2017

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Journal of Sea Research xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Journal of Sea Research

journal homepage: www.elsevier.com/locate/seares

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ARTICLE INFO

Keywords:
Invasive species
Protists
Microorganisms
Water quality
Ballast water

ABSTRACT

Living organisms ≥ 10 μm and < 50 μm in ballast water discharged from ships are typically collected by filtering samples through a monofilament mesh net with pore openings sized to retain organisms ≥ 10 μm. This (or any) filtering method does not result in perfect size fractionation, and it can induce stress, mortality, and loss of organisms that, in turn, may underestimate the concentration of organisms within samples. To address this loss, the retention efficiency (*RE*) was determined for six filtration approaches using laboratory cultures of microalgae and ambient marine organisms. The approaches employed a membrane filter or mesh nettings of different compositions (nylon, stainless steel, polyester, and polycarbonate), nominal pore sizes (5, 7, and 10 μm), and filtering sequences (e.g., pre-filtering water through a coarse filter). Additionally, in trials with polycarbonate track etched (PCTE) membrane filters, water was amended with particulate material to increase turbidity. Organisms ≥ 10 μm were counted in the material retained on the filter (the filtrand), the material passing through the filter (the filtrate), and the whole water (i.e., unfiltered water). In addition, variable fluorescence fluorometry was used to gauge the relative photochemical yield of phytoplankton—a proximal measurement of the physiological status of phytoplankton—in the size fractions. Further, the mesh types and filters were examined using scanning electron microscopy, which showed irregular openings. The *RE* of cultured organisms—calculated as the concentration in the filtrand relative to combined concentration in the filtrand and the filtrate—was high for all filtration approaches when laboratory cultures were assessed (> 93%), but *RE* ranged from 66 to 98% when mixed assemblages of ambient organisms were evaluated. Although PCTE membrane filters had the highest *RE* (98%), it was not significantly higher than the efficiencies of the 7-μm polyester, Double 7-μm polyester, and Dual 35-μm and 7-μm polyester approaches, but it was significantly higher than the 5-μm nylon and 5-μm stainless steel techniques. This result suggests that PCTE membrane filters perform comparably to 7-μm polyester meshes, so that any of these approaches could be used for concentrating organisms. However, the potential for handling loss is inherently lower for one rinsing step rather than two. Therefore, it is recommended that, either PCTE filters or 7-μm polyester mesh could be used to concentrate organisms ≥ 10 μm and < 50 μm. In trials conducted using a 10-μm PCTE filters with water amended to increase the particulate concentration, no significant difference in *RE* of ambient organisms was found compared to unamended water. Finally, photochemical yield did not vary significantly between organisms in the filtrand or filtrate, regardless of the filtration approach used.

1. Introduction

Filtration of organisms is integral in biological oceanography, specifically for collecting and quantifying organisms for purposes including, but not limited to, elucidating population dynamics, conducting biomass estimations, determining primary production, and quantifying aquatic chemical composition. Fractionation using filtration is often required to separate components by size for measuring feeding rates (Miller and Wheeler, 2012) and separating suspended and particulate matter (Wright and Colling, 2013). Another important use for filtration is for fractionation of various sized organisms in ballast water discharged from ships, as national and international standards

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http://dx.doi.org/10.1016/j.seares.2017.06.005
Received 14 October 2016; Received in revised form 31 May 2017; Accepted 9 June 2017

Table 1
Attributes of cultured organisms used in experiments. Culture numbers were designated by the National Center for Marine Algae and Microbiota (NCMA, formerly CCMP); cell dimensions and colony descriptions were reported by NCMA.

Organism	Culture number	Cell dimensions (μm; length × width)		Morphology
		Minimum	Maximum	
Prorocentrum donghaiense (Dinoflagellate)	CCMP3122	12 × 10	16 × 14	Unicellular
Prorocentrum micans (Dinoflagellate)	CCMP2794	28 × 14	48 × 30	Unicellular
Tetraselmis marina (Flagellate)	CCMP898	8 × 9	9 × 15	Unicellular
Melosira octogona (Diatom)	CCMP483	16 × 14	24 × 26	Chain forming
Skeletonema tropicum (Diatom)	CCMP788	5 × 8	10 × 10	Chain forming

limit the concentrations of living organisms in ballast water discharged from ships. These limits are based upon organisms' size: < 10 cells mL^{-1} for organisms ≥ 10 and < 50 μm (nominally protists) and < 10 cells m^{-3} or organisms ≥ 50 μm (nominally zooplankton) (IMO, 2004; USCG, 2012) (the three bacterial indicator organisms and pathogens have varying allowable concentrations).

It is expected that most ships will use a ballast water management system (BWMS) to meet these limits. Quantifying the concentration of living organisms is central to determine the efficacy of BWMS in both land-based and shipboard testing (i.e., verification testing), and a measure of organisms' concentrations will likely be used to verify that ships comply with the discharge limits. For shipboard compliance monitoring, organism concentrations in the ≥ 10 and < 50 μm size class are ideal targets for analysis, primarily because the sample volume needed is small (i.e., liters) relative to the ≥ 50 μm size class, which would require large volumes (i.e., on the order of 1 m^3) for analysis. Nevertheless, sparse organism concentrations (here, 10 mL^{-1}) are difficult to resolve in the small sample volumes that will likely be assessed. In the tools commercially available to measure compliance, typically, volumes ≤ 5 mL are examined, and they may require sparsely dispersed organisms to be concentrated prior to analysis.

