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Review

Approaches for determining the effects of UV radiation on microorganisms in ballast water

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Abstract

To minimize the dispersal of aquatic nuisance species through shipping, ballast water can be treated to kill, remove, or inactivate organisms. Ultraviolet (UV) radiation is used in some ballast water management systems to address this goal. Because UV treatment renders cells non-viable (by sterilizing them, preventing reproduction) and does not necessarily kill organisms instantaneously, the efficacy of UV treatment has been verified historically by regrowth assays in which microorganisms are cultured (and thus, reproduce) under optimal growth conditions. Although regrowth assays are definitive, they are time consuming—lasting for days or weeks—and, importantly, are applicable only to organisms amenable to culturing. Furthermore, these estimates of cell concentrations are often accompanied by large error estimates. In this paper, several rapid alternatives to regrowth assays are described and evaluated. An ideal approach would shorten or simplify the analysis burden and, potentially, could be used for shipboard testing to determine compliance with national and international ballast water standards. Complicating this task is the requirement that compliance with the ballast water standards will be determined by quantifying the number of *living* organisms in ships' ballast water, and while organisms may be living following UV treatment, they may not be *viable* (i.e., they may not reproduce). To address this dichotomy , alternatives to regrowth assays were categorized based upon the complexity of the analysis and the means used to determine the status of microorganisms (either as viable or living): 1. Instantaneous growth and cell replication, 2. Cell activity and metabolic rates, 3. Cell structural integrity, and 4. Biomolecule presence and status. With the suite of approaches currently available, it is not possible to determine the viability of organisms rapidly, that is, within minutes of collecting a ballast water sample. Measurements of the photosystem integrity via variable fluorescence and the presence of adenosine triphosphate (ATP) are currently the most promising for rapidly estimating concentrations of living cells in compliance testing of ballast water discharges; however, extensive validation is required to verify the applicability of these approaches for the complexity of real-world samples.

Key words: ultraviolet radiation; invasive species; shipping; methods; protists; ballast water management systems

Introduction

Throughout the past century, ultraviolet (UV) radiation has been used to kill microorganisms or inactivate them (that is, sterilize or render cells non-viable, preventing reproduction; Hijnen et al. 2006). Because UV light is effective across different types of microorganisms (including viruses, bacteria, and pathogenic microeukaryotes), it has been used as a secondary treatment (following, for example, filtration) of both wastewater and drinking water (Wolfe 1990). UV light is also used as component of some ballast water management systems to reduce the transfer and release of potential aquatic nuisance species in ballast water discharged from ships (e.g., Gregg et al. 2009). Treating ballast water with UV light, in contrast to dosing water with "active substances", such as chemical compounds, is advantageous because operators do not have to generate (or store and handle) large volumes of potentially harmful compounds, nor do these compounds need to be neutralized prior to discharge. Thus, vessel operators can limit the potential exposure of both the vessel and crew to chemicals, many of which are strong oxidizing chemicals and may also lead to corrosion in ballast tanks.

Sterilization with UV light is typically achieved by exposure to low- or medium-pressure mercury lamps. Although other cellular components can be damaged via UV radiation (including both cell membranes and cytoplasmic proteins; Schwartz 1998), damage to DNA is the main mode of

sterilization. In this case, exposure to UV radiation generates pyrimadine dimers (linkages between pyrimidine bases), which interfere with DNA replication (Goodsell 2001; Oguma et al. 2002). The total UV dose (i.e., the fluence) is calculated as the product of the flux of radiation over time per unit area and the exposure time. In flow-through chambers, UV exposure time is dependent upon water flow (slower flow rates will expose particles to higher UV doses). While flow rate can be controlled, fluence is difficult to quantify throughout a three-dimensional flow chamber (Qualls and Johnson 1983). Particularly in ballast water applications, characteristics of the ambient water taken up in ports (such as turbidity and the concentration of chromogenic dissolved organic matter) can attenuate the fluence (Hijnen et al. 2006), so these parameters must be accounted for in designing ballast water management systems.

