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Multi-site validation of a method to determine the viability of organisms ≥ 10 µm and < 50 µm (nominally protists) in ships' ballast water using two vital, fluorescent stains

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1.0 INTRODUCTION

The International Maritime Organization (IMO) is in the process of ratifying a Convention for the Control and Management of Ships' Ballast Water and Sediments (2004), which includes guidelines for approving ballast water management systems. Similarly, the United States Environmental Protection Agency (EPA) is finalizing a Protocol for the Verification of Ballast Water Treatment Technologies through the Environmental Technology Verification (ETV) Program (Lemieux et al., in review). Both documents categorize organisms by size classes based on minimum dimensions: ≥ 50 µm (nominally zooplankton), ≥ 10 µm and < 50 µm (nominally protists), and < 10 µm (nominally bacteria); and for the two larger size classes, these documents specify the maximum number of viable organisms permitted in a vessel's discharged ballast water. The smallest size class is represented by three indicator species of bacteria with limits on the number of colony-forming units found in discharged water. The IMO G8 guidelines define "viable organisms" as "organisms and any life stages thereof that are living" (2005).

Determining viability of protists has long been a difficult task that is further complicated by several factors when testing ballast water treatment technologies. The analyses must be relatively affordable and easy to replicate at all testing facilities, they must produce results within a short time frame (on the scale of hours, not days or weeks), and they must quantify viability in terms of organisms per volume (rather than an indirect measure). Finally, they must be applicable to a diverse assemblage of organisms present in ballast water.

To this end, the Naval Research Laboratory in Key West (NRLKW) has developed a method for determining viability in the ≥ 10 µm and < 50 µm size class that utilizes a combination of the vital stains fluorescein diacetate (FDA) and 5-chloromethylfluorescein diacetate (CMFDA; Drake et al., in review). Fluorescein diacetate is a non-fluorescent molecule that can pass freely through cell membranes. Within cells, non-specific esterases cleave the acetate groups from the molecule to create the product fluorescein, which is both fluorescent under blue light excitation and largely membrane-impermeable and thus is retained within living cells with intact membranes. The second stain, CMFDA, has similar characteristics, but its methyl group is mildly thiol-reactive, so the molecule binds to thiol groups within the cells and leads to better cellular retention than FDA. The fluorescent signal is not as intense as FDA, however.

This combination of stains was successful at the laboratory in Key West: only 1% of the stained ambient organisms in the ≥ 10 µm and < 50 µm size class were false negatives (the organisms moved but were not stained), and 5% of the organisms were false positives (the organisms were heat-killed but still fluoresced) (Drake et al., in review). Given the oligotrophic environment of the laboratory, which is unique among test facilities, this method of staining needed to be validated at other locations with different water characteristics and different communities of organisms. Four sites were chosen for trials: Baltimore, MD (brackish), Sequim, WA (marine), West Boothbay Harbor, ME (marine), and the Great Lakes, MI (fresh).

2.0 METHODS

2.1 Site Locations and Sample Collection

The samples analyzed in Key West were collected from the flowing seawater system at the Naval Research Laboratory (NRLKW; Drake et al. in review). Ten samples were collected at NRLKW between 5 May 2009 and 27 May 2009. Because the water adjacent to the laboratory is oligotrophic, it was necessary to filter a large volume of water to collect a diverse sample of protists with abundant organisms. To do so, water was filtered through a 50 µm mesh sieve situated above a 10 µm mesh filter bag, and the material retained in the 10 µm filter bag (filtrand) was stained and evaluated. These freshly collected samples were referred to as 'live' (as opposed to the 'heat-killed' samples described in the next section). This method of sampling was used at all locations.

The M/V *Cape Washington* is currently moored in Baltimore, MD and houses the testing facility of the Maritime Environmental Resource Center (MERC). The facility directly utilizes the ship's ballast tanks and pumps for testing ballast water treatment technologies. On 2 July 2009, a plankton sample was collected and concentrated from a pipe located 1 m upstream of the testing facility, and on 23 July, 28 July, and 06 August 2009, surface water was collected with a bucket from the dock adjacent to the ship. The concentrated samples were transported in a cooler to the Naval Research Laboratory (NRL) in Washington, DC for analysis.

Seawater samples from Sequim, WA were collected daily on 14-16 September 2009 at the Pacific Northwest National Laboratory (PNNL) located at the mouth of Sequim Bay. Surface water was collected with a bucket from a floating dock, carried to the laboratory on site, and analyzed.

At Bigelow Laboratory for Ocean Sciences (BLOS) in West Boothbay Harbor, ME, three samples were collected each day on 30 September – 2 October 2009 from a floating dock, carried to the laboratory on site, and analyzed.

Samples from the Great Lakes were collected from three locations in Michigan: Luna Pier (Lake Erie), Bay City (Lake Huron), and St. Joseph (Lake Michigan) on 25-27 August 2009. One sample from each location was concentrated immediately after collection, transported in a cooler, and then analyzed at the Great Lakes Environmental Research Center (GLERL) in Ann Arbor, MI.

2.2 Sample Staining and Preparation

One milliliter subsamples from the filtrand in the 10 µm filter bag were stained with FDA (5 µM) and CMFDA (2.5 µM) and incubated for 10 minutes in the dark before being loaded onto a Sedgewick Rafter (SR) counting chamber with etched measurement grids. Each organism in a randomly selected row was classified as moving or non-moving and fluorescing or non-fluorescing. Previous research in Key West showed that even a weak fluorescent signal was proof of viability, so any detectable signal was scored as fluorescing. All intact organisms

examined were scored in one of four categories: moving and fluorescing, moving and non-fluorescing, non-moving and fluorescing, and non-moving and non-fluorescing.

In addition to examining live samples, heat-killed samples (heated to 50°C for 10 min) were prepared to evaluate fluorescence in dead organisms. Three SR counting chambers were enumerated for each live sample and three for each killed sample. Samples from PNNL and BLOS were also freeze-killed (-20°C for over 1 hour) and analyzed.

Previous research had shown a marked increase in background fluorescence as fluorescein leaked out of the cells (MKS, pers. obs.), so all counts were made within 30 minutes after the stain was added to the sample whether or not the randomly selected row had been entirely counted. Not all samples had a drastic increase in background noise, but the time limit ensured consistency among sites.

2.3 Microscope Configurations

Both FDA and CMFDA have excitation and emission maxima of 495 nm and 515 nm respectively, and at each location, a blue excitation bandpass filter cube (e.g., 480/40; 505; 530/30) was used. Samples were analyzed at 100x magnification, although each location had a slightly different microscope: an Olympus BX61 compound microscope (MERC samples analyzed at NRL), a Leica DM6000 B compound microscope (GLERL), a Leica DM IRB inverted microscope (PNNL), and a Zeiss Axioskop compound microscope (BLOS). Digital cameras and imaging software varied at each location as well, but this equipment did not directly affect data collection, as an organism with any green fluorescence was scored as living.

2.4. Green Autofluorescence

Some organisms may contain natural pigments that fluoresce in the same wavelengths as fluorescein emissions. In order to quantify the effects of green autofluorescence on the accuracy of FDA and CMFDA, additional samples from PNNL and BLOS (both live and heat killed) *without* stain were evaluated using the microscopy methods described above.