For the ≥ 10 and < 50 μm size class, organisms are generally concentrated by filtering water through monofilament mesh with pore openings sized to retain organisms ≥ 10 μm. This (or any) filtering method does not result in perfect size fractionation (e.g., Wainwright et al., 2002; Wang et al., 2007). Organisms, especially pliable, soft-bodied protists, can pass through the mesh pores that are only slightly smaller than the organism's physical dimensions (Carrias et al., 2001; Stockner et al., 1990). It has also been reported that varying size and morphology of copepods effected the *RE* using Continuous Plankton Recorder silk (Hays, 1994). In addition, the concentration process—including filtering, collecting organisms on the mesh in a thin layer of water, and rinsing them into a collection vessel—can induce stress, mortality, and loss of organisms. Further, organisms may become lodged within the pore spaces of the mesh net and become unable to be removed in the rinsing process. When passing organisms through stacked or sequential filters, it was reported that organisms were retained in fractions both smaller and larger than the organism's size (Pitta and Karakassis, 2005). Concerns of organism loss when using size fractionation by filtration to estimate microbial diversity have also been reported (e.g., Padilla et al., 2015). These factors contribute to the mortality and loss of organisms that, in turn, may underestimate the concentration of organisms within samples. Determining the extent of organism loss, or inefficiency of filtration, is necessary for both selecting optimal materials and protocols and, potentially, calculating the systematic error of an analytical method.

Because size-selective filtration is conducted prior to sample analysis, identifying any materials and methods that minimize organism loss is paramount, particularly with respect to the stringent ballast water discharge limits, where the loss of a small percentage of organisms may represent the difference between complying and not complying with regulations. The goal of this study was to investigate six filtration approaches used to concentrate organisms ≥ 10 to < 50 μm to determine retention efficiency (*RE*)—the total concentration of organisms retained on the filter or mesh compared to the total concentration of organisms present—to enumerate organisms as prescribed in the Environmental Technology Verification (ETV) Program Generic Protocol for the Verification of Ballast Water Treatment Technology (ETV Protocol, U.S. EPA, 2010). To address the loss of organisms, the *RE* was examined using laboratory cultures of microalgae and ambient assemblages of marine organisms ≥ 10 and < 50 μm. Dissolved and particulate matter was added to test the hypothesis that higher concentrations of suspended material could affect the *RE* of suspended organisms; the material was added in concentrations specified in the ETV Protocol (U.S. EPA, 2010), which prescribes conditions to "challenge" ballast water management systems during verification testing. In addition, variable fluorescence fluorometry was used to evaluate the physiological status of organisms (by measuring photochemical yield) following filtration by the various approaches. Scanning electron microscopy (SEM) was also performed to evaluate the geometry of mesh and membrane filters.

2. Materials and methods

2.1. Organisms

Cultured organisms used in experiments were selected for size, taxonomic diversity, and chain-forming capability (Table 1); they were visually distinguishable from each other and were easily identified at the magnification used for counting (100 ×). Cultures, which were obtained from The National Center for Marine Algae and Microbiota (NCMA, Bigelow Laboratory for Ocean Sciences, East Boothbay, ME) were maintained in 72 μmol quanta $m^{-2} s^{-1}$ lighting at 23 °C under a 16:8 h light:dark cycle, and they were transferred to fresh media every two weeks to sustain them in exponential growth phase. At the beginning of each experiment with cultured organisms, aliquots of different monocultures were combined to allow for concurrent treatment of a mixed community of organisms within the test. For experiments using a mix of unicellular and chain-forming, cultured organisms, a mixture was prepared with four microalgae: *Prorocentrum micans*, *Prorocentrum donghaiense*, *Melosira octagona*, and *Skeletonema tropicum* (Table 1). First, individual cultures were sampled to determine their initial concentrations. Next, volumes of cultures were added to 1 L of FSW so that each culture's concentration was 250 mL^{-1}, so the final concentration of microalgae—which included all four organisms—was approximately 1000 mL^{-1}. For experiments using only unicellular cultured organisms, a concentration of 10 cells mL^{-1} of each of two organisms (*T. marina* and *P. micans*) in 1 L of FSW was used, for a final concentration of 20 cells mL^{-1}.

In separate experiments, natural assemblages of ambient organisms in seawater, collected at the U.S. Naval Research Laboratory in Key West, FL (24.575° N, 81.7944° W), were used. Water was collected using a diaphragm pump, which was attached to a flexible tube leading to a polyvinyl chloride (PVC) pipe with holes to collect water along its length (0.5 m). The PVC pipe was submerged ~0.5 m below the water surface. Twenty-liter volumes were dispensed into individual 20-L

Table 2
Filtration approaches used in experiments. ETV = Environmental Technology Verification Program Protocol, N = nylon, SS = stainless steel, PCTE = polycarbonate track-etched, and PE = polyester.

Filtration approach	Pre-filter	Secondary filter	Open area (pre-filter, secondary filter [if used]) (%)	Target organism size range	Tested organisms	ETV challenge water (if used)
5-μm N	–	5-μm N	1	≥ 10 μm	Ambient	No
5-μm SS	–	5-μm SS	3	≥ 10 μm	Ambient	No
7-μm PE	–	7-μm PE	2	≥ 10 μm	Ambient	No
					Cultured	No
Double 7-μm PE	7-μm PE	7-μm PE	11, 11	≥ 10 μm	Ambient	No
Dual 35 and 7-μm PE	35-μm PE	7-μm PE	11, 2	≥ 10 μm and < 50 μm	Ambient	No
					Cultured	No
10-μm PCTE	–	10-μm PCTE	8	≥ 10 μm	Ambient	Yes
					Cultured	Yes

carboys, one for each filtration method being tested and one for a whole water (control) sample. Collected water was held at room temperature (~21 °C) for the duration of each experiment, which was < 6 h.