Under national and proposed international regulations, very sparse concentrations of living organisms (e.g., <10 individuals mL^{-1} of organisms ≥10 μm and <50 μm) may be discharged in ballast water (International Maritime Organization, 2004; U.S. Coast Guard 2012). Importantly, the U.S. Coast Guard final rule and the International Maritime Organization Guidelines for Approval of Ballast Water Management Systems (G8; International Maritime Organization 2004) apply to organisms that are *living* at the time of discharge, not *viable* ones. This distinction is relevant because at the doses used in ballast water management systems, UV light is generally used to prevent reproduction but does not necessarily kill cells immediately following exposure. Thus, when considering compliance monitoring of ballast water, which will likely occur quickly, while a compliance officer is onboard the vessel, the lack of rapid and reliable approaches to determine whether UV-treated cells that are living at the time of discharge will eventually be non-viable complicates the use of UV technologies for ballast water treatment. Even during "verification testing" of ballast water management systems, which is needed before systems are installed on board ships, determining the relationship between living and viable cells is difficult: the traditional approach to determining viability, a regrowth assay, requires that microorganisms are cultured under laboratory conditions, but many microorganisms cannot be cultured. For completeness, this paper considers approaches to determine cell viability as well as whether cells are living following exposure to ballast water treatment, specifically UV radiation. While this paper is focused on testing UV-treated water, these approaches may also be applicable to other treatment technologies that do not lead to instantaneous mortality. Furthermore, additional research will need to be conducted to reconcile the difference between viable and living cells with respect to ballast water testing.

Obstacles to effective sterilization of ballast water by UV radiation

The use of UV light to treat ballast water introduces several challenges: First, many vessels transport large volumes of water for ballast (1,000s to 10,000s of m^3), and water must be treated at a fast rate to minimize the period of ballasting or deballasting or both, depending on the operation of the ballast water management system. Higher water flows through UV systems will allow larger water volumes to be processed, but high flow rates will reduce the effective UV dosage. Second, dissolved organic matter or suspended particulates can effectively decrease incident irradiation by absorbing UV light or shielding organisms from direct light, respectively.

Aquatic organisms display different tolerances to UV light: bacteria, in general, are considered highly susceptible to low doses of UV, whereas protistan cysts and certain viruses, in some cases, resist high UV doses (Hijnen et al. 2006). Bacterial spores in the environment display increased resistance to UV light relative to laboratory cultures, as aggregated spores in the environment receive protection through shading (Mamane-Gravetz and Linden 2005). Aquatic microorganisms, especially phototrophic eukaryotes, have strategies for both protecting against UV light damage and repairing cellular damage. One strategy for UV protection is the production of UV-resistant compounds, which minimize UV damage in the photic zone. For example, mycosporine-like amino acids and scytonemins are compounds found in eukaryotic microalgae and cyanobacteria that absorb UV light and protect cellular components most sensitive to UV damage (Gao and Garcia-Pichel 2011). These compounds are produced by phytoplankton, however, they may be transferred to heterotrophic organisms through grazing (Hylander and Jephson 2010). While these compounds provide the majority of protection for longer wavelengths (UV-A and UV-B), certain mycosporine-like amino acids may have maximal absorbance in short wavelengths used for sterilization (here, UV-C; Gao and Garcia-Pichel 2011). Mechanisms for

repairing DNA damage, such as light-dependent and light-independent repair, allow organisms to correct or remove DNA damaged by UV light (Goosen and Moolenaar 2008; Lesser et al. 1994).

In light of organisms' biological protection and repair mechanisms and in consideration of the mechanical hurdles facing ballast water management systems, an approach to verify sterilization is needed. Typically, "biodosimeters" (that is, test organisms with a known tolerance to UV) are used to test the efficacy of a treatment regime, for example, in the drinking and wastewater fields. Biodosimeters can be a single organism of particular interest (*Giardia* sp. for testing drinking water; Linden et al. 2002) or a class of organisms (fecal coliform bacteria for testing wastewater; Oguma et al. 2002). In addition to well-established tolerances to UV light, a biodosimeter, critically, must be able to regrow under laboratory conditions. Because ballast water originates from different geographical locations (each with a unique assemblage of organisms and water characteristics), a universal biodosimeter—one suited for all aquatic environments, across all global ports, from freshwater to saltwater—is unlikely to exist. Additionally, aquatic organisms may be fastidious and not exhibit growth with standard techniques for culturing organisms. Thus, the absence of regrowth in laboratory incubators seems a poor predictor of effective treatment. Particularly for ballast water management systems, which are relatively new technologies and need to process a wide range of water types and biotic assemblages, alternatives to standard regrowth assays are needed.