2.5. Data Analysis

Every Sedgewick Rafter counting chamber evaluated was considered a subsample, and three live and three killed subsamples were counted for each sample. The subsamples for each day were pooled into percentages for each of the four categories (e.g., moving and fluorescing). Daily percentages were averaged so that days with more total organisms, perhaps caused by a bloom, did not influence the results more than days with fewer total organisms. Statistical analyses were performed using SPSS (v16.0).

3.0 RESULTS

3.1. Naval Research Laboratory Key West (NRLKW) - Key West, FL

The original series of trials at NRLKW looked at stained plankton samples collected over ten non-sequential days in May 2009 (Drake et al., in review). The majority of organisms in the live samples were fluorescing (87%), and of the non-fluorescing organisms, only 1% were moving (false negatives; Figure 1). Of the heat-killed samples, none of the organisms were moving, and 5% of the organisms were fluorescing (false positives). Organisms that were non-moving and non-fluorescent were considered dead. The densities of organisms in the samples and the sediment load varied by day, but in general, the assemblages were evenly divided among dinoflagellates, diatoms, and ciliates.

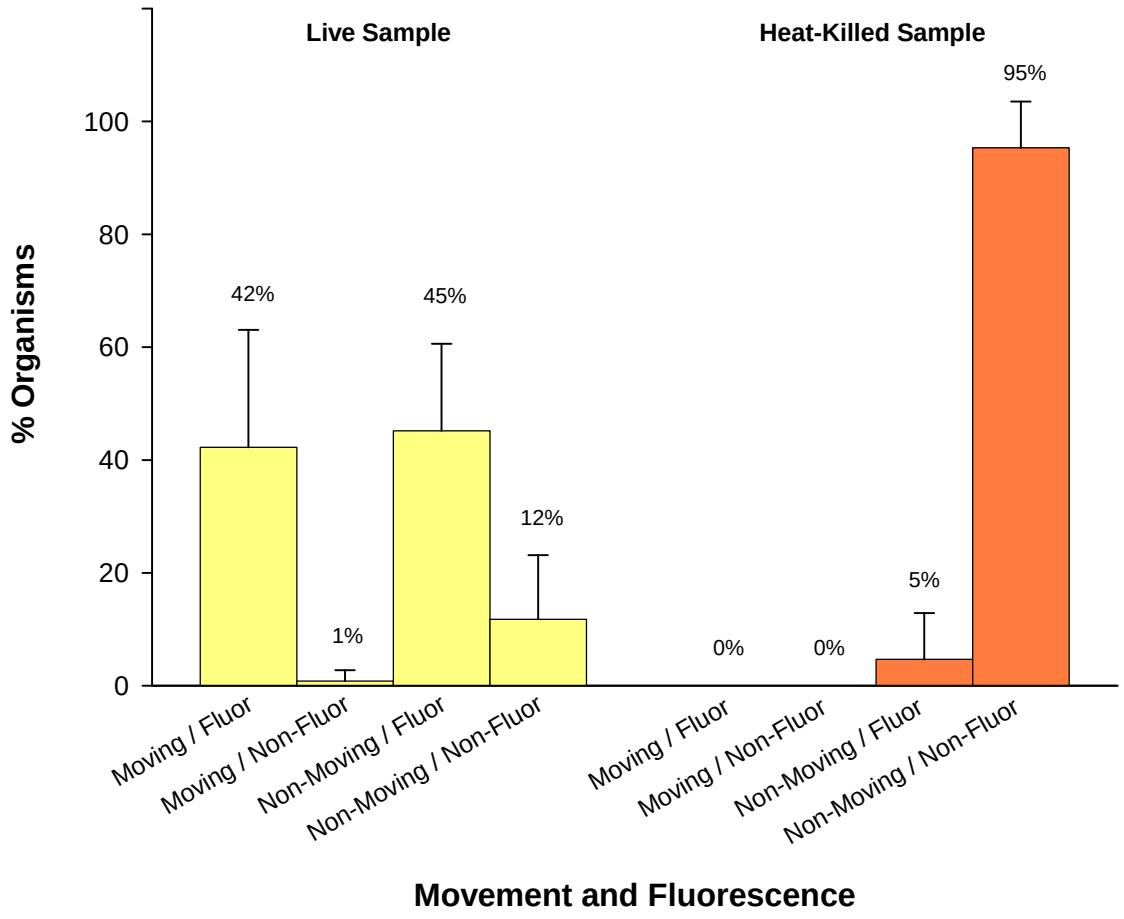

Figure 1. Live and heat-killed NRLKW samples stained with FDA and CMFDA. Error bars represent one standard deviation, and the numbers above the bars represent mean values of daily percentages (n=10). A total of 1025 organisms were counted in the live samples, and 501 organisms were counted in the heat-killed samples.

3.2 Maritime Environmental Research Center (MERC) – Baltimore, MD

Four stained samples were analyzed from MERC, and the results were similar to NRLKW; only 1% of the live organisms were moving and non-fluorescing (false negatives), and 3% of the heat-killed organisms were fluorescing (false positives; Figure 2). There were more small dinoflagellates at MERC compared to NRLKW, and the MERC assemblage had many discoid diatoms.