2.2. Filtration approaches

Filtration experiments were performed using custom sieves composed of different mesh types: woven nylon (Small Parts, Inc., Logansport, IN), stainless steel (Utah Biodiesel Supply, Clinton, UT), or woven polyester (Saati, Fountain Inn, SC). Nylon and polyester mesh netting was rated by the nominal pore size (determined by the manufacturer as the retention rate using microbeads rather than the physical size of the openings) (Saati, Fountain Inn, SC), and the percent open area (Table 2), that is, the total area of the mesh openings not occupied by filament, thus potentially allowing for the sample to pass. The percent open area was either provided by the manufacturer or calculated as described by SaatiTech, Somers, NY. Sieves were constructed by placing a sheet of mesh (pulled taut) between two sections of 20-cm diameter, PVC pipe. Polyurethane-based sealant (3M Marine Sealant, 5200, Fast Cure; 3 M, St. Paul, MN) held both the top and bottom PVC sections together (with the mesh sandwiched between the sections). Sealant was also applied around the inner border of the sieve to minimize open areas at the interface of the sieve and the PVC pipe. The sealant was allowed to cure for > 24 h prior to use. Testing was also performed using 47-mm diameter polycarbonate track-etched (PCTE) membrane filters (Sterlitech Corporation, Kent, WA). A standard filtration apparatus was used in experiments with PCTE filters.

Six filtration approaches were examined using monofilament mesh and PCTE filters (Table 2). In addition, the effectiveness of multi-stage filtration approaches was evaluated. They were used to remove larger organisms (pre-filtered through a 35-μm mesh) or to assure the complete capture of organisms (reprocessing the filtered water [i.e., the filtrate] through a second stage of filtration). Filtration approaches were selected following the results from previous trials. The filters were selected based on the trials measuring the *RE* of woven mesh, which were succeeded by trials using metal and track etched filtration approaches. Each experimental trial for a given filtration approach was conducted on a separate day.

2.3. Experimental design

The cultured organism mixture was independently processed by two treatment approaches—7-μm PE and Double 7-μm PE—and a control, using 0.5 L of sample for each approach. For the treatment approach, the 0.5 L of the cultured organism mixture was mixed by gently inverting it 3 times, and it was then manually poured through the sieve(s) so that the height of the water above the mesh was at least ~0.5 cm and at most 10 cm. The organisms captured on the mesh (i.e., the filtrand) were then rinsed from the mesh into a beaker until the target volume (~75 mL) was reached. Next, the filtrand was measured gravimetrically on a laboratory balance to accurately calculate the volume of the sample: mass was converted to volume using standard equations for seawater state based upon the measured temperature and salinity (Fofonoff and Millard, 1983). Next, the water that passed through the mesh (the filtrate) was collected in a beaker, and then the sample was concentrated on a 0.22-μm membrane filter (Whatman Inc., Clifton, NJ) to a target volume of ~75 mL. After concentrating, the filtrate sample was measured gravimetrically. For the control, the water sample was not processed by filtration. Rather, three subsamples (each 5 mL) were taken from 0.5 L of the cultured organism mixture. These samples were used to compare filtered (treated) samples to non-filtered (control) samples. From each concentrated filtrand and filtrate sample, three 1-mL subsamples were removed for epifluorescence microscopy (and organisms enumerated using a Sedgewick Rafter counting chamber), and from control samples, three 5-mL subsamples were removed for microscope counts using a Bogorov chamber. The Bogorov chambers—acrylic plates with a single, winding chamber—were used for the control (non-filtered) samples because they allow a greater volume to be analyzed in a single sample than a Sedgewick Rafter counting chamber, which holds a 1-mL volume.

Trials using ambient organisms were conducted in a similar fashion as those with cultured organisms. Twenty liters of well-mixed (by gently inverting 3 times) ambient seawater was processed following one of the six filtration approaches described in Table 2. After filtration of the 20-L sample, the filtrand was collected from the mesh to a target volume of ~75 mL. The filtrand was then measured gravimetrically as described above. The filtrate was collected in a 20-L bucket and was well mixed by gently pouring it into a second bucket and then back into the first bucket 3 times. After mixing, a 2-L sample of the filtrate was poured into a beaker and then concentrated on a 0.22-μm membrane filter until the target value of ~75 mL was reached. For control samples, the water sample was not processed by filtration. Rather, three 5-mL subsamples were taken from the 20-L, well-mixed, whole water sample. These whole water samples were used to compare filtered (treated) samples to non-filtered whole water samples. From each concentrated filtrand and filtrate sample, 3 1-mL subsamples were removed for epifluorescence microscopy, and from control samples, 3 5-mL subsamples were removed for microscope counts using a Bogorov chamber.

After determining the *RE* of monofilament mesh, 10-μm PCTE membrane filters were tested to examine the *RE* of membranes with uniformly track-etched pores. To determine the effect of dissolved and suspended particles on the *RE* of 10-μm PCTE membrane filters, separate trials were conducted with cultured and ambient organisms. These experiments were conducted with cultured organisms with water amended following the ETV Protocol, and ambient organisms with and without ETV additives. Water was amended by adding materials to 1 L of 0.22-μm filtered sea water (FSW) to achieve final concentrations of 6 mg L^{-1} dissolved organic carbon (DOC; Nestlé [decaffeinated iced tea], Glendale, CA), 4 mg L^{-1} particulate organic carbon (POC; Mesa Verde Resources, Placitas, NM), 20 mg L^{-1} mineral matter (MM; Powder Technology Inc.), for a total of 24 mg L^{-1} total suspended

solids (TSS = POC + MM). Experiments with cultured organisms used 1 L of FSW amended with the ETV additives, and a concentration of 10 cells mL^{-1} of each of two organisms (*T. marina* and *P. micans*), representing the smallest and largest of the ≥ 10 to < 50 μm size class, respectively. From the 1 L sample mixture, three 50-mL well-mixed subsamples were filtered by gravity, and the filtrand was raised to a volume of 10 mL (the volume was verified as described above) using FSW. The remaining filtrate (50 mL) was concentrated to 10 mL on a 0.22-μm membrane filter.

