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DEVELOPMENT OF A METHOD TO DETERMINE THE NUMBER OF VIABLE ORGANISMS ≥ 50 µm (NOMINALLY ZOOPLANKTON) IN SHIPS' BALLAST WATER: A COMBINATION OF TWO VITAL, FLUORESCENT STAINS

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1.0 Introduction

Federal and international agencies charged with regulating aquatic nuisance species (ANS) face many obstacles when assessing the quality and efficacy of ballast water management systems (BWMS). Some of these challenges are physical, such as handling and testing large volumes of water. Other challenges are biological or chemical, such as differences in water quality and plankton assemblages at ports throughout the world. Because determining the efficacy of a BWMS hinges on the ability to identify living organisms in the discharged water, developing methods to assess diverse and dynamic plankton assemblages has become a major focus of investigation at the national and international level.

In 2004, the International Maritime Organization (IMO) adopted the International Convention for the Control and Management of Ships' Ballast Water and Sediments, which is undergoing ratification. The United States Environmental Protection Agency (EPA), through the Environmental Technology Verification (ETV) Program, published a Draft Protocol for the Verification of Ballast Water Treatment Technologies. Both documents categorize organisms by size classes based on the minimum dimension: ≥ 50 µm (nominally zooplankton), ≥ 10 µm and < 50 µm (nominally protists), and < 10 µm (nominally bacteria). The IMO convention specifies the maximum number of viable organisms permitted in a vessel's discharged water, as shown in Table 1 (IMO, 2005).

Table 1. Adopted IMO discharge standard. These numbers resprent the maximum concentrations of viable organisms permitted in discharged ballast water as per Regulation D-2 of the IMO Convention for the Control and Management of Ships' Ballast Water and Sediments (2004).

	Organisms ≥ 50 µm in minimum dimension	**Organisms < 50 µm and ≥ 10 µm in min. dimension**	**Vibrio cholera**	**Escherichia coli**	**Intestinal Enterococci**
IMO D-2 Discharge Standard	< 10 viable organisms m^{-3}	< 10 viable organisms ml^{-1}	< 1 colony-forming unit (cfu) 100 ml^{-1}	< 250 cfu 100 ml^{-1}	< 100 cfu 100 ml^{-1}

For the ≥ 50 µm size class, movement is commonly used as an indicator of viability. There are also numerous reports of using vital and mortal stains (e.g., Onji et al. 2000). Previous work at the Naval

Research Laboratory in Key West, FL (NRL) showed that movement was a useful tool for determining viability of cultured brine shrimp (*Artemia franciscana*) and cultured rotifers (*Brachionus plicatilis*). This technique, however, has not been as successful with zooplankton collected from ambient seawater since many of the species do not move as frequently as *A. franciscana* and *B. plicatilis*. Also, protists comprise a small – but consistently present – fraction of the organisms found in the ≥ 50 µm size class, and many of these protists do not move (MKS personal obs.).

To supplement using mobility as an indicator of viability in the ≥ 50 µm size class, this study investigated the addition of four stains: neutral red (NR), aniline blue (AB), fluorescein diacetate (FDA), and 5-chloromethylfluorescein diacetate (CMFDA; CellTracker™ Green). Neutral red is a vital stain that is selectively retained by lysosomes and other acidic organelles (Horobin and Kiernan 2002) and is commonly used for copepod viability studies (Dressel et al. 1972; Crippen and Perrier 1974; Tank et al. 2006). Neutral red has also been used with oyster pediveligers and juvenile freshwater mussels (Phelps and Warner 1990; Jacobson et al. 1993), although bivalves require a longer staining period than copepods.

Aniline blue (also known as water blue and methyl blue) stains collagen and callose in dead cells and has been used to stain a wide variety of organisms ranging from mysid shrimp and larval fish to copepods and algae (Seepersad et al. 2004; Bicket et al. 2008). Both aniline blue and neutral red have weak fluorescent signals and are predominantly used under brightfield illumination.

