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Research Article

Applicability of a cell proliferation assay to examine DNA concentration of UV- and chlorine-treated organisms

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Abstract

Ultraviolet radiation (UV) and total residual oxidant (TRO) treatments have been used to control microorganisms in drinking water, and are commonly used in ballast water management systems designed to reduce the transport and delivery of invasive organisms. Because treatments may not result in immediate cell death but may render organisms unable to reproduce, standard approaches to determine if a cell is living or dead (i.e., based on movement or cellular function) may not identify treated organisms. This scenario particularly applies to UV-treated cells that are rendered non-viable but still living. Thus, alternative approaches are needed to determine an organism's viability (ability to reproduce). This study evaluated a cell proliferation assay measuring DNA concentrations in 96-well plates to determine proliferation of UV- and chlorine-treated organisms. Treatments included UV doses of 300 and 800 mWs cm^{-2} or an applied ClO^- concentration of 10 mg L^{-1}. To determine optimal cell proliferation thresholds and detection limits, two phytoplankton species, *Tetraselmis marina* (UV and ClO^-) and *Prorocentrum micans* (ClO^-), were evaluated immediately following treatment and after 1-, 5-, and 14-day incubations in light or dark conditions. *T. marina* exhibited cell proliferation in all UV treatments, although no cell proliferation was detected for *T. marina* or *P. micans* in any ClO^- treatment. This type of assay allows for increased sample replication, smaller sample volumes, quicker preparation, and faster measurement than traditional growth assays. In conclusion, DNA staining assays may be useful to detect changes in cell proliferation over time in the context of ballast water testing.

Key words: most probable number, cell growth, ballast water

Introduction

The transfer of aquatic invasive species transported by ships' ballast water has resulted in global actions to reduce the transfer and delivery of these organisms. The International Maritime Organization (IMO) adopted the Ballast Water Management (BWM) Convention (IMO 2004). Now that the BWM Convention has entered into force, ships are required to meet a discharge standard prescribing the number of living organisms allowable

in ballast water. To meet this standard, ballast water management systems (BWMS) have been developed for installation onboard commercial vessels. They use combinations of technologies routinely employed for the treatment of drinking water and wastewater, most commonly filtration, electrochlorination, and UV radiation. Some of these approaches, used to treat ballast water, such as UV radiation, will not necessarily result in immediate cell death, but will render organisms unable to reproduce (although cells may still be living) (Oguma et al. 2001; First and Drake 2014), although regrowth of cells after treatment has been reported (Liebich et al. 2012; Stehouwer et al. 2015).

Notably, while UV can be effective by inducing DNA lesions, such as cyclobutane pyrimidine dimers (CPDs) and 6–4 photoproducts (6–4PPs) (Sinha and Häder 2002), some UV-treated organisms may be capable of recovering from exposure by various mechanisms, including direct repair by photolyases. These photolyases cause a break in the cyclobutane rings of pyrimidine dimers (Sancar and Sancar 1988; Sinha and Häder 2002; Liebich et al. 2012; Martinez et al. 2013). This mechanism, known as photoreactivation, has been seen in various organisms, including marine viruses (Weinbauer et al. 1997; Hester et al. 2000), coral zooxanthellae (Reef et al. 2009), zooplankton (Zagarese et al. 1997), and algae (Grad et al. 2003). Light-independent mechanisms of DNA repair have also been reported (Zagarese et al. 1997).

To address the capability of treatment to alter the viability of cells, alternative methods are needed to detect organisms that are living, but are unable to reproduce. One such method is the Most Probable Number (MPN) method (Cullen and MacIntyre 2016). This method is based on a bacterial MPN technique, which entails cell detection following serial dilutions. This approach, which was adapted for use with microalgae ≥ 10 and < 50 μm in size, requires numerous test tubes and an incubation time of 14 days. A more streamlined assay and a decrease in testing time would provide a quicker indication of treatment efficacy. Rapid testing for treatment efficacy is required to prevent ships from being delayed due to testing (Drake et al. 2014) including schedule reliability and increased service bill costs (Sun et al. 2015). Therefore, it is of interest to evaluate additional methods to determine viability that employ smaller sample volumes and shorter incubation times.

