6130/1011
18 Mar 2010

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DEVELOPMENT OF A METHOD TO DETERMINE THE NUMBER OF LIVING ORGANISMS ≥ 10 µm AND < 50 µm (NOMINALLY PROTISTS) IN SHIPS' BALLAST WATER: A COMBINATION OF TWO VITAL, FLUORESCENT STAINS

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1 INTRODUCTION

Standards dictating the number of viable organisms discharged in ships' ballast water are in the process of being enacted: the International Maritime Organization's (IMO) International Convention for the Control and Management of Ships' Ballast Water and Sediments is undergoing ratification (IMO 2004), and the US Coast Guard recently published a Notice of Proposed Rule Making (NPRM) outlining ballast water discharge standards (Federal Register 2009). To meet the new standards, vendors are developing technologies to treat ballast water. Subsequently, the efficacy of these technologies must be verified by test facilities in order for a port state to approve their use. Internationally, the IMO has promulgated the G8 guidelines for Approval of Ballast Water Management Systems (IMO 2005) to provide direction to test facilities. Nationally, the Environmental Protection Agency's Environmental Technology Verification Program has developed the Protocol for the Verification of Ballast Water Treatment Technologies (Lemieux et al. 2009) to meet this need.

Three size classes are defined by the Convention and the ETV protocol. The smallest, < 10 µm in minimum dimension, represents heterotrophic bacteria, which can be enumerated by standard grow-out techniques or, in the case of specific bacteria indicators (e.g., *Vibrio cholerae*, agent of cholera), using commercially available assays. The largest two size classes, ≥ 10 µm and < 50 µm in minimum dimension and ≥ 50 µm in minimum dimension, nominally represent protists and zooplankton, respectively. Both size classes typically contain both autotrophs and heterotrophs. Thus, the method used to determine the status of protists and zooplankton must be applicable to a community of organisms with different energetic pathways and pigments.

It should be noted that although the IMO Convention uses the adjective 'viable' to describe the number of discharged organisms, the IMO G8 guidelines define 'viable' organisms as 'organisms and any life stages thereof that are living' (IMO 2005). Likewise, the US NPRM uses the adjective 'living'. Therefore, the work described here focused on methods to determine if an organism was living at the time of observation, not its ability to reproduce in the future.

Intact organisms that are moving can be classified as 'living' with confidence. Most zooplankton are motile and thus easily categorized as living or non-living. In contrast, the status of protists, which are often non-motile—at least on scales in which we observe them in the laboratory—is a question that has bedeviled microbiologists for decades (e.g., Kemp et al. 1993). Furthermore, coupling the question of protist condition with ballast water technology testing places additional constraints on analyses to determine protists' status: the analyses must be relatively affordable and uncomplicated because they should not inordinately inflate the cost of technology testing, they should produce results in a short time frame (on a scale of hours, not days or weeks), and they must be applicable to a community of organisms present in ballast water samples (as mentioned above) rather than a monoculture grown in the laboratory.

Many of the traditional methods used to evaluate protist communities are not applicable to technology testing because they are indirect measures or require an extended period to complete. For example, a well-used indirect method, uptake of a radiolabeled carbon substrate, measures the production of radiolabeled molecules (e.g., $^{14}CO_2$), which, in turn, must be converted to a number of organisms. The conversion factor varies by species, and within a species, by an organism's physiological state. There is simply too much variability inherent in a population, let alone in a

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community, to enumerate confidently the number of organisms in a ballast water sample using this method. Another technique commonly used in bacteriology, the most probable number (MPN) approach, may be applied to protists, but this dilution-based method requires samples to be cultured over a period of weeks (e.g., Cochran 1950), a timeframe that is undesirable for testing. Finally, the technique requires organisms in the sample to be grown under inherently artificial laboratory conditions. We know the vast majority of bacteria are not culturable (ZoBell 1947), and they have simpler growth requirements than the more evolutionarily advanced protists. Therefore, using the MPN to determine the number of living protists will likely result in an underestimate. Both drawbacks prevent the MPN method from being a viable option.