FDA is a non-fluorescent, cell-permeable molecule. After entering a live cell, the acetate groups are cleaved by intracellular esterases, and the product, the non-permeable, fluorescent molecule fluorescein, is retained in the cell. CMFDA is similar to FDA, but the added methyl group is mildly thiol-reactive and binds to structures inside the cell, aiding in cellular retention. FDA and CMFDA are commonly used for phytoplankton viability and metabolism studies and have also been used with bacteria (e.g., De Rosa et al. 1998; Garvey et al. 2007).

In the work presented here, each stain was evaluated with ambient zooplankton as well as with cultured, standard test organisms.

2.0 Materials and Methods

2.1 Study Site and Ambient Zooplankton

The Naval Research Laboratory (NRL) is located on Fleming Key in Key West, FL (24° 34.5’ N; 81° 47.7’ W) and is surrounded by oligotrophic water. Seawater for the facility is pumped directly from Garrison Bight, which was the source for all ambient plankton samples used in this study. Zooplankton (≥ 50 µm size class) densities ranged from 38 to 325 organisms l^{-1} from July 2008 to July 2009, and the community was consistently dominated by crustaceans except during summer protist blooms (Steinberg et al., in

review). Plankton samples were collected from output hoses connected to the flowing seawater system and concentrated using a 50 µm nylon mesh sieve.

2.2 Standard Test Organisms

The brine shrimp *Artemia franciscana* has been used extensively in aquaculture and research for decades due to the widespread retail sale of cysts and the ease of hatching (Brine Shrimp Direct, Ogden, UT). To hatch the nauplii, cysts were incubated in 5-µm-filtered, aerated seawater for 24 h in 25°C with a 12:12 light:dark cycle (fluorescent bulbs: 72 µM Einsteins m^{-2} s^{-1}). Newly-hatched nauplii were approximately 300-400 µm long.

The rotifers *Brachionus plicatilis* and *B. calyciflorus* (Reed Mariculture Inc., Campbell, CA) are also commonly used in aquaculture and are easy to acquire as cysts or as adults. Adult rotifers were approximately 100-300 µm in length and held in the dark at 4°C prior to use.

2.3 Microscopy

One-ml samples were loaded into Sedgewick-Rafter counting chambers and viewed on an Olympus AZH10 stereoscope (brightfield only), a Nikon E600 compound microscope (epifluorescence and brightfield), or a Nikon AZ100 stereoscope (epifluorescence and brightfield). Fluorescein is excited by blue light and emits green light, and standard FITC filter cubes are suitable for FDA and CMFDA as well (480/30; 505; 535/40). By using a bandpass filter, chlorophyll *a* autofluorescence, which interferes with the emitted green light, was blocked from view.

Photomicrographs were collected with a QImaging Retiga 1300i cooled digital camera with a 2.5 cm CCD sensor and 1280 x 1024 pixel resolution (Surrey, Canada). Images were monochromatic unless an external color wheel was added to the system to generate color images.

2.4 Neutral Red (NR)

A 1:2000 stock of NR (Fisher Scientific©, Pittsburgh, PA) was created by adding 5.0 mg dry NR to 10 ml Type II water (filtered to nominally remove particles >0.5 µm and deionized; Fleming and Coughland 1978). Ambient plankton samples (≥ 50 µm) were stained with a final concentration of 1:100,000 NR for 1-3 hours before being rinsed with Instant Ocean® artificial seawater (Atlanta, GA) and viewed in a Sedgewick-Rafter counting chamber under brightfield illumination. To serve as a negative control group, ambient samples were heat killed (50°C for 5-10 min) and stained with NR.

A. Franciscana nauplii in two age classes – newly hatched and 3 days old – were each stained for two hours at two concentrations of NR (1:100,000 and 1:50,000). A heat-killed sample was also stained and evaluated as mentioned above.