Rapid alternatives to regrowth assays

The purpose of this review is to summarize available approaches used to quantify viable and living microbial eukaryotes following UV treatment. Prokaryotes are not considered here because the assays for the indicator organisms have been used for decades in the drinking and wastewater industries. Specifically, this review focuses on rapid approaches used to detect or predict cell viability (the capacity to regrow), which can potentially be used as an alternative to regrowth assays, as well as approaches to detect living cells. Relative to regrowth assays, the approaches reviewed here can be completed rapidly (minutes to hours), and some can detect characteristics of organisms that could be predictive of their viability; thus, they may be suitable for shipboard assessment of ballast water. Approaches using sophisticated equipment or materials that could potentially cause health problems or require specialized training (such as $^{14}CO_2$ incorporation to measure photosynthesis) were also excluded. Potential techniques based upon DNA amplification, while currently requiring specialized training and equipment, are included in this review because the innovations in this field may permit the development of rapid evaluation tools.

This review is primarily focused on free-living microeukaryotes, which include both phototrophs and heterotrophs. These organisms have demonstrated more resistance to UV sterilization than bacteria (Waite et al. 2003; Hijnen et al. 2006), and, relative to larger organisms, microeukaryotes are more difficult to remove from water by physical separation in ballast water management systems, which typically strive to remove organisms ≥50 µm, a size generally larger than microeukaryotes. This list of potential approaches is not exhaustive but identifies the major categories for establishing whether a unicellular organism is viable or living and provides examples for each category. Among this list are approaches that, with the emergence of novel technologies and engineering, could provide continuous and automated analysis for remote and real-time monitoring of organisms in ships' discharges.

Candidate approaches for detecting viable or living cells

The approaches reviewed are grouped into general categories based upon their target measurement: instantaneous growth and cell replication, cell activity and metabolic rate measurements, cell structural integrity, and biomolecule detection, and although some detect viability, most determine solely if a cell is living (Table 1; see Table 2 for definitions). The advantages and disadvantages of each approach are discussed. Additionally, ancillary factors (such as portability of instruments and cost of analysis) are also considered.

Instantaneous growth and cell replication

Cell division. Organisms that reproduce asexually undergo morphological changes indicative of their reproductive phase. The most obvious of these changes are binary fission in prokaryotes and mitosis in eukaryotes, which can be observed using microscopy (Hagström et al. 1979). To that end, the "frequency of dividing cells" assay uses

Table 1. Summary of approaches that could potentially be used following UV treatment to detect viable or living organisms ≥10 and <50 µm, a size class dominated by free-living, single-celled eukaryotes. In the three columns on the right-hand side of the table, each approach is evaluated as described in Table 2. Approaches are grouped into broad categories (instantaneous growth and cell replication, cell activity and metabolic rates, etc.).

Approach	Parameter measured (viable or living cells detected)	Reagents	Equipment	Suitability to detect UV damage	Approach complexity	Analysis time
Instantaneous growth and cell replication						
Cell division	Frequency of dividing cells (viable cells)	Fluorescent labels	Epi	Highly suitable	Moderately complex	Lengthy[1]
Growth phase analysis	Frequency of cells in various growth phases (viable cells)	Fluorescent labels	FC	Highly suitable	Highly complex	Moderate[1]
Cell activity and metabolic rates						
Community respiration	Oxygen dynamics in light and dark incubations (living cells)	O_2-sensitive compounds	O_2 detectors	Minimally suitable	Simple	Short[1]
Motility	Cell or organelle movement (living cells)	Fluorescent labels	Light microscope or Epi	Minimally suitable	Simple	Moderate to lengthy
Thermogenesis	Heat production and metabolic responses to temperature changes (living cells)	None	Micro-calorimeter	Minimally suitable	Simple	Short[1]
Cell structural integrity						
Photosystem integrity	Photochemical yield (living cells)	None	Variable fluorescence fluorometer	Moderately suitable	Simple	Short
Lysosome integrity	Direct count of cells with intact lysosomes (living cells)	Fluorescent labels	FC	Moderately suitable	Moderately complex	Moderate
Membrane integrity	Direct count of cells with intact membranes (living cells)	Fluorescent labels	FC	Moderately suitable	Moderately complex	Moderate
Enzyme integrity	Direct counts of single cells or community enzyme activity (living cells)	Fluorescence labels transformed by enzymes	Epi or FC	Moderately suitable	Moderately complex	Moderate
Biomolecule presence and status						
DNA detection	Relative concentration of intact, intracellular DNA (living cells)	Numerous reagents (PMA, DNA polymerase, etc.)	qPCR thermocycler	Highly suitable	Highly complex	Lengthy
ATP detection	Relative concentration of intracellular ATP (living cells)	Luciferin, luciferase, and lysis buffers	Luminometer	Moderately suitable	Simple	Short

