with the 520 nm emission wavelength of Sytox®. The initial experiment involved the use of homogenous samples of either live or dead cells. The samples were stained using the same procedure and were introduced to the FlowCAM® to determine the florescent properties of the detected particles.

Figure 15 provides the results that were obtained during this initial experiment. The data in the figure are provided in normalized histogram format with the x-axis being the florescent intensity levels and the y-axis being the normalized count (i.e., the number of particles detected with that florescent property divided by the total number of particles detected in the sample). A review of the data provided in Figure 15 clearly shows that there are two distributions for cells, one for the viable cells at low florescent intensity level and one for the non-viable cells at a higher florescent intensity level. These data clearly indicate the potential of using the cell impermeant dye Sytox® in conjunction with the FlowCAM® as an automated means of assessing cell viability in BWTTF phytoplankton samples.

Additional work is required to fully demonstrate the applicability of this dye system to the automated detection of live/dead ratios of BWTTF phytoplankton samples. The work required to bring this technology to the operational level is described in Section 4.5 of this report.

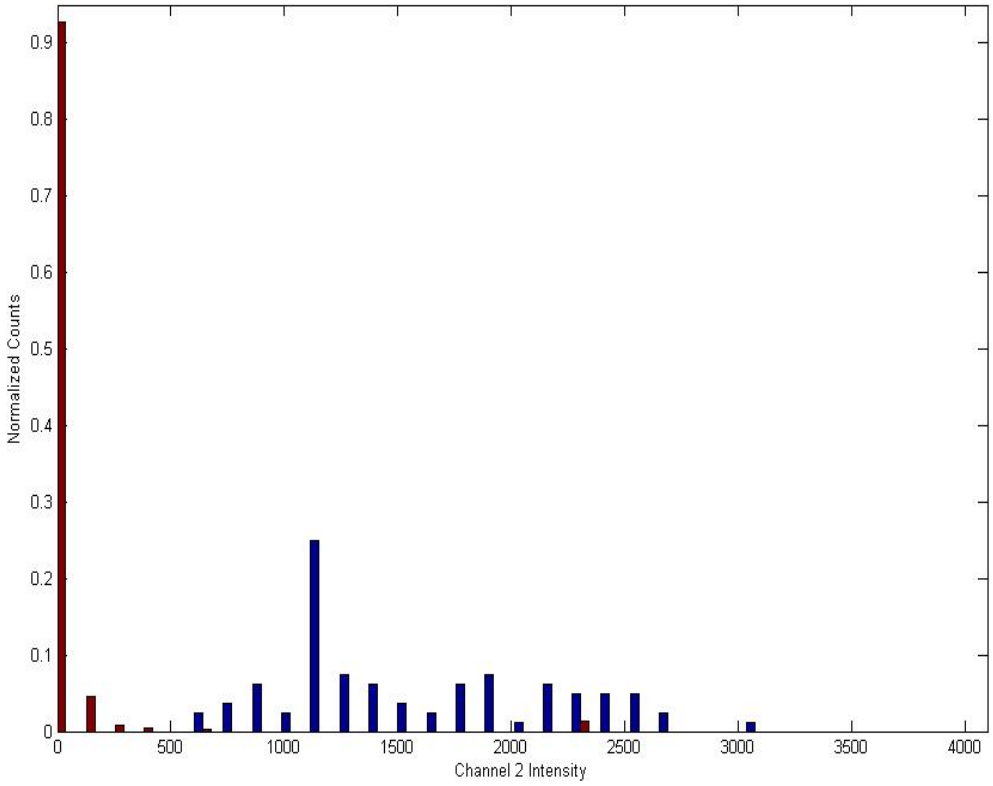

Figure 15. Normalized histograms for Sytox® stained viable (red) and non-viable (blue) phytoplankton samples.

3.2.2.3 Image Processing and Classification Algorithms

Some work was conducted on developing image processing and classification algorithms for the discrimination of various zooplankton and phytoplankton species using FlowCAM®. This section describes work that was performed to demonstrate the potential use of these methods for zooplankton discrimination. In Section 4.2 of this report, initial work that was performed to demonstrate the ability to discriminate between surrogate phytoplankton and debris is described. Both the work described in this section and in Section 4.2 was performed using data that were generated using the original 2-channel FlowCAM® system.

Table 1 provides a list of feature values that are extracted directly by the FlowCAM® when evaluating samples. The FlowCAM® extracts features from the imagery associated with each detected particle. These features include geometrical information regarding the detected particle including its size (Cell Area, Cell ESD (Equivalent Spherical Diameter), CellX and CellY), its shape (CellX, CellY, Feret maximum diameter, Feret minimum diameter, as well as parameters like aspect ratio that can be derived by taking the ratio of some of the above parameters), and the intensity levels of the chlorophyll florescence channel (Channel 1 Peak) and the intensity level measured by the forward scatter detector (Channel 2 Peak). The Feret maximum diameter and Feret minimum diameter are the longest major and shortest minor axes of all possible ellipses

that could encapsulate a detected blob. (Note that a 4-channel system also provides the florescence intensity information at 520 nm and that recent improvements in the FlowCAM® software have resulted in more and improved features for discrimination.) Also shown in the table is the particle number (assigned for each particle detected) as well as the particle Class. The particle Class was specified based on a manual review of the FlowCAM® image data. The *Artemia* occur in three separate stages; nuplii (particle Class art), umbrella (particle Class umb) and cyst (particle Class cyst). Also occurring in the sample is debris that is approximately in the same size range as the *Artemia* (particle Class junk). The features associated with the umbrella stages are highlighted in Table 1.