2.5 Aniline Blue (AB)

A 1:25 stock of AB (Fisher Scientific©, Pittsburgh, PA) was created by adding 2.0 g AB to 50 ml Type II water. Ambient zooplankton samples were stained with a final concentration of 1:100 AB for 15-25 min before being rinsed with Instant Ocean® and examined on a Sedgewick-Rafter counting chamber under brightfield illumination. A sample of heat-killed ambient zooplankton was stained with AB, incubated under ambient light and temperature for 30 min, and examined again to identify any leaching or bleaching of the stain.

Live and heat-killed *A. franciscana* ranging in age from newly-hatched to 4 days old were stained with a final concentration of 1:100 AB for 15 min. *A. franciscana* were also killed by exposing them to chlorine for 24 hours at 16 ppm and for 5 hours at 32 ppm. Samples were rinsed before staining and analysis.

2.6 Fluorescein Diacetate (FDA)

A 50 mM primary stock of FDA (Invitrogen©) was created by adding 104.1 mg FDA to 5 ml dimethyl sulfoxide (DMSO). A 1 mM working stock was created by adding 20 µl primary stock to 980 µl DMSO. Ambient plankton samples were stained with final FDA concentrations of 10 µM, 5 µM, 1 µM, or 0.1 µM for 10 min and analyzed on a Sedgewick-Rafter counting chamber using fluorescent microscopy. Heat-killed (as above) and chlorine-killed (23 ppm chlorine for 24 h) samples were also analyzed.

Live and heat-killed *A. franciscana* ranging in age from newly-hatched (0 days after hatch), 1 day after hatch, and 5 days after hatch were stained with a final concentration of 1 µM, 4 µM, and 8 µM FDA. Live and heat-killed samples of *B. plicatilis* and *B. calyciflorus* were also stained with 10 µM FDA. All samples were incubated in the dark for 10 min after FDA was added, and the samples were not rinsed prior to analysis.

2.7 5-Chloromethylfluorescein Diacetate (CMFDA)

A 250 µM working stock of CMFDA (Invitrogen©) was created by adding 430 µl DMSO to a vial of 50 µg CMFDA. Live and heat-killed *A. franciscana* ranging in age from 0 days, 1 day, and 5 days after hatch were stained with a final concentration of 2.5 µM CMFDA. Since previous experiments at NRL found FDA and CMFDA complimented each other when staining protists (Drake et al. 2010), testing the combination of stains with *A. franciscana* was warranted. The same experiment was repeated using FDA and CMFDA together with final concentrations of 5 µM and 2.5 µM, respectively.

3.0 Results

3.1 Neutral Red (NR)

A qualitative assessment of NR showed inconsistent staining of ambient organisms, ranging in color from light pink to dark red (Figure 1). Some organisms were entirely stained while others were patchy with dark red viscera. Several species of crustacean larvae and dinoflagellates were moving – and clearly living – but remained unstained. These inconsistencies were present regardless of the stain incubation time or the stain concentration. This result, a false negative, led us to question whether non-moving, unstained organisms were, in fact, dead. Most organisms and debris in the heat-killed sample were unstained. Regardless of age, neither live nor heat-killed *A. franciscana* stained with NR.