[1]Additionally, this analysis requires an incubation time (minutes to hours). ATP = adenosine triphosphate, DNA = deoxyribonucleic acid, Epi = epifluorescence microscope, FC = flow cytometer, PMA = propidium monoazide, and qPCR = quantitative polymerase chain reaction.

the relative portion of individuals undergoing reproduction as a proximal measure of population growth. Here, the number of cells in the total population is counted, and then the number of organisms undergoing division is quantified. This approach provides a relative measurement of the population's viability, and it is commonly used to compare two populations (or a single population at multiple time points) to assess the response to treatment or measure relative production (First and Hollibaugh 2008; Sherr et al. 1992). While most commonly used for prokaryotes, the approach has also been adapted to single-celled eukaryotes (Jahan et al. 2001). To evaluate organisms following treatment with UV light, samples could be analyzed immediately after treatment, held for a short time period (for example, 2 – 4 h), and reanalyzed. Provided that growth stimuli (such as labile carbon for bacteria or light and nutrients for algae) are provided during the interim period, increased frequency of dividing cells indicates viable organisms.

Table 2. Three criteria for evaluating the approaches used to quantify viable or living organisms ≥10 and <50 μm following UV treatment (as listed in Table 1).

Criterion	Appraisal	Description
Suitability for detecting UV damage	Highly suitable	Directly measures DNA replication
	Moderately suitable	Measures other cellular components (e.g., organelles, photosystems) that may not be critically affected by UV light
	Minimally suitable	Measures bulk community activities that may continue after sublethal exposure to UV light
Approach Complexity	Highly complex	Lengthy and intricate protocol; sophisticated equipment required; intricate data analysis and interpretation necessary; extensive training of analysts required
	Moderately complex	Rapid or minimal sample preparation; sophisticated equipment, but straightforward data analysis and interpretation; moderate training of analysts required
	Simple	Minimal steps in the protocol; portable, facile instrumentation; uncomplicated data analysis; little training of analysts required
Analysis time	Lengthy	Analysis requires several hours
	Moderate	30 – 60 min
	Short	<30 min

The frequency of dividing cells approach requires an epifluorescence microscope with the appropriate magnification for the population of interest. Portable, field epifluorescence microscopes are available (Seaver et al. 2001; Albert et al. 2010; Miller et al. 2010), and automation can facilitate analysis (Culverhouse et al. 2003). The major drawback with this approach is that absence of cell division is not necessarily indicative of inability to grow; the experimental conditions may be sub-optimal, precluding reproduction. Additionally, the incubation time precludes rapid analysis, the number of organisms counted must be large ($>10^2$) to reduce uncertainty in the estimates, and counting times can be substantial for sparse concentrations.

Growth phase. The use of flow cytometry reduces some of the problems of epifluorescence microscopy, such as long counting times and the need for cells to be concentrated. Using flow cytometry, furthermore, provides an additional metric of growth: cellular DNA content. The concentration of cellular DNA, along with relative cell size, can be used to indicate the growth phase of a population (Binder 2000). Organisms duplicate cellular DNA prior to division, and DNA (labeled with a fluorescent probe) is quantified by flow cytometry. Therefore, DNA concentration is a preliminary forecast of frequency of dividing cells. Similar to frequency of dividing cells, the approach requires analysis at two time points (at a minimum) to observe relative changes. Cell populations need to be abundant (and thus easily distinguishable on the plots of light scatter produced by the flow cytometer) for proper estimates.

Cell activity and metabolic rates

Community-based metabolism. Determining changes in oxygen concentration is a well-established approach to measure both primary production (Pomeroy 1959; Pomeroy et al. 1981) and respiration (Pomeroy and Johannes 1966). The biological oxygen demand, for instance, is a standard technique to measure microbial activities in the environment (Dye 1980) and in wastewater (Kim et al. 2003; Liu and Mattiasson 2002). Oxygen can be readily estimated with optical probes (Hasumoto et al. 2006) or with redox-sensitive chromophores (Créach et al. 2003; Karakashev et al. 2003) and fluorophores (Czekanska 2011). Standard techniques for measuring total community activity, such as biological oxygen demand, require long incubation times (days), and during this time, "bottle effects" could lead to anomalous measurements (Bender et al. 1999). Bottle effects include all the factors associated with laboratory growth that drive organisms to respond differently *ex situ* (e.g., Murrell and Hollibaugh 1998), such as an artificially high surface area to volume ratio. Shorter incubations (min to h) require sensitive instrumentation, but reducing the incubation time mitigates bottle effects. Following UV treatment, oxygen demand and production could be measured

