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Research Article

Evaluating the response of freshwater organisms to vital staining

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Abstract

The unintentional introduction of nonindigenous species by ballast water discharge is one of the greatest threats to biodiversity in freshwater systems. Proposed international regulations for ballast water management will require enumeration of viable plankton in ballast water. In this study we analyze the efficacy of vital stains in determining viability of freshwater taxa.

The efficacy of vital stains fluorescein diacetate (FDA) and FDA+5-chloromethylfluorescein diacetate (CMFDA) was evaluated with freshwater macroinvertebrates, zooplankton, and phytoplankton. Macroinvertebrates were cultured in laboratory, while plankton were collected from Hamilton Harbour and ballast tanks of commercial vessels. Organisms were subjected to various treatments (i.e., heat, NaClO, and NaOH) to establish efficacy of stains for viable and non-viable organisms. No significant difference in accuracy rate was found between stains, regardless of treatment, within groups of organisms, indicating that the addition of CMFDA is superfluous in the sample region studied. False positive errors, in which dead organisms fluoresced similarly to live organisms, occurred in most groups and were significantly different between test groups. False positive error rates were 2.3% for phytoplankton, 20% for ballast water zooplankton, 35% for Hamilton Harbour zooplankton and 47% for macroinvertebrates.

Response to stains varied between taxonomic groups. Low (< 10%) false positive error rates were observed with phytoplankton, soft-bodied rotifers, oligochaetes, and *Bosmina* spp., while rates between 20% and 50% were observed for *Daphnia* spp., *Hexagenia* sp., and *Chironomus riparius*. False positive rates of copepods, *Hyalella azteca*, and *Hemimysis anomala* were between 70% and 100%. The FDA/FDA+CMFDA vital staining methods provide useful tools for viability analysis of freshwater phytoplankton, soft-bodied invertebrates and zooplankton, and may be used for viability analysis of the ≥ 10 µm to < 50 µm size fraction in compliance testing of ballast water. However, viability analysis of larger freshwater crustaceans with vital stains should be undertaken with caution.

Key words: 5-chloromethylfluorescein diacetate, fluorescein diacetate, ballast water, macroinvertebrates, plankton

Introduction

Aquatic nonindigenous species (NIS) are organisms that have established populations outside of their native range, through either intentional or unintentional means of introduction. NIS that successfully establish in a new environment may inflict negative impacts on the receiving ecosystem, and are considered by many to be the greatest threat to biodiversity in freshwater ecosystems and the second greatest cause of global extinction (Sala et al. 2000; MEA 2005; Lawler et al. 2006). The unintentional introduction of aquatic NIS through ballast water discharge from commercial vessels is a primary vector for aquatic NIS introductions in freshwater systems such as the Great Lakes and St. Lawrence River (Ricciardi and MacIsaac 2000; de Lafontaine and Costan 2002; Holeck et al. 2004; Ricciardi 2006). Viability of organisms upon discharge of ballast

water may be dependent on various factors such as length of voyage, physical-chemical conditions, occurrence of mid-ocean exchange, and application of ballast water treatment systems (Olenin et al. 2000; McCollin et al. 2007; Klein et al. 2010).

In 2004, the International Maritime Organization (IMO) adopted the International Convention for the Control and Management of Ships' Ballast Water and Sediments, which, when ratified, will govern the maximum allowable concentrations of viable organisms in discharged ballast water. In relation to plankton and invertebrates, the Convention states that maximum discharge densities must be less than 10 viable organisms ≥ 50 μm per m^3, and less than 10 viable organisms ≥ 10 μm to < 50 μm per mL (IMO 2004). A variety of treatment systems are being developed to meet these discharge limits, which require accurate, quantitative testing to verify their effectiveness in removing or exterminating viable organisms.

Traditional methods of collecting plankton in the field, preserving, and enumerating total numbers of organisms rely on the assumption that all visibly intact organisms were viable at the time of collection. However this assumption is not supported for many organisms in natural environments (Tang et al. 2006; Bickel et al. 2009) and may not prove true in ballast tanks where environments may be harsh and transit times may be too long for many organisms to survive (McCollin et al. 2007; Klein et al. 2010). Moreover, the application of biocides to inactivate organisms shortly before sample collection results in little time for decomposition, likely resulting in overestimation of viable plankton abundance.

The need to quantify organisms in ballast water to determine compliance with new IMO discharge standards therefore requires the development of viability assessment protocols. The use of vital stains to assess viability of phytoplankton and zooplankton in marine and coastal environments has been well established in recent years: SYTOX green (Veldhuis et al. 2001; Baudoux et al. 2008); fluorescein diacetate (FDA)(Brookes et al. 2000; Garvey et al. 2007; Peperzak and Brussaard 2011; Villac and Kaczmzrska 2011); FDA + 5-chloromethyl-fluorescein diacetate (CMFDA) (Steinberg et al. 2011); neutral red (Elliott and Tang 2009; Zetzche and Meysman 2012). In contrast, there have been few studies examining appropriate methodologies for freshwater communities (Seepersad and Crippen 1978; Bickel et al. 2009; Reavie et al. 2010).