Table 1. FlowCAM® generated features for potential discrimination between *Artemia* life stages.

Class	Part Num	Cell Area	Cell ESD	Cell X	Cell Y	Feret_max_ diameter	Feret_min_ Diameter	Channel 1 Peak	Channel 2 Peak
art	68	47941	247	78	236	422	288	547	3292
umb	17	47199	245	198	256	396	188	429	2980
art	36	46908	244	119	188	365	324	4040	3326
art	9	46669	243	265	164	370	226	440	2576
art	61	45026	239	202	219	391	231	503	1171
art	26	44629	238	104	238	350	247	437	2864
art	24	44152	237	92	240	355	247	514	2538
art	35	43781	236	247	225	355	226	477	2786
art	11	43648	235	111	90	427	319	445	1629
art	52	43171	234	207	247	411	200	408	2596
umb	79	43039	234	539	215	350	190	541	2388
art	44	42032	231	304	222	314	262	518	791
art	62	42032	231	349	230	370	267	4095	1672
art	19	41660	230	145	239	370	314	498	2439
umb	105	41422	229	277	246	339	231	425	2829
umb	43	40229	226	492	219	344	185	415	2251
junk	6	39963	225	124	240	607	267	511	1088
junk	2	39434	224	95	190	1029	236	511	1088
umb	20	39673	224	449	256	370	169	562	3472
art	32	38825	222	543	83	314	263	477	2958
umb	92	38825	222	363	222	350	175	425	2529
umb	8	35565	212	538	184	344	149	396	1498
art	45	35380	212	41	252	262	200	465	2441
art	47	35486	212	318	247	365	154	415	1349
art	93	33524	206	114	215	365	185	285	1200
art	71	31245	199	51	236	293	149	428	1559
art	27	29894	195	354	256	344	154	598	3060
umb	31	29894	195	59	73	252	149	500	862
art	56	28118	189	328	247	226	195	3973	2876
art	89	26051	182	152	231	303	159	424	2926

A review of the features provided in Table 1 shows that these features can not be used to separate the art/umb/junk particle classes. Additionally it was not possible to discriminate between the cyst/junk particle classes with the features that are directly generated using the FlowCAM®. In order to supplement the features used for *Artemia* life stage separation, an in-house developed GUI was configured for extracting additional features from the FlowCAM® imagery. Figure 16 provides a screen shot generated from this GUI. The particle being worked with is indicated by the red T in the upper left corner of the image. This image is rendered with different degrees of contrast enhancement in the two sub-images that are shown directly under the box containing 7 (the number of the particle being worked with).

