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Validation of Additives used to Meet Challenge Water Criteria

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14. ABSTRACT

Procedures for land-based verification testing of Ballast Water Management Systems (BWMS) are referenced in the U.S. Coast Guard's final rule on ballast water discharge. The International Maritime Organization (IMO) has codified similar procedures. Both procedures define minimal concentrations of dissolved and particulate organic matter (DOM and POM, respectively) and mineral matter (MM) in test water to provide an appropriate challenge for the BWMS. This report examines the additives used to meet "challenge water" criteria, summarizing additives proposed or in use to supplement DOM, POM, and MM. Validation efforts have primarily examined the response of organisms directly to the additives. Critically, validations should demonstrate that additives reflect materials found in natural waters and verify that (relative to natural water constituents) additives do not impact the survivability of organisms during test incubations, change organism concentrations, or make organisms more susceptible or resistant to treatment. We also recommend that the secondary impacts on organisms, such as the stimulation of bacterial growth and respiration, be examined for new additives or mixtures. Periodic testing of stock material would assure the stability of the additives.

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1 INTRODUCTION

Procedures for land-based verification testing of Ballast Water Management Systems (BWMS) are described in the Environmental Technology Verification (ETV) Program Protocol (EPA 2010), which was incorporated by reference into the U.S. Coast Guard's final rule on ballast water discharge (U.S. Coast Guard 2012). Both the U.S. procedures and the guidelines published by the International Maritime Organization (IMO) set minimal characteristics for ballast water used in Type Approval (TA) testing to assure the test water provides an appropriate challenge for the BWMS. The U.S. and IMO set the same challenge water limits for living organisms ≥50 μm (>10^5 m^{-3}, including 5 species across 3 phyla) and living organisms ≥10 and <50 μm (10^3 mL^{-1}, including 5 species across 3 phyla). However, the U.S. and IMO differ in their limits for heterotrophic bacteria in challenge water (10^3 or 10^4 mL^{-1}, for the U.S. and IMO, respectively) and their requirements for dissolved and particulate material. In addition, IMO limits do not specify minimum values for mineral matter (MM), only total suspended solids (TSS). Finally, the U.S. has the same concentration limits regardless of water salinity, whereas the IMO limits differ based upon the salinity of the test water (Table 1).

Table 1. Challenge water limits for U.S. and International Maritime Organization (IMO) type approval testing. All values are in mg L^{-1}.

	Fresh (<1 PSU)		Brackish (10-20 PSU)		Marine (28-36 PSU)	
Challenge water component	**U.S.**	**IMO**	**U.S.**	**IMO**	**U.S.**	**IMO**
Dissolved Organic Carbon (DOC)	6	5	6	5	6	1
Particulate Organic Carbon (POC)	4	5	4	5	4	1
Mineral Matter (MM)	20	-	20	-	20	-
Total Suspended Solids (TSS)	24	50	24	50	24	1

Both the U.S. and IMO allow augmentation of test water to meet the challenge water characteristics, but both test protocols require that:

- "…a [Test Facility] TF must assess the effect of additives on the ambient and test organisms (if used) before using," (EPA 2010, §5.2.1.1 and 5.2.1.2),
- "The [Test Organization] TO should verify that, whatever source of augmentation or delivery system is used, the addition of the material should minimize to the extent possible biocidal or growth stimulate response of ambient organisms," (EPA 2010, §5.2.1.3), and
- "Any augmentation of test water with dissolved organic carbon (DOC), particulate organic carbon (POC) or total suspended solids (TSS) to achieve the minimum required content should be validated and approved by the Administration. As natural DOC constituents are complex and primarily of aromatic character, the type of added DOC is particularly critical to the evaluation of BWMS performance. The validation should ensure that relevant properties of the augmented water (such as the oxidant demand/ [Total Residual Oxidant] TRO decay and [Ultraviolet] UV absorption in the range of 200 to 280 nm, the production of disinfectant by-products and the particle size distribution of suspended solids) are equivalent, on a mg L^{-1} basis, to that of natural water that would quantitatively meet the challenge conditions. In addition, the validation should ensure that augmentation does not bias a test for or against any specific treatment process. The test report

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should include the basis for the selection, use and validation of augmentation." (IMO 2016, Marine Environmental Protection Committee [MEPC] 70/18/Add.1, §2.4.21).