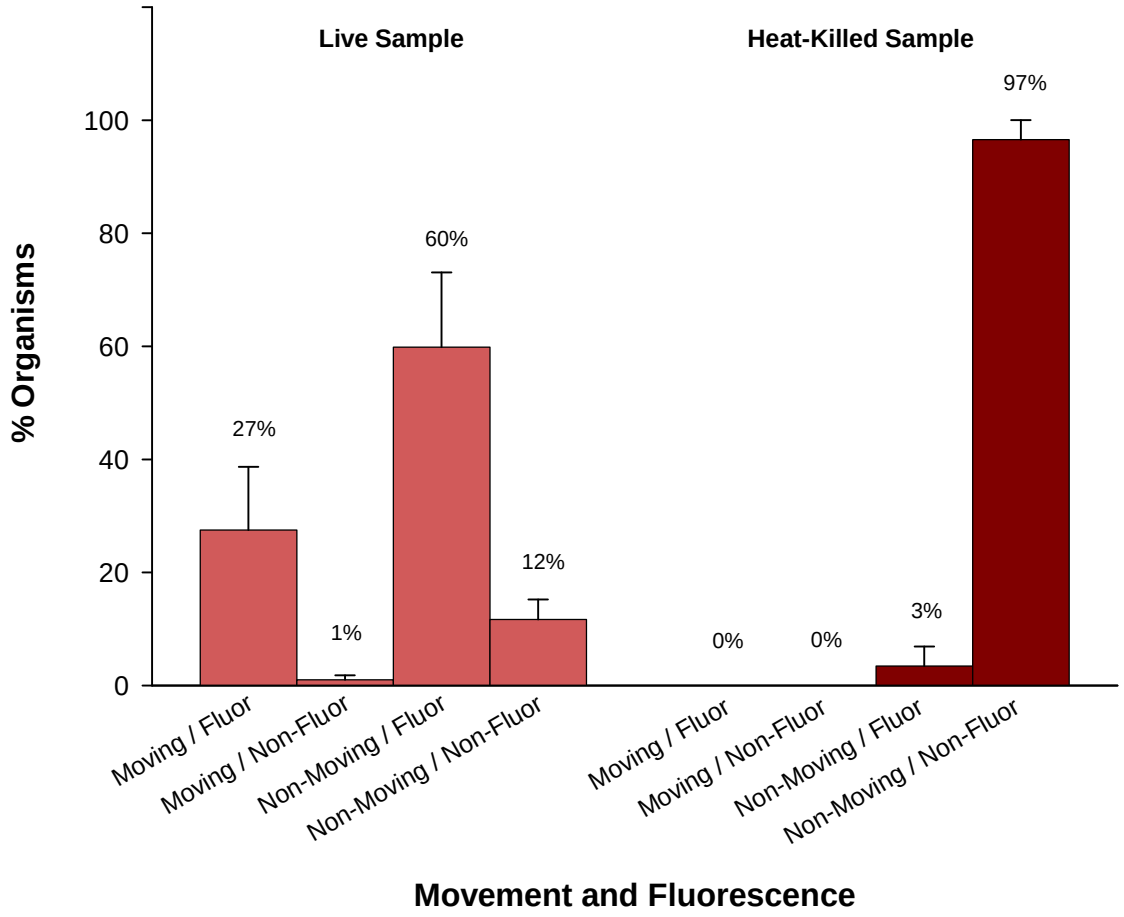

Figure 2. Live and heat-killed MERC samples stained with FDA and CMFDA. Error bars represent one standard deviation, and the numbers above the bars represent mean values of daily percentages (n=4). A total of 391 organisms were counted in the live samples, and 453 organisms were counted in the heat-killed samples.

3.3 Pacific Northwest National Laboratory (PNNL) – Sequim, WA

Three stained samples were analyzed from PNNL, which had a significantly lower percentage of moving organisms in the live samples than KW and MERC (t-test p=0.001). There were no false negatives (moving and non-fluorescing), and 36% of the heat-killed organisms were fluorescing (false positives; Figure 3). There was a high diversity of dinoflagellates, and the heterotrophic dinoflagellates were the most common organisms to fluoresce when dead. There were also many discoid diatoms such as *Coscinodiscus* sp. and *Thalassiosira* sp.

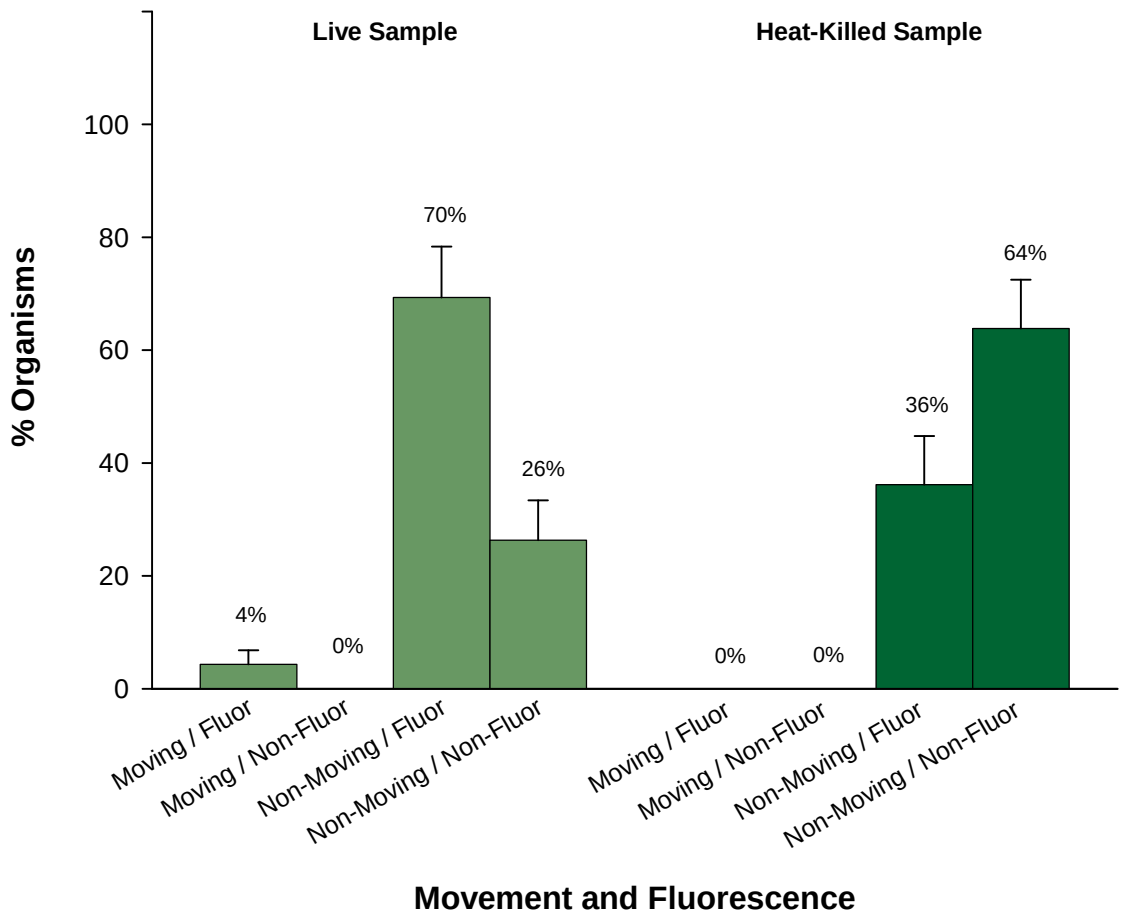

Figure 3. Live and heat-killed PNNL samples stained with FDA and CMFDA. Error bars represent one standard deviation, and the numbers above the bars represent mean values of daily percentages (n=3). A total of 394 organisms were counted in the live samples, and 465 organisms were counted in the heat-killed samples.

On the third day, three live and three heat-killed subsamples were analyzed without stain to identify green autofluorescence in the sample (Figure 4). The percentage of fluorescing heat-killed organisms (false positives) was 11% when stain was not added; approximately one third of the false positives could be attributed to GAF. The percentage of moving organisms in the live samples also increased to 29% when stain was not added.