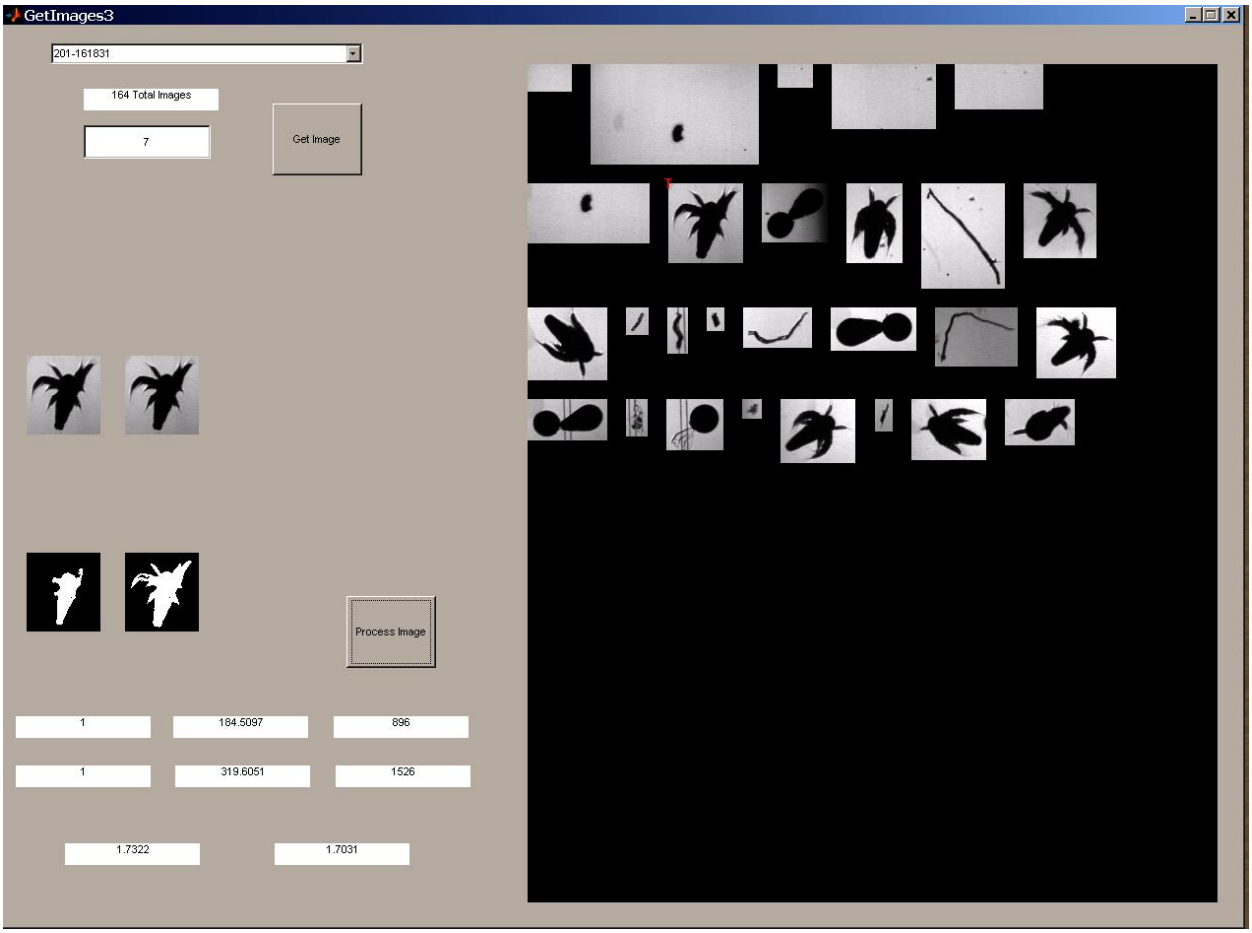

Figure 16. Screen Shot of an in-house developed GUI for extracting additional features from FlowCAM® imagery used to discriminate between *Artemia* life stages and debris. The right half of the screen displays individual images. Information boxes in the upper left include sample number, total number of images in sample, and number of image being investigated. The paired images below the boxes result from different contrast levels. The paired binary images are the results of different thresholds. Features of the imaged organism are shown below the binary images.

In order to develop a feature that can be used for discrimination, two binary images are created from the sub-image. This is accomplished by applying two thresholds to the sub-image. The application of the first threshold (higher value of the two) highlights the body section of the

particle being detected. The application of the second threshold highlights both the body section of the detected particle as well as its appendages, in the case of *Artemia* nauplii. Note that in the case of umbrella and cysts there is very little difference in the two binary images that are created by applying the two threshold values. In order to exploit this, a new feature is generated. This feature is the ratio of the perimeter of the two objects in the binary images. In the case of nauplii, this ratio tends to be high as a result of the differences in the two binary images. In the case of umbrella stages and cysts, the value of this perimeter ratio is generally close to 1 since there is little difference in the binary images. In the case of debris, the perimeter ratio that is produced is in general of a very low value.

Table 2 provides many of the features that were previously provided in Table 1 supplemented by the perimeter ratio feature (perim ratio) that was just described. The data in the table were sorted in descending value based on this ratio. Particles of the *Artemia* umbrella stage are again highlighted in Table 2.

Table 2. A supplemental feature (Perim Ratio) was required to separate *Artemia* life stages.

Class	Part Num	Cell Area	Cell ESD	Cell X	Perim Ratio
art	93	33524	206	114	2.76
art	45	35380	212	41	2.59
art	11	43648	235	111	2.28
art	36	46908	244	119	2.18
art	26	44629	238	104	2.04
art	19	41660	230	145	2.02
art	62	42032	231	349	1.99
art	35	43781	236	247	1.95
art	52	43171	234	207	1.82
art	9	46669	243	265	1.8
art	61	45026	239	202	1.8
art	68	47941	247	78	1.77
art	44	42032	231	304	1.73
art	24	44152	237	92	1.58
art	32	38825	222	543	1.57
umb	105	41422	229	277	1.48
art	27	29894	195	354	1.48
art	89	26051	182	152	1.43
art	47	35486	212	318	1.33
art	56	28118	189	328	1.32
art	71	31245	199	51	1.17
umb	31	29894	195	59	1.13
umb	92	38825	222	363	1.08
umb	79	43039	234	539	1.07
umb	43	40229	226	492	1.06
umb	20	39673	224	449	1.04
umb	8	35565	212	538	1.04
umb	17	47199	245	198	1.03
junk	6	39964	225	124	0.1
junk	2	39434	224	95	0.1

A review of the data provided in Table 2 shows that the supplemental feature, the perimeter ratio, affords the reliable separation of the two *Artemia* life stage classes as well as separation of the two stages from debris in the sample. The single umbrella stage (particle 105) that overlaps with the *Artemia* nauplii stage was actually an umbrella stage that was in the process of hatching. A combination of particle size parameters and the perimeter ratio also provided excellent separation between the umbrella stages, cysts and other debris that were in these samples. The data

provided in Table 2 clearly demonstrates that a combination of the FlowCAM®-generated features and supplemental features that can be generated through in-house developed image processing algorithms can be used to separate classes of particles that are detected by the FlowCAM®. An example of the results obtained using FlowCAM® features to separate detected phytoplankton from detected debris is provided in Section 4.2.

4.0 Results and Discussion

4.1 Zooplankton Characterizations

4.1.1 Artemia

The following provides an example of running the algorithms described in Section 3.1.3.2 on a complex sample. The time-resolved high-resolution TIFF image series was collected as part of a toxicity evaluation that was being performed on *Artemia* and other zooplankton surrogates. The sample is a "real world" sample and includes significant *Artemia* cysts and debris particles.

Figure 17 provides the raw image of the TIFF frame specified for analysis during this evaluation. The figure clearly shows that this is a dense *Artemia* sample with a lot of debris present. Furthermore, this sample contains a mixture of very mobile, slightly mobile, and dead *Artemia*.