Experiments using ambient organisms were conducted with and without ETV-amended water. Ambient seawater (20 L) was collected using a diaphragm pump, and from that water, a 1-L sample was removed for each amended and unamended subsample. From each of these 1 L samples, 50 mL was filtered through a 10-μm PCTE membrane filter. The filtrand was rinsed from the filter until reaching the target volume of (~10 mL). To concentrate the filtrate, the filtrate was filtered through a 0.22-μm filter, and the material retained on the filter was rinsed and brought up to a target volume of ~10 mL. The volumes and organism concentrations used for the amended water filtration approach were chosen based on guidelines for the ETV Protocol (U.S. EPA, 2010).

2.4. Sample analyses

Samples were labelled with fluorescein diacetate (FDA) and chloromethylfluorescein diacetate (CMFDA). Specifically, FDA (5 μL) and CMFDA (10 μL) were added to a 985-μL sample to achieve a final concentration of 5 μM and 2.5 μM, respectively (U.S. EPA, 2010). The sample was incubated in the dark for 10 min prior to analysis. After incubation, fluorescent, polystyrene microbeads (8 and 50 μm in diameter; Chromosphere, Fisher Scientific, Pittsburgh, PA) were added to each subsample as a size reference. The labelled sample with microbeads was then transferred to a gridded Sedgewick Rafter counting chamber and counted by epifluorescence microscopy. Five to seven randomly chosen rows were manually scanned for organisms in the ≥ 10 to < 50 μm size class (size was determined by comparing organisms to microbeads) and were counted as living if they were fluorescent, moving, or both (Steinberg et al., 2011; U.S. EPA, 2010). For recordkeeping, the samples containing a cultured organism mixture were tallied and grouped by species. The samples of ambient organisms were also tallied and categorized into broad taxonomic groups (e.g., copepod nauplii, ciliates, flagellates) and, when possible, into species- or genus-specific groups (e.g., *Prorocentrum lima*, *Navicula* sp.).

In the ambient trials, due to the sparse concentration of organisms in the samples, larger sample volumes (5 mL) were analyzed. The samples were labelled with FDA and CMFDA and loaded into a Bogorov counting chamber. Living organisms were then quantified using an epifluorescence stereomicroscope and categorized in the broad and species-specific taxonomic groups, as described above.

Measurements of variable fluorescence were taken for the filtrand and filtrate samples using a pulse amplitude modulated (PAM) fluorometer (WaterPAM™, Walz, GmbH, Effeltrich, Germany). This analytical approach measures photochemical yield or the ratio of variable (F_V) to maximum (F_M) fluorescence. Photochemical yield (hereafter, F_V/F_M) is a relative measure of the physiological status of the community of microalgae within a sample and—when fluorescence is within detectable ranges—is independent of organism concentration. Each 3-mL subsample was read by the instrument three times (with 10 s between readings).

Photomicrographs were collected for all mesh types and the membrane filter using a tabletop SEM (Hitachi TM3000; Hitachi HTA, Schaumburg, IL) at 1500 × magnification. Mesh swatches (~1 cm diameter) were cut from the bolting, mounted on adhesive tape, and placed in the vacuum chamber of the SEM. Images were collected from random fields of view near the center of the sample (avoiding the fray around the edges).

2.5. Data analysis

The population of living organisms ≥ 10 and < 50 μm (P, organisms L^{-1}) was calculated using the following formula (Eq. (1)):

$$P = \frac{ICD}{AS} \quad (1)$$

where I was the count of organisms, C was the volume of concentrated sample (20 or 75 mL), D was the dilution factor (1.015 mL^{-1}) resulting from adding fluorophores to the sample aliquot (A, 1 mL), and S was the total sample volume (e.g., 2 or 20 L). The concentration for each sample was calculated as the mean concentration from the analysis of three subsamples. These mean values and their measurements of error, were propagated throughout the calculations. Because the concentrations in the filtrand and filtrate may vary by orders of magnitude, all organism concentrations were log-transformed (Sokal and Rohlf, 2011). Log-transformation occurred prior to calculating retention efficiency (*RE*, %), which was based upon the concentrations in the filtrand ($P_{Filtrand}$) compared to concentrations in whole water (P_{Whole}), as determined via microscope counts of 5-mL Bogorov chambers (Eq. (2)):

$$RE = \frac{\log[P_{Filtrand}]}{\log[P_{Whole}]} \quad (2)$$

In addition to the approach above (Eq. (2), "whole water" approach), an alternative approach ("combined filtrand and filtrate" approach) was used to calculate *RE*. The alternative approach was used because the depth of the Bogorov chamber (4–5 mm depth) was greater than in the Sedgewick Rafter slide (1 mm depth), and therefore, analysts needed to scan the entire depth of the water column and focus on organisms at different strata. The method, which is different from the Sedgewick Rafter method (where the entire water column is visible at one focal depth), may have led to missing organisms or "double counting" highly motile organisms and, therefore, high variability among population estimates of living organisms. Thus, in the "combined filtrand and filtrate" approach, the concentrations of organisms in the filtrand were normalized to the sum of concentrations in the filtrand and the filtrate, with all measurements collected using Sedgewick Rafter counting chambers ($P_{Filtrate}$; Eq. (3)):

$$RE = \frac{\log[P_{Filtrand}]}{(\log[P_{Filtrand}] + \log[P_{Filtrate}])} \quad (3)$$

