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Algal DNA Repair Kinetics Support Culture-Based Enumeration for Validation of Ultraviolet Disinfection Ballast Water Treatment Systems

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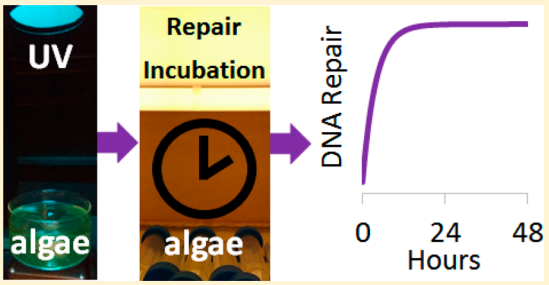
ABSTRACT: To mitigate the potential spread of invasive species and pathogens, the International Maritime Organization and U.S. Coast Guard (USCG) adopted discharge performance standards for ballast water management that will take full effect in 2017. The USCG requires staining methods for enumerating ballast water treatment system (BWTS) efficacy. These stains do not detect DNA damage, the primary mechanism for ultraviolet (UV) disinfection, and neglect potential DNA repair after discharge. These factors necessitate investigation of enumeration methods for accurate validation and approval of UV-based BWTSs. To molecularly assess DNA damage and repair kinetics, UV-induced DNA lesions were quantified by an enzyme-linked immunosorbent assay in *Tetraselmis suecica* control and UV-treated samples that were cultured and tested over time. Most DNA repair occurred within 6 h, was essentially complete within 24 h, and was insensitive to light or nutrient conditions during incubation. Asymptotic repair kinetics indicated a maximum of 67% of DNA damage inflicted by 300 mJ/cm^2 was repairable. These data provide a novel UV dose response for DNA damage in *T. suecica* and indicate that enzymatic DNA repair kinetics are not affected by culture conditions. Because DNA is rapidly photorepaired, culture-based enumeration can be used to accurately validate UV BWTSs.



INTRODUCTION

The International Maritime Organization (IMO)[1] and U.S. Coast Guard (USCG)[2] regulate discharge of ballast water used by ships to maintain balance with and without cargo. Intake and discharge of huge water volumes in divergent ports can transport pathogens or nonindigenous species (NIS). Size-delineated discharge performance standards that take full effect in 2017 were set by the IMO in 2004 and adopted by the USCG in 2012. Ballast water treatment systems (BWTSs) can be installed to mitigate NIS and pathogen transport and comply with new regulations.

IMO type approval can be obtained by demonstrating BWTS efficacy using various biological enumeration methods for each of the regulated criteria.[1] Approval by USCG, however, requires enumeration of organisms in the ≥10 to <50 μm size fraction (which is dominated by phytoplankton[3]) using "vital" stains FDA and CMFDA that detect esterase enzyme activity in cells with intact membranes.[2,4−6] These stains are appropriate for oxidative disinfectants that affect metabolism and membranes, but they cannot detect DNA damage, the primary mechanism of treatment by ultraviolet (UV) light.[7] Absorption of UV by DNA induces formation of cyclobutane pyrimidine dimers (CPDs) that inhibit reproduction.[8] Just as pathogens must reproduce to cause an infection,[9] multiplication of viable cells is necessary for NIS invasion.[3,10]

Because the UV dose required to inflict enzyme and membrane damage that can be detected by vital stains is much higher (up to 11 times)[7] than the dose necessary to render organisms unable to reproduce,[11] UV BWTSs have struggled to obtain USCG approval without the use of exorbitant doses.[10] This disconnection necessitates urgent investigation of accurate enumeration techniques. Besides FDA and CMFDA and other stains coupled with microscopy or flow cytometry,[5−7,11−14] other biological methods such as microscopic examination of morphology and motility,[11,15] active fluorescence measurements for photosynthetic state,[16,17] and ATP measurements for metabolic activity[15,18] have been suggested for enumerating phytoplankton but can differ by taxon and/or disinfection mechanism. Additionally, these methods may underestimate concentrations when they are applied to UV-treated discharge by neglecting possible growth after photorepair of CPD by the photolyase enzyme.[3,8,19−22]

Culture-based methods, however, are consistent with all treatment mechanisms and should provide a conservative assessment by allowing repair during incubation.[4,10,15,23−25] A serial dilution culture method known as most probable number (MPN) has been proposed to allow accurate enumeration of viable ≥10 to <50 μm phytoplankton in discharge water for