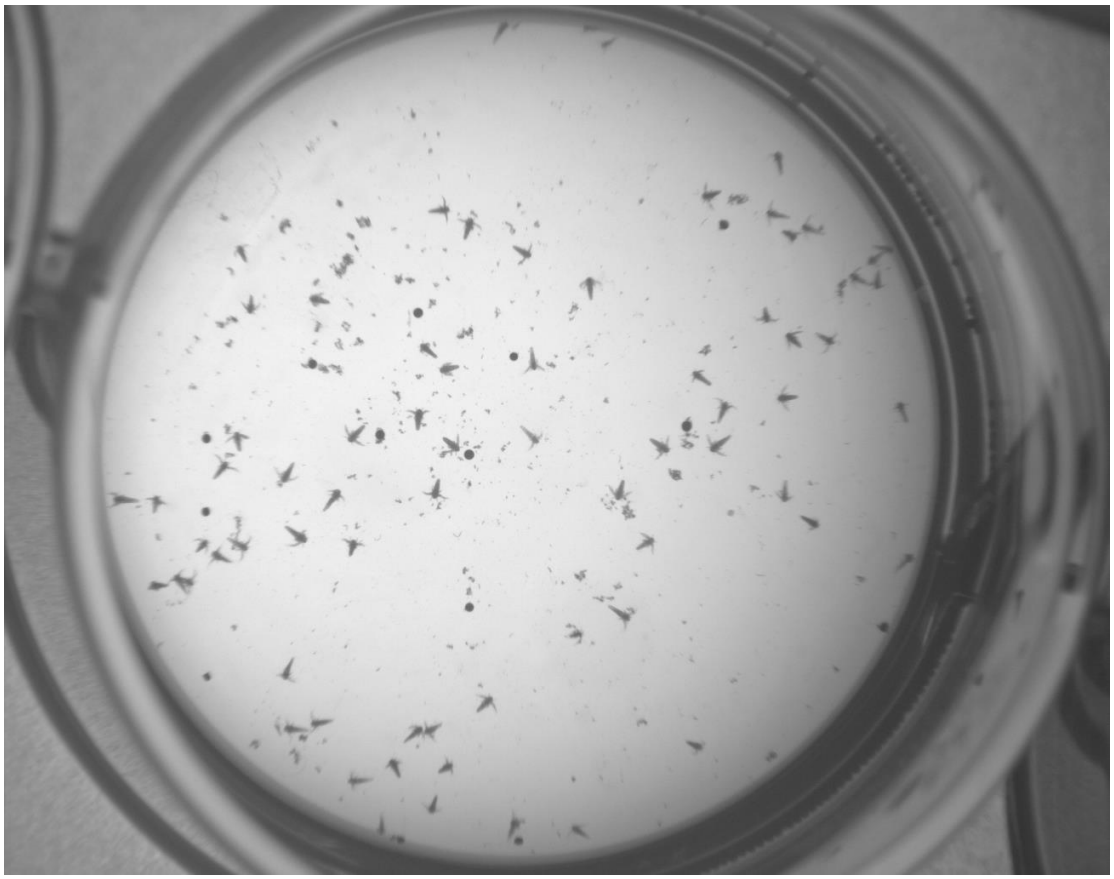

Figure 17. Raw image of a sample well evaluated by the algorithms described in this paper.

Figures 18 and 19 provide the output images that were produced in the analysis of the image shown in Figure 17 (and the associated TIFF image series). Figure 18 provides the output image and tabulated results and Figure 19 provides the binary output image and tabulated results.

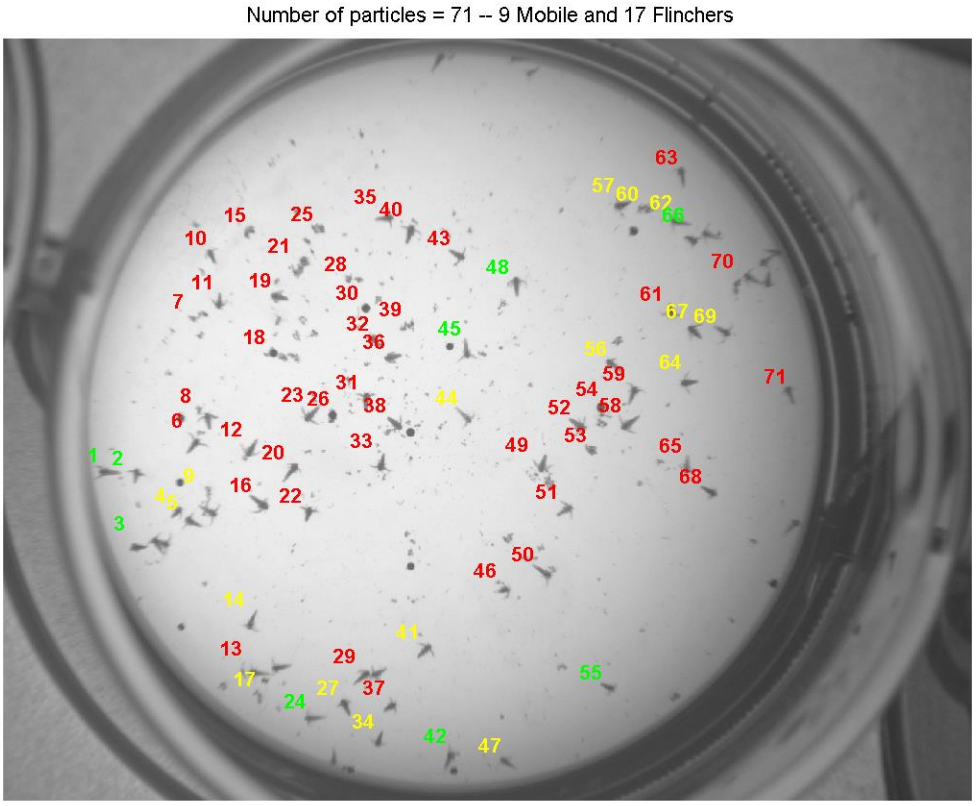

Figure 18. Image of sample well with labeled detected *Artemia* and classification results.

