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INTERNATIONAL Ocean Systems

November/December 2008

The Magazine for Ocean Professionals

SURFACE & SUBSEA NAVIGATION AND PRECISE POSITIONING

Testing ballast water for zooplankton

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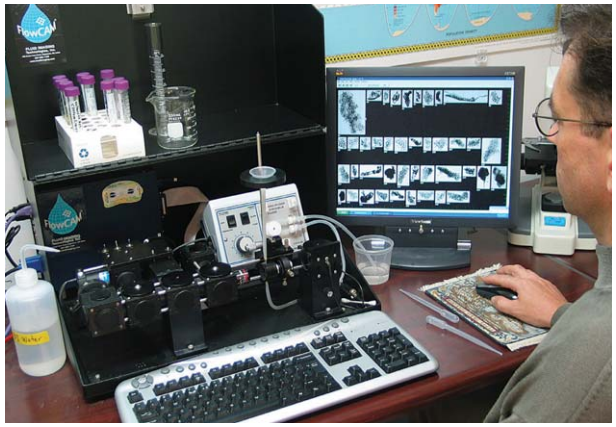
The problems associated with the transfer of non-indigenous species in ships' ballast water is well known. The IMO and researchers around the world continue to work towards a situation where effective treatments can be specified, and their efficacy verified. The Fluid Imaging FlowCAM is emerging as an important tool for both aspects of this work and is establishing itself as an essential tool in the search for a simple, automatic and verifiable device for this important area of marine monitoring.

The objective of this study was to test the applicability of the FlowCAM for ballast water analysis of micro-organisms with minimum diameters equal to or greater than 50 microns (zooplankton). This is done using the viability stain, fluoroscein diacetate (FDA), for detecting live cultured rotifers (*Brachionus plicatilis*).

METHODS

Fluoroscein diacetate (FDA) is a compound that has been widely used in various biological systems as a vital stain for fluorescent microscopy, fluorometers and flow cytometry. As an electrically neutral or near-neutral molecule, the substrate freely diffuses into most cells. Once inside the cell, the non-fluorescent substrates are converted by nonspecific intracellular esterases into a green fluorescent product (two molecules of



fluoroscein) that are retained by cells with intact plasma membranes.

In these tests a live sample of cultured rotifers (*Brachionus plicatilis*) was obtained and size fractioned. The culture was first filtered through a 300um mesh to remove large particles from the sample, then the rotifers were collected onto a 55um mesh and re-suspended in filtered seawater.

An initial primary stock solution of FDA was prepared by mixing with reagent grade dimethylsulfoxide (DMSO) to a final concentration of 5mg/ml. This solution was stored at 4°C (note – DMSO will freeze at this temperature).

A working stock solution was prepared by diluting the primary DMSO solution 400 fold (2.5ul/ml) into filtered seawater. During preparation of the working stock the sample was mixed to prevent precipitation.

All solutions were kept cold and in the dark until use. Each live or dead sample was stained for a minimum of ten minutes prior to the FlowCAM analysis. Analysis took place soon after staining was complete – after prolonged periods of time the stain will dissipate from the cell.

A FlowCAM from Fluid Imaging Technologies equipped with a blue laser (488nm) and black and white high-resolution camera was tested to determine if it could distinguish between live and dead rotifers that were stained with FDA. In this case, the stain was fluorescent, and a scatter trigger was used for particle detection (light scatter by each particle as it passes through the laser beam).

Once a sample of cultured rotifers was prepared the sample was divided into six equal volumes (~50mls each). Two samples were preserved with formulin (0.5% final concentration – low concentration to minimise pH changes) and represented one of the "dead tests" or a dead control. This preserved sample was stored at 4°C for one hour to ensure complete preservation.

Two other sub-samples were heat killed by incubating the culture in a 60°C water bath for 30 minutes. This represented a second "dead test" or dead control. The remaining two samples were not treated and were considered the "live test".

After one hour (preservation time required), a 10ml sub-sample was removed from each of the samples (two preserved, two heat killed, and two live). Beginning with the live samples, 250ul of the FDA working stock was added to each tube and stored in the dark and 4°C for a minimum of 10 minutes. After staining, each was analysed using a FlowCAM equipped with a blue laser, black and white camera and a 4x objective with a 300um depth quartz flow cell (new design). The pump speed selected was FAST 5 and each sample was processed in approximately 20 minutes.

RESULTS

The data results and images of the live FDA stained and unstained rotifers are presented in Figure 1. Upon using the FDA stain with live rotifers, there was a dramatic increase in Ch2 fluorescence (green fluorescence – mean 3114.88 - Figure 1A). In Figure 1B the rotifers are unstained and all cells had a baseline Ch2 mean green fluorescence of 146.43. It is expected that staining variability among different species and genera will occur within natural field samples, however, this particular species of rotifer (typically used as feed in aquaculture) appears to utilise the FDA stain efficiently.