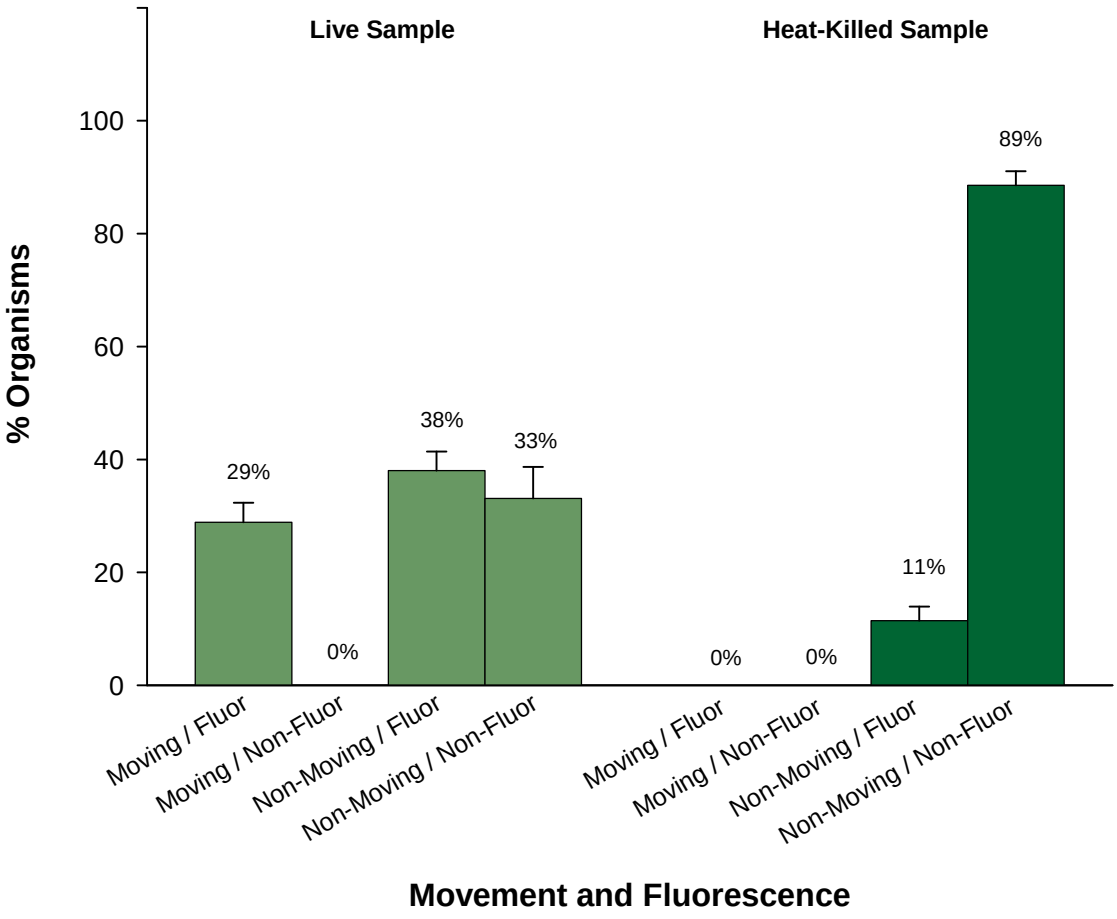

Figure 4. Live and heat-killed PNNL samples with no stain. Error bars represent one standard deviation, and the numbers above the bars represent mean percentages of three subsamples. A total of 142 organisms were counted in the live samples, and 131 organisms were counted in the heat-killed samples.

Since green autofluorescence could possibly be attributed to a protein, and thus be denatured when the organisms are heat-killed, a fourth sample was analyzed without stain: three live subsamples, three heat-killed subsamples, and three freeze-killed subsamples. Slightly more organisms in the freeze-killed subsamples were fluorescent than in the heat-killed subsamples, but this result was not significant (t-test p=0.407) (Figure 5).

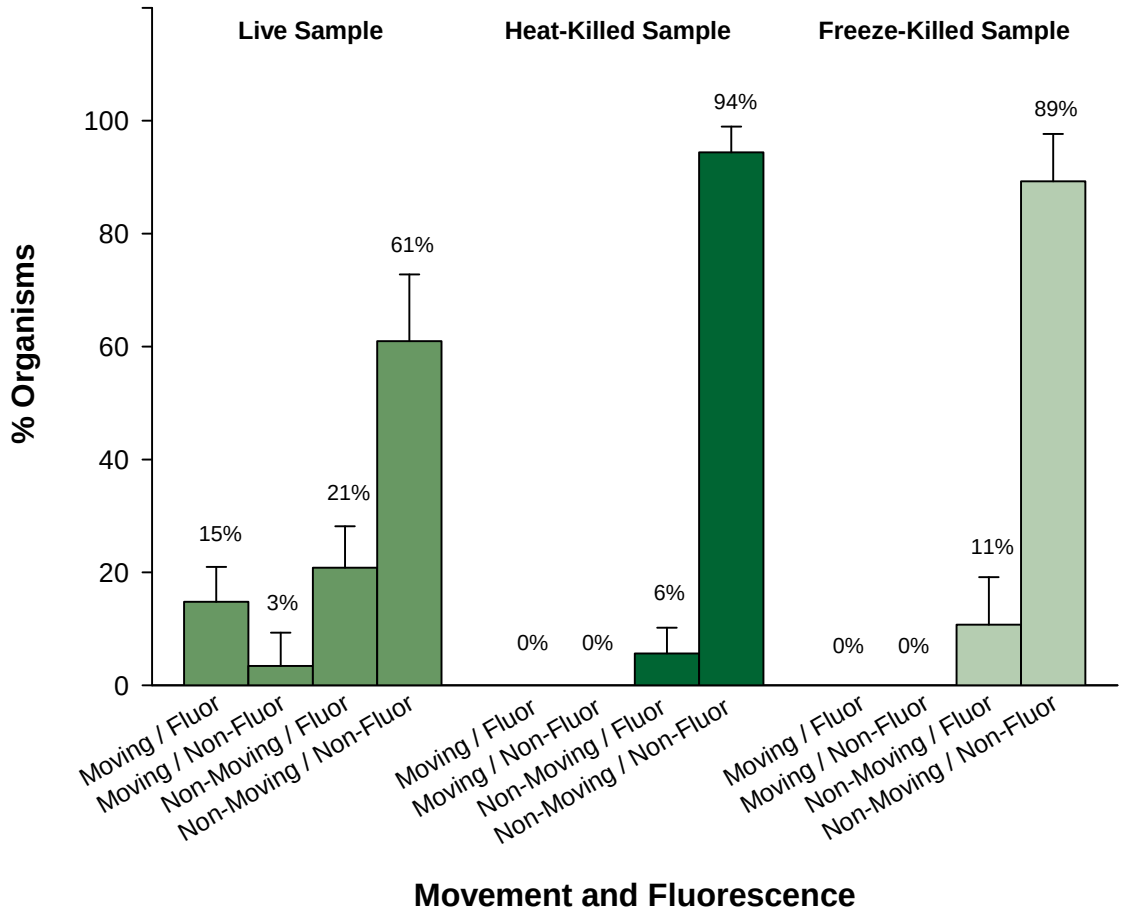

Figure 5. Live, heat-killed, and freeze-killed PNNL samples with no stain. Error bars represent one standard deviation, and the numbers above the bars represent mean percentages of three subsamples. A total of 126 organisms were counted in the live samples, 87 in the heat-killed sample, and 79 in the freeze-killed sample.

3.4 Bigelow Laboratory for Oceanic Sciences (BLOS) – West Boothbay Harbor, ME

Like PNNL, the stained samples from BLOS had few moving organisms. There were no false negatives (moving and non-fluorescent), and 19% of the heat-killed organisms fluoresced (false positives; Figure 6). There was a dinoflagellates bloom of *Prorocentrum micans* on the first day of sampling, and the percentage of false positives for that day was 32%. On the next two days of sampling, the number of *P. micans* decreased, as did the number of false positives (6% and 18% on days two and three, respectively). There was also a bloom of the chain-forming diatom *Eucampia* sp. during sampling, and the diatoms represented over 50% of the total organisms. These organisms, however, were rare among the heat-killed, fluorescing organisms (false positives), which were dominated by *P. micans*.