The vital stain FDA reacts to non-specific enzymatic activity within cells, is non-toxic, and inexpensive. CMFDA also reacts with non-specific enzymatic activity and is mildly thiol reactive, allowing the compound to remain within the cell longer, but is more expensive. Reavie et al. (2010) tested the accuracy of FDA with phytoplankton assemblages from Lake Superior and several small lakes in northern Minnesota (USA). The vital fluorescent stain was found to be suitable for organisms in the 10–50 μm size range, however, it is unknown if FDA would be useful for determining viability of larger freshwater organisms potentially found in ballast water, or for freshwater phytoplankton outside of Lake Superior. In contrast, the stains FDA and CMFDA were found insufficient for viability assessment of marine phytoplankton when used individually due to differential staining across species (Steinberg et al. 2011). Yet it was observed that the combination of stains provided complimentary staining of the majority of phytoplankton (Steinberg et al. 2011).

In this study, we assess the use of FDA and FDA+CMFDA in determining viability of freshwater organisms. We evaluate the accuracy of the fluorescent vital stains in differentiating between live and dead organisms for different size classes and taxonomic groups across different treatments (kill methods). Our null hypotheses are that: i) no difference in staining efficacy will be observed between the two vital stains; ii) the varying treatments applied will not have an influence on the outcome of staining; and iii) no difference in staining accuracy will be present between different taxonomic groups of freshwater organisms. Finally, we evaluate the method in terms of its potential application for assessment of ballast water from ships transiting the Laurentian Great Lakes, and compare results with traditional methods of plankton assessments for ballast water.

Methods

Test groups and sample collection

Five sample groups were subjected to testing: macroinvertebrates, harbour and ballast plankton (zooplankton and phytoplankton). Macroinvertebrates consisted of primarily benthic, laboratory-grown cultures including two species of oligochaetes (*Lumbriculus variegatus* Mueller, 1774 and *Tubifex tubifex* Mueller, 1774), midge larvae *Chironomus riparius* (Meigen, 1804), the amphipod *Hyalella azteca* (Saussure, 1858), and mayfly larvae *Hexagenia* sp. With the exception of *L. variegatus,* which was purchased from a commercial vendor

(Merlan Scientific Ltd, Mississauga, Ontario), all cultures were reared/ hatched in a laboratory at the Canada Centre for Inland Waters, Burlington, ON. Included in the macroinvertebrates group is the large planktonic invasive amphipod, *Hemimysis anomala* (Sars, 1907), which was sampled from Lake Ontario and maintained in the laboratory for up to 2 weeks post-collection. The zooplankton (> 50 µm) and phytoplankton (10–50 µm) samples consisted of collected species from Hamilton Harbour and ballast water tanks of commercial ships transiting the Great Lakes-St. Lawrence Seaway.

Zooplankton samples were collected from Hamilton Harbour (Lake Ontario; 43°N, 79°W) on seven occasions between April and July 2012 by a single vertical net haul in 9 metres of water, using a 35 µm mesh net (50 µm diagonal). Samples were collected and concentrated into a 35 µm cod end and rinsed into a 500 mL plastic sample bottle. Phytoplankton samples were collected from Hamilton Harbour on two occasions during October 2012. Whole surface water samples were collected using a 20 L bucket and sieved through a 35 µm mesh, with the filtrate collected and further size fractionated using a vacuum filtration system fitted with a 5 µm mesh cloth (7 µm diagonal). The 5 µm mesh cloth was then rinsed into a 300 mL beaker using a small amount of filtrate water.

Ballast water samples were collected from three domestic and three foreign ships on arrival to the Port of Hamilton, ON or while in transit in the Welland Canal between September and November 2012. Domestic ships were transporting ballast water sourced from Montreal, Quebec, Tracy, Quebec, and Cote Ste-Catherine, Quebec, while all foreign ships had undertaken mid-ocean exchange in the Atlantic Ocean. Approximately 1000 L of water was filtered from a single tank of each ship for collection of zooplankton using a 35 µm mesh net. Samples were then concentrated in a 35 µm codend and rinsed into a 1000 mL sample bottle. Phytoplankton samples were collected as whole water samples from the tank surface.

Vital stains

Laboratory trials were performed to assess the accuracy and efficacy of vital stains FDA (Sigma-Aldrich Canada, Oakville, Ontario) and CMFDA (Invitrogen Canada, Burlington, Ontario). Cultured and ambient plankton were stained either with FDA-only or a combination of CMFDA+FDA. A primary solution of FDA was made by combining 50 mg of solid powder FDA with 10 mL of reagent grade dimethyl-sulfoxide (DMSO; Sigma-Aldrich Canada, Oakville, Ontario), for a final concentration of 12.0 mM. Further, FDA working solution was made through the addition of 10.0 µL of FDA primary solution to 1.0 mL of distilled water, for a final working solution concentration of 120 µM. Primary solutions of CMFDA were created through the addition of 10.7 µL DMSO to 0.05 mg of powdered CMFDA, resulting in a final concentration of 10 mM. Twenty-five µL aliquots of the CMFDA primary solution were added to microcentrifuge tubes containing 1 mL distilled water, resulting in working solutions with final concentration of 250 µM. Primary solutions were stored at 4°C in the dark, while working solutions were prepared new with every use.