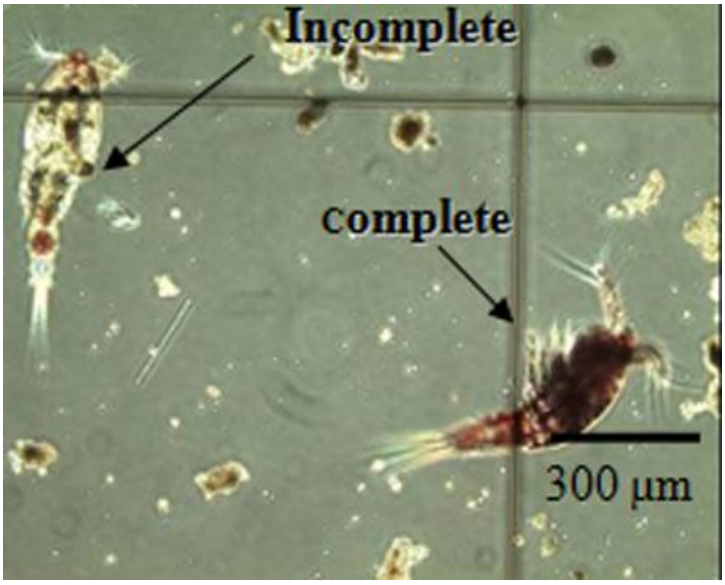

Figure 1. Live copepods stained with the vital stain neutral red (NR); staining varied from incomplete with only a few internal organs stained red (left copepod) to complete (right copepod). This image was taken at 100x magnification.

3.2 Aniline Blue (AB)

The mortal stain AB also inconsistently stained ambient zooplankton (Figure 2). The color of stained organisms varied from light to dark blue with occasional dark spots in the viscera; however AB never stained moving organisms. Some organisms in heat-killed samples did not stain with AB (false

negatives), including several copepods, dinoflagellates, and a bivalve veliger. Additionally, photobleaching, leaching, or both were evident after 30 min of exposure to ambient light (Figure 3).

Aniline blue yielded inconsistent staining of 12-hour old heat-killed *A. franciscana* with some individuals staining completely while others showed patchy staining. Heat-killed *A. franciscana* that were 3-4 days old stained more consistently and thoroughly than newly hatched nauplii. Chlorine-killed samples stained poorly with AB (SHR personal obs.).

Figure 2. Live and heat-killed *A. franciscana* stained with the mortal stain aniline blue (AB). Live nauplii did not stain blue (1st and 3rd from left), however heat-killed nauplii showed inconsistent staining (2nd and 4th from left). These images were taken at 7x magnification.

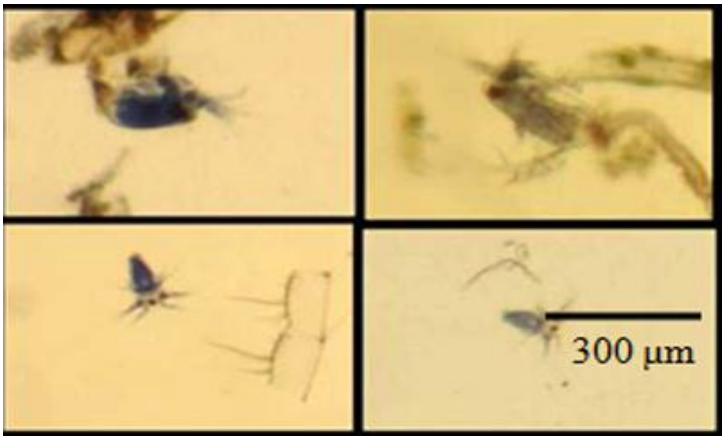

Figure 3. Time-lapse image series of heat-killed zooplankton stained with AB. Images taken immediately after staining (left images) and zooplankters after exposure to ambient light for 30 min (right images). These images were taken at 150x magnification.

3.3 Fluorescein Diacetate (FDA)

The vital stain FDA consistently produced a strong fluorescent signal in ambient zooplankton, but higher concentrations of FDA also yielded higher background fluorescence (Figure 4). This result was likely because fluorescein was produced at higher rates within the organisms, and thus more fluorescein leaked out of the cells and into the water than zooplankton stained with lower concentrations of FDA. Although some dead organisms were stained (false positive), the fluorescent signal made it easy to identify organisms in samples with high concentrations of debris where movement, a reliable indicator of viability, could be obscured. The presence of 23 ppm chlorine did not immediately kill all the organisms and did not appear to interfere with FDA staining of ambient, living organisms.