A review of the data provided in Figures 18 and 19 clearly show that the algorithms described in this paper are capable of working with complex microscope samples. The majority of the debris and *Artemia* cysts are "ignored" by the algorithm and not counted. A small number of particulates (not *Artemia*) were counted and classified as dead *Artemia* in this sample. A few groups of *Artemia* are classified as individual *Artemia* as they were physically (or very close to physically) in contact with each other. A few *Artemia* are additionally not counted as they were "masked" as a result of the masking operations.

A total of 71 *Artemia* were detected in this sample well (a fairly dense sample). Of the 71 total *Artemia*, 9 *Artemia* were classified as mobile (very) and 17 were classified as "Flinchers" or slightly mobile. Consequently, the algorithm detected 45 dead *Artemia* in this complex sample. These results agree very well with the observed number of particles and their viability in the multi-frame TIFF imagery. These automated results however are obtained in a fraction of the time required to do manual microscope observations and there is a "hard" data archive of the analysis and its results.

As mentioned earlier (Section 3.1.3.1) the time required to position the sample well and collect the required imagery for enumeration and viability assessment for *Artemia* in the well is less than

two minutes. This is almost a 20-minute savings over the manual microscopy method, and an archive record is obtained. The complete characterization of a BWTTF water sample (10 wells at typical sample densities) takes approximately 20 minutes as compared to the 3½ hours of continuous manual microscope observations presently required to do the same work. Again, the manual method does not provide an archive of observations as the automated method does.

The above-mentioned discrepancies (grouping of *Artemia*, not detecting *Artemia* due to masking, classifying debris as *Artemia*) have minimal impact on the overall results, and the results compare favorably to manual microscopy and counting. These and any other discrepancies in the automated analyses can be easily resolved through review of the image shown in Figure 18.

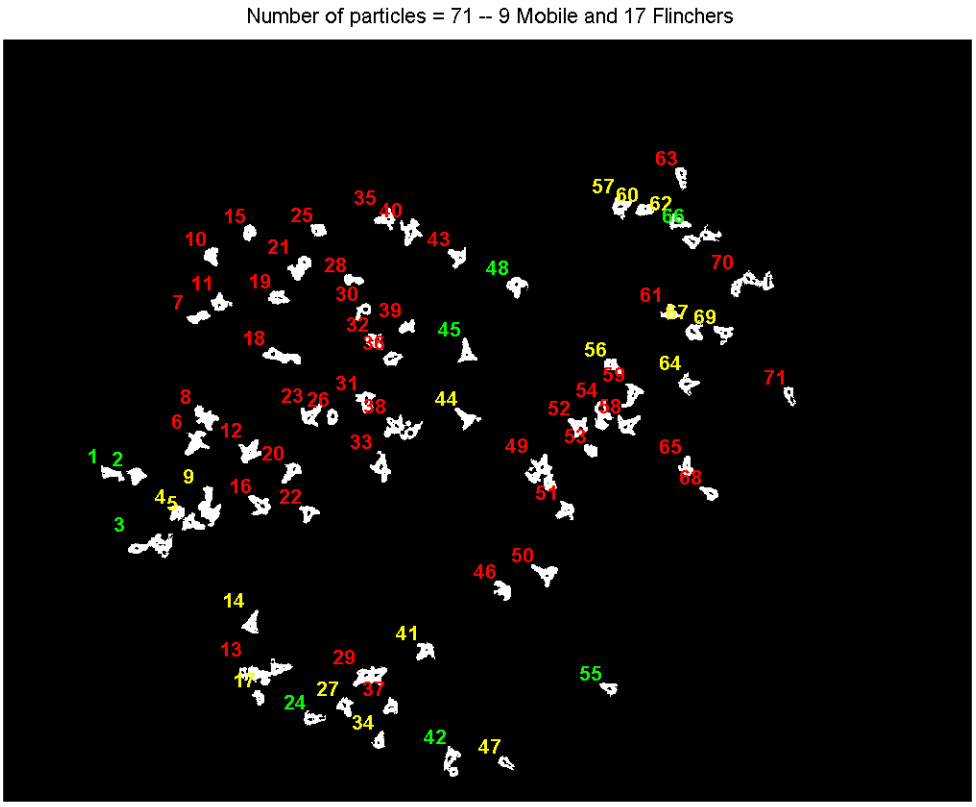

Figure 19. Binary image representation of detected particles in the sample well with labeled detected *Artemia*, and classification results.

Lastly, as previously mentioned, the final classification results provided in Figures 18 and 19 can be both validated and fine tuned, if necessary, through microbiologist or technician review of the GUI's video and image outputs.

4.1.2 Rotifers

The algorithm for the enumeration and live/dead classification of *Artemia* has, with minor modification, also been applied to zooplankton rotifer samples. Rotifers are smaller than 50 microns but larger than 10 microns. 50-µL, 100-µL and 1-mL samples have been evaluated in 3-mL sample wells and 1-mL samples have been evaluated in 7-mL sample wells. It should be noted that the smaller volume samples were evaluated primarily to reduce the water column

depth and that the surface area of the standard 7-mL sample well is approximately two times that of 3-mL sample wells. The data presented in this section were generated using 100-µL samples in 3-mL sample wells.