Currently in the U.S., land-based and ship-based validation testing of BWMS must follow the requirements of the Generic Protocol for the Verification of Ballast Water Treatment Technology (NSF International 2010) This protocol requires the analysis of organisms with a minimum dimension of ≥ 10 and < 50 μm (nominally protists) using a combination of two vital, fluorescent probes: fluorescein diacetate (FDA) and 5-chloromethylfluorescein diacetate (CMFDA). Enzyme-activated fluorophores have a long history of

marking eukaryotic cells (Rotman and Papermaster 1966). The probes accumulate within living organisms, where they are transformed by cellular enzymes, producing fluorescence that can be detected using epifluorescent microscopy. Fluorescing (or non-fluorescing, but moving) organisms are considered living. This microscopy-based method, described in Steinberg et al. (2011), targets organisms with intact enzymes and cell membranes, and it cannot differentiate between living organisms that are capable of reproducing and living organisms that have irreparable damage causing sterility. That is, living and non-viable organisms are indistinguishable following this method. This method was adopted as the required method for quantifying organisms to ensure treatments are effective to the level of the standards required for the number of living organisms allowable in discharge ballast water (USCG 2012).

Using cultured organisms and simulated ballast water treatment, this study used a cell proliferation well-plate assay—a highly sensitive fluorescence-based method normally used for quantifying cells and assessing cell proliferation and cytotoxicity—to examine DNA concentrations over time to assess the utility of cell proliferation assays for determining the effect of ballast water treatment on the viability of cells. The aspect of cellular damage— the potential for photo repair—was also assessed.

Materials and methods

Organisms

Cultures of two marine flagellates, *Tetraselmis marina* (Cienkowski) R.E. Norris, Hori and Chihara, 1980 (~ 12–15 µm) and *Prorocentrum micans* Ehrenberg, 1834 (~ 20–40 µm), obtained from The National Center for Marine Algae and Microbiota (NCMA, Bigelow Laboratory for Ocean Sciences; East Boothbay, ME), were cultured in 72 µmol quanta m^{-2} s^{-1} lighting at 24 °C under a 12:12 hour light:dark cycle. To maintain the cultures in the exponential growth phase, every two weeks, they were aseptically transferred to fresh media containing 0.22-µm filtered seawater (FSW) enriched with nutrients (Guillard's F/2; Guillard and Ryther 1962). Separate culture vessels (300 mL volumes) were used for each sample. Common ballast water standard test organisms, *Tetraselmis* sp. and *Prorocentrum* sp., were chosen for experimentation for robustness against UV treatment, and large cell size, respectively.

In preliminary trials, increased DNA concentrations were detected following treatment. To determine if they were due to increasing bacterial concentrations, three independent trials were conducted with *T. marina* and *P. micans* samples that were filtered through 5-µm nitrocellulose filters to reduce bacteria. Samples were incubated in the dark.

Experimental Design

Initial cell concentrations

For *T. marina*, samples were labeled with the two vital fluorescent probes, fluorescein diacetate (FDA) and 5-chloromethylfluorescein diacetate (CMFDA), and initial cell concentrations were determined via flow cytometry (BD Accuri C6, BD Biosciences; San Jose, CA) using light scattering (FTIC/GFP channel [533/30 nm]), and NIST-traceable microbeads for size reference (1–15 µm, Molecular Probes; Eugene, OR). For *P. micans*, epifluorescent microscope counts (Steinberg et al. 2011) were used to enumerate cells because the organism exceeded the flow cytometer's detection limit (≤ 30 µm particles). Cell counts for *T. marina* ranged from 8.8 ± 1117 × 10^3 to 113.0 ± 33,144 × 10^3 cells mL^{-1}, and counts for *P. micans* were 54.5 ± 16 cells mL^{-1}.