Given the limitations imposed by technology testing and the suite of microbiological tools available at this time, a traditional microscopy approach is warranted. Some researchers advocate using chlorophyll *a* autofluorescence to enumerate live cells (Pouneva 1997, Garvey et al. 2007), but the pigment can autofluoresce for weeks or months after a cell is fixed in formalin (Garvey et al. 2007). Alternatively, using a stain that emits a fluorescent signal only when an enzymatic reaction occurs within a organism has several advantages: the fluorescence is indicative of a living organism (not merely the presence of a molecule such as chlorophyll *a* or DNA), the required instrumentation is available in most research laboratories, staining techniques are commonplace and straightforward, and sample incubation times are short, thereby generating results immediately. If samples are evaluated by a researcher, however, the data are subject to errors from operator-specific biases as well as from fatigue effects during extended observation periods. To this end, automated data collection is desirable.

We stained ambient plankton samples as well as algal monocultures with numerous biological stains, both vital and mortal, and over 100 trials showed that no stain, when used alone or in combination and various staining times or concentrations, was adequate. The vital stains were faint or did not stain a large portion of the moving (living) organisms; the mortal stains were faint or erroneously stained moving (living) organisms. Combining the two vital stains Fluorescein Diacetate (FDA) and 5-chloromethylfluorescein diacetate (CMFDA, CellTrackerTM Green); however, was a promising indicator of living organisms in both of the two larger size classes. When non-specific esterases in living cells cleave the stains, the resultant molecules fluoresce green when excited with a blue light (e.g., Selvin et al. 1988, Li et al. 1996). The results concerning protists will be discussed in detail in this report; zooplankton will be addressed in another report (in preparation).

2 MATERIALS AND PROCEDURES

The sections below provide a description of materials and protocols designed to evaluate protists.

2.1 Stains

Multiple concentrations of the stains were tested, and because the fluorescence signal at higher concentrations (up to 15 µM for FDA and 50 µM for CMFDA) was not superior to the signal at lower concentrations, the lower concentrations were adopted. The following protocol was developed: the stains FDA and CMFDA (Molecular Probes-Invitrogen Carlsbad, CA; final concentration 5 µM and 2.5 µM, respectively) were added to a 1 ml sample that was incubated in the dark for 10 min. Next, the

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sample was loaded into a gridded Sedgewick Rafter Counting Chamber (1 ml volume), and it was examined under epifluorescence using a Fluorescein Isothiocyanate (FITC) narrow pass filter cube. Samples were examined for a maximum of 20 min because preliminary experiments showed the signal faded and background fluorescence increased as stain leaked from the organisms after a total of 30 min. If an organism was labeled by either FDA or CMFDA as exhibited by a characteristic fluorescent green color when excited with a blue light, it was scored as 'fluorescent'.

2.2 Microcoscopy

Samples were viewed on a Nikon E-600 compound microscope under brightfield (white light) illumination and under fluorescence using a FITC narrow pass filter cube (excitation 465-495 nm, dichroic mirror wavelength 505 nm, barrier filter 515-555 nm). A narrow pass filter cube was installed because preliminary trials showed when a long pass filter was used, chlorophyll *a* autofluorescence largely masked the fluorescent signal from the stains.

Most photomicrographs were collected with a QImaging Retiga 1300i cooled, digital camera having a charged coupled device sensor with a 2.5 cm detector with 1.3 x 106 pixel resolution. Images were collected in black and white or, by attaching an external color wheel, in color. The images in Figure 3 were collected with an mvBlueFOX 121 C CCD color, digital camera with a 0.84 cm detector with 7.9 x 10^5 pixel resolution. For analysis, it was determined the exposure time should be set at 50 ms or less to keep the background florescence low.

2.3 Ancillary Staining of Test Dust

Because the IMO Convention and ETV Protocol specify amounts of suspended material that must be present in challenge water used in validation tests, it seemed prudent to determine if FDA or CMFDA stained inorganic matter. Commercial test dust in two sizes was used: ISO 12103 -1, A3 Medium (size range 0.808 µm - 81.48 µm) and ISO 122-1, A4 Coarse (0.827 µm - 174.1 µm; Powder Technology Inc., Burnsville, MN). Test dust was added to a sample of ambient protists, which was stained with a combination of FDA and the mortal stain SYTOX® Orange (Molecular Probes-Invitrogen Carlsbad, CA) as well as the FDA-CMFDA combination and examined under epifluorescence.