Figure 20 provides one of the image outputs from a run conducted with rotifer zooplankton. The algorithm correctly identified the 35 rotifers that were present in the sample and, as indicated, identified 12 potentially dead rotifers in this sample. The results provided here are similar to those that were obtained using manual microscopic methods, and a review of the image shows that the algorithm has detected the majority of particulates that were present in the sample. Figure 21 provides the binary representation of the image that is shown in Figure 20. A review of this image shows that a few of the rotifers were grouped together (in the vicinity of # 19) and that the indicated concentrations are indicative of the sample.

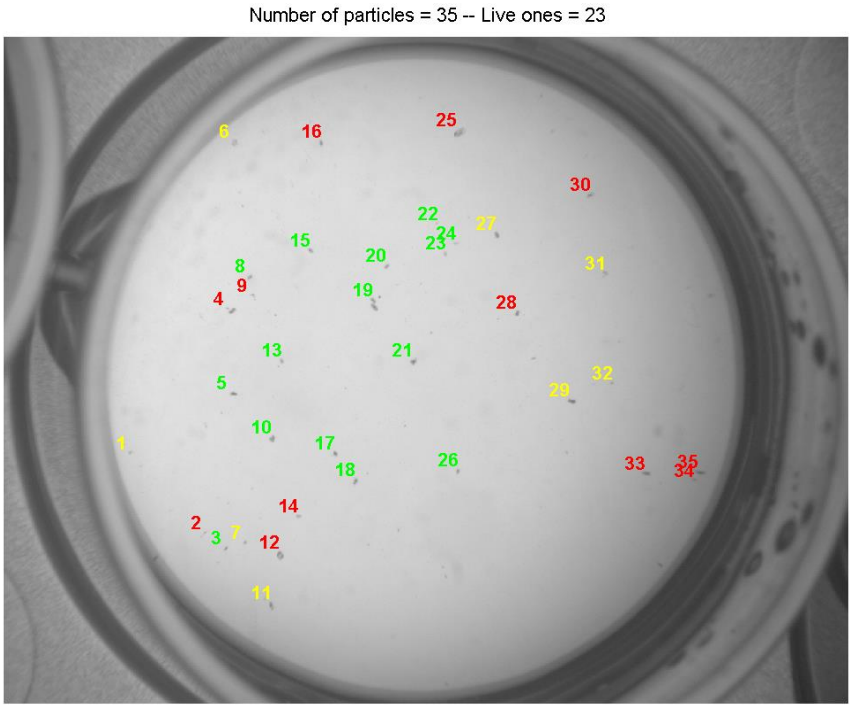

Figure 20. Image of sample well with labeled detected rotifers and classification results.

The data provided in Figures 20 and 21 provide very encouraging results that the methods that have been primarily developed for *Artemia* can be modified or directly utilized for the detection, enumeration and viability of rotifers. For these images the GUI's video outputs show imagery of rotifers at very high resolution. A review of the video also validates the classification results that are provided in these two figures.

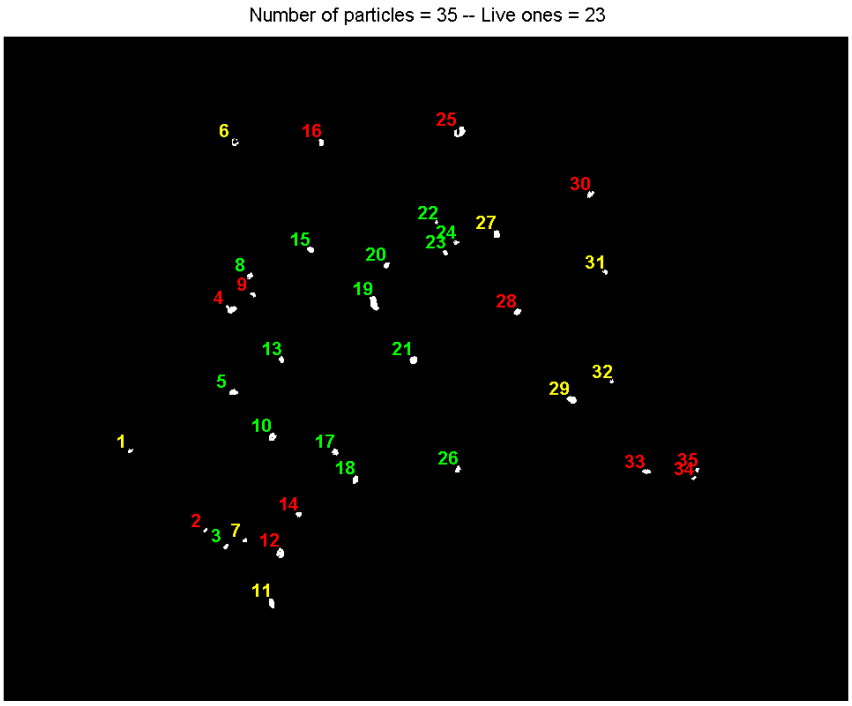

Figure 21. Binary image representation of detected particles in the sample well with labeled detected rotifers, and classification results.

When the rotifers are evaluated in 1-mL samples in 7-mL sample wells (as they normally will be in BWTTF zooplankton samples), the spatial resolution of the detected particles will be reduced by approximately a factor of two. The detection of the much smaller ciliates in the rotifer samples provides a good indication that zooplankton of rotifer size should be readily detectable in 1-mL samples that contain *Artemia*, rotifers and potentially other indigenous zooplankton.