Validation experiments are critical, as the selection of additives used to augment test water could affect the biological community, altering their susceptibility or resistance to treatment and, in turn, the outcomes of the verification testing. For example, the efficacy of BWMS employing UV radiation will be reduced by dissolved organic matter (DOM[1]), which absorbs and attenuates UV light (Moran et al. 2000).

Here, we examine the additives used to augment test water in both U.S. and IMO TA Testing. We summarize additives for DOM, particulate organic matter (POM), mineral matter (MM, also, TSS) that are in use (or proposed for use) in land-based testing, using published protocols, TA reports from testing organizations, and publically available literature. We review published validation efforts for additives—as well as other validations conducted for TA testing—and summarize the critical components of a validation test. Finally, we propose a parameter to measure the biological lability of the organic additives in future validation studies: biological oxygen demand (BOD). The BOD assay can be used as a preliminary approach to screen additives, additive mixtures, and—for organic materials—verify the stability of the additives over time.

2 REVIEW OF AVAILABLE ADDITIVES

2.1 Additive Selection

Adding DOM or POM introduces compounds that may affect test organisms, warranting careful consideration of additives to be selected to meet DOM and POM concentrations. First, the additives should not cause a shift or restructuring of the biological community either directly (by inducing mortality, osmotic stress, or flocculation of planktonic organisms) or indirectly, by stimulating bacterial growth and oxygen depletion. Among planktonic marine microorganisms, heterotrophic bacteria will respond rapidly and directly to the pool of organic matter, which is metabolized at different rates corresponding to the molecular weight or structure of the compounds in the carbon pool (Amon and Benner 1996). It is critical that the compound does not overstimulate bacterial growth, as this can lead to oxygen depletion and mortality of aerobic organisms. Glucose, e.g., is readily metabolized by bacteria, leading to rapid bacterial growth and oxygen depletion. Ideally, additives should represent the complexity of dissolved and particulate material in the ambient water used in testing. Typical coastal and estuarine ecosystems will have high input of dissolved and particulate materials introduced from terrestrial sources (Wang et al. 2004; Cai et al. 2012).

Second, additives should be selected with consideration of their impacts on key parts of the BWMS: the size and composition of particulates will affect the rates filters are loaded, the UV absorbance spectra will affect transmissivity of UV radiation, and the molecular structure and composition will impact reactivity with chlorine and other oxidants. Finally, the additives used should be appropriate for the characteristics of the test water, as the temperature, salinity, and pH will affect whether the compounds readily dissolve or precipitate. The additive must also be commercially available in bulk quantities due to the large water volumes necessary for TA testing. The additive should be appropriate for the water salinities and temperatures defined for required testing as low, brackish, and high salinities may effect dissolution efficiency.

[1] Organic carbon (DOC and POC) and organic matter (DOM and POM) are often used interchangeably. The former is the mass contribution of carbon only; the latter includes the mass of all constituents (carbon, hydrogen, oxygen, etc.).

2.2 Additives Used for DOC

***Camellia sinesis* (unsweetened decaffeinated iced tea mix)**

The U.S. Naval Research Laboratory (NRL) began using instant iced tea mix to supplement DOM, and its use was suggested in the ETV Program Protocol (EPA 2010), with the caveat that a TF must assess the impact of the additive on the ambient community. As this additive is derived from the leaves of *C. sinesis*, the pool of organic molecules is complex, and it includes chromophoric material (plant pigments), polyphenols, organic acids, and cellular biomolecules, such as amino acids, nucleic acids, and proteins (Harbowy and Balentine 1997). Each of the constituent compounds will range in reactivity, solubility, and bioavailability. Decaffeinated, instant iced tea is difficult to procure in quantities needed for large-scale testing. Further, commercial brands will often have additives and preservatives (e.g., artificial lemon flavor, maltodextrin). Consequently, *Camellia sinesis* has not been widely adopted for use as an additive.

Lignosulphonate

Lignins describe a variety of structural biomolecules in plant matter. Lignin is removed from lignocellulose as part of the sulfite pulping process, and the byproduct of pulping—lignosulphonate—is readily commercially available. As they are heterogeneous mixtures of high-molecular weight, aromatic compounds, lignosulphonates do not overstimulate bacterial growth, respiration, and oxygen depletion. Because lignosulphonates are opaque when dissolved, their use in test facilities will decrease transitivity of test water, which will affect the UV transmissivity (UVT), reducing the effective UV dose for BWMS using UV light. Two TF—DHI and NIVA—use calcium lignosulphonate (CAS Registry 8061-52-7) to supply a portion of the augmented DOC.