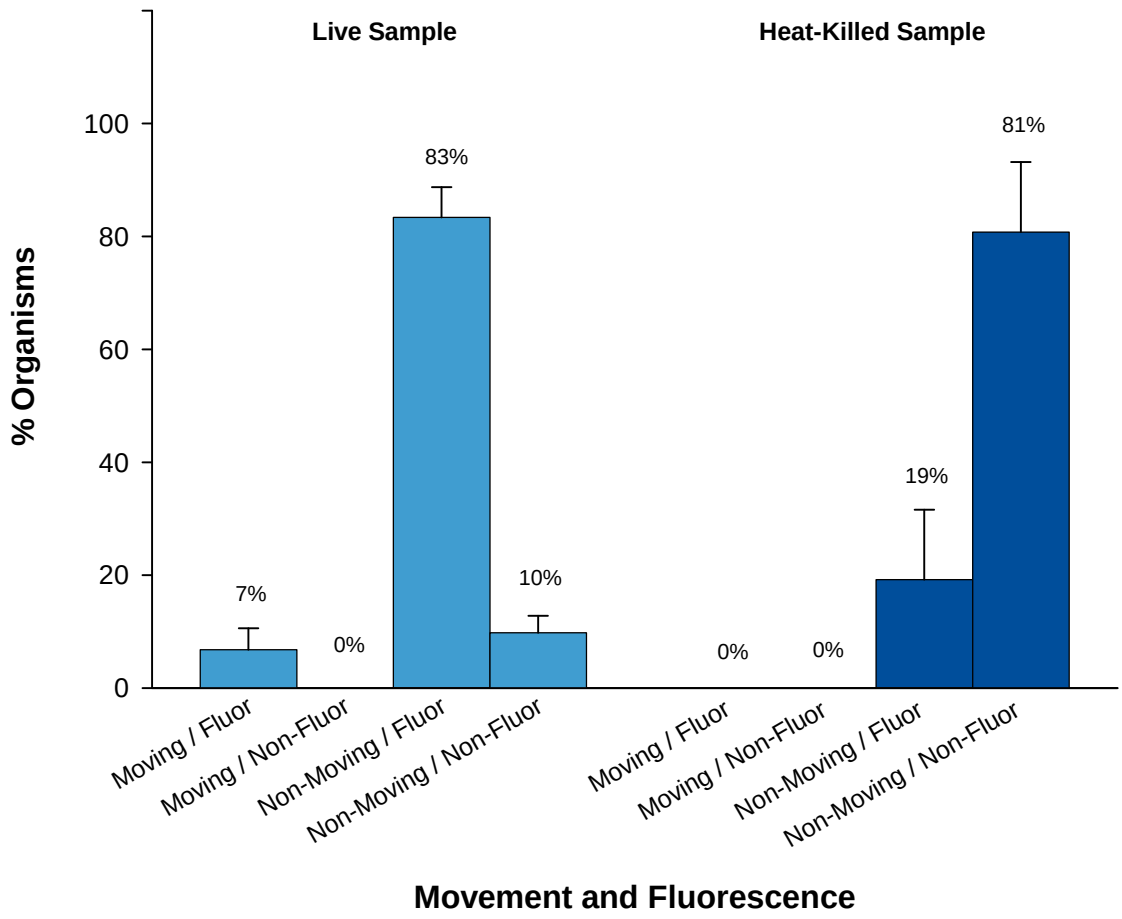

Figure 6. Live and killed samples from BLOS stained with FDA and CMFDA. Error bars represent one standard deviation, and the numbers above the bars represent mean values of daily percentages (n=3). A total of 793 organisms were counted in the live samples, and 1030 organisms were counted in the heat-killed samples.

Three live and three heat-killed subsamples were analyzed without stain on the first day. There was no GAF in the heat-killed subsample, suggesting any heat-killed fluorescence was attributed to the stains (Figure 7). Freeze-killed subsamples were also analyzed on the second day, and there was no significant difference between the heat-killed and freeze-killed subsamples (t-test p=0.547) (Figure 8). Similar to the samples analyzed at PNNL, the BLOS samples that did not have stain added had more moving organisms than samples that were stained.

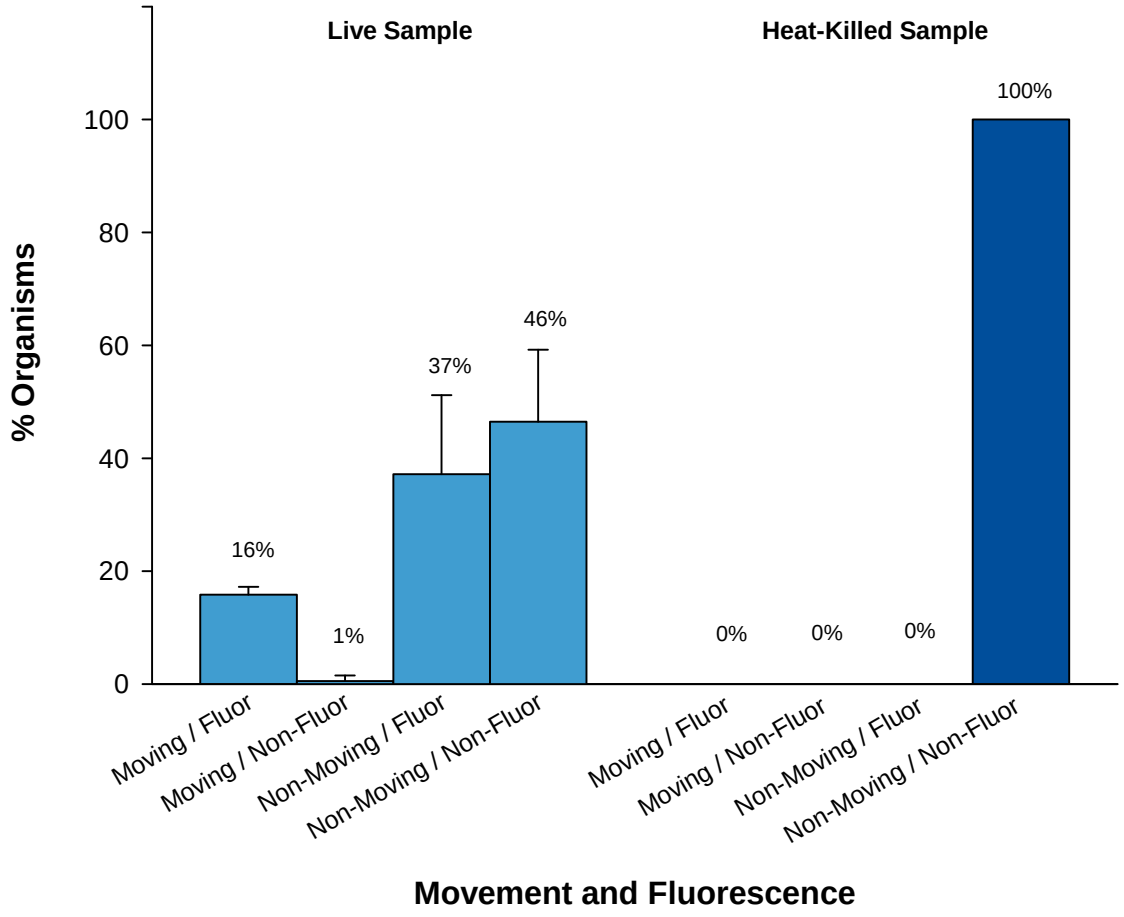

Figure 7. Live and heat-killed BLOS samples with no stain. Error bars represent one standard deviation, and the numbers above the bars represent mean percentages of three subsamples. A total of 183 organisms were counted in the live samples, and 179 organisms were counted in the heat-killed samples.