Independent, replicate trials were conducted for each treatment: n = 6 for 7-μm PE, and Dual 35 and 7-μm PE, and n = 3 for all others. The calculated *RE* values for each treatment were checked for normality using a Shapiro-Wilk test prior to performing an analysis of variance (ANOVA, $\alpha = 0.5$) to detect significant differences among groups of organism or filtration approach. Values for subsamples within each trial were averaged and used in calculations among trials. When significant differences were found, a pair-wise, post-hoc test (Holm-Sidak method, $\alpha = 0.05$) was used to determine significant differences between filtration approaches. An ANOVA was also used to detect significant differences in F_V/F_M among the different treatments. All statistical analyses were performed using SigmaPlot (V12.5; Systat Software, Inc.; San Jose, CA).

3. Results

The retention of living, cultured organisms, using 7-μm PE mesh, was high when calculating *RE* using both the whole water (range: 93–102%; Fig. 1A) and the combined filtrand and filtrate approaches (range: 96–100%; Fig. 1B). In both cases, the dinoflagellate *P. donghaiense* had the lowest retention of all the organisms tested. The unicellular dinoflagellate *P. micans* and the two chain-forming diatoms, *M. octogona* and *S. tropicum*, were retained with high efficiency (> 99%). Efficiencies above 100% are an artifact of variance between subsamples.

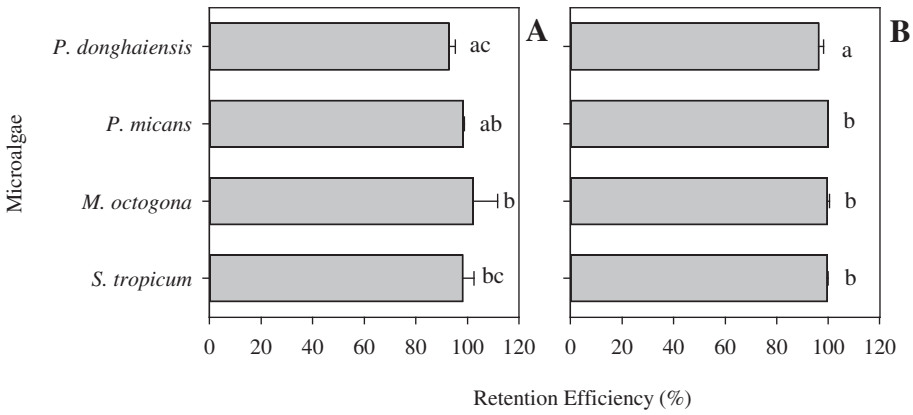

Fig. 1. Mean percent retention efficiency (*RE*) of cultured microalgae collected on 7-µm polyester mesh. Calculations of *RE* were performed by comparing the organism concentration in the filtrand to: A. the organism concentration in the whole water (counted using a Bogorov chamber as calculated with Eq. (2), the "whole water" approach) or B. the organism concentration in the combined filtrand and filtrate (counted using a Sedgewick Rafter slide as calculated with Eq. (3), the "combined filtrand and filtrate" approach). Different lower-case letters indicate significant differences, e.g., all bars marked with only "a" were not significantly different from each other but were significantly different from bars marked "b"; a bar marked "ab" was not significantly different from bars marked either "a" or "b." Error bars represent the mean and one standard deviation of independent trials (n = 6).

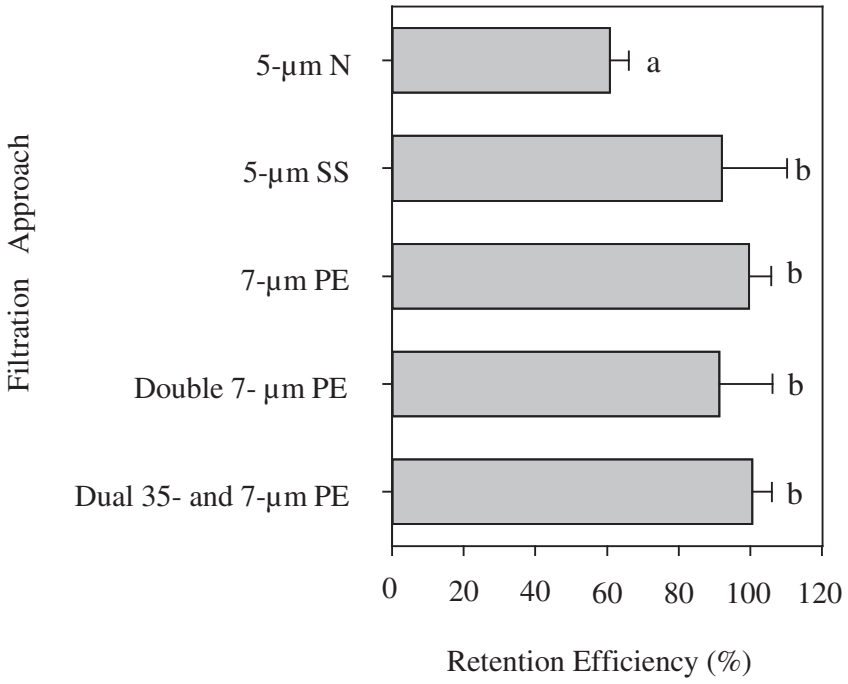

Fig. 2. Retention efficiency (*RE*) of ambient organisms using different filtration approaches calculated by comparing the organism concentration in the filtrand to the organism concentration in whole water (i.e., the "whole water" approach). Different lower-case letters indicate significant differences. Bars show the mean and standard deviation of independent trials (n = 3 to 6). N = nylon, SS = stainless steel, and PE = polyester.