4.2 Phytoplankton Characterization

The FlowCAM®, as a result of the significant modifications implemented in this study, has been made suitable for the reliable automated detection of phytoplankton. Initial results (Section 3.2.2) clearly demonstrate the feasibility of using the FlowCAM® for the detection, classification of phytoplankton, and for viability classification.

An early assessment of the applicability of FlowCAM® (the original 2-channel system) for performing phytoplankton sample characterization involved the use of a sample that contained 240 total particles. Of these particles approximately half were *Tetraselmis* and half were debris. A combination of the cell ESD (equivalent spherical diameter), aspect ratio (derived from the ration of the Feret maximum to Feret minimum dimensions), and the chlorophyll florescence intensity level was used to discriminate between *Tetraselmis* and the debris in the samples. In this sample 98 percent of the *Tetraselmis* were correctly classified as *Tetraselmis* and 96 percent of the debris signatures were correctly classified.

The results presented above as well as those presented in Section 3.2.2 strongly indicate that when the data collection, algorithm development and validation experiments described in the next section of this paper are completed, the methods that have been described for the automated characterization phytoplankton can be brought on-line as the principal methods for characterizing phytoplankton in BWTTF samples.

4.3 Additional Work Required to Maturate Algorithms and Validate Automated Methods

Additional work is required to bring the technologies described in this report on-line for the routine and automated characterization of BWTTF samples.

At this time, the in-house developed method used for the automated characterization and classification of zooplankton is more mature than the techniques used with phytoplankton. The largest technical question to be answered and demonstrated through technical work is whether the much smaller rotifers can be reliably detected and their viability classified in samples that also contain *Artemia*. Work performed during the course of this program strongly indicates that the potential for hardware and image processing and classification algorithms that have been developed and demonstrated during this program should be successful, with minor modifications, in working with the more complex samples that need to be analyzed in support of BWTTF zooplankton sample characterization. This is strongly indicated by the types of samples that have been analyzed to date (the same volume and concentration of zooplankton in the same sample wells). The largest outstanding technical issue is the ability to accurately characterize zooplankton of the variable size range (*Artemia* to rotifers) in the same samples.

Specifically, the in-house developed system described in this report needs to be used to collect multi-frame TIFF image sets of the same sample wells that are currently being evaluated using manual microscopy methods. This would provide a direct comparison of results. These data will allow for modifications of the existing algorithms to ensure reliable performance with the more complex samples. Following adjustments in the algorithm, the results obtained using the automated methods need to be validated by direct comparison to the results obtained using manual microscope methods. There is high confidence that these technologies can be maturated to required levels.

With the significant modifications that have been implemented in the FlowCAM® system during the course of this program, this system is finally at a level where its suitability to the automated detection and classification of phytoplankton can be further optimized, fully evaluated and validated. Maturating this system in this application will require some additional basic data collections followed by algorithm development and validation. This activity will allow the suitability of this instrument to the characterization of phytoplankton in BWTTF samples to be fully assessed.

Specifically, the forward-scattering detector triggering needs to be fully optimized and tested. During this program the sensitivity of the forward-scattering detector and its circuitry were modified to ensure reliable triggering on all objects in the size range of the indigenous and surrogate phytoplankton. At this time, the unit is successfully triggering on all of these particles but it is also triggering on particles that are significantly smaller than the surrogate and indigenous phytoplankton. Further optimization of this triggering circuit is required to ensure

that the unit triggers reliably on particles in the phytoplankton size range while not triggering on particles that are significantly smaller or larger than the phytoplankton.

Following the optimization of the triggering, data must be collected for mixed *Thalassioira weissflogii*/*Tetraselmis* samples (of known relative concentration) followed by samples from the BWTTF that contain *Thalassioira weissflogii* and *Tetraselmis* as well as indigenous phytoplankton and debris. These data are required to further develop and validate phytoplankton classification algorithms that allow the concentrations of each surrogate as well as that of the indigenous phytoplankton to be measured using the FlowCAM®. The high quality of the current image data, the improved geometrical features that are now extracted from imagery by FlowCAM® directly (in a recent software release), in-house developed features that benefit from the higher quality imagery, and reliable captured values of the chlorophyll and 520 nm florescence intensity values provide high confidence that the current instrument with appropriate classification algorithms will be able to provide this needed capability.

Following the optimization of the triggering, data must also be collected from samples with a variety of known live/dead ratios (through fixing some of the cell populations) to fully demonstrate that the DNA staining dyes and FlowCAM® can determine cell viability in non-homogenous samples. Early work performed using the florescent microscope and this same dyes system provides a good indication that this work will be successful.

Following the algorithm optimizations and data collections described above, additional BWTTF water samples should be collected and run through the system and evaluated by the algorithms. These results will need to be compared to those obtained by manual microscopy. In this fashion the instrument and its algorithms can be fully validated for the automated detection, classification, enumeration (at the species level) and the viability classification of BWTTF phytoplankton samples.

There is high confidence that both the in-house developed microscope system plus algorithms for evaluating zooplankton samples and the modified FlowCAM® plus dye system and algorithms for evaluating phytoplankton samples will provide efficient means for automating the characterization zooplankton and phytoplankton samples during standardized testing of BWTE performed at shore side test facilities.

5.0 Conclusions

During the course of this program methods for the automated detection, classification, enumeration and viability classification of zooplankton and phytoplankton were explored. The overall objective of this activity is to bring automated methods on-line for the characterization of zooplankton and phytoplankton samples during standardized testing of BWTE at shore-based test facilities such as the BWTTF located at NRLKW.