2.4 Validation at the Naval Research Laboratory

After the method was developed, it was validated using protist monocultures as well as samples of ambient protists collected nearby the laboratory.

2.4.1 Positive and Negative Controls

To serve as positive controls, algal monocultures were purchased from commercial vendors (Reed Mariculture [Campbell, California]; Provasoli-Guillard National Center for Culture of Marine

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Phytoplankton [West Boothbay Harbor, Maine]). Aliquots of cultures were stained with the combination of fluorophores and tested alone; that is, they were not mixed with ambient samples. Cultures evaluated were the motile green flagellate *Tetraselmis* sp. (strain PLY 429); the pennate diatoms *Melosira octogona* (strain CCMP483) and *Amphora* sp.; the motile pennate diatom *Cylindrotheca closterium* (strain CCMP340); and the motile dinoflagellate *Prorocentrum hoffmannianum* (strain CCM2804).

As a negative control, samples of algal monocultures or ambient organisms (described below) were heat killed by placing the sample in a beaker on a hot plate and raising the temperature to 50 ºC, which was maintained for 5-10 min. The sample was cooled to room temperature before being stained.

2.4.2 Collection of Ambient Protists

The Naval Research Laboratory (NRL) in Key West, Florida is situated on Garrison Bight. Because the waters surrounding Key West are oligotrophic and contain low numbers of ambient organisms (Steinberg et al. 2009), it was necessary to filter a large volume of water to collect a diverse sample of protists with abundant organisms. To address this issue, a two-tier filtration device was constructed with a 50 µm mesh sieve (15.2 cm diameter) situated above a 10 µm mesh filter bag (15 cm x 41 cm). The sieve contained a port by which a seawater hose could be attached above the sieve, and in this manner, when the filtration device was placed outdoors with a hose turned to a very low flow rate, a seawater sample from Garrison Bight was collected overnight and analyzed the following morning.

Samples were collected on 11 days, and the protists were classified as described below. These samples were referred to as 'live'—even though a portion of the ambient community was dead—because the protists were not heat killed after they were collected. On 9 days, part of the 'live' sample was heat killed; these samples were referred to as 'heat killed'). Every Sedgewick Rafter Counting Chamber evaluated was considered a subsample, and each day, one or two observers evaluated multiple chambers. Because the number of subsamples varied widely (from 1-9 per day), the data were pooled by day to prevent a disproportionate weighting of the results by a day with a large number of subsamples.

2.4.3 Categorization of protists

Depending on a organism's fluorescence signal and movement, each protist was identified in one of the following groups: fluorescent and moving, fluorescent and unmoving (enumerating only organisms that appeared to be living, e.g., had intact organelles; empty frustules were not counted), non-fluorescent and moving, non-fluorescent and unmoving (enumerating only organisms that appeared to be living, e.g., had intact organelles; empty frustules were not counted).

3 RESULTS

The results using the staining method with ambient protists, test dust, and monocultures are described in the following sections.

3.1 Staining

The dyes had different staining patterns, with some microalgal nuclei intensely stained with FDA and the other portions of the cell—not the nuclei—stained with CMFDA (see the diatoms in Fig. 1). Most, but not all, moving organisms (both ambient and cultures) were fluorescent (see section 3.3 below).