Sample treatment

Three kill methods were applied to each of the five test groups (macroinvertebrates, Hamilton Harbour (HH) zooplankton, HH phytoplankton, ballast water (BW) zooplankton, and BW phytoplankton) in replicates of five, in order to perform staining trials on live and dead organisms, and to compare influence of kill methods on staining results: heat, NaClO, and NaOH. In kill method #1, samples were placed in a water bath of 95°C for 15 minutes. Samples were then allowed to return to room temperature prior to staining. Kill method #2 involved 24 hour incubation with NaClO, for a final Cl^- concentration of 23 ppm. Kill method #3 entailed the addition of NaOH to increase the sample pH to 12.0 for 24 hours, or for 1 hour (*L. variegatus* only). Samples were kept in the dark during incubation, and NaOH was subsequently neutralized by addition of HCl. NaOH kill method trials could not be performed on *T. tubifex*, as addition of the strong base to the sample resulted in immediate disintegration of the animal. Following all kill methods, all macoinvertebrate and zooplankton samples and all phytoplankton samples were decanted onto 35 µm and 5 µm filter mesh, respectively, and gently rinsed with filtered (<5 µm) ambient water to eliminate residual chemicals used by the kill methods. Heat treated samples were also rinsed to maintain a consistent methodology across treatments. Untreated samples were stained and analyzed within 2 hours of collection, while treated samples were stained and analyzed immediately following treatment.

Sample staining and analysis

Each treated (5 per kill method per test group) and untreated (5 per test group) replicate for the five test groups were stained with the vital stains. Macroinvertebrates were stained in 20 mL scintillation vials along with 5 mL of culture water, at densities of 5 organisms/sample (*L. variegatus* and *T. tubifex*), 10 organisms/ sample (*Hexagenia* sp. and *C. riparius*), or 20 organisms/sample (*H. azteca* and *H. anomala*). Zooplankton and phytoplankton were stained by transferring 5 mL of each sample to 20 mL scintillation vials. Macroinvertebrates, zooplankton, and phytoplankton were stained with 417 µL of the FDA working solution and, for the combination method, 100 µL of the CMFDA working solution, for a final concentration of 10 µM and 5 µM, respectively.

Stained samples were incubated in the dark at room temperature for 10 minutes. Following incubation with the stain, macroinvertebrates were loaded into well-plates, zooplankton samples were loaded onto a gridded (5mm^2) zooplankton counting chamber measuring approximately 6 cm × 3 cm, and phytoplankton samples were loaded onto gridded (1 mm^2) Sedgewick–Rafter counting chambers measuring 7.6 cm × 2.5 cm, with a total cell size of 5 cm × 2 cm × 0.1 cm. Phytoplankton samples were allowed to settle for 2 minutes prior to observation. Macroinvertebrates were enumerated at 10× magnification, while zooplankton were enumerated at 40× magnification. Both macro-invertebrates and zooplankton were observed using a Nikon AZ100 compound epifluorescent microscope with blue light excitation-green bandpass emission filter cubes (FITC; excitation 465–495 nm, dichoric 505 nm, barrier 515–555 nm). Phytoplankton were enumerated at 200x magnification using a Zeiss Axiovert A1 inverted epifluorescent microscope with the same blue light excitation-green bandpass emission filter cubes. Transitions between brightfield and epifluorescence were employed for zooplankton and phytoplankton observations for simultaneous taxonomic identification and viability analysis. Phytoplankton were examined under epifluorescent light for a maximum of 20 minutes, as it was assessed during preliminary trials that prolonged exposure to light in combination with stain leakage over time resulted in increased background fluorescence and fading of stain, leading to difficulty distinguishing between fluorescing plankton and background.

A minimum of 100 individuals were enumerated for each HH zooplankton sample, while HH phytoplankton samples were enumerated to either a minimum of 500 individuals, or until the maximum observation time of twenty minutes was reached. In the case of ballast water samples, phytoplankton and zooplankton were enumerated until the appropriate minimum numbers were reached, or the entire sample was analyzed.

Preliminary trials indicated that even a weak fluorescence signal may indicate a live organism, therefore any detectable signal observed was considered a positive result and that individual was counted as 'live'. Individuals in both untreated and treated samples were analyzed for movement and fluorescence simultaneously to determine error rates for each stain. As all organisms were considered dead in treated samples, any organism emitting a fluorescence signal was considered a false positive. Organisms in untreated samples which had movement but did not fluoresce were considered false negatives. Organisms that either moved or fluoresced were considered live, while organisms were considered dead when they neither moved nor fluoresced. To determine the effect, and potentially confounding issue, of green autofluorescence on vital staining (Tang and Dobbs 2007), in addition to the effect of kill methods on fluorescence, each sample type included a negative control to which no stain was applied.

Statistical analysis

Statistical analysis was conducted using Systat v.11 (Systat Software, Inc.). Variations in percent of organisms stained in untreated samples were compared using a one-way multivariate analysis of variance (MANOVA), where test groups (macro-invertebrates, HH zooplankton, HH phytoplankton, BW zooplankton, and BW phytoplankton) were dependent variables and stains (FDA and CMFDA+FDA) were independent variables. Variations in the rate of false positives among kill methods and stains were compared using two-way MANOVA, where test groups (macro-invertebrates, HH zooplankton, HH phytoplankton, BW zooplankton, and BW phytoplankton) were dependent and kill methods (heat, NaClO, and NaOH) and stains (FDA and CMFDA+FDA) were independent variables. Furthermore, one-way analysis of variance (ANOVA) was performed to test for differences among test groups. To determine if taxonomic groups responded differently to various kill methods and stains, variation in the rate of false positives among kill methods and

Table 1. Results of multivariate analysis of variance (MANOVA) with untreated test groups as dependent (macroinvertebrates, Hamilton Harbour (HH) zooplankton, HH phytoplankton, ballast water (BW) zooplankton, and BW phytoplankton) and stain (FDA and CMFDA+FDA) as independent variable.