UV Treatment

UV treatment was performed as described in First and Drake (2014). Briefly, samples (30 mL) were placed in plastic Petri dishes (10 cm diameter, 1.5 cm deep, with lids removed) and situated in a chamber with a 254-nm wavelength, low-pressure, mercury lamp (UV Crosslinker; UVP, LLC; Upland, CA). A plastic cylinder (10 cm in height) placed on the rim of the Petri dish acted as a light collimator, decreasing indirect and scattered light. The intensity of UV light (300 or 800 mWs cm^{-2}) was measured with a radiometer (UVP, LLC; Upland, CA), and the dose was determined by the length of exposure time. UV intensities were chosen based on reported values used in ballast water treatment (Sassi et al. 2005; First and Drake 2014; Olsen et al. 2016). For each experiment, three independent samples (30 mL) were exposed, and each sample was transferred into a sterile, 50-mL centrifuge tube after UV treatment. Samples were allowed to acclimate for 30 min at room temperature and ambient light before dilution into 96-microwell plates.

Chlorine treatment

Samples were treated with chlorine by adding chlorinated seawater that was generated electrolytically. A current (~ 1 A) was impressed through platinum-clad titanium electrodes suspended in 1 L of FSW. After electrolysis, the concentration of oxidizing compounds (which included hypochlorite [ClO-], hypochlorous acid [HClO], as well as oxidizing bromides and chloramines) in the stock solution were measured with a Pocket Colorimeter™ II (HACH; Loveland, CO). The chlorinated water was added to samples to achieve an applied dose of 10 mg L^{-1} Total Residual Oxidant (chlorine). Chlorine dose was chosen based on reported effective values in ballast water treatment (First and Drake 2014). Samples were held

for 30 min at room temperature and ambient light before dilution into 96-microwell plates. To determine the chlorine demand of Guillard's F/2 media, chlorine concentrations were measured in three replicate trials by adding 10 mg L^{-1} to media without organisms. At an applied dose of 10 mg L^{-1}, the initial residual concentration was 7.3 mg L^{-1}, and after 30 min, the concentration was 6.0 mg L^{-1}. This indicated a chlorine demand of the media was 4.0 mg L^{-1} after 30 min.

Cell proliferation assay

CyQuant® (ThermoFisher Scientific; Waltham, MA) DNA assays in 96-well plates were used. From a UV- or chlorine-treated sample, a well-mixed aliquot was aspirated and dispensed into the first row of a black-bottomed 96 well-plate containing Guillard's F/2 media. From this row, a dilution series was performed by transferring the required amount of sample from a starting well to a receiving well with the appropriate volume of F/2 media within it. This dilution process was repeated 4 times with each sample, so there were 4 replicate wells for each dilution. Additionally, 4 replicate positive controls (untreated) and 4 replicate negative controls (only Guillard's F/2 media) were included within each plate.

Preliminary experiments (not shown) were conducted using sample dilutions to 0.25% of the initial cell concentration, but the results showed low detection at dilutions below 5%; thus, dilutions less than 5% of the initial concentrations were not used in successive experiments. An individual dilution plate was made for each incubation time period (0- [immediately after treatment], 1-, 5-, and 14-d). After initial experiments with 5-d incubation in lighted conditions, additional experiments were conducted using 14-d incubations in the dark. Dark, 14-d incubation experiments were conducted to simulate dark incubation in ballast holding tanks and 14-d MPN incubations. All plates were covered with transparent covers and incubated after dilutions were complete. Light-incubated plates were incubated with a 12:12, light:dark cycle at 24 °C for their respective incubation periods, while dark-incubated plates were stored in the dark at 24 °C. At the end of each incubation period, plates were centrifuged at 3000 rpm for 2 min, and media was removed following the CyQuant® protocol by blotting on laboratory wipes, and stored at −20 °C until all plates reached their incubation periods. Day 0 plates were immediately stored at −20 °C sans incubation.

Experimental design

Experiments were conducted as described in Table 1. Initial experiments to determine the effect of 300 mWs cm^{-2} UV dose on *T. marina* incubated in both light and dark conditions were conducted to assess possible photorepair of DNA damage that could lead to increased DNA concentrations.

Table 1. Treatments used within the experiments

		Applied Dose	Incubation	Filtration
T. marina	UV	300 mWs cm^{-2}	Light	No
		300 mWs cm^{-2}	Dark	No
		800 mWs cm^{-2}	Dark	No
	chlorine	10 mg L^{-1}	Dark	No
		10 mg L^{-1}	Dark	Yes
P. micans	chlorine	10 mg L^{-1}	Dark	Yes

For light-incubated plates, the light cycle remained the same as described above for culture conditions. Next, a dose of 800 mWs cm^{-2} UV with dark incubation was used to determine if an increased UV dose would affect *T. marina* DNA replication.