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various BWTS technologies.[10,24] In 2015, the USCG rejected MPN,[2] deeming it less protective because it measures viability (ability to reproduce) instead of vitality (ability to live)[26] and citing uncertainties about false negatives (nondetection of viable cells) and potential repair after UV treatment.[27] Neither stains nor MPN provides a complete assessment of living status, as both measure some but not all properties of life, and recent work shows neither method to be more susceptible to false negatives.[5,10,24,28] However, MPN should be equally protective for preventing NIS invasion, because neither nonviable nor nonvital organisms can successfully colonize an environment.[10] The contribution of viable cells that repair but may not resuscitate is poorly understood for any enumeration method.[29−31] However, culture-based methods that enumerate both undamaged and repairable cells should be more accurate than existing methods in BWTS validation. To molecularly test the hypothesis that culturing avoids underestimating the viable cell concentration by including DNA repair, *Tetraselmis suecica* (a phytoplankton species commonly used for BWTS studies)[7,11] were treated with UV, and DNA damage was measured over incubation time after UV treatment. Repair was compared for harsh (seawater only) and favorable (seawater with additional nutrients) culture conditions, over a range of light levels to simulate environmental discharge.

MATERIALS AND METHODS

Cultivation of Algae. *T. suecica* (CCMP 904) marine phytoplankton were cultured in 0.2 μm filter-sterilized artificial seawater (Crystal Sea) supplemented with "nutrients" (Guillard's f/2 Marine Water Enrichment Medium, Sigma-Aldrich) under visible light-emitting diodes (LEDs) emitting a photosynthetically active radiation (PAR) intensity of 100 μmol m^{-2} s^{-1} (LightScout Quantum light meter with a PAR sensor, Spectrum Technologies, Inc.). The relative spectral output (Maya USB 2000, Ocean Optics) of LEDs is shown in Figure 1.

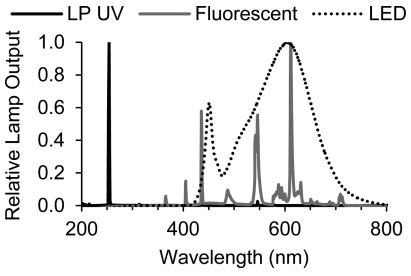

Figure 1. Relative lamp output of light sources used for UV irradiation (LP UV), cultivation of algae (LED), and MPN and repair incubation (Fluorescent).

For UV experiments, cells were cultured to a target concentration of ~60000 cells/mL. The UV absorbance at 254 nm (A_{254}) and pulse amplitude-modulated (PAM) fluorometry F_v measurements (Hach BW680) were used to estimate concentrations based on previous correlations with cell counts (Coulter Multisizer 4). Cells grown to this density had a PAM F_v/F_m ratio of 0.65 (indicating good health) and an A_{254} of 0.1 cm^{-1} in seawater with nutrients.

UV Irradiation and DNA Repair Incubations. For collimated low-pressure (LP) UV exposures, incident irradiance (IL1700 radiometer) was corrected for Petri (PF), water (WF), divergence (DF), and reflection factors (RF = 0.9750) to determine average irradiance, which was used to calculate exposure time to deliver a chosen dose to a well-mixed sample.[32] Though BWTSs utilize both LP and medium-pressure (MP) UV lamps (which can additionally damage proteins[33]), LP was used here to pinpoint DNA damage. To determine the DNA damage dose response of *T. suecica* to LP UV, samples of algae (A_{254} = 0.1013 cm^{-1}) were exposed to a range of doses from 0 to 400 mJ/cm^2. An incident irradiance of 0.294 mW/cm^2 resulted in an average irradiance of 0.139 mW/cm^2 after corrections (WF = 0.59; DF = 0.89; PF = 0.92). Analysis of DNA extracted from these samples immediately after UV irradiation indicated that a LP UV dose of 300 mJ/cm^2 induced sufficient DNA damage to assess repair in subsequent visible light incubations, without complete algal inactivation.

For UV exposure and repair experiments, algae were harvested on 0.5 μm nylon net filters (Millipore) by gravity filtration and resuspended in artificial seawater without nutrients. Absorbance was used to match cell density between the UV dose−response and repair experiments. Because nutrients added 0.02 cm^{-1} of absorbance, the target A_{254} in seawater without nutrients for UV exposures was 0.08 cm^{-1} (actual A_{254} = 0.0803 cm^{-1}). An incident irradiance of 0.224 mW/cm^2 resulted in an average irradiance of 0.127 mW/cm^2 after corrections (WF = 0.68; DF = 0.91; PF = 0.94). Samples were collected before and after UV irradiation for DNA analysis and MPN enumeration.