Stain	Test Group	df	F	*p*
	Macroinvertebrates	1	1.000	0.347
	HH Zooplankton	1	1.060	0.333
	HH Phytoplankton	1	0.242	0.636
	BW Zooplankton	1	0.003	0.954
	BW phytoplankton	1	0.011	0.919
	Wilks' lambda = 0.752	5	0.264	0.912

Table 2. Results of multivariate analysis of variance (MANOVA) with treated test groups as dependent (macroinvertebrates, Hamilton Harbour (HH) zooplankton, HH phytoplankton, ballast water (BW) zooplankton, and BW phytoplankton) and stains (FDA and CMFDA+FDA) and treatment (heat, NaClO, and NaOH) as independent variables.

Stain	Test Group	df	F	P
	Macroinvertebrates	1	2.407	0.134
	HH Zooplankton	1	0.092	0.764
	HH Phytoplankton	1	1.704	0.204
	BW Zooplankton	1	0.042	0.839
	BW phytoplankton	1	0.330	0.571
	Wilks' lambda = 0.745	5	1.369	0.277
Treatment	Test Group	df	F	P
	Macroinvertebrates	2	0.428	0.657
	HH Zooplankton	2	2.047	0.151
	HH Phytoplankton	2	3.360	0.052
	BW Zooplankton	2	1.284	0.295
	BW phytoplankton	2	0.957	0.398
	Wilks' lambda = 0.604	10	1.159	0.354

stains was compared between taxonomic test groups conducting two-way MANOVA, where taxa (*H. azteca*, *H. anomala*, *C. riparius*, *Hexagenia* sp., oligochaetes, copepods, copepod nauplii, rotifers, cladocerans, and phytoplankton) were dependent and kill methods and stains were independent variables. Treated taxonomic data did not fit assumptions of parametric testing and were subsequently arcsine transformed to meet assumptions. Significance levels for statistical comparisons were adjusted for multiple pair-wise comparisons by Bonferroni-type correction with a family-wise error rate of 0.05.

Results

Untreated samples

Regardless of vital stain applied, untreated cultures stained correctly 98.6% of the time. False negative errors occurred during one experiment with *Hyalella azteca* cultures, during which 10% (2/20) of individuals did not stain, but were mobile. Untreated HH plankton stained correctly 100% of the time as assessed through the observation of movement and fluorescence (Figure 1). No significant difference ($p > 0.05$; Table 1) was observed between the two vital stains for accurately identifying living organisms of plankton from either Hamilton Harbour or ballast water.

Vital staining results indicated that untreated HH zooplankton samples contained 89% to 100% viable organisms and an average of 5% non-viable organisms, while phytoplankton samples contained 56% to 100% viable organisms and an average of 18% non-viable organisms. Viable organisms comprised 40% to 100% of any single BW zooplankton sample, and 27% to 100% of any single phytoplankton sample. Non-viable organisms comprised on average 30% samples for both BW zooplankton and BW phytoplankton.

Treated samples

The rates of false positive occurrences within and between test groups were evaluated to determine the overall performance of the vital

stains (Figure 2). The results showed no significant differences in error rates between FDA and CMFDA+FDA within any test group – macroinvertebrates, HH zooplankton, BW zooplankton, HH phytoplankton or BW phytoplankton ($p > 0.05$; Table 2). Furthermore, no significant difference occurred between the three kill methods used, within test groups ($p > 0.05$; Table 2).

However, between the test groups, false positive rates differed significantly ($p < 0.001$; Figure 3). Rates of false positives were significantly lower amongst phytoplankton when compared to zooplankton and macroinvertebrate groups ($p < 0.05$), where phytoplankton false positive rates were 2.3% for both BW and HH samples. Furthermore, rates of false positives were significantly higher for HH zooplankton than for BW zooplankton with error rates of 35% and 20%, respectively ($p < 0.001$). Macroinvertebrates exhibited the highest rates of false positives at 47%, and were not significantly different from HH zooplankton error rates ($p > 0.05$).

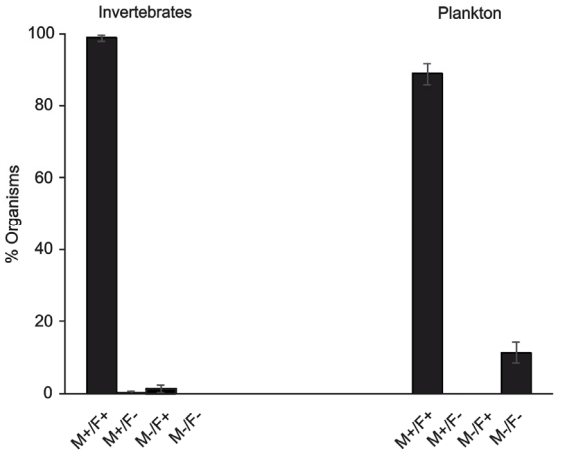

Figure 1. Mean (± standard error) total percentage (%) of untreated organisms stained with vital stains for macroinvertebrate and HH and BW plankton (total zooplankton and total phytoplankton). M+, F+, M-, and F- indicate movement, fluorescence, no movement, and no fluorescence, respectively.