A number of methods were initially investigated that showed promise for the characterization of zooplankton and phytoplankton samples. These included an in-house developed system including advanced image processing and classification algorithms for working with zooplankton samples, the LemnaTEC Scanalyzer systems for characterizing zooplankton samples, and the

Fluid Imaging Technologies FlowCAM® for characterizing both zooplankton and phytoplankton samples.

Based on initial laboratory investigations, the in-house developed system was determined most suited for automated zooplankton sample characterizations. The FlowCAM® system was investigated for zooplankton analysis, but it was not able to sense motion in viable organisms. It was also not possible to use dyes with *Artemia* zooplankton surrogates (as a result of their exoskeleton) for viability assessments with respect to the FlowCAM® system. This left the LemnaTEC and in-house developed systems for zooplankton analysis. The LemnaTEC Scanalyzer systems provided no benefit over the in-house system. Its use of the camera zoom rather than a microscope zoom did not provide as good a resolution, and the system cost significantly more than the in-house developed system. Thus a cost/performance analysis led to the selection of the in-house system for zooplankton analysis.

The LemnaTEC and FlowCAM® systems were investigated for sue with phytoplankton samples. The LemnaTEC Scanalyzer system did not have the necessary resolution for analyzing phytoplankton (10 – 50 micron size range). The FlowCAM® system had the required resolution and initial laboratory investigations showed that it was suitable for the automated characterization of phytoplankton samples. This work also showed that when the FlowCAM® system is used in conjunction with cell impermeant DNA staining dyes, also identified and evaluated under this program, a real-time determination of cell viability is possible.

As a result of initial laboratory investigations, these in-house developed system and the FlowCAM® system were selected for further development and evaluation to ensure their applicability to zooplankton and phytoplankton sample analyses at the scale required for shore-based approval tests of BWTE.

The in-house developed system was further developed and extensively evaluated during the course of this program. The developed instrumentation and the image processing and classification algorithms have shown an ability to enumerate the number of organisms in sample wells and to determine their viability based on cell activity or movement. Samples of approximately the same organism densities as those encountered in BWTTF investigations have been evaluated with relatively homogenous zooplankton species. These data provide a strong indication of the applicability of the developed methods for zooplankton BWTTF sample characterization. The largest outstanding technical issue will be the ability to accurately characterize zooplankton of the variable size range (*Artemia* to rotifers) in the same samples.

There are many advantages to the in-house developed system. First and most important, this system offers the potential of reducing the zooplankton sample analysis times in support of BWTTF testing by an order of magnitude. Current protocols take at least 24 hours to complete zooplankton characterization work using manual microscopic methods. This has resulted in the biologists who support BWTTF testing spending almost full time performing microscope observations when production testing is ongoing at the facility. The use of the in-house developed system and its algorithms promises to reduce both the time and man-hours required to characterize zooplankton samples. This should free up the time of biologists assigned to the program to work in other areas that are important to standardized testing of BWTE. Second, the methods that have been developed and demonstrated during this program produce a number of outputs that provide a digital archive of all observations conducted to determine the

characteristics of zooplankton samples. These outputs include still imagery, video imagery, and the complete high-resolution time-resolved image set that is generated. This data archive is valuable in that it can be used to validate specific test results and also can be reviewed by trained biologists in the event that the results from automated analyses are questioned.

Significant modifications were implemented in the Fluid Imaging Technologies FlowCAM® system during the course of this program. At this program's end, these modifications have resulted in this unit being suited for the automated characterization of phytoplankton samples. Work performed over the course of this program has shown the potential for the FlowCAM® system and its outputs (image geometrical features and florescence intensity values) combined with features extracted from FlowCAM® imagery using in-house developed image processing and classification algorithms to perform species and life-stage classification and enumeration of phytoplankton samples. Work performed with the FlowCAM® and with cell impermeant DNA staining dyes has clearly demonstrated the potential of using this approach for phytoplankton cell viability analysis. It is also important to note that the dye identified during the course of this program is currently being used in conjunction with a florescent microscope system for the manual determination of phytoplankton cell viability. Lastly, it is important to note that the current BWTTF phytoplankton samples that are being manually analyzed are at an ideal organism concentration level for direct introduction to the FlowCAM® system. With the work suggested in this report, it is likely that the FlowCAM® and supplemental algorithms can be maturated to the required levels for routine automated characterization of BWTTF phytoplankton samples.

There are also significant advantages to the use of FlowCAM® for making these measurements during standardized testing at the BWTTF. These methods offer the potential to reduce the current times required to analyze phytoplankton samples from the current 4½ hours to approximately ½ hour including all sample preparation time. As was the case with the zooplankton, this time savings allows biologist program personnel to perform functions other than sample characterization during production testing. The FlowCAM® additionally produces a digital archive of images of all detected phytoplankton cells (and other particulates that were encountered during phytoplankton sample characterization).

The automated methods that have been developed and demonstrated during this program offer significant benefits to sample characterization during standardized testing of full-scale BWTE at shore-based test facilities. The major advantage of the methods developed and demonstrated during this study is the significant reduction in the time and man-hours required to fully characterize zooplankton and phytoplankton samples such as those routinely processed at the BWTTF. A secondary advantage of these technologies is the digital data archive that is created of sample observations as well as conclusions drawn by the automated algorithms. It is anticipated that with the additional experiments/validation work that is described in this report that these automated methods can be brought on-line for routine analyses during production standardized testing at the BWTTF and other shore-based facilities.

6.0 References

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