in whole water or size-fractionated samples. Alternating light and dark treatments could be used for phototrophs to decouple primary production from respiration, and potentially, growth substrates could be added to stimulate activity. While community-based measurements are difficult to translate to cell concentrations, these approaches may be suitable for relatively quick, indicative tests of treatment efficacy.

Motility. Organism motility can be used to indicate living cells or organisms, and motility has been used to distinguish between living and dead microinvertebrates during land-based verification testing of ballast water management systems (U.S. Environmental Protection Agency 2010; Veldhuis and Fuhr 2008 [motility was used in conjunction with the vital stain Neutral Red]). In this case, movement may be induced by physical contact, such as gentle touching or prodding with a probe. Three-dimensional movements of copepods can be captured with high-speed video (Yen and Okubo 2002). Regarding microorganisms, motile protists can be tracked via video microscopy, and movement of the entire cell (or movement of organelles) can indicate living cells (Boenigk et al. 2001; Capriulo and Degnan 1991). Motility, however, is not universal among living cells. For example, planktonic diatoms, while capable of buoyancy regulation (Moore 1996), would not be expected to exhibit visible movement, even in response to a tactile stimulus. Likewise, living microinvertebrates may be motionless, for example, during molts.

Thermogenesis. Heat generated through metabolism can be detected and measured via isothermal microcalorimetry. In principle, a sample (<10 mL) is held at a constant temperature, and heat generated via biological processes can be detected and used to indicate the biomass or activity of organisms (Braissant et al. 2010). For bacteria, microcalorimetry has been used to detect contamination in food, measure the efficacy of antibiotics, and determine the optimal growth yield on various substrates (Traore et al. 1982). Microcalorimetry has also been used to measure heat production by phototrophic protists. When normalized to biomass, heat production appears constant between protists of different species and size (Johnson et al. 2009), which could be an ideal trait for estimating the biomass of living protists in samples of unknown composition. The time required to detect metabolically active organisms is proportional to the concentration and activity of organisms in the target size class. Heat production is measured instantaneously, and the sensitivity of the instrument would determine whether microcalorimetry is a valid approach to detect sparsely concentrated organisms. Often, heat production is measured over a relatively long period to determine time-integrated heat production. For example, analyzing bacterial samples requires several hours to measure the heat production relative to control samples (Wadso 2002). Not surprisingly, larger organisms require less time (~1 h) to measure time-integrated heat production (Johnson et al. 2009). The expense, size, and sophistication of isothermal microcalorimeters limit their use as portable field instruments. However, design innovations have decreased costs and increased portability of microcalorimeters, which may allow for increased use in field environments (Braissant et al. 2010).

Cell structural integrity

Photosystem integrity. Chlorophyll *a* is a photopigment universally used by photosynthetic organisms, and its concentration (measured either by autofluorescence or light absorbance) is commonly used to estimate algal biomass (Lorenzen 1967; de Jonge 1980). However, chlorophyll *a* persists after cell death and, therefore, when reported without supplementary information, does not appear to be an appropriate metric for quantifying living phototrophs in many environments, including from water discharged from ballast water management systems. Nonetheless, it may be useful as an ancillary metric to determine system efficacy. Variable fluorescence, such as pulse amplitude modulated (PAM) fluorometry, uses variable, *in vivo* fluorescence to evaluate photosystem efficiency. Chlorophyll *a* associated with intact photosystems is capable of using light with high efficiency, and by varying the excitation intensity, the relative status of the photosystems can be quantified (Genty et al. 1989). High photosystem efficiency, measured via variable fluorescence, correlates with algal productivity (Barranguet and Kromkamp 2000). Because variable fluorescence provides both a relative concentration of total chlorophyll *a* and a measure of the physiological status of the algal community, it has been suggested as a potential tool to assess compliance of ballast water with discharge standards (Gollasch and David 2011; Stehouwer et al. 2009). Furthermore, samples require no prior processing or manipulation, and readings of chlorophyll *a* fluorescence and

photosynthetic yield are rapidly collected (within seconds). Other types of approaches based upon variable, *in vivo* fluorescence (such as fast repetition rate fluorometry) are described elsewhere (Cosgrove and Borowitzka 2010).