Sodium citrate

Sodium citrate—a term that describes either monosodium, disodium, or trisodium citrate —is a soluble, low molecular weight carbon compound. Trisodium citrate, dihydrate (CAS Registry 6132-04-3; formula: $C_6H_5Na_3O_7 \cdot 2H_2O$) is used to augment DOC at TF in both Denmark (DHI) and Norway (NIVA). When dissolved, sodium citrate is transparent, so it will augment DOC in test water to meet challenge water conditions without decreasing UVT. Similar to lignosulphonate, sodium citrate is readily available commercially, as it is a common food additive. The homogenous, low molecular weight compound would be readily metabolized by bacteria, supporting bacterial growth and respiration, and in turn, oxygen depletion.

Glucose

Glucose (CAS Registry 50-99-7; formula: C6H12O6) is a simple sugar, widely commercially available as a food additive. It is used by the TF in the Republic of Korea (KOMERI) to augment DOC in test water. Similar to sodium citrate, glucose is a homogenous, low-molecular weight compound that is transparent when dissolved.

2.3 Additives Used for POC

Starch
Starch (CAS Registry 9005-25-8; formula: $(C_6H_{10}O_5)n•H_2O$) is polysaccharide, derived flow plants (typically as cornstarch), and widely commercially available as a food additive. As it is insoluble in water at ambient temperatures, it contributes to the pool of POM, and three test facilities—DHI Denmark, NIVA, and KOMERI—use starch to augment test water. In suspension, particulate and colloidal starch is likely readily colonized by planktonic bacteria, which metabolize the polymer by releasing extracellular enzymes to break the macromolecule into monomers that are transported across the cell wall.

Humic substances
Humic substances are derived from decomposing plant matter, and thus they contain a complex mixture of plant substances. Humics contain a heterogeneous mixture of macromolecules, low molecular weight compounds, and organic acids and bases. This mixture, when introduced to test water, will likely contribute to the dissolved, colloidal, and particulate organic matter pools. Characteristics of the test water influence the solubility of the humic substances, with low salinity and low pH (as well as higher temperatures) increasing the solubility of organic acids. The ETV Program Protocol suggested using humic micromates, which are pulverized humic substances screened to conform to a narrow particle size range (EPA 2010). In this form, with particles 1 to 3 mm in diameter, humic substances remain suspended and well-dispersed throughout the volume of test water.

2.4 Additives Used for Mineral Matter

Kaolin
Kaolin (CAS Registry 1332-58-7; $Al_2Si_2O_5\ (OH)_4•2H_2O$) is clay mineral used to supplement particulate inorganic matter (i.e., MM). As the density of clay particles exceed seawater, Kaolin (and other MM) are kept suspended by continuous mixing. Continuous mixing prevents settling and flocculation, i.e., binding and settling of plankton, colloids, and POM. The U.S. and IMO protocols do not specify the size range of particles added, but implicitly, MM additives should reflect those in natural waters. Smaller particle size spectra (<10 µm) would best represent MM suspended in the water column in natural systems. Smaller-sized MM will also prevent clogging of filters designed for 50-µm organisms.

Ultra-fine Test Dust
The particle size range of ultrafine Arizona Test Dust (ATD) is specified, such that ~70% of particles are <5.5 µm and ~98% are <11 µm (ISO 12103-1). Ultrafine ATD was suggested as an appropriate additive in the ETV Program Protocol (EPA 2010). Like kaolin, it is a clay mineral composed mostly of silica and aluminum oxides.

3 SUMMARY OF VALIDATION EFFORTS

3.1 Korean Testing and Research Institute (KTR)

Korea Testing and Research Institute (KTR) examined the effect of seven additives on total residual oxidant (TRO) consumption, carbon yield, disinfection byproducts (DBP), and toxicology (KTR 2016). Study

results were reported to the International Maritime Organization (IMO 2017; MEPC 71/INF.6). Most of the additives examined are described above; exceptions include sodium acetate (formula: $C_2H_3NaO_2$), methylcellulose, and psyllium fiber (brand name: Metamucil®). Similar to lignosulphonate and humic matter, methylcellulose and psyllium fiber are not chemically homogenous, but heterogenous mixtures of polymers of different lengths and plant-derived biomolecules. Starch was the only substance designated to augment POC, so mixtures of starch and the other compounds (designated to augment DOC) were used in experiments. In experiments, measured pools of DOC and POC were compared to theoretical yields. Dissolved carbon was *greater* than expected concentrations, and POC was *less* than expected; together, these observations suggest that a portion of starch dissolved in the test samples.