Taxonomic responses

Rates of false positives were consistently low amongst phytoplankton groups, which included primarily diatoms (both centric and pennate), dinoflagellates, cyanobacteria, and chlorophytes. However, false positive errors were much more common and variable amongst macroinvertebrates and zooplankton, which consisted primarily of several varieties of copepods and copepod nauplii, and several families of rotifers and cladocerans (Figure 4; Figure 5). Oligochaetes stained correctly in 100% of trials. Rotifers and cladocerans had moderately high false positive rates of 29% and 22%, respectively. Furthermore, variation existed between rotifer genera, as *Asplanchna*, *Polyarthra*, and *Synchaeta* were less likely to produce false positives than *Keratella* or *Kellicottia*. Likewise, *Bosmina* and *Eubosmina* spp. had a low mean false positive rate of 7%, while *Daphnia* spp. had a higher false positive error rate of 41%. Insect larvae also exhibited moderately high false positive rates at 23% for mayfly larvae *Hexagenia* sp. and 49% for midge larvae *C. riparius*. Finally, high rates of false positives were observed for the larger crustaceans: copepod nauplii (47%), copepods (71%), *H. anomala* (94%), and *H. azteca* (98%).

Accuracy typically did not vary significantly between stains or kill methods (Table 3, Figure 6). However, accuracy between FDA and CMFDA+FDA varied significantly for treated *H. azteca* and *C. riparius* ($p<0.05$). *H. azteca* had consistently high rates of false positives regardless of stain used, with error rates of 98% with FDA and 97% with CMFDA+FDA. *C. riparius* ($p < 0.05$) had lower overall rates of false positives; FDA error rates (32%) were significantly lower than error rates for CMFDA+FDA (62%).

Within two taxonomic groups, false positive error rates differed significantly between kill methods (Table 3, Figure 7; $p < 0.05$). Larvae of the insect *C. riparius* ($p < 0.05$) had significantly lower rates of false positives with heat or NaOH as kill method, than when killed with NaClO (30%, 39%, and 79%, respectively). Similarly, copepod nauplii ($p < 0.001$) had significantly lower rates of false positives when killed with NaOH (14%), than when killed with heat or NaClO (64% for both heat and NaClO).

The possibility that delayed staining following kill methods may yield better results in macroinvertebrate cultures and zooplankton was investigated during preliminary trials. Trials included staining and observation at 24, 48, and 72 hours following heat, NaClO, and NaOH kill methods with *H. azteca, C. riparius,* and copepods. Observations indicated no difference in the rate of false positive errors when organisms were stained with either FDA or CMFDA+FDA following any of the prescribed wait periods.

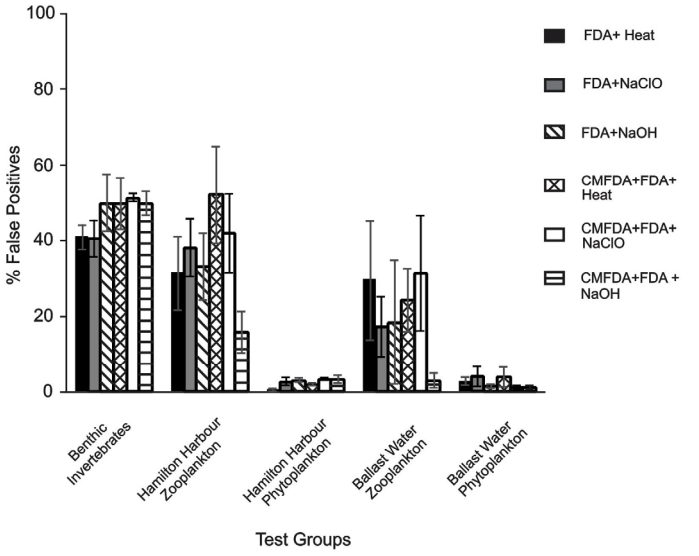

Figure 2. Mean (± standard error) total percentage (%) of false positive errors for treated samples within each test group across all combinations of stains and treatments.

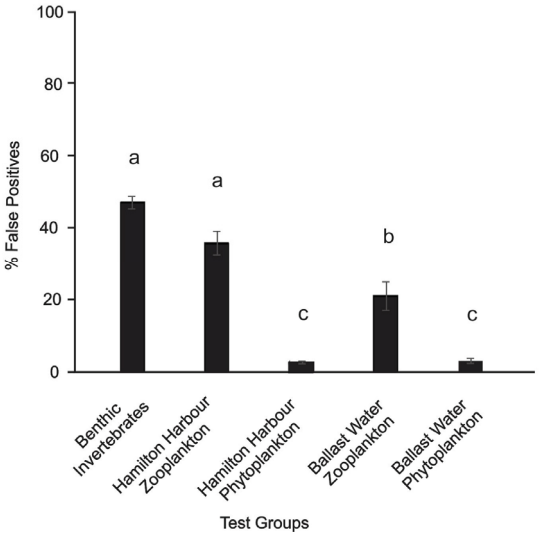

Figure 3. Mean (± standard error) total percentage (%) of false positive errors for each test group. Different letters denote significant difference at 0.05 in false positive rates between groups

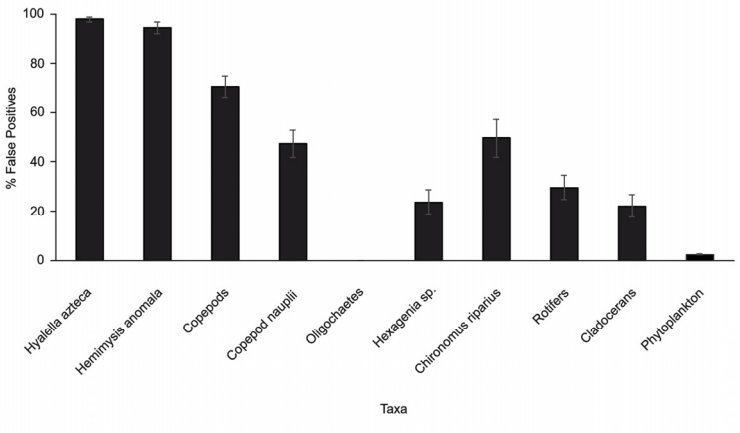

Figure 4. Mean (± standard error) total percentage (%) of false positive errors for each taxon.