The main disadvantage for this approach is that only phototrophic organisms are detected. In some systems, algae may constitute the majority of total biomass. However, for ballast water not exposed to light, a community shift from autotrophic to heterotrophic dominated assemblages could be expected, as rapid shifts in community structure have frequently been observed in short-term (1 – 2 d) experimental incubations (Agis et al. 2007; Kim et al. 2011). Consequently, variable fluorescence would underestimate the total concentration of organisms in these scenarios. While measurements of photosystem efficiency are highly indicative of UV exposure, chlorophyll *a* fluorescence, in some cases, increases after irradiation (First and Drake 2013). This increase results in overestimations of algal concentrations. Finally, these measurements are proxies for cell concentrations and thus would likely be suitable in detecting gross non-compliance with discharge standards (for example, concentrations 10× or 100× greater than the standard).

Lysosome integrity. Detection techniques based upon chlorophyll *a* autofluorescence will not capture the heterotrophic portion of the community. Specific probes that target acidic structures in the cell cytoplasm, such as lysosomes, can, however, be used to label and detect viable heterotrophic protists in ballast water samples. Lysosomes acidify food vacuoles of actively feeding protists, and acidification occurs rapidly upon food vacuole formation (Capriulo and Degnan 1991; Fok et al. 1982). Thus, pH-sensitive fluorescent probes will target actively feeding organisms that are capable of maintaining membrane potential. For example, LysoTracker Green™ (Invitrogen, Carlsbad, CA) can detect the heterotrophic protists in unpreserved samples via flow cytometry, and this approach yields concentration estimates similar to manual microscopy counts (Rose et al. 2004). In combination with chlorophyll *a* fluorescence, these probes can be used to differentiate between heterotrophic and autotrophic protists (Heywood et al. 2011). Other pH-sensitive probes, such as LysoSensor™ (Invitrogen), have also been used to detect food vacuoles (Carvalho and Granéli 2006).

Membrane integrity. Another approach to quantifying living organisms is to count the abundance of dead organisms (relative to the total organisms) within a sample and determine the number of live organisms by subtraction. Membrane-permeable fluorophores bind to nucleic acids within organisms, but because they cannot permeate intact cell membranes, they label the dead or moribund organisms within a sample. The fluorophore SYTOX® Green (Invitrogen, Carlsbad, CA) has been used to label dead bacteria (Schumann et al. 2003) and eukaryotic algae (Steinberg et al. 2011a). Other membrane-permeable labels (such as TO-PRO®-1; Invitrogen) have been shown effective for labeling microinvertebrates, specifically, copepod eggs (Gorokhova 2010). Another approach must be used concurrently on the sample to detect the total number of cells. For example, cells can be counter-labeled with a DNA probe that penetrates the membranes of both living and dead cells. Alternatively, the total count of target organisms can be measured by light microscopy. This requirement for a secondary count, however, increases analysis burden and potential for measurement error. Additionally, the physiological state of the cell affects the labeling efficiency (Lebaron et al. 1998). Similar to other approaches using fluorescent probes, membrane permeable-labels are detected by flow cytometry (Peperzak and Brussaard 2011), epifluorescence microscopy (Steinberg et al. 2011a), or with plate readers that measure fluorescence in microwells (Peeters et al. 2008). Other molecular probes are available for use to indicate the presence and integrity of cellular components, such as mitochondria (Hirst et al. 2011).

Enzyme integrity. The integrity of cell enzymes can be measured using compounds that are transformed into fluorescent molecules by non-specific cellular enzymes. For example, fluorescein diacetate (FDA) has a long history of use for identifying intact cellular enzymes (Rotman and Papermaster 1966). While FDA has been widely used to measure the enzymatic activity (and thus, whether a cell is living) of microorganisms (e.g., Chand et al. 1994), a concern with FDA is the occurrence of non-enzymatic transformation of the molecule, which results in fluorescence in the absence of intact enzymes (Clarke et al. 2001). Additionally, fluorescent FDA is highly permeable across the cell membrane and diffuses out of the cytoplasm over time (Prosperi et al. 1986). Derivatives of these fluorescent probes have been developed to minimize the permeability across the cell membrane, notably, chloromethyl fluorescein diacetate (CMFDA, Invitrogen). Once enzymatically

transformed into a fluorescent compound, CMFDA is non-permeable across the cell membrane. CMFDA has been used to label both bacteria (Schumann et al. 2003), phytoplankton (Steinberg et al. 2011b), and metazoans (Bernhard and Bowser 1996). The label is retained within living cells, and it can be retained within the cytoplasm of daughter cells after several generations. Additionally, CMFDA also persists in preserved cells, and it can be used to visualize cells after extensive manipulation (including dehydration and epoxy-embedding; Bernhard et al. 2003; First and Hollibaugh 2010).