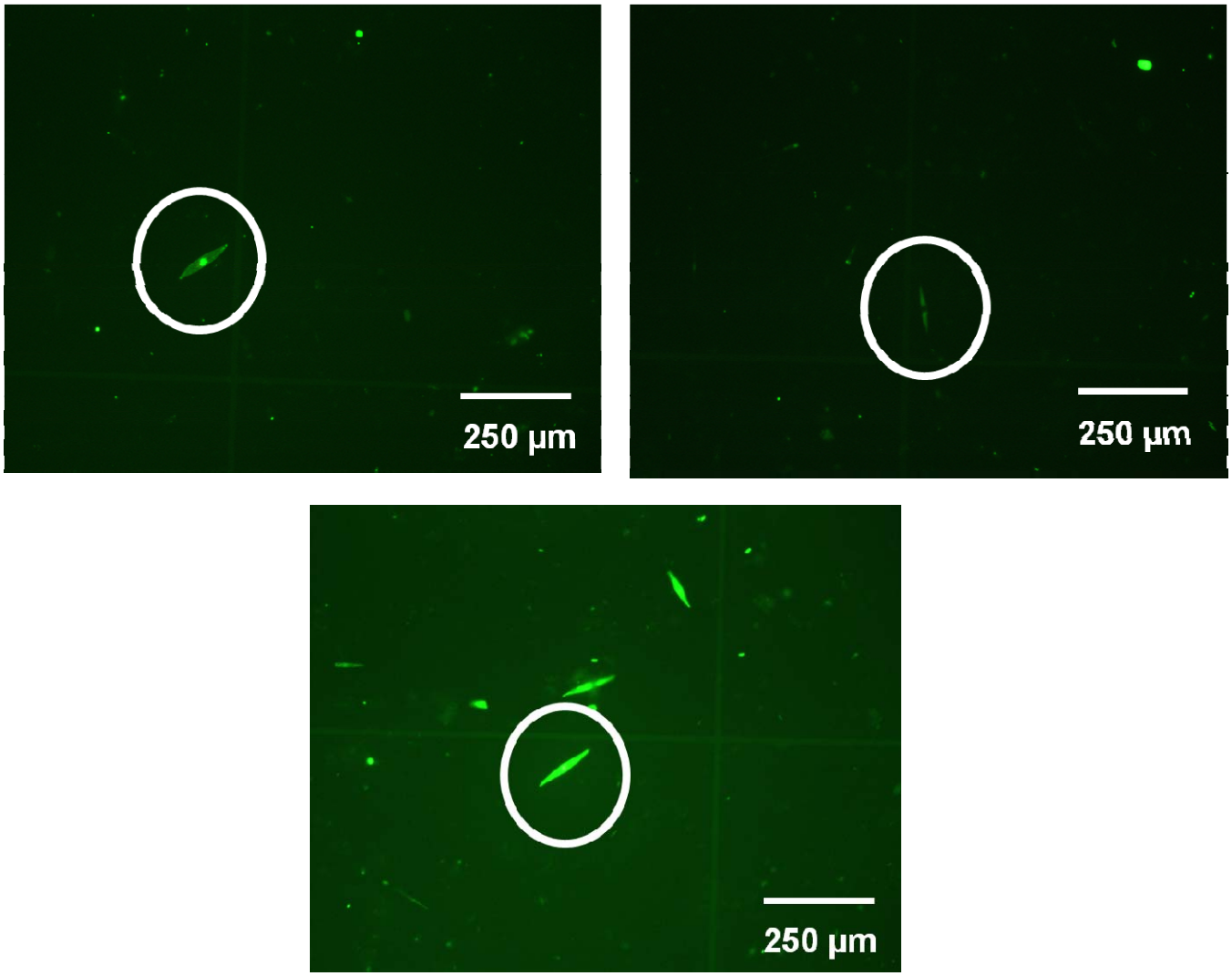

Figure 1. Ambient sample stained with FDA (10 µM final concentration, top left panel) or CMFDA (5 µM final concentration, top right panel). Ambient sample stained simultaneously with FDA and CMFDA (10 µM and 5 µM final concentration, respectively; bottom panel). Epifluorescence microscopy; the original magnification was 100x.

Although organisms stained with FDA were sometimes brighter than organisms stained with both FDA and CMFDA, using the stains together allowed some organisms that would not stain with one of the dyes alone to fluoresce when both stains were applied. Figure 2 shows the dinoflagellate

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Prorocentrum hoffmannianum and the diatom *Melosira octogona* fluoresced poorly with CMFDA but were much more visible when treated with the combination of stains. In contrast, the diatom *Cylindrotheca closterium* was not visible with FDA but fluoresced when stained with both dyes. Increasing the exposure time did not increase the visibility of the cells, as the background fluorescence became more pronounced.