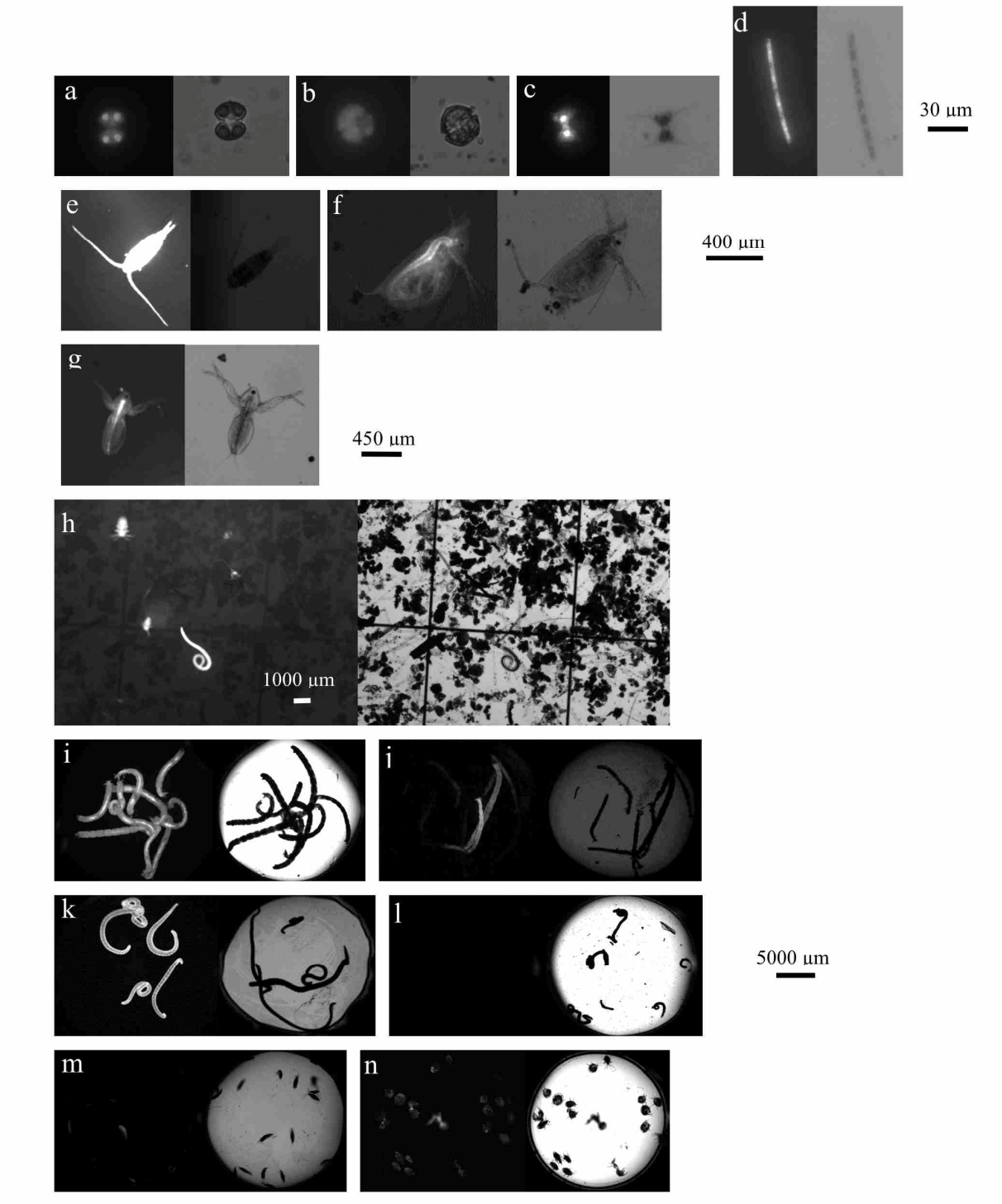

Figure 5. Paired epifluorescent (left)/brightfield (right) photomicrographs of select phytoplankton: *Cosmarium* sp. (a), *Peridinium* sp. (b), *Staurastrum* sp. (c), filamentous green algae (d), zooplankton: copepod (e), *Daphnia* sp. (f), *Diaphanosoma* sp. (g), mixed assemblage (h), and macroinvertebrates: *C. riparius* untreated (i), *C. riparius* treated (j*), L. vareigatus* untreated (k), *L. variegatus* treated (l), *H. azteca* untreated (m), and *H. azteca* treated (n) recovered from samples. Each epifluorescent photo was taken using blue light excitation on samples stained with FDA. Scale bars are included with set of images.