Biomolecule presence and status

DNA extraction and detection. Extracting and amplifying environmental DNA for quantification of microbes is problematic, as DNA can persist after cell death, and extracellular DNA can be more than one order of magnitude more abundant than intracellular DNA (Corinaldesi et al. 2005). In sediments, DNA binds to particles and resists degradation (Lorenz and Wackernagel 1987). Several techniques, however, are employed to distinguish between DNA from active and inactive organisms. Propidium monoazide, when added to a sample, penetrates cells with compromised membranes and binds to DNA. Then, DNA bound to propidium monozaide is destroyed by exposure to infrared light, allowing only DNA from living organisms to be extracted and amplified (Nocker et al. 2007). While this technique has been primarily used for environmental bacteria, it should be applicable to single-celled protists. Other techniques, including approaches to digest extracellular DNA, have also been used to distinguish between the intracellular and extracellular pools (Dell'Anno et al. 2002). Once "dead" DNA has been degraded or removed, the quantity of target organisms can be measured through the quantitative polymerase chain reaction assay. Extraction and amplification of DNA is especially valuable for interrogating a sample for the presence of a known pathogen (e.g., Ishii et al. 2006). Problems with DNA quantification, however, still hinder its usage as a rapid and straightforward approach to estimate concentration of live organisms. For example, organisms may contain multiple gene copies, which will lead to an overestimation of organisms (Not et al. 2009). Additionally, the protocols for extracting and amplifying DNA are time-consuming and required specialized training. Therefore, a rapid approach to evaluate the status of UV- treated organisms using DNA extraction is currently not practicable.

Detection of Adenosine Triphosphate (ATP). Universal to all living cells, ATP is commonly used as a proximal measure of cellular biomass. The light-producing reaction of ATP with luciferin and luciferase allows for rapid, sensitive detection of ATP by measuring light, which has been used to estimate concentrations of organisms in aquatic environments (Hodson et al. 1976). The ATP assay seems well-suited for ballast water testing (Waite et al. 2003), and the development of liquid-stable reagents and handheld luminometers allow the analysis to be performed in field environments (such as aboard ships). Estimations of ATP are also possible through automated, microfluidic approaches (Liu et al. 2005; Tran et al. 2007), which further accelerate and simplify the analysis.

One difficulty with the ATP assay is that the presence of dissolved metals can inhibit the reaction, leading to underestimation of biomass (Sudhaharan and Reddy 1999). In contrast, free ATP in the environment (e.g., Azam and Hodson 1977) will lead to overestimations of biomass. A way to circumvent these problems is to concentrate organism on a filter membrane – a process that eliminates the confounding effects of dissolved compounds on ATP measurements. Another problem is that cellular ATP in bacteria increases in response to UV radiation (Villaverde et al. 1986), and a similar response has been observed in mixed assemblages of marine organisms (First and Drake 2013). This response complicates the interpretation of ATP readings from UV-treated samples. Additionally, it seems likely that this approach would be used as an indicator of gross non-compliance with the discharge standard, given that the concentrations of ATP will vary with cells size, and the composition of the community in ballast water will be unknown during compliance testing.

Practicability for shipboard testing of ballast water

The approaches described above were evaluated for their use in detecting UV damage (Table 1), and the criteria used to assess the approaches are defined in Table 2. Approaches that focus on DNA replication and molecular integrity are the most highly suited for detection of UV damage, as DNA is the target of UV radiation used for sterilization (Hijnen et al. 2006). However, these approaches are highly complex and require

extensive analysis. A suite of approaches is available to measure the integrity of cellular components by detecting fluorophores that selectively label cells or cell structures; however, these approaches currently require sophisticated instruments or data interpretation, rendering them unsuitable for shipboard use.