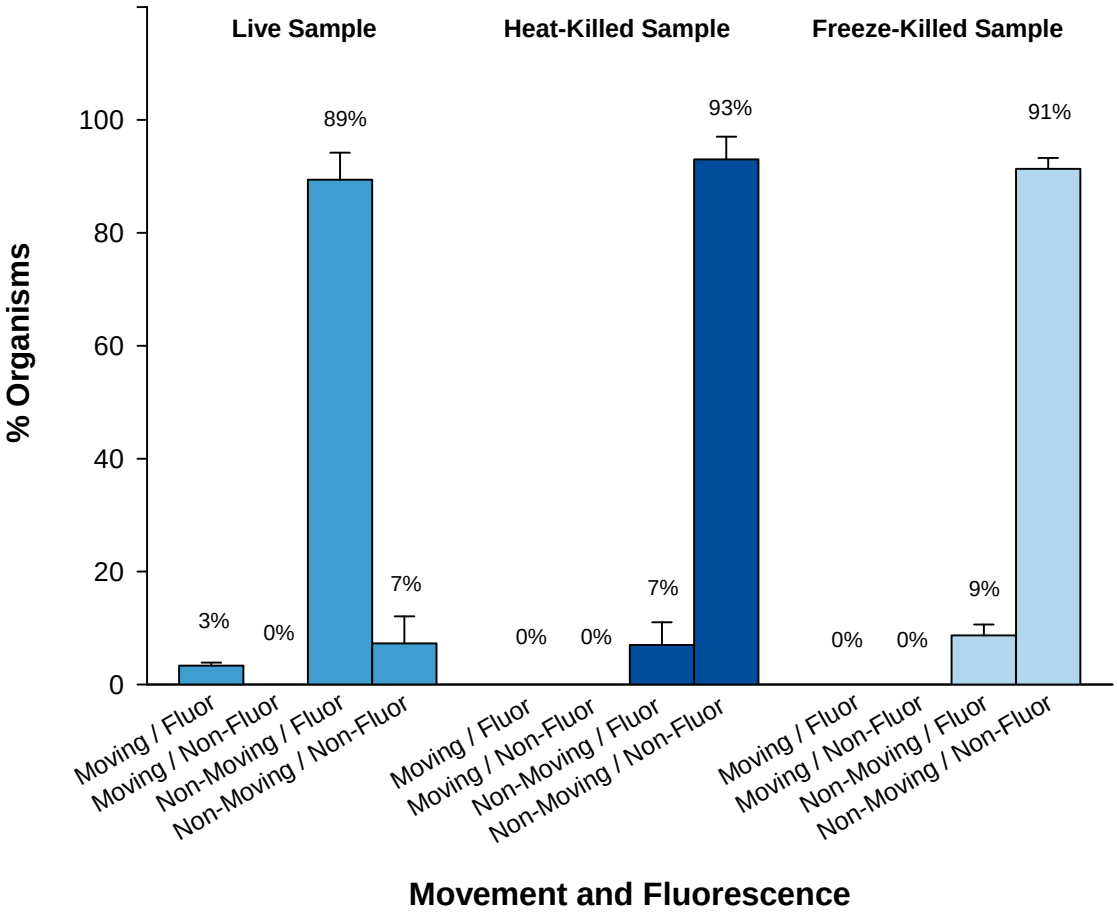

Figure 8. Live, heat-killed, and freeze-killed BLOS samples stained with FDA and CMFDA. Error bars represent one standard deviation, and the numbers above the bars represent mean percentages of three subsamples. A total of 392 organisms were counted in the live samples, 342 in the heat-killed sample, and 315 in the freeze-killed sample.

3.5 Great Lakes Environmental Research Laboratory (GLERL) – Ann Arbor, MI

The Lake Erie and Lake Huron samples were taken in shallow areas of the lakes and had more sediment and organic material than the samples from Lake Michigan. Though there were no moving organisms that did not fluoresce (i.e., no false negatives), 48% of the heat-killed organisms did fluoresce (false positives). Nearly all of these false positives had a very faint signal and were thus scored as fluorescent. For consistency, the Great Lakes samples were analyzed in the exact same manner as samples from the other sites, but it is possible that different intensity thresholds will need to be set for freshwater samples (i.e., only strong fluorescent signals are considered indicators of life).

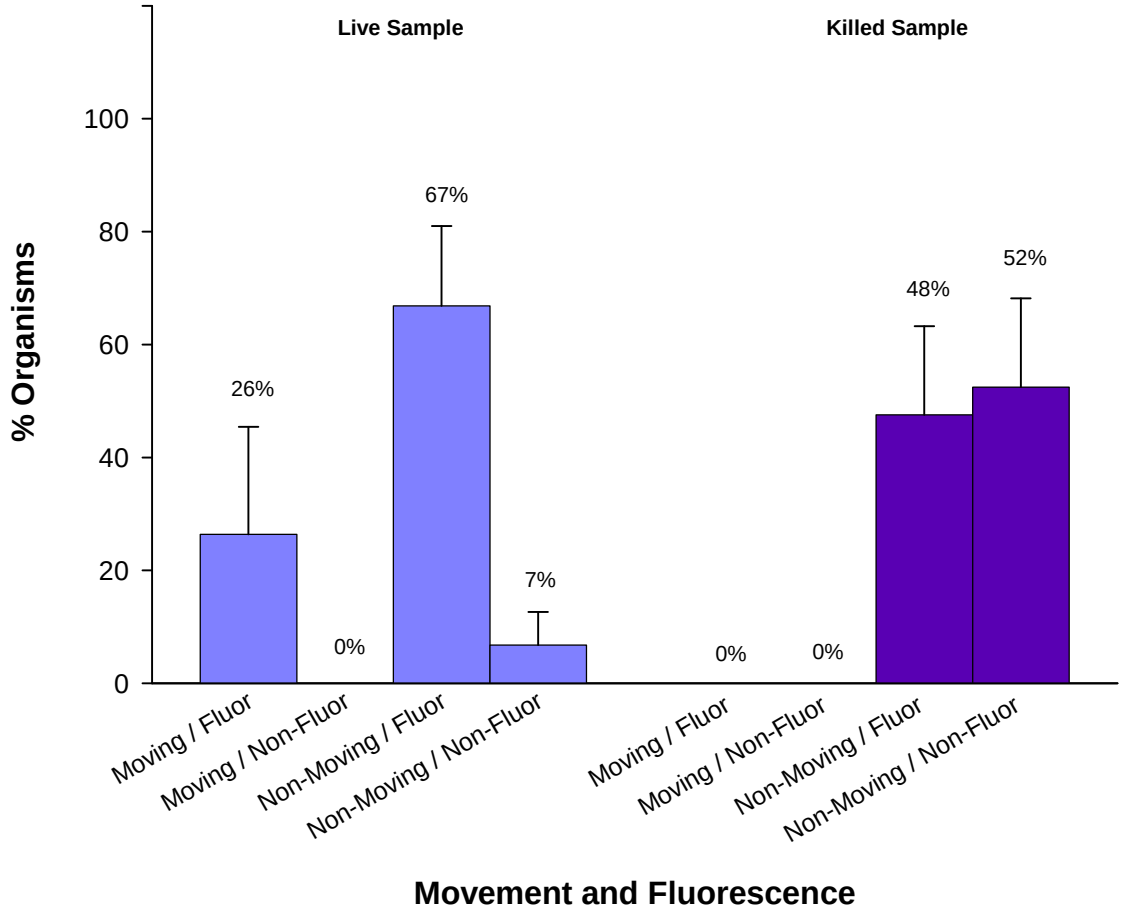

Figure 9. Live and heat-killed GLERL samples stained with FDA and CMFDA. Error bars represent one standard deviation, and the numbers above the bars represent mean values of daily percentages (n=3). A total of 205 organisms were counted in the live samples, and 192 organisms were counted in the killed samples.