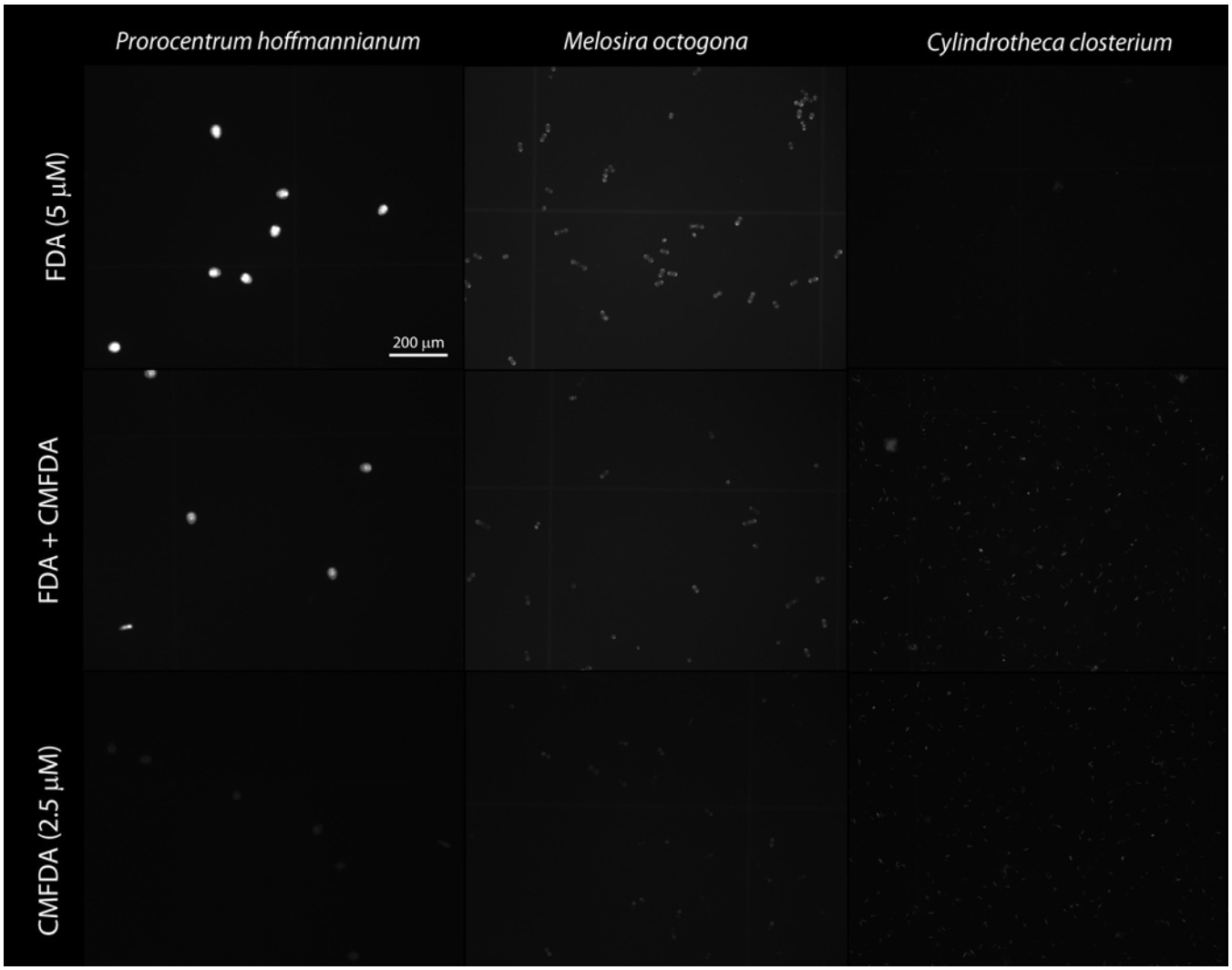

Figure 2. Three protist monocultures stained with FDA (top row), a combination of FDA and CMFDA (middle row), or CMFDA (bottom row). Epifluorescence microscopy; the original magnification was 100x.

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3.2 Test Dust

Initial trials with FDA and the mortal stain SYTOX Orange showed neither size class of test dust was fluorescent (Fig. 3); subsequent evaluations of samples containing test dust and stained with the FDA-CMFDA combination also showed no fluorescent signal by test dust (data not shown).