The *RE* of living, ambient organisms, using the whole water approach, was, counterintuitively, significantly lower for 5-µm nylon (60 ± 5%) than the other mesh types, which ranged from 92 to 100% (Fig. 2). When using the combined filtrand and filtrate approach, 5-µm nylon and 5-µm stainless steel yielded *RE* values significantly lower (66 ± 2% and 80 ± 10%, respectively) than the other filtration approaches (Fig. 3). The *RE* for other mesh types (excluding 5-µm nylon, and 5-µm stainless steel) ranged from 90 to 98% (Fig. 3). Of the different filtration approaches, 10-µm PCTE membrane filters had the highest *RE* at 98% of the combined filtrate and filtrand concentrations (Fig. 3).

Trials conducted using 10-µm PCTE filters with ETV challenge water amendments revealed no significant differences in *RE* when compared to non-amended water for laboratory cultured or ambient organisms ($p = 0.865$ and $p = 0.421$, respectively; data not shown). Retention efficiencies were > 97% for all ETV-amended water filtration approaches using 10-µm PCTE membrane filters (data not shown).

There was no significant difference in the mean F_V/F_M between organisms in the filtrand and organisms in the filtrate in any of the filtration approaches; all values ranged between 474 and 600 ($p < 0.05$; data not shown). This result suggests that the physiological status of the phytoplankton community did not vary significantly among filtration approaches. Likewise, there were no significant differences between F_V/F_M in the filtrand among the approaches ($p > 0.05$), nor were there significant differences in F_V/F_M in the filtrates among filtration approaches ($p > 0.05$). Because organism concentrations were low in samples amended according to the ETV Protocol, variable fluorescence measurements were below detection limits, and therefore, they were not included in this analysis.

Scanning electron microscopy revealed the potential causes of organism loss: irregular geometries of openings in tightly-woven mesh and overlapping pores of membrane filters. Imaging the monofilament mesh showed the pore openings of the nylon, stainless steel, and polyester meshes were 3-dimensional, asymmetrical, constrictive pathways, in contrast to 2-dimensional "squares" that are more indicative of mesh with larger (e.g., > 50 µm) nominal dimensions (Fig. 4). Imaging the PCTE membrane filters showed circular pores, although interconnections among of some pores created openings > 10 µm (Fig. 4E).

4. Discussion

Using mesh netting to concentrate organisms has a long history in aquatic biology, particularly for collecting zooplankton (e.g., as reviewed by Wiebe and Benfield, 2003). For organisms ≥ 10 and < 50 µm, the challenges of physically separating suspended organisms vary

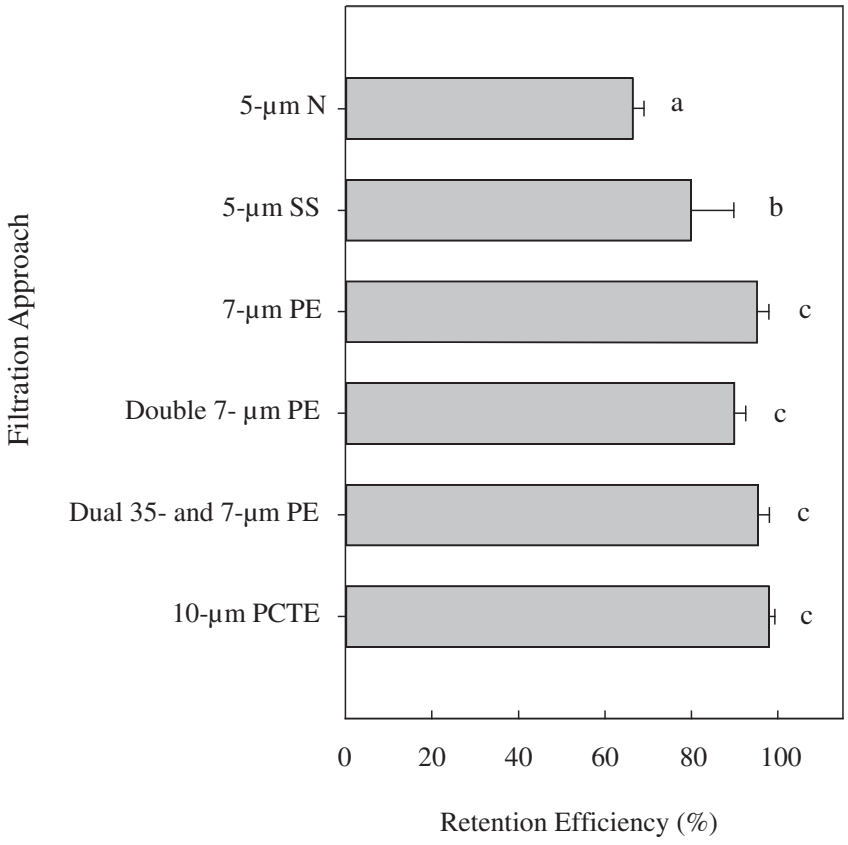

Fig. 3. Retention efficiency of ambient organisms using different filtration approaches calculated by comparing the organism concentration in the filtrand to the organism concentration in the combined filtrand and filtrate (i.e., the "combined filtrand and filtrate" approach). Different lower-case letters indicate significant differences. Error bars show one standard deviation (n = 3 to 6). N = nylon, SS = stainless steel, PCTE = polycarbonate track-etched, and PE = polyester.

with the size and structure of the target organisms. Variable dimensions of chain-forming phytoplankton, as well as factors independent of the organism, such as mesh size and suspended particulate matter, may increase the measurement uncertainty (Raymont, 1980). On a single-cell (individual) level, the likelihood of the cell passing through the mesh netting is affected by cell rigidity and how close the body dimensions are to the mesh size. Protists, in general, demonstrate an array of cellular plasticity, ranging from amoebae that alter their cytoskeletons to deform their plasma membranes to diatoms that have rigid silica frustules that would seemingly maintain their structure under shear stress. Further, protists can escape through pores smaller than their body dimensions: nanoflagellates with minimum dimensions > 2 µm have been found in water filtered through pores as small as 0.4 µm (Cynar et al., 1985).