Experiments were conducted by applying a 10 mg L^{-1} chlorine treatment to either *T. marina* or *P. micans* and incubating samples in the dark. To reduce bacterial DNA, prior to incubation, samples were filtered through a 5-µm, 25-mm nitrocellulose filter to retain phytoplankton cells while allowing bacteria to pass through the filter. *P. micans* was used only in initial chlorine experiments with filtration because its larger cell dimensions ensured cells were retained on the sample filter. In addition, samples were visually examined with epifluorescence microscopy from filtered samples for comparison of CyQuant® measurements to physical cell presence.

Sample analyses

Plate-based assay

All plates were thawed at room temperature and light. Once thawed, 200 µL of 200-fold diluted CyQuant® GR dye/cell-lysis buffer was added to each sample well as suggested by the manufacturer. Plates were then incubated for 5 min at room temperature, protected from light. Fluorescence was measured using a microplate reader (Gemini XPS, Molecular Devices; Sunnyvale, CA) set with an excitation and emission maxima optimal for the CyQuant® GR dye (480/520 nm).

Data Analysis

The relative fluorescence unit (RFU) 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 between incubation days. When significant differences were found, a pair-wise, post-hoc test (Holm-Sidak method, $\alpha = 0.05$) was used to determine significant differences among days. All statistical analyses were performed using SigmaPlot (V13.0; Systat Software, Inc.; San Jose, CA).

Results

Based on RFUs, the mean DNA concentration of *T. marina* increased from Day 1 to Day 5 for all dilutions in trials using 300 mWs cm^{-2} UV dose with