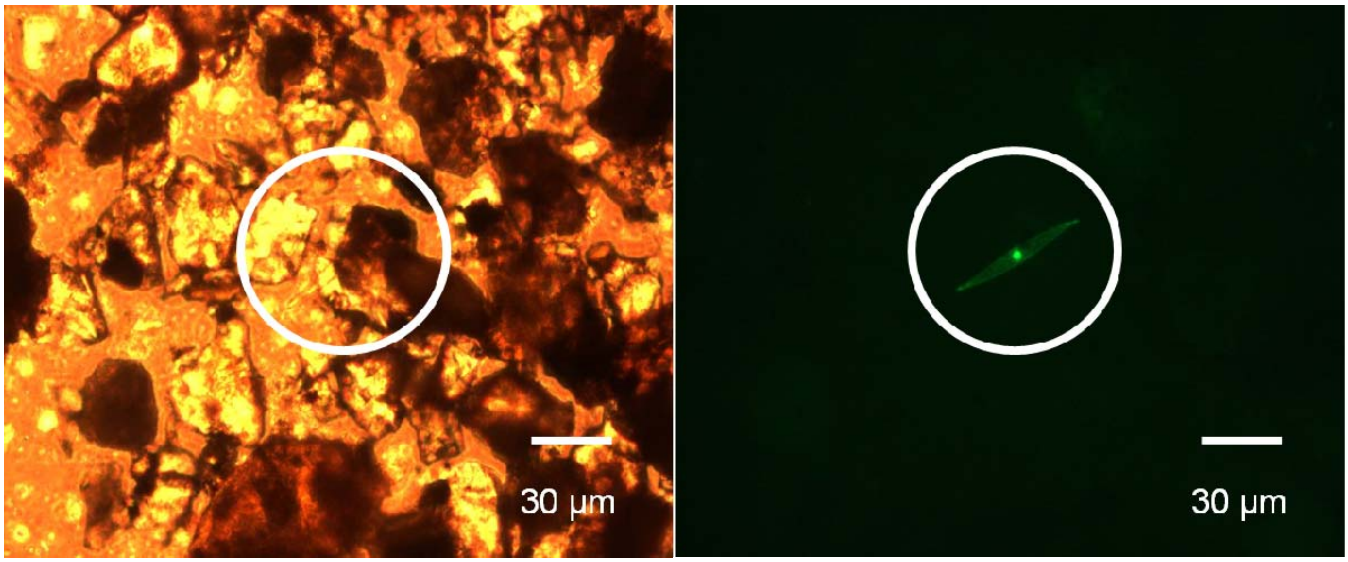

Figure 3. Diatom and test dust stained with FDA and SYTOX® Orange (10 µM final concentration for both stains). The field is visible in brightfield illumination (left) and epifluorescence (right).

3.3 Validation at the Naval Research Laboratory

The protist monocultures fluoresced as expected: when stained, the cells fluoresced green, and when cells were heat killed, they did not. To verify heat killing did, indeed, kill organisms, ambient, heat-killed protists were examined one day after they had been heat killed. No organisms moved.

The results of the field data using ambient protists were, in general, consistent with the results from the monocultures (Fig. 4). In the 'live' samples, most (87%) of the organisms were fluorescent, with roughly half moving and half non-moving. In a technology validation test, they would be scored as living. A small percentage (1%) of organisms was moving but non-fluorescent; clearly, they were living despite lacking a fluorescent signal. Twelve percent were neither moving nor fluorescent and would be scored as dead.

The combination of stains worked well on the heat-killed samples: nearly all (95%) were non-moving and non-fluorescent (dead). A small percentage (5%) was non-moving yet fluorescent and, in a validation test, would mistakenly be scored as living. Organisms in this category, however, were often rotifers or oligocheate worms, which are very mobile when alive. If they were unmoving yet stained, an experienced biologist could, with a large degree of confidence, score them as dead, thus mitigating the error.