Changes in TRO concentrations were measured by adding oxidants to approximately 10 mg L^{-1} and then measuring the final concentration after a 5-day incubation. Absolute and relative changes in TRO concentrations listed in the report (KTR 2016) are summarized below (Table 2). Control samples were unamended samples of ambient water with 1.44 mg L^{-1} and 0.05 mg L^{-1} of DOC and POC, respectively. Starch alone had little impact on TRO relative to control samples: starch consumed 22% of TRO, where control water consumed 21% of TRO. Starch + sodium citrate (34%) and starch + lignin (43%) had the highest consumption rates.

Table 2: Changes in TRO concentration from Day 0 to Day 5 for the tested additives (reproduced from KTR 2016).

	TRO Concentration (mg L^{-1})			
Additive	**Initial**	**Final**	**Absolute change**	**Relative change (%)**
Control	9.9	7.8	2.1	21%
Starch	9.8	7.7	2.1	22%
Starch + glucose	10.1	7.7	2.4	24%
Starch + sodium acetate	10.5	7.8	2.7	25%
Starch + Metamucil	10.1	7.1	3.0	30%
Starch + sodium citrate	9.4	6.2	3.2	34%
Starch + methylcellulose	10.0	8.2	1.8	18%
Starch + lignin	9.5	5.4	4.1	43%

Changes in pH due to additives caused only minor shifts from the baseline of ambient seawater (7.4). The largest changes in pH were caused by addition of starch + methylcellulose (7.02) and starch + lignin (8.06). Disinfection byproducts (DBP) were measured after neutralizing chlorinated samples. Both trihalomethanes (THM) and halocetic acids (HAA) were measured with gas chromatography and electron capture. In addition to DBP, the relative concentrations of humic acids, fulvic acids, and proteins were measured in samples with additives. In general, the relative contribution of the three components were similar to unamended seawater: the exception was starch + lignin, which had higher concentrations of fulvic acids or humic acids in untreated or chlorinated water, respectively. Starch alone did not increase DBP concentrations, but the highest concentrations of DBP were observed with starch combined with sodium

citrate or lignin.

The microalgae *Isochrysis galbana* and the bacteria *Vibrio fischeri* were used to determine the ecotoxicity of samples that were treated with the seven tested additives. Treated samples were sent to a separate laboratory, NeoEnBiz Laboratory (Neoenbiz Inc., Bucheon, Korea) to examine toxicity by measuring population growth. Results showed *I. galbana* exhibited toxicity for all treated samples, but when the treated samples were neutralized, the toxicity was eliminated for all except the lignin samples. Ecotoxicity was also measured using *V. fischeri*, a bioluminescent, non-pathogenic, marine bacterium, whose respiratory process is disrupted as a response to toxicity, resulting in a change in bioluminescence. Similar to the *I. galbana*, samples neutralized with sodium thiosulfate were not toxic to *V. fischeri*.

3.2 DHI Denmark

DHI Denmark (a sub-laboratory of DNV-GL) evaluated materials to augment the DOC, POC, and MM in test water: calcium lignosulphonate, sodium citrate, cornstarch, and kaolin. The additives were evaluated for their effect on two of the major BWMS treatment processes: UV, by measuring UVT, and chlorination, through measuring TRO consumption rates (DHI 2016). UV-T was measured by dissolving the DOC additives in purified water then measuring transmittance in the 200-280 nm spectra using a spectrophotometer. Sodium citrate did not impact UVT, as 5 mg C L^{-1} allowed ~100% of UV light (254 nm), whereas lignosulphonate decreased UVT to 77% at 254 nm. For comparison, natural freshwater and brackish marine water decreased UV-T to 65% and 77%, respectively. TRO consumption was used by measuring the decline on TRO initially (0 min), 5, 30, 60, 90 minutes, over longer times 2, 4, and 24 hour following the addition of 9 mg Cl_2 L^{-1}. In addition to lignosulphonate and sodium citrate, D-glucose and sucrose were tested for comparison. Lignosulphonate had the highest rate of TRO consumption (~4 mg Cl_2 L^{-1} in 4 hours). Other compounds showed consumption rates <1 mg Cl_2 L^{-1} in 4 hours. Cornstarch, used to supplement POM, and kaolin, used to supplement MM, had negligible impacts on UVT or TRO consumption.