Table 3. Results of multivariate analysis of variance (MANOVA) with taxon (*Hyalella azteca, Hemimysis anomala*, Oligochaetes, *Hexagenia* sp., *Chironomus riparius*, copepods, copepod nauplii, rotifers, cladocerans, and total phytoplankton) as dependent and stain (FDA and CMFDA+FDA) and treatment (heat, NaClO, and NaOH) as independent variables. Significant *p*-values are presented in bold.

Stain	Taxa	df	F	*p*
	Hyalella azteca	1	5.475	**0.029**
	Hemimysis anomala	1	2.699	0.115
	Copepods	1	1.312	0.265
	Copepod nauplii	1	0.447	0.511
	Oligochaetes	1	----	----
	Hexagenia sp.	1	0.020	0.889
	Chironomus riparius	1	5.753	**0.026**
	Rotifers	1	0.518	0.480
	Cladocerans	1	3.521	0.075
	Phytoplankton	1	4.001	0.059
	Wilks' Lambda			**0.043**
Treatment	Taxa	df	F	*P*
	Hyalella azteca	2	1.929	0.170
	Hemimysis anomala	2	0.976	0.393
	Copepods	2	0.335	0.719
	Copepod nauplii	2	5.262	**0.014**
	Oligochaetes	2	----	----
	Hexagenia sp.	2	2.864	0.079
	Chironomus riparius	2	5.541	**0.012**
	Rotifers	2	0.855	0.439
	Cladocerans	2	1.710	0.205
	Phytoplankton	2	2.422	0.113
	Wilks' Lambda			**<0.001**

Discussion

While ecological assemblages of soft-bodied aquatic worms, *T. tubifex* and *L. variegatus*, several rotifer genera, and *Bosmina* and *Eubosmina* spp. were reliably stained by both FDA and FDA+CMFDA, vital stains proved problematic with freshwater copepods and amphipods. False negative errors were not prevalent amongst those taxa, however false positive errors were common, as most treated individuals displayed fluorescence regardless of live or dead status as assessed through movement, and would hence be misidentified as live. Seepersad and Crippen (1978) and Bickel et al. (2009) attributed errors in aniline blue staining of copepods and cladocerans to individuals entering a moribund state following exposure to a stressor (such as heat, NaClO, or NaOH in our study). Such individuals would be on the verge of death, but potentially still possess enzymatic activity, hence the observed fluorescence. However, we did investigate the possibility that delayed staining following a kill method may yield better results in macroinvertebrates and zooplankton. Observations indicated no difference in the number of treated organisms stained with either vital stain up to 72 hours following treatment, unlike Elliott and Tang (2009) who found that marine zooplankton allowed to sit in room temperature water for 5 minutes would no longer display false positive staining with neutral red.

Insect larvae, though not as prone to false positives as copepods and amphipods, displayed intermediate rates of false positives. Observations of *C. riparius* indicate increased accuracy with the use of the single vital stain, FDA, over the combination of vital stains. Quality of vital staining in treated, dead *C. riparius* differed from live, stained organisms. In treated individuals, stain consistently appeared to be superficial, with only the outer wall of the organism picking up the stain. However, in untreated individuals the stain is more internal than external. Similar degrees of staining were seen in treated *Hexagenia* sp., which also displayed a staining pattern following exposure to vital stain differing from that of live *Hexagenia* sp. Dead *Hexagenia* sp. that did fluoresce with the vital stain exhibited the fluorescence primarily on the legs and tail (cerci), while the gills and most of the abdomen and thorax did not fluoresce, whereas the legs of live *Hexagenia* sp. did not fluoresce and the gills, abdomen, and thorax fluoresced brightly.

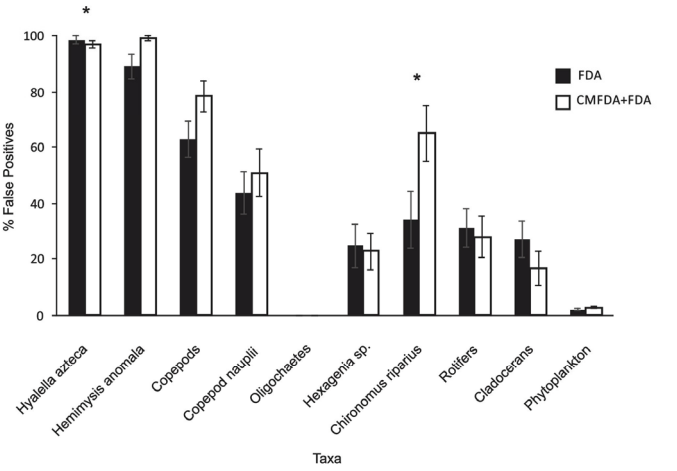

Figure 6. Mean (± standard error) total percentage (%) of false positive errors for each taxon and different vital stain. * denotes significant difference at 0.05 in false positive rates between the two stains.