Although not the focus of this study, it is worthy to note that filtration rate may influence *RE*. This effect has been demonstrated with organism collection in relation to plankton tow speed (e.g., Hays, 1994; Colton et al., 1980) and fish egg collection efficiency as a function of the ratio of filtration to the mouth area of a net (Favero et al., 2015). For this study, the filtered volumes (20 L) of ambient organisms filtered through monofilament mesh are representative of the sample volumes that would be manageable as ballast water was collected during shipboard operations to determine a vessel's compliance with the discharge standard. This volume, while large enough to provide a representative

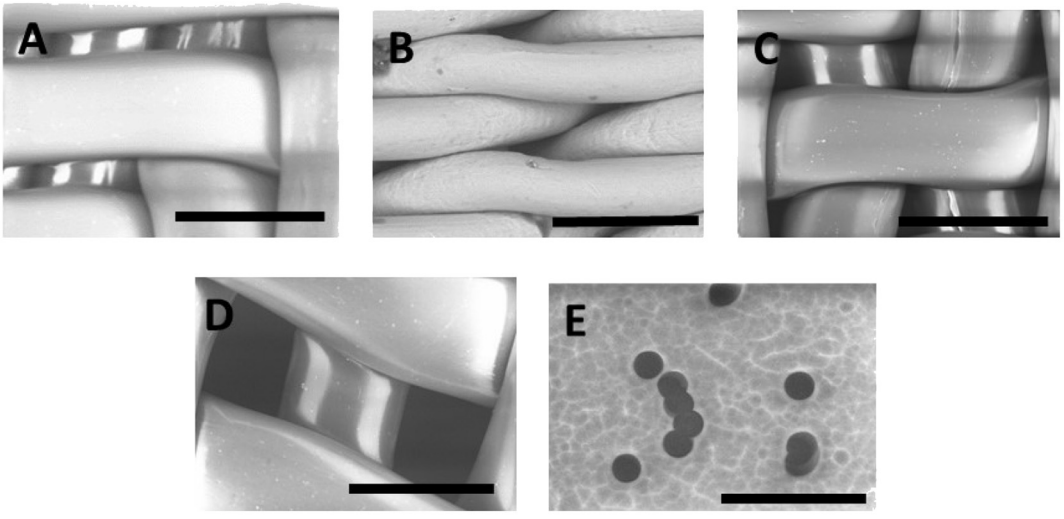

Fig. 4. Scanning electron microscope (SEM) photomicrographs of mesh: A. 5-µm N B. 5-SS C. 7-µm PE, D. 35-µm PE, and E. 10-µm PCTE. Scale bars are 50 µm. N = nylon, SS = stainless steel, PCTE = polycarbonate track-etched, and PE = polyester.

concentration of organisms within a ballast tank, was small enough to allow for controlled filtration rates, which decrease mesh clogging, which could lead to unintentional cell death.

The *RE* for 7-μm polyester mesh was high for cultured organisms: > 99% for both chain-forming diatoms as well as one dinoflagellate species (*P. micans*). The dinoflagellate *P. donghaiense*, which is smaller than its congener *P. micans* (the species have maximum sizes of 16 × 14 μm and 48 × 30 μm, respectively), was relatively poorly retained (93%). This result empirically shows unicellular organisms near the 10-μm size threshold can have a lower *RE* than chain-forming organisms.

Colonial and chain-forming phytoplankton, on the other hand, can remain on filters during pre-filtration steps ostensibly used to remove zooplankton (Bidigare et al., 2005). These types of organisms (e.g., diatoms and raft-forming cyanobacteria) may be caught on filters with pore sizes larger than their individual cell size, which can fall below 1 μm in diameter. Aggregated cells, when abundant, may interfere with the detection of single-celled organisms by blocking the view of cells within the desired size range, which may also clog filters, causing increased sheer pressure. In addition, another concern associated with chain-forming phytoplankton is the integrity of the chains themselves. When partitioning phytoplankton with various sizes of screens and filters, no colony breakage was reported for 7 of 8 species evaluated (Runge and Ohman, 1982). The relative solidity of the chain-forming organisms, i.e., their tendency to remain intact as a chain, indicates they will be collected and concentrated with high efficiency; however, it complicates the analysis when using manual microscopy, especially when chain-forming organisms include individuals with sizes above and below the 10-μm threshold.

Biological composition of ballast water from various locations may differ in chain-forming species concentrations, which may lead to inconsistencies in organism capture and analysis. Several of the filtration approaches showed comparable *RE* and the highest *RE* for ambient organisms were > 90% for the whole water approach as well as the combined filtrand and filtrate approach. This *RE*, however, would likely vary among locations with different assemblages of ambient organisms. Effectively, the *RE* of organisms (both ambient and cultured) was similar to the inert, latex particles used by manufacturers to test the efficiency of mesh netting; organisms much greater than the nominal pore size are retained with nearly 100% efficiency, but *RE* decreases to ~90–95% for organisms (or microbeads) approaching the nominal pore size (Saati, Fountain Inn, SC). These inert, rigid particles, of course, will behave differently from pliable organisms (e.g., Carrias et al., 2001).