The particle size distribution was analyzed in natural, fresh, and brackish waters prior to and following addition of the additives. For both water types, the particle load (i.e., the total number of particles) increased, but the size distribution of particles did not change substantially. Finally, the presence of two disinfection byproducts (aldehydes and haloacetic acids) were measured for natural marine water, natural water with sodium citrate, and natural water with a 85:15 ratio of lignosulphonate and sodium citrate. "Slightly elevated" concentrations of the disinfection byproducts were found in test solutions with lignosulphonate, however, concentrations were not reported (DHI 2018a).

The responses of live organisms to additives were examined in a separate study (DHI 2018b). Experiments were performed using harvested live brackish-water organisms held in 1-m^3 tanks. Organisms ≥10 and <50 μm and ≥50 μm were analyzed in unamended tanks, tanks with brine additions, and tanks with additives for TSS, DOC, and POC (each analyzed in separate tanks). Initial samples were collected upon tank filling and upon discharge after a 5-day hold. Analysis included water chemistry, standard microscopy-based techniques used in type approval tests, and taxonomic analysis performed on preserved samples. Additions

of brine to yield either 28 PSU (practical salinity units) or 34 PSU did not result in notable differences in organism concentrations relative to unamended control tanks. After 5 days, concentrations declined in both control tanks versus tanks with brine added to 28 PSU (59% versus 57%, respectively) and 34 PSU (62 and 67% versus 64 and 72%, respectively). Likewise, differences in concentrations due to addition of starch, lignosulphonate, sodium citrate, and cornstarch relative to unamended controls were minor. Organisms ≥10 and <50 µm displayed similar trends, except the total changes in concentration in control and treatment tanks—in these cases, over a 1-day incubation—were generally minor, and total concentrations at discharge were generally within 10% of inlet concentrations.

DHI also examined the percent survivability of organisms ≥50 µm and ≥10 and <50 µm in full-scale tests. Survivability was greatest for freshwater organisms ≥50 µm (median ~55%) compared to brackish (~35%) and marine (~25%) tests. Organisms ≥10 and <50 µm showed a similar trend in survivability, although the range of survivability was larger than for organisms ≥50 µm: in some cases, taxa increased in concentrations (survivability >100%).

3.3 NRL Key West

Additives used by NRL—*Camellia sinesis* (decaffeinated iced tea mix), pulverized humic matter, and clay minerals—were examined to determine mass yields of the ETV recommended additives at different temperatures and salinities (First et al. 2014). The decaffeinated iced tea mix was effectively completely dissolved into solution, except for high salinity (35 PSU), cold water (4°C) treatments, where 93 ± 2% of the DOM was dissolved, which was significantly lower than observed in other treatments and salinities (range 96-99%; First et al. 2014). A portion of the mass of humic matter (3-5% of total mass) dissolved in solution, contributing to the DOM pool. The combined suite of additives were also used in a mesocosm experiment to determine the response of the biological community (relative to unamended controls). For mesocosm experiments, organisms ≥50 µm, ≥10 and <50 µm, and bacteria (measured as culturable, heterotrophic bacteria) were examined as the start and after 5 days of an incubation. Significant changes in concentrations were observed only for heterotrophic bacteria: concentrations in amended water were more than one order of magnitude greater than in control tanks.

4 REVIEW OF OTHER VALIDATION EFFORTS

In some cases, validation procedures are outlined in TF protocols. For example, NIVA's procedure on analysis method validation describes the key parameters required for validation: method specification, sensitivity, limit of detection, measuring range, linearity, precision, accuracy and bias (NIVA 2015). Findings of these validations are (in some cases) published and publically available. DHI examined the effects of water temperature on TA testing (Drillet et al 2013). This study observed high rates of biological activity when warm, tropical, eutrophic water is held in aboveground, dark tanks. Validation of different configurations of sample probe and relative flow velocities widened the range of potential sampling configurations and scenarios (Wier et al. 2015).