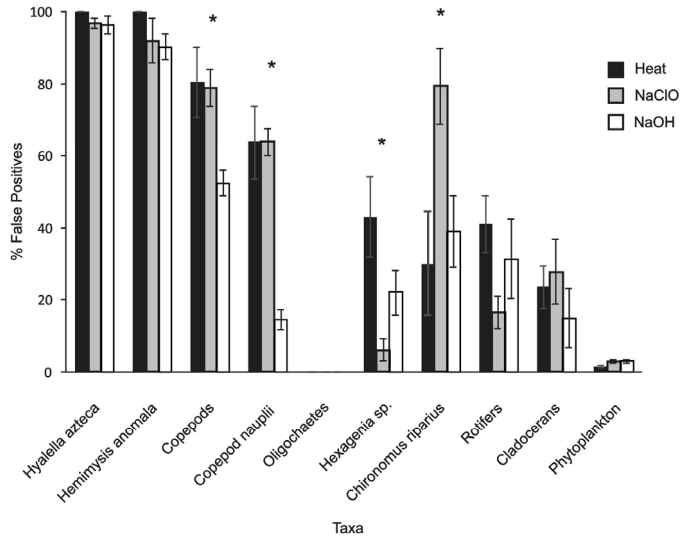

Figure 7. Mean (± standard error) total percentage (%) of false positive errors for each taxon and type of treatment tested. * denotes significant difference at 0.05 in false positive rates between the three treatments.

Patchy staining has also been observed in live marine zooplankton, particularly copepods and molluscs stained with neutral red (Elliott and Tang 2009; Zetsche and Meysman 2012), however precise patterns of staining were not as predictable as were seen here for *Hexagenia* sp. Based on the high rates of false positives seen in copepods and amphipods, and high degree of variability observed in insect larvae, we recommend FDA or CMFDA+FDA be used with caution on samples containing such assemblages.

Precise reasons as to why the vital stains would continue to stain zooplankton and macro-invertebrates several days following death remains unknown. However it is possible that the presence of a carapace or exoskeleton in such organisms is related to the occurrence of false positives, as could be evidenced by the differences in the patterns and appearance of staining between live and dead *C. riparius* and *Hexagenia* sp., and lower rates of false positives amongst soft bodied plankton and invertebrates. Future investigations into the physiological reactions of zooplankton and macroinvertebrates with fluorescent stains after death could potentially aid in the search for appropriate viability assessment techniques for these groups of organisms.

Our laboratory testing indicated that the two vital staining methods appear to be appropriate for use with freshwater phytoplankton. Reavie et al. (2010) and Steinberg et al. (2011) also recently investigated the utility of fluorescent vital stains with phytoplankton communities. While Reavie et al. (2010) conclude that FDA alone is useful

for freshwater phytoplankton of Lake Superior, Steinberg et al. (2011) indicate the need for the combined staining method with FDA+CMFDA for use with marine phytoplankton taxa. Our analysis of mixed phytoplankton assemblages show no significant differences in accuracy rates between the two staining methods for freshwater taxa and supports the findings of Reavie et al. (2010), concluding that FDA alone will provide accurate and consistent viability results in freshwater phytoplankton communities. Furthermore, the lack of significant difference observed between HH and BW phytoplankton is an indication of the wide applicability of FDA/FDA+CMFDA for use with phytoplankton, as it seems that the utility of these stains can be applied across a range of locations and sample types. Nonetheless, we suggest that an initial round of testing of any stain be employed prior to use in a new region.

Our results indicate that an overestimation of viable plankton density is likely to occur through the use of traditional preservation methods alone when sampling ballast water. Traditional methods for analysis of ballast water samples consider the degradation status of individuals as a means of determining viability at time of collection, however, organisms recently killed by treatment or other means may not exhibit noticeable decomposition prior to collection. Tang et al. (2006) and Bickel et al. (2009) indicate that abundances of zooplankton carcasses in natural samples may be 29% and between 6% and 8% for marine and freshwater environments, respectively. The abundances of freshwater zooplankton carcasses found by Bickel et al. (2009) are similar to abundances found in Hamilton Harbour samples reported here (5%), as determined by the vital stains. However ballast water samples appear to have elevated abundances of dead zooplankton (30%), relative to harbour communities, for reasons possibly including but not limited to harsh environments of ballast tanks and long travel times between source and recipient ports. Therefore assessments of plankton communities in ballast tanks should include viability testing to determine compliance with discharge standards, as traditional methods will likely overestimate density of viable organisms.

Concluding remarks

Determining compliance with impending IMO standards for ships discharging ballast water will require precise knowledge of viable plankton densities present in ballast water to be discharged (IMO 2004), yet, traditional assessments of plankton present in ballast water do not take into account viability of organisms. Our study confirmed the findings of Reavie et al. (2010) that vital stains could be a useful tool for testing efficacy of ballast water treatment systems, particularly with phytoplankton. However, our findings suggest that vital stains FDA and CMFDA are not suitable as viability assessment methods for mixed assemblages of freshwater or marine zooplankton samples, particularly for those samples containing large crustaceans such as amphipods or copepods, as gross overestimates of live organisms are likely to occur due to the high occurrences of fluorescing dead organisms. This finding may prove problematic for the use of ballast water test kits in determining compliance with new IMO-D2 standards. Ballast water test kits are designed to provide a rapid on-board assessment of ballast water compliance, often measuring the bulk FDA fluorescence in a small subsample of ballast water to determine the presence of viable organisms. An assumption with using such kits would be that any type of error associated with the stain would be negligible. Additionally, our findings indicate that traditional methods of assessing plankton in ballast water may overestimate the true viability status of communities. The vital stains are efficient at accurately determining viability status of phytoplankton, many types of rotifers, soft-bodied aquatic worms, and some cladocerans from Lake Ontario, the St. Lawrence River, and ships which have undertaken mid-ocean exchange. These results therefore increase the confidence of using FDA and CMFDA across a variety of kill methods and illustrate the range of applicability of these vital stains with natural freshwater assemblages.

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