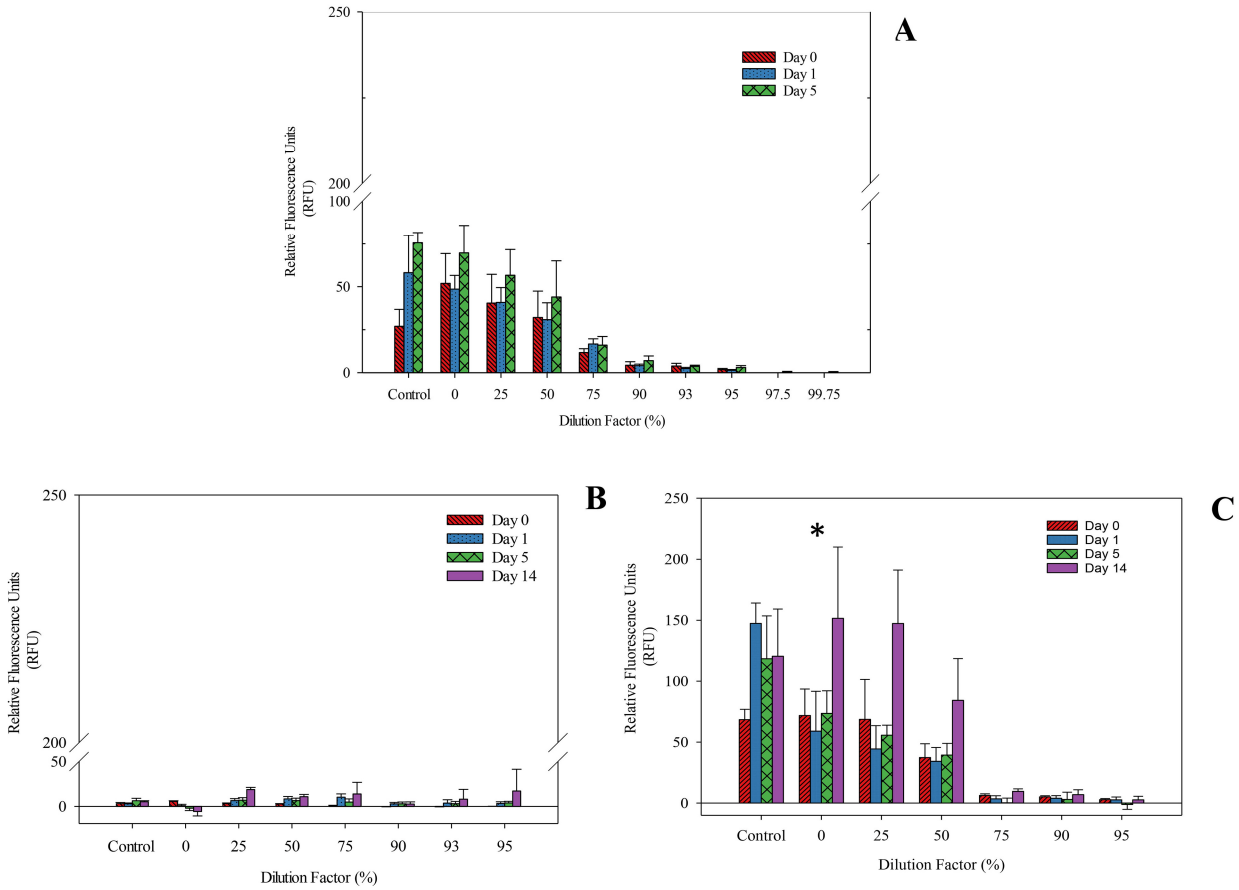

Figure 1. Relative fluorescence for *T. marina* with: A. 300 mWs cm^{-2} UV dose and light incubation over 5 days, B. 300 mWs cm^{-2} UV dose and dark incubation over 14 days, and C. 800 mWs cm^{-2} UV dose and dark incubation over 14 days. Controls are for a 25% dilution factor. Error bars represent the mean and one standard deviation of independent trials (n = 3). Asterisks indicate a significant difference between Day 0 and Day 14.

light incubation, although there were no significant differences between days for all dilutions ($p > 0.05$; Figure 1A). The same experiment was repeated, but plates were held in the dark for 14 days. In these samples, DNA concentration increased at Day 14 for all dilutions, excluding samples with no dilution, although no significant differences were found ($p > 0.05$; Figure 1B). To determine if a higher UV dose would prevent DNA replication, *T. marina* cells were exposed to a UV dose of 800 mWs cm^{-2}, with dark incubation, and they also showed an increased mean concentration of DNA for all dilutions at Day 14, yet only the 75% dilution showed a significant difference between Day 0 and Day 14 ($p < 0.05$; Figure 1C). Cell proliferation results are summarized in Table 2. Negative controls were also included in all treatments and had a RFU value of 0.487 ± 0.660.

To evaluate chlorine treatment, *T. marina* was treated with an applied dose of 10 mg L^{-1} chlorine and incubated in the dark. Mean fluorescence values in all dilutions increased over time with a decline on Day 14, although only the 50% dilution showed a significant difference between Day 0 and Day 14 ($p < 0.05$; Figure 2A). In an attempt to minimize bacterial DNA, an experiment with 3 replicate trials was then conducted

Table 2. Cell proliferation within each experiment

		Applied Dose	Incubation	Filtration	Significant differences Among Days at Any Dilution
T. marina	UV	300 mWs cm^{-2}	Light	No	No
		300 mWs cm^{-2}	Dark	No	No
		800 mWs cm^{-2}	Dark	No	Yes*
	chlorine	10 mg L^{-1}	Dark	No	Yes**
		10 mg L^{-1}	Dark	Yes	No
P. micans	chlorine	10 mg L^{-1}	Dark	Yes	No