The data presented in Figure 2 is similar to the organisms in Figure 1, however, due to the formulin preservation and the heat killing process, the rotifers did not acquire the FDA stain. Since no green fluorescence

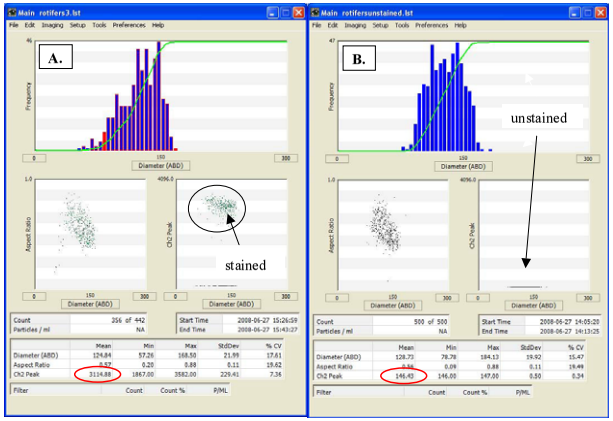


*Figure 1 A&B. LIVE stained vs unstained rotifers (*Brachionus plicatilis *)*

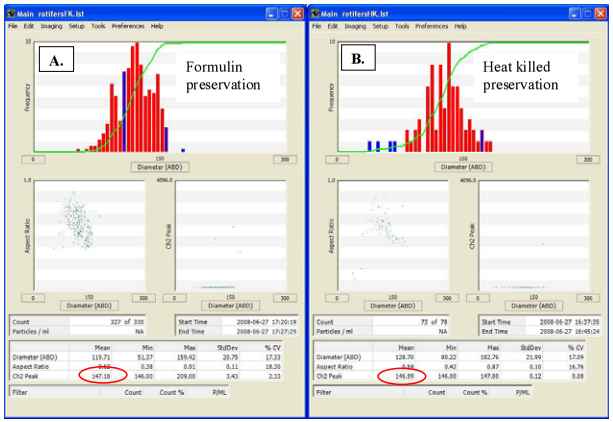


*Figure 2 A&B. Dead stained formulin and heat killed rotifers (*Brachionus plicatilis *)*

was observed these rotifers were not viable. The heat killing process appeared to destroy many of the rotifers as determined from the particle density differences between the samples. The primary difference between Figures 1 and 2 is the presence or absence of green fluorescence (Ch2), which will determine which organisms are viable and which are nonviable within a treated culture sample.

Based on the results from the size and Ch2 parameters specific filters can be used to determine the number of viable (or non-viable) organisms within a treated sample.

In summary, Table 1 presents the overall data results for determining whether different FDA stained samples (live and dead) could distinguish between viable and non-viable cultured rotifers. Both replicate live samples demonstrated a high FDA (green) fluorescence and the majority of the culture appeared to be viable (80-74%) when compared to the dead samples, where very little was detected as viable (0.5%).

CONCLUSIONS

This study used the viability stain, FDA, in combination with both live and dead (preserved and heat killed) cultured rotifers. We examined both a live and two treated samples (formulin and heat killed) – and in only the live test did the FlowCAM successfully detect the presence of the viability stain (an increase in the green fluorescence parameter, Ch2). It should be noted that for all viability stains of different organisms in a natural field sample some will stain more effectively than others. It would be recommended that cultured organisms (such as rotifers or others) be tested for viability using either a visual probe, such as, neutral red or a fluorescent probe (FDA) as in this experiment. By using cultured organisms an appropriate selection of organisms that stain effectively can be determined for use with testing ballast water treatment systems.

	Live		Dead – Formulin		Dead – Heat Killed	
	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2
Avg FDA fluor. Peak	3013	3115	147	148	147	147
Live Count (FDA +)	352	356	3	22	1	1
Dead Count (FDA -)	126	86	327	405	78	47

Table 1. FDA fluorescence results and count data from both live and dead rotifer samples

In addition to the viability tests with regard to the applicability of the FlowCAM for ballast water treatment viability analysis, the FlowCAM will be shipped in the future with flow cells and objectives that are paired in order to determine the concentration or abundance of cells within a sample when using fluorescence based triggering. The FlowCAM will also be shipped with a calculation for minimum dimension. Minimum dimension is determined by using an algorithm that orients the particle on its longest axis and slices the particle into many thin sections. The smallest section or "dimension" will determine the minimum dimension of the particle. This dimension must be known in order to determine if the organisms passing through the FlowCAM meet the IMO's D2 regulations. ■

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