The findings of these studies, however, are not often published or publically available. DHI Denmark provided NRL with reports of several validations, including, a validation of a sampling point location (DHI Denmark 1738-01). For this study, organism concentrations were measured at three different sample locations (prior to the pump, immediately downstream of the pump, and near the discharge). Significant differences in organism concentrations were not observed in these three samples. DHI Denmark also performed validation studies to determine the storage length for samples preserved in Lugol's iodine and whether the volume of control water sampled (whether 100 or 200 m^3) affects the concentration estimate.

5 A COMPREHENSIVE APPROACH FOR VALIDATING ADDITIVES

5.1 General Approaches

Challenge water criteria were set recognizing that BWMS would experience a range of natural water types, and anticipating that some test facilities may—even during certain test cycles—use ambient water with low concentrations of organisms and or constituents that interfere with treatments. Successful test outcomes with "unchallenging" water would not be predictive of the BWMS performance in typical water conditions. Implicitly, organisms or abiotic additive used to meet challenge water conditions should be reflective of natural water constituents. Explicitly, the testing organization should verify that additives "should minimize to the extent possible biocidal or growth stimulant response to the ambient organisms" (EPA, 2010). With this stated requirement, the key validation experiments should determine the organisms' response directly to the additives, observing whether the additives directly or indirectly affect test organisms. Other validation studies would be helpful to characterize the materials added to the test water to determine if they are reflective of natural waters in how they interfere with or respond to treatment (including the formation of DBP).

The primary and secondary validation tests are summarized below (Table 3). Tests are categorized by scale: Mesocosm-scale (≥ 1 m^3) tests will examine the response of all size classes of organisms simultaneously, but these tests are also appropriate for examining changes in water characteristics that respond directly or indirectly (through the activity of organisms) to additives. Laboratory-scale tests will use small experimental manipulations (typically <10 L) to determine the properties of the additives (either individually or in combinations), including transmissivity of dissolved materials, turbidity of suspended solids, and reaction with typical biocides leading to the formation of DBP.

Table 3. Validation tests categorized by experimental scale target effect.

Experimental Scale			
		Effects on Test Organisms	
Mesocosm		• Organisms ≥50 µm • Organisms ≥10 and <50 µm • Total Heterotrophic Bacteria	Organism concentrations in amended samples are compared to controls (unamended) after 1 or 5 days of incubation. For organisms ≥50 µm, mesocosm studies (>1 m^3) are appropriate.
		Effects on Water Quality	
		• Oxygen Demand • pH and Conductivity	Water characteristics are affected—both directly and indirectly—by additives, and these should be compared to controls or samples enriched with natural materials.
		Effects on Treatment Efficacy	
		• UV Transmissivity • Oxidant Demand • Turbidity and Filtration Efficiency	Materials added will affect treatment processes. The characteristics suspected to impact treatment (e.g., UVT on efficacy of UV-based systems; particle size for filtration efficiency) should be compared to natural waters.
	Laboratory	**Characteristics of the Additives**	
		• Generation of DBP • Chemical Composition • Dissolution and Mass Yield	Properties of the materials, including how they respond to waters of different temperatures and salinities, and whether their use generates relatively more DBP than natural waters should be investigated.

5.2 Test Protocol: Mesocosm-Scale Validations

General Test Design

The response of organisms to additives is most appropriately examined at the mesocosm scale (volumes ≥1 m^3), as maintaining natural communities of organisms ≥50 µm in laboratory-scale volumes (e.g., <50 L) for a 1- or 5-day test cycle would be challenging. Laboratory tests—focused on bacteria or organisms ≥10 and <50 µm —may be appropriate for some studies, such as for BOD assays. In general, the response of organisms ≥50 µm, ≥10 and <50 µm, and total heterotrophic bacteria are examined simultaneously in two mesocosms: one amended with the full suite of additives, and one control tank without amendments. Organism concentrations are measured at the start of the experiment, either during the simultaneous filling of two tanks from a common source, or from grab samples of the two tanks prior to treating the test tank with additives. The incubation conditions should mimic those in full-scale testing, e.g., if the full-scale test water is continuously mixed and shielded from direct light, those conditions should be applied to the mesocosms. After 1 and 5 days (and periodically throughout the incubation, as needed), both tanks should be sampled with standard methods appropriate for the size class, and organisms in the three size classes should be quantified using standard methods (e.g., First et al. 2014).