After the remaining UV-irradiated sample had been split and supplemented with equal volumes of either artificial seawater or nutrients (for harsh or favorable incubation conditions, respectively), 50 mL aliquots were distributed into polypropylene tubes for repair incubations under fluorescent lamps emitting in the active region (350−450 nm) of photolyase.[8,19,20] Different light intensities were achieved by varying the number of fluorescent lamps and the distance between samples and lamps. The relative spectral outputs of the fluorescent and LP UV lamps are shown in Figure 1. The 50 mL samples were incubated for 0.5, 3, 6, 24, or 48 h under fluorescent lamp intensities of 25, 50, 100, or 200 μmol m^{-2} s^{-1} and frozen rapidly at −80 °C after the specified incubation time.

MPN Enumeration. Samples of untreated and UV-treated algae were enumerated in duplicate by MPN.[10,24] For each sample, five replicate subsamples of serial 10-fold dilutions were incubated in media with nutrients under 100 μmol m^{-2} s^{-1} fluorescent lamps. Algal growth was measured by fluorescence (Promega Quantas fluorometer, red channel) after incubation for 7, 14, and 21 days. The subsample fluorescence was scored positive for growth when the increase between consecutive measurements exceeded 4 times the standard deviation of five replicate media blanks. Positive scores were used to calculate MPN.[34] Consecutive fluorescence measurements of positive subsamples were used to calculate exponential growth rates (n = 7 subsamples).

DNA Analyses. Frozen samples were thawed rapidly, and 10 mL duplicates were aliquoted and centrifuged at 4696g for 30 min to pellet algae. After the supernatant had been discarded, the pellet was suspended in 200 μL of 1× PBS, and DNA was extracted with a Qiagen DNeasy blood and tissue kit,[35] with an elution volume of 100 μL. DNA was quantified by Picogreen (Invitrogen) on a Quantus fluorometer (Promega) and by absorbance (Nanodrop 1000). CPD−DNA was quantified spectrophotometrically (Epoch, BioTek) using

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dimer-specific antibodies (OxiSelect UV-Induced DNA Damage ELISA Kit, Cell Biolabs, Inc.) without mass normalization.

RESULTS AND DISCUSSION

The dose-dependent CPD−DNA damage concentration in LP UV-treated algae is shown in Figure 2. Total DNA measured by

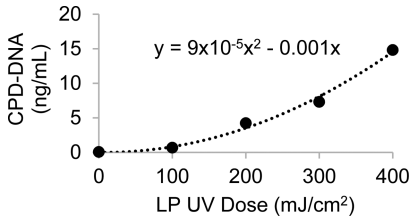

Figure 2. CPD−DNA damage concentration in UV-treated algae and the fitted dose−response equation (R^2 = 0.99).

picogreen (3.0 ± 0.5 ng/μL) did not vary with dose and was near the optimal enzyme-linked immunosorbent assay (ELISA) loading value of 4 ng/μL, negating the need for mass normalization. At 300 mJ/cm^2, CPD−DNA comprised 0.24% of the total DNA, or approximately 10^9 dimers per cell, assuming a genome mass of 0.7 pg and a base pair mass of 650 Da or 1.67×10^{-24} g.[35,36] Shielding by the high lipid content of these algae could have contributed to slight shouldering at the smallest doses.

For the repair study, the total DNA concentration was measured by both Nanodrop absorbance and Picogreen fluorescence for all samples to ensure accuracy of comparisons between longitudinal samples for CPD−DNA data, by verifying constant total DNA and equal ELISA mass loadings. DNA was quantified by both methods because the effect of UV treatment and DNA damage on these quantification mechanisms is not known. Untreated control and UV-treated sample DNA concentrations (n = 2 for each) did not differ by nanodrop (t test p = 0.39) or picogreen (t test p = 0.08), indicating that DNA structural changes did not affect quantification for either method. By the more specific picogreen assay, neither nutrients [analysis of variance (ANOVA) p = 0.54, and n = 20 each], time (ANOVA p = 0.40; n = 8 each), nor light (ANOVA p = 0.21; n = 10 each) affected the DNA concentration (average = 1.7 ± 0.6 ng/μL), validating ELISA results and comparisons between samples. The concentrations of viable cells determined by MPN in untreated and UV-treated samples were 29000 and 0.20 cells/mL, respectively, resulting in 5.2 log reduction by 300 mJ/cm^2 LP UV and an apparent UV resistance of 58 mJ/cm^2 per log. This is consistent with the results of recent culture-based studies in which no growth was detected either 21 days after irradiating ~10^4 *T. suecica* cells per milliliter with 400 mJ/cm^2 MP UV[11] or 7 days after irradiating ~10^3 cells of another *Tetraselmis* species per milliliter with 500 mJ/cm^2 LP UV.[15] On the basis of untreated MPN and assuming a genome mass of 0.7 pg,[35] the DNA extraction efficiency was 85%.