There was more GAF in both the organisms and the debris in the Lake Erie and Lake Huron samples than in any other samples analyzed during these trials. Even when heat-killed, there was still faint GAF, which contributed to the false positives. Further complicating the samples was background GAF, which was exacerbated when stain was added (Figure 10). This background noise intensified while the sample was kept in the dark, and exposure to light quickly quenched the fluorescence. It is important to note that fluorescein will also fade under constant exposure to light (MKS, pers. obs.).

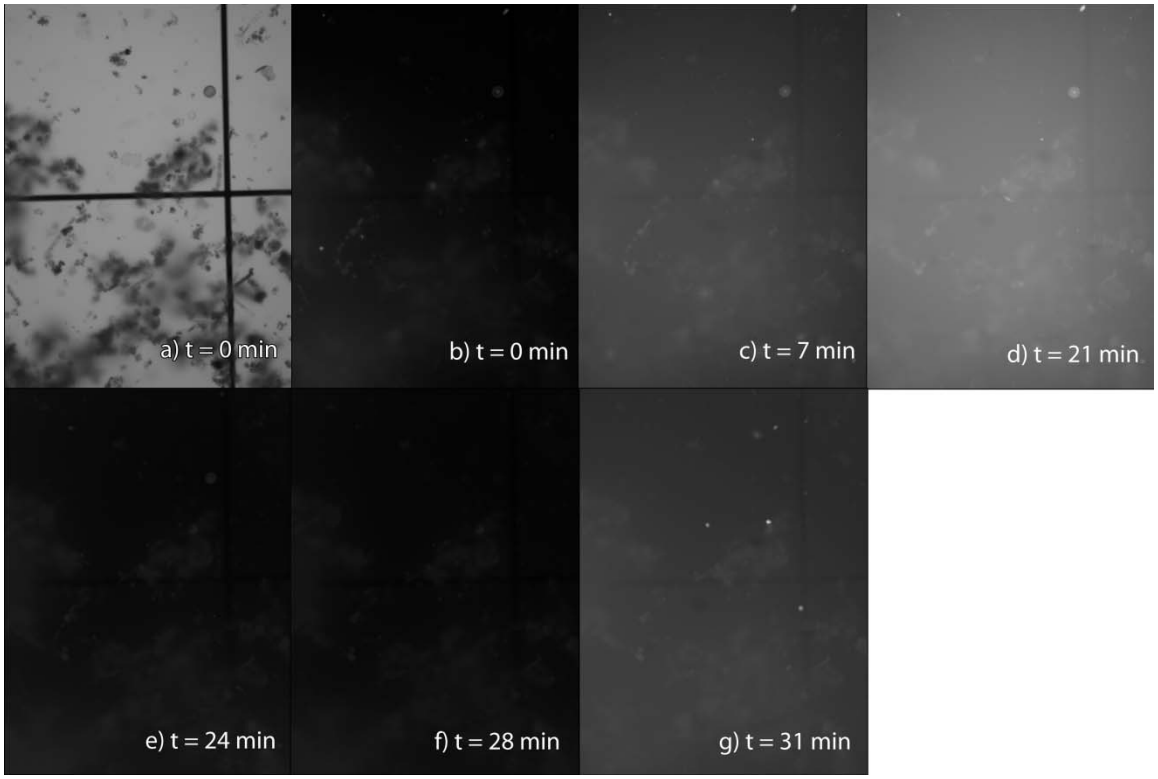

Figure 10. Heat-killed sample from Lake Huron with no stain. The subsample was loaded on a Sedgewick-Rafter counting chamber and immediately imaged under brightfield (a) and epifluorescence (b). The chamber remained on the stage in the dark except to take epifluorescent photomicrographs at 7 and 21 min (c-d). The blue excitation light remained on from 21-28 min, and photomicrographs were taken at 24 and 28 min (e-f). The light was turned off, and a final image was taken at 31 min (g).

3.6 Summary of Sites

For comparison, the results from all live and heat-killed stained samples are grouped in Figures 11 and 12. All stations had very low false negatives (Fig. 11; Table 1). Regarding the live samples, at all sites, the preponderance of organisms was categorized as non-moving and fluorescing. Agreement of live samples was good between the NRLKW and MERC sites.

Among heat-killed samples, agreement between NRLKW and MERC results was also good, with the incidence of false positives the lowest at these two sites (Fig. 12; Table 1). The highest false positives were at GLERL (48%), followed by PNNL (36%).