Measurements of total community activity provide relative estimates of living biomass (e.g., using FDA, as discussed in Welschmeyer and Maurer 2011), require less-sophisticated instruments and simplify the interpretation of results. The most rapid and simplest approaches are measuring community respiration, quantifying heat produced from metabolism, evaluating photosystem integrity, and detecting ATP. Of these approaches, variable fluorescence and ATP detection can generate measurements without the need to incubate or hold organisms. For these reasons, these approaches show the most promise for use in evaluating treated ballast water for compliance testing. However, fluorometry only detects a subset of the total population (i.e., phototrophs) and will not capture high concentrations of heterotrophs. The concentration of cellular ATP has been shown to increase in response to UV radiation (Villaverde et al. 1986). For these reasons, validation studies are needed for these (or any) approaches before they are used in compliance testing.

Validation

In addition to regrowth assays (e.g., Liebich et al. 2012), approaches directly measuring UV damage can be used to benchmark the performance of the rapid approaches. For example, the endonuclease sensitive site assay directly measures dimerization from UV light and can be used to track the repair of treated organisms (Oguma et al. 2002). Incorporation of synthetic nucleotides, which can be easily detected in DNA, is used to identify replication, and thus regrowth, at a molecular scale (Hamelik and Krishan 2009). Regardless of the approach, the validation process must consider the range of organism types and water characteristics of ballast water. A "geovalidation" will incorporate, at least, major water types (such as oligotrophic and hypereutrophic waters) and typical endpoints of the typical temperatures (4–35°C) and salinities (0–35 psu) encountered in the world's ports. A new suite of biodosimeters would be useful, as standard biodosimeters that are well-suited for evaluation of municipal water (e.g., Linden et al. 2002) are not relevant for ballast water testing.

Candidate approaches for continuous and automated analyses

The approaches described herein apply to discrete sampling and analysis. In the future, measuring viable or living organisms could potentially be integrated into the ship's piping system. Prior to this use, the approach must be simplified and automated. For example, "lab-on-a-chip" analyses allow for sophisticated routines of reagent mixing and fluid transferring (Liu et al. 2005; Tran et al. 2007). Generally, the future iterations of these tools must reduce the number of reagents required for analysis or simplify their use. For example, determining pH using pH-sensitive compounds embedded on litmus paper (rather than in solution, which requires careful titration) could be one of many models of analysis simplification. Ideally, approaches with minimal processing are ideal and are most adaptable to real-time, in-line monitoring of ballast water. Two techniques, variable fluorescence and microcalorimetry, meet the requirements of rapid analyses without the need for extensive processing or reagents. While variable fluorescence is limited to phototrophs, the production of heat is universal for all organisms. Approaches for rapid and high-throughput analysis must be developed before microcalorimetry is a practicable approach for compliance testing or in-line measurements.

Conclusions

The approach best suited to examine UV-treated organisms will: 1. Distinguish between living and dead organisms, and 2. Distinguish between living organisms that are capable of reproducing (viable) and those with irreversible damage (non-viable). Regrowth assays satisfy these requirements and have been used to evaluate UV-treated water (Liebich et al. 2012). Alternatives to regrowth assays, however, are necessary for rapid appraisals of the efficacy of UV treatment, and alternatives (or a combination of approaches) are needed to ensure that aquatic organisms in ballast water that are both living and viable can be quantified, as many aquatic organisms are not culturable.

Approaches described herein could be partitioned into two groups: one set includes approaches that detect proximal measures of biomass and viability; the other set includes direct counting approaches. The proximal measures require extensive validation to assure that readings are reflective of cell concentrations and that measurements, such as photosynthetic yield or respiration, are appropriate indicators of living organisms. The latter (direct measurements, such as the frequency of dividing cells) generally require more sophisticated equipment, reagents, and sample preparation. However, direct approaches produce measurements directly comparable to numerical standards (U.S. Coast Guard 2012). Regardless, the optimal approach will also consider ancillary factors, such as portability, cost, and ease of use. From all of these considerations, at present, there is no practicable approach for rapidly measuring the *viability* of UV-treated microorganisms in shipboard compliance testing of ballast water. Regarding the detection of *living* microorganisms, variable fluorescence and ATP detection appear to be the best candidates for shipboard compliance testing at present. The promise of automated approaches may be fulfilled after current approaches are simplified, new ones are developed, or both. Nonetheless, any successful tool for determining compliance with discharge standards during compliance testing must be rigorously validated and tested by independent parties with robust quality assurance programs, and tests must be conducted under conditions representative of shipboard environments.

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