Two approaches used to calculate *RE*—whole water vs. the combined filtrand and filtrate approach—resulted in different outcomes. Analysis of organisms in whole water would be ideal to determine the total concentration of organisms in the filtrand and the filtrate combined, yet the sparse populations of organisms in the unprocessed whole water require large analysis volumes. Bogorov chambers, having a deep water column (~0.5 cm) were used for analysis of whole water samples. While the depth of the Bogorov chamber was scanned, it was possible that organisms could move in or out of the focal plane. While the two approaches showed similar trends, the whole water approach yielded broader (i.e., more variable) ranges of *RE*. Thus, to enumerate organisms and to calculate *RE*s in this size class, using the combined filtrand and filtrate approach is recommended when possible.

The comparison of different filtration approaches using both the whole water and combined filtrand and filtrate approaches revealed, unexpectedly, that the *RE* of the 5-μm nylon mesh was significantly lower than all other meshes. As the nominal opening for this mesh was the smallest, the retention was expected to be among the highest, but this was not the case. One explanation is that the mesh, which has a lower percentage of open area (2%, compared to 3% for 7-μm polyester mesh), potentially led to higher flow velocities and pressures through the smaller area, thus forcing organisms through the holes. In addition, due to the structure of the mesh (as determined by SEM), organisms embedded in the mesh may have been more difficult to remove via rinsing than when other, larger meshes were used. Considering the same pore size, but using a different material (stainless steel), the *RE* of stainless steel was significantly higher than the 5-μm nylon mesh's *RE*. The difference between these results may be attributed to the more rigid and consistent weave pattern and pore spaces of the stainless steel mesh compared to the more flexible nylon mesh. The *RE* of the 5-μm stainless steel mesh was similar to the other three filtration approaches using the whole water approach.

Comparisons between ETV-amended and non-amended water, using the combined filtrand and filtration approach, revealed high *RE* regardless of the type of organism tested (i.e., cultured or ambient organisms), indicating that the introduction of particulates and dissolved organic carbon, representing challenging water quality conditions, does not affect *RE* when using a 10-μm PCTE membrane filter. Because the concentrations of organisms—as well as particulate, dissolved, and mineral matter used here (which are within the middle to upper range of concentrations observed on coastal and estuarine waters; First et al., 2014)—were representative of those outlined within the ETV Protocol, these results can be considered comparable to possible outcomes in collected ships' ballast water samples.

Regarding filtration-induced stress on organisms, no physiological changes were evident when comparing the F_V/F_M microalgae measurements for all filtration configurations. Comparing the filtrands and filtrates of all treatments, all F_V/F_M comparisons revealed no significant differences—that is, there was no evidence that any of the processing methods resulted in a change in F_V/F_M. However, it should be noted that this metric only provides information on one biological system, the photosystem, and it only gauges the status of photoautotrophs, not heterotrophic organisms. It is important that sample processing—for the analysis of live organisms—not induce cell mortality. As photochemical yield is an indicator of photosystem integrity, cell damage–but necessarily cell loss–may be detectable by diminished F_V/F_M of the microalgal community. Although F_V/F_M measurements were not collected prior to filtration, measurements taken after each filtration treatment were within ranges typically observed for living organisms (e.g., Stehouwer et al., 2010). Although additional measurements were outside the scope of this study, measurements of F_V/F_M indicated that large-scale damage to microalgal cells did not occur in any of the filtration approaches.

Although PCTE membrane filters had the highest *RE* (98%), the efficiency was not significantly higher than efficiencies of the 7-μm polyester, Double 7-μm polyester, and Dual 35-μm and 7-μm polyester approaches, and it was significantly higher than 5-μm nylon and 5-μm stainless steel. Based on these conclusions, PCTE membrane filters perform comparably to 7-μm polyester meshes, so that any of these approaches could be used for concentrating organisms. Of the 7-μm polyester filtration approaches, the 7-μm mesh with no secondary filter would be a faster, less cumbersome approach than those requiring two stacked sieves. Also, the potential for handling loss is inherently lower for one rinsing step rather than two. Therefore, it is recommended that, either PCTE membrane filters or 7-μm polyester mesh could be used to concentrate organisms ≥ 10 μm and < 50 μm.

The examination of the PCTE membrane filters by SEM revealed that overlapping pores formed openings larger than the mean pore size. These asymmetrical pore openings have been reported to play a role in retention efficiency (Nayar and Chou, 2003) and could potentially allow for organisms larger than the selected size range to pass through the filter. This observation has been made previously to alert aquatic ecologists about “leaky” membrane filters and the potential problems they pose (Stockner et al., 1990). Based upon the results from this study, this warning should be expanded to include “leakage” through mesh netting. However, as demonstrated here, the loss of organisms can be minimized and, if necessary, estimated.

Acknowledgements

This work was supported by the U.S. Coast Guard (USCG) Office of Operating and Environmental Standards (CG-OES-3, contract HSCG23-14-X-MMS010, Task 5.1), although it does not represent official policy of the USCG or the U.S. Navy. We are grateful to Richard Everett and Regina Bergner (USCG) for their advice and programmatic guidance. This research was supported by Diane Lysogorski (Section Head, Naval Research Laboratory Code 6136 and Director, Corrosion and Marine Engineering, Key West, FL). The reviews of this paper by Edward Lemieux (Director, Center for Corrosion Science and Engineering, Code 6130), Warren Schultz (Acting Superintendent, Chemistry Division, U.S. Naval Research Laboratory), and two anonymous reviewers improved it—thank you.

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