Additional Considerations

At least three independent trials should be performed, with mesocosms filled with natural water on different days, assuring that (even if similar) the community of natural organisms in the test water is unique, and thus would respond independently to the additives. If surrogate organisms are typically used to achieve challenge water concentrations, they should also be added to the experimental mesocosms at the same relative density as used in full-scale tests. Temperature and salinity of the tanks should be monitored throughout the experimental incubation to assure that the water conditions are similar, assuring that the tanks are not heated at different rates due to their exposure to sunlight (or other heat sources for indoor trials). Secondary effects of the additives can influence organisms' growth or mortality rates. For example, bacterial respiration may deplete dissolved oxygen at higher rates relative to the control. Oxygen and other variables (such as pH and conductivity) are ideally monitored continuously or periodically throughout the incubation period. Tracking these variables could be helpful in identifying potential drivers of significant changes in organism concentrations.

5.4 Test Protocol: Laboratory-Scale Validations

General Test Design

Characteristics of the additives are best studied at the laboratory scale. In general, these tests demonstrate how the additives affect the test water, influence treatment efficacy, and react to treatment. Essentially, these tests verify whether the additives chosen are reflective of natural water constituents, and although they do not measure the direct impact on organisms, these studies still illustrate the behavior of the additives in test water, their potential impacts on ballast water treatments, and their fate in the environment. Validation tests will, in general, treat natural test water with one or a combination of additives and then compare the response variable (UV transmissivity, oxidant demand, etc.) in the tests samples to unamended controls. As part of a general understanding of the materials added, their dissolution rates and mass yields should be determined, especially when tests are performed on a range of water types, with differing salinities and temperatures.

Additional Considerations

For measuring BOD, an ideal comparison of material added (especially dissolved and particulate organic matter) would compare the additive to an equal concentration of natural DOM and POM. Natural POM (as well as suspended minerals) can be easily concentrated from natural water by size fractionation. Extracting DOM to enrich samples requires a more sophisticated approach, such as solid-phase extraction, where filtered test water is passed through a media that binds DOM (e.g., Dittmar et al. 2008). The concentrated DOM is then "released" from the media with a chemical solvent, the solvent is evaporated, and the natural DOM can be added to test samples.

6 DISCUSSION

Challenge water conditions are in place to assure BWMS under test can handle some of the typical conditions observed in eutrophic locations, such as inland ports and estuaries. Compounds added should reflect natural compounds in these locations. Additives, especially particulates, may directly influence organism concentrations in the ≥50 μm and ≥10 and <50 μm size class, for example, by affecting their filter-feeding efficiency (Boenigk and Novarino 2004). Adding organic matter through DOM and POM will primarily affect bacterial communities, stimulating growth and respiration, which can affect organisms in larger size classes. Additional bacterial biomass and activity will deplete oxygen, increase carbon dioxide, and sequester available nutrients. Additional bacteria also may benefit bacterivorous plankton, which may

respond quickly to the increased biomass (Kim et al., 2011). For these reasons, newly proposed materials should be first screened in a set of laboratory experiments, where the key test would measure the response of the bacterial community to the additives, either directly through bacterial growth or indirectly through BOD. Standard techniques for measuring bacterial biomass and activity can be performed with reasonable effort in laboratory environments (Reuschenbach et al. 2003). Increases in bacterial biomass and activity will amplify the direct effects of additives, driving significant changes in the water characteristics, and in turn, the susceptibility of organism to treatment. Bacteria, for example, though initially affected by UV, returned to pre-treatment concentrations within 5 days of treatment (First et al. 2016). Therefore, bacterial activity during tank holds—driven by the selection of DOM and POM—may drive change in the population of organisms in the larger size class, and these changes may not be reflective or representative of natural waters. For these reasons, understanding the bacterial response to organic matter is critical.

Potential additives must be commercially available in large quantities to support full-scale testing. This requirement limits the potential list of candidate materials, and for DOM and POM, where natural constituents are complex, chemically homogenous compounds (e.g., glucose) do not reflect the heterogeneity of natural organic matter. For these reasons, an alternate material, supplementary to those identified by test facilities, was not identified. Likely, alternate or "new" materials will be mixtures of existing compounds, created to support the testing needs, commercial availability, and costs of the test facilities. The mixtures should be treated as alternates and undergo the full set of validation tests. Storage conditions could affect the chemical the composition of complex compounds in humic materials and plant extracts; it is likely that these changes are ongoing through the shelf life of the material. This and other factors, such as lot-to-lot variation, suggest that certain validation tests should be performed periodically to assure that the additives, especially the organic materials, remain similar to the additives validated in mesocosm-scale experiments.

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