A. Franciscana was stained with FDA at several time points after hatching (0 days [T0], 1 day [T1], and 5 days [T5]) and at final stain concentrations of 1 µM, 4 µM, and 8 µM (Figure 5). The first naupliar stage of *A. franciscana* did not stain with FDA regardless of concentration; however, larvae in the second instar stage had a narrow line of fluorescein along the central axis. All samples of heat-killed *A. franciscana* did stain with FDA despite the fact that FDA is a vital stain that should only hydrolyze in the presence of active intracellular enzymes (false positive). There was a positive relationship between the age of *A. franciscana* and the intensity of the FDA fluorescence, and higher concentrations of FDA also resulted in a stronger signal in the T1 and T5 samples. Untreated (presumably live) *A. franciscana* cysts did not stain with FDA; however, heat-killed (dead) cysts had a faint, green signal (false positive).

Live samples of the marine rotifer, *B. plicatilis,* stained with FDA were very bright, and the heat-killed sample had a very faint signal (false positive). Live samples of the freshwater rotifer, *B. calyciflorus*, stained very brightly, too, but the heat-killed samples did not fluoresce.

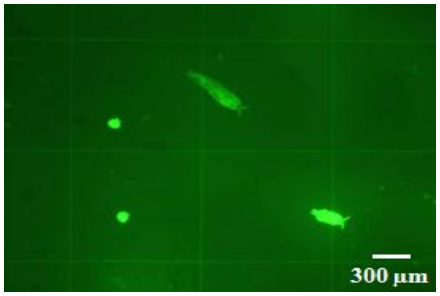

Figure 4. Live, ambient zooplankton stained with FDA. Samples were analyzed using blue-light excitation on epifluorescent microscopes. This image was taken at 40x magnification.