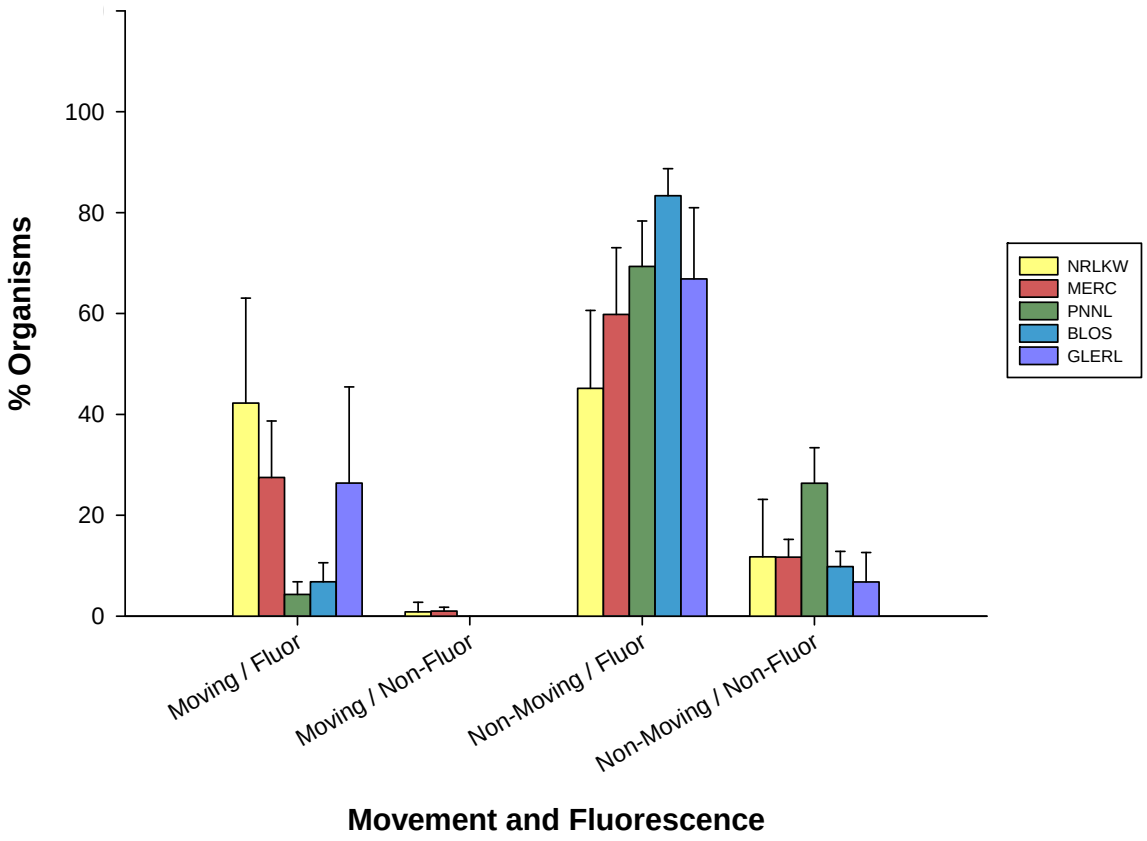

Figure 11. Live, stained samples from all sites. Error bars represent one standard deviation. NRLKW = Naval Research Laboratory Key Wet, MERC = Maritime Environmental Resource Center, PNNL = Pacific Northwest National Laboratory, BLOS = Bigelow Laboratory for Ocean Sciences, GLERL = Great Lakes Environmental Research Laboratory.

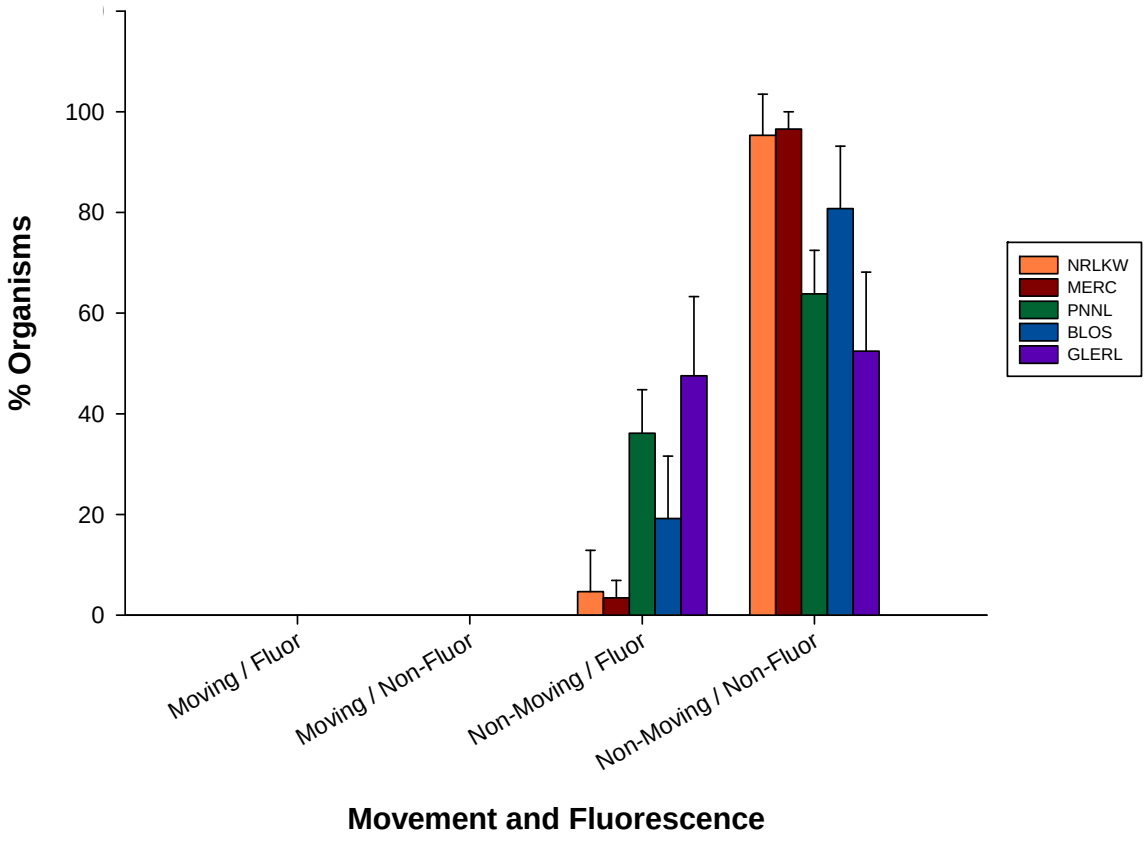

Figure 12. Heat-killed, stained samples from all sites. Error bars represent one standard deviation. NRLKW = Naval Research Laboratory Key Wet, MERC = Maritime Environmental Resource Center, PNNL = Pacific Northwest National Laboratory, BLOS = Bigelow Laboratory for Ocean Sciences, GLERL = Great Lakes Environmental Research Laboratory.

Error		
Site	False Positive (Type I)	False Negative (Type II)
NRLKW	5%	1%
MERC	3%	1%
PNNL	36%	0%
BLOS	19%	0%
GLERL	48%	0%

Table 1. Error rates for all sites. False positive organisms, which were fluorescing when heat-killed, represent Type I errors. False negative organisms, which were moving but non-fluorescing, represent Type II errors.

4.0 DISCUSSION

While the combination of FDA and CMFDA stains was a successful method for determining viability at NRLKW and MERC, it was less successful at PNNL and BLOS where heterotrophic dinoflagellates were more abundant. Because these dinoflagellates hydrolyzed the stains and fluoresced when heat-killed or freeze-killed, the percentage of false positives was high at these sites. Research is underway to devise an alternative viability technique for these problematic species.

Although applying the FDA and CMFDA technique to samples collected from the Great Lakes was problematic, the errors appear to be due to the threshold at which a fluorescent signal was considered to represent a viable organism. By selecting a different threshold for these freshwater sites, these stains will likely be much more accurate, although it seems that pollution and debris may have an effect on the background noise. Scientists using FDA with live and heat-killed samples collected from Duluth/Superior Harbor (oligotrophic Lake Superior) successfully identify viable protists (Euan Reavie, pers.com.). More samples from multiple freshwater sources will be needed to identify limitations of this technique in freshwater samples.

Unstained live samples from PNNL and BLOS showed more movement than samples that had been stained, which suggests that the stains or the DMSO in which they are suspended may have affected movement of the organisms. In the NRLKW and MERC live samples, 43% and 30% of the stained organisms were still moving. In contrast, only 5% and 6% of the live, stained organisms at PNNL and BLOS were moving. This is likely due to differences in the ambient plankton assemblages at each site, and future experiments are planned to evaluate using stain working stocks with higher concentrations that would facilitate using less DMSO.

Although staining with FDA and CMFDA was not perfect at every site, combining these stains remains the best viability analysis tested to date for the ≥ 10 µm and < 50 µm size class of organisms in ballast water samples. The success of the staining depended on the protist assemblage at each location, and any new methods designed to evaluate protist viability should undergo similarly rigorous testing at various locations.

5.0 ACKNOWLEDGEMENTS

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