*75% dilution
**50% dilution

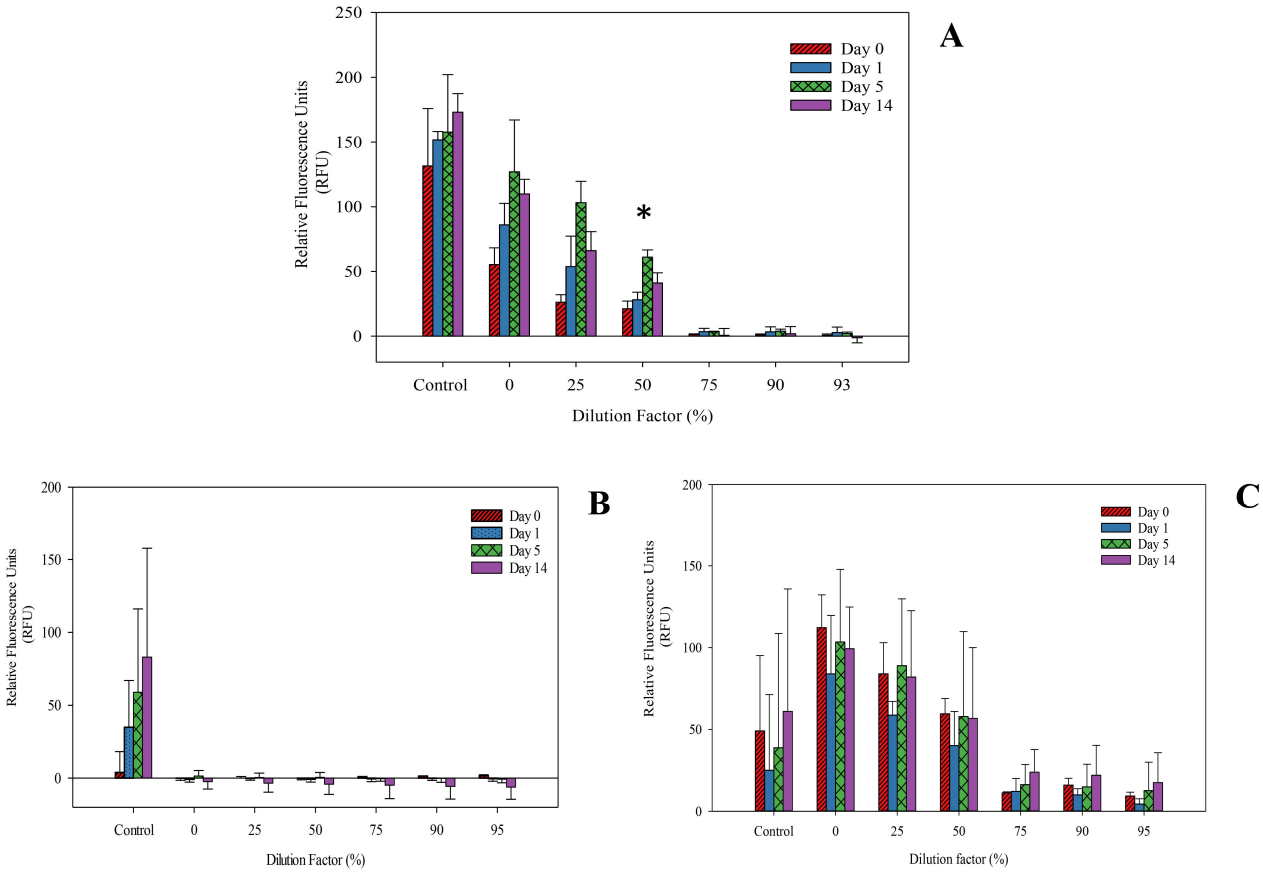

Figure 2. Relative fluorescence with 10 mg L^{-1} TRO treated samples with dark incubation over 14 days and A. *T. marina* with no filtration, B. *P. micans* with filtration, and C. *T. marina* with filtration. Controls are for a 25% dilution factor. Error bars represent the mean and one standard deviation of independent trials (n = 3). Asterisks indicate a significant difference between Day 0 and Day 14.

using *P. micans* filtered through a 5-µm nitrocellulose filter and dosed with an initial applied concentration of 10 mg L^{-1} chlorine. Here, mean DNA concentrations decreased to or at near zero for all dilutions (Figure 2B). For comparison, this treatment was repeated with *T. marina* cells, and DNA content did not significantly change over 14 days for all dilutions ($p > 0.05$; Figure 2C). Cell proliferation results are summarized in Table 2.

For experiments in which filtration was used to remove bacteria, samples were viewed under epifluorescence microscopy to compare CyQuant® measurements to visual cell concentrations. This evaluation

revealed that for *P. micans*, very few living cells were present at Day 0 and Day 1 in the treated samples. *T. marina* samples indicated population growth for all experimental days.