No CPD−DNA was detected in the repair study for the untreated sample of algae. To demonstrate DNA repair kinetics in 300 mJ/cm^2 LP UV-treated samples, relative CPD−DNA (R_{CPD}) was calculated for each time point and condition as the ratio of CPD−DNA in the repair-incubated sample to CPD−DNA in the time zero UV-treated sample. Figure 3 shows the average relative CPD−DNA concentrations over time in UV-treated samples incubated (a) in seawater with and without nutrients and (b) at each light level. As shown by overlapping

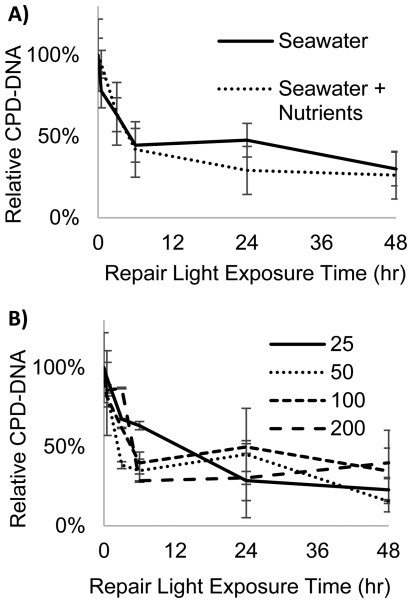

Figure 3. Average CPD−DNA damage concentration in UV-treated relative to unexposed algae by (A) nutrient condition or (B) fluorescent lamp PAR light intensity (micromoles per square meter per second). After results of duplicate samples had been averaged, standard deviations shown by error bars were calculated for (A) all light levels or (B) both nutrient conditions.

standard deviations, neither nutrient condition nor light level affected relative CPD−DNA concentrations.

The accuracy of culture-based enumeration requires detection of all viable cells, including those that are damaged and have an initial lag time to repair and regain the ability to reproduce. This requires monitoring for a sufficient time for a subsample with a single viable cell to grow to the detection limit. For exponentially growing cells, at a given time (t, in hours), the number of cells (N_t) growing in a subsample can be expressed as a function of the initial cell number (N_0), growth rate (k, in inverse hours), and lag time for a damaged cell to repair (l, in hours) (eq 1).

$$N_t = N_0 e^{k(t-l)} \quad (1)$$

On the basis of fluorescence data for untreated algae (where l = 0), growth rates ranged from 0.028 to 0.042 h^{-1}, which concur with those of another *Tetraselmis* species with doubling times of 24−48 h.[37] At the slowest growth rate, it would take 82 h (3.4 days) for each subsequent 10-fold diluted subsample to reach the detection limit.

The same calculation can be applied to UV-treated cells to determine the additional incubation time required for accurate detection of damaged cells. Lag times for individual cells can vary after UV treatment because some cells are more damaged than others, some cells can reproduce before complete DNA repair, and the DNA of some cells cannot be repaired. Algal CPD−DNA repair kinetic data were modeled to determine the maximal lag time in UV-treated samples. The relative amount of CPD-DNA (R_{CPD}) versus incubation time (t, in hours) for UV-treated algae was modeled (root-mean-square error of 0.23) by exponential decay (eq 2).

$$R_{CPD} = 0.33 + 0.62e^{-0.26t} \quad (2)$$

The asymptote of this model represents the minimum expected R_{CPD} (33%, with a 95% confidence interval of 25−

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41%) or the maximum capacity for CPD−DNA repair. On the basis of this expected limit of DNA repair capacity, 87% of repairable CPD−DNA inflicted by 300 mJ/cm^2 LP UV was repaired in the first 6 h of incubation, and repair was essentially complete within 24 h. Therefore, a conservative estimate of maximum lag time for UV-treated cells is l = 24 h. A similar lag was reported for complete restoration of photosynthetic activity in *T. suecica* 24 h after UVB exposure.[38] This lag time would increase the time required to ensure no further detection of growth in subsequent subsample dilutions to 106 h (4.4 days). In untreated and UV-treated samples, the most dilute subsamples that scored positive did so by day 14, and no subsamples of greater dilution showed growth in the following 7 days. Thus, 14 days was sufficient to detect growth of the most dilute subsamples containing viable cells, and the