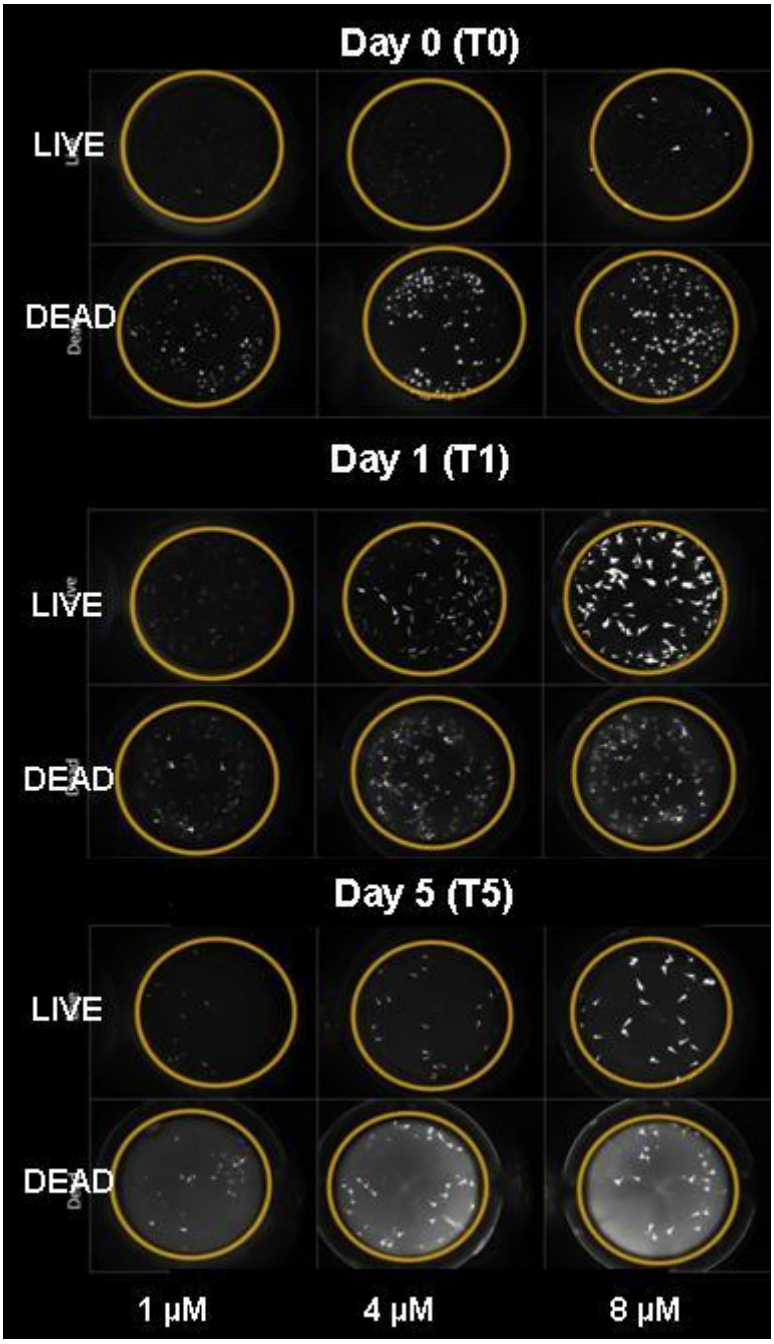

Figure 5. Live and dead (heat-killed) *A. franciscana* stained with FDA. Larvae ranged from newly-hatched (T0) to 1 day after hatch (T1) to 5 days after hatch (T5). FDA concentrations were 1 µM, 4 µM, and 8 µM. These images were taken at 7x magnification.

3.4 5-Chloromethylfluorescein Diacetate (CMFDA)

Newly-hatched, live samples of *A. franciscana* did not stain with CMFDA (false negative), and heat-killed samples stained very faintly (false positive). On T1, live samples were faintly stained, and heat-killed samples likewise became brighter (false positive). By Day 5, both live and heat-killed *A. franciscana* had strong fluorescent signals.

3.5 Fluorescein Diacetate (FDA) and 5-Chloromethylfluorescein Diacetate (CMFDA)

Samples of *A. franciscana* stained with both FDA and CMFDA were identical to the FDA-only results.

4.0 Discussion

Results of the staining trails varied among the vital and mortal stains used. The vital stain neutral red (NR) showed the least potential of all the stains tested, largely due to the large number of moving (living) organisms that were inconsistently stained. It should be noted that only marine organisms were evaluated, and therefore, NR efficacy with freshwater species was not assessed. The mortal stain aniline blue (AB) did not stain organisms that were visibly mobile, but the usefulness of this stain diminished due to variability in staining intensity. Previous research has found that the quality of the staining varies by species and by health of the organism (Seepersad and Crippen 1978; Seepersad et al. 2004). Also, some groups of zooplankton require staining incubations of up to 3 hours for bivalve larvae (Horvath and Lamberti 1999). These results coupled with photobleaching, leaching, or both over time, suggest that AB is an unacceptable method for assessing zooplankton viability in ballast water samples.

Combining the two vital stains FDA and CMFDA showed the most potential as a method for zooplankton viability assessments. Final stain concentrations of 5 µM and 2.5 µM for FDA and CMFDA, respectively, and an incubation time of 10 min consistently produced a strong fluorescent signal in ambient zooplankton, which made the organisms easily visible in samples with abundant debris. The fact that FDA and CMFDA occasionally stained dead organisms can be overcome by combining it with a mobility analysis to ultimately determine viability. The two analyses could also be applied to assessing the standard test organisms *A. franciscana* and *B. plicatilis*, which are very active swimmers. FDA and CMFDA were found to be the best stains for ambient plankton due to the bright, fluorescent signal and easy preparation, and when paired with mobility assessments, represent the best option to date for determining viability in the ≥ 50 µm size class.

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