Discussion

Using the cell proliferation assay, the viability of treated cells was detectable. The two standard ballast water testing organisms illustrated plausible differences between treatment outcomes for different test species. Surprisingly, population maintenance or growth was evident in *T. marina* after UV treatment at two doses. On the other hand, a negative response was detected in *P. micans* for chlorine treatment, but not for *T. marina*. The cell proliferation assay performed similarly to the traditional MPN assay, but with the advantages of requiring smaller sample volumes and possibly shorter incubations.

We hypothesized that the higher dose of UV (800 mWs^{-1}) would prevent an increase in DNA concentration (population growth), but this was not the case for nearly all dilutions among days. Species adaptation, availability of abundant nutrients present in phytoplankton dilutions, and temperature within this study may have promoted UV repair leading to sustained and increased concentrations of DNA. This phenomenon has been shown in previous phytoplankton and zooplankton response studies, and UV repair mechanisms have been reported to increase with increasing temperature and decrease with limited nutrients (Hadar et al. 2015). Additionally, the unexpected increased DNA concentrations with increased UV dose may depend on varying tolerances of species due to adaptations and environmental conditions.

In this study, photoreactivation could have been attributed to increased mean DNA concentrations in tests incorporating light incubation (Figure 1A). Natural defense mechanisms can reduce cell-damaging effects of UV radiation (e.g., Ha et al. 2016), and because of this known response, additional testing was conducted with dark incubation. Although it was thought that performing testing with dark incubation would eliminate photoreactivation, unexpectedly, results nevertheless revealed an *increase* in DNA concentration after UV treatment. Response of organisms to UV treatment may be conditional to the robustness of the individual organisms being treated. This effect has been reported in previous studies showing UV to be ineffective in inactivating higher organisms and in totally eliminating dinoflagellates (Matousek et al. 2006). This is of particular importance for ballast water treatment that may require a holding time in dark ballast tanks in which cells may undergo repair even in the absence of light. Although the response to UV radiation may vary by treatment, the use of DNA assays to proximally measure cell proliferation may give insight to the efficacy of UV treatment, with reduced effort relative to traditional MPN assays.

Differing tolerances to chlorine between species have been previously reported (Sun et al. 2015). Chlorine exposure effects algal cells when chlorine penetrates and damages the cell membrane, which then allows for chlorine to enter the cytoplasm (Sun et al. 2015). After attacking the cell envelope, chlorine enters the cell, and intracellular components (including the photosynthetic apparatus), are disrupted (Sukenik et al. 1987). Some cells may contain exclusion mechanisms that would increase tolerance to chemical treatment (Hall et al. 1979). This, along with a decrease in available chlorine concentration over time, can lead to increased cell survival for certain organisms. Species present in mixed assemblages of organisms, such as those treated by ballast water treatment systems, may not all respond to chlorine treatment similarly.

As seen in this study, comparable results for each dilution series within each experimental treatment would allow for use of fewer dilutions in future testing. Results suggest that 100%, 75%, and 50% dilutions could be used in further experiments although most dilutions did not show a significant difference among days for different dilutions, those that did differ were contained within this range, suggesting that the removal of lower dilutions would not interfere with results. Although using fewer dilutions reduced the volume of sample needed and decreased experimental preparation time, while still allowing for representative dilutions and replication, additional testing with other species may be warranted. Using smaller sample sizes in a 96-well plate assay format is easy to use, as dilutions can be multichannel pipetted from well to well. Further, rapid measurement using a fluorescent plate reader drastically reduces the analysis time as compared to individual tube readings required by the traditional MPN method. This provides for high through-put readings for quick detection of changes of cell concentrations. In conclusion, DNA staining assays—such as CyQuant®—represent a promising approach to detect temporal changes in cell proliferation. This type of assay requires relatively small sample volumes, allows quick sample preparation, and facilitates rapid measurement. The ability to use this assay to determine significant growth over periods of time shorter than 14 days would greatly affect ballast water testing. Additional testing is justified for the applicability of a cell proliferation assay using ambient and mixed algal assemblages after UV and chlorine treatment, and for comparison to the traditional MPN assay.

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