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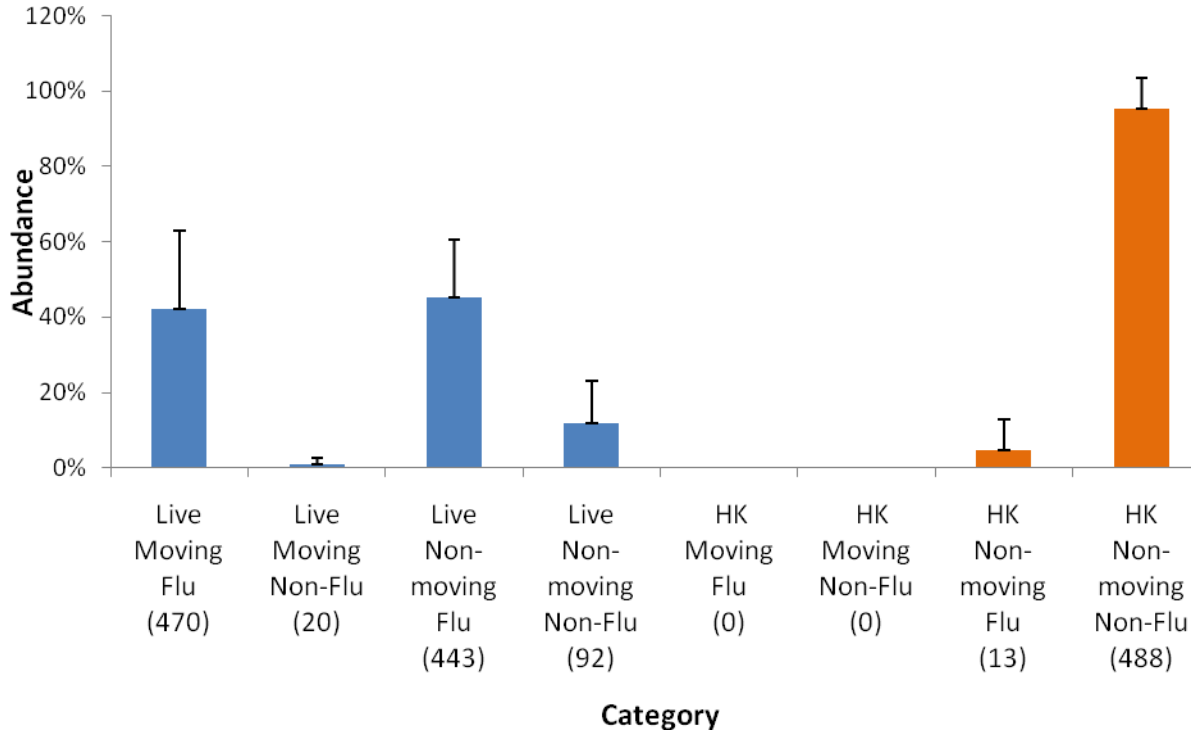

Figure 4. Categorization of ambient protists stained FDA and CMFDA (5 µM and 2.5 µM final concentration, respectively). Error bars represent one standard deviation; numbers below axis labels represent the total number of organisms in each category.

4 CONCLUSIONS

Developing a method to determine accurately whether an assemblage of protists is living or non-living is a deceptively complicated task. Our research showed a combination of vital stains, FDA and CMFDA, has potential to be a reliable indicator of the number of living organisms in the ≥ 10 µm and < 50 µm size class. To validate the method, ambient organisms collected nearby Key West, Florida were stained and scored as moving or non-moving and fluorescent or non-fluorescent. Encouragingly, preliminary results showed low error rates: the Type I error rate was 5%, and the Type II error rate was 1%.

Type II errors have been found by other researchers as well. Dorsey et al. 1989 showed when cultures stained with FDA were held in darkness for more than two days, they showed little to no fluorescence, but when they were subsequently exposed to light, the fluorescence signal returned. These results indicate cells had been quiescent rather than dead. Garvey et al (2007) showed FDA was ineffective on five of eight species of phytoplankton tested. One problem was that red autofluorescence obscured the FDA signal, but this issue can likely be ameliorated by using a narrow pass filter cube, as

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red and green autofluorescence were assessed simultaneously in the Garvey et al. study. Another problem was that the fluorescent signal rapidly faded, but this issue may be addressed using an automated technique. Despite these drawbacks with laboratory cultures, our field results with natural communities show the error rates are minimal.

For technology testing, it would be extremely valuable to have a visual record of organisms scored as living. Therefore, we highly recommend taking photomicrographs of any such organisms under brightfield illumination as well as fluorescence (to ensure the fluorescence is due to a protist). As mentioned above, if the data are collected manually by an observer, they are subject to error due to observer bias or fatigue or both. To this end, we recommend and are developing means to automate data collection. Although this investigation had the specific aim of developing a method for ballast water technology testing, the method could be employed more broadly, that is, in general investigations in aquatic ecology. This method for assessing protists is currently being tested at several other locations in the United States encompassing freshwater, marine, and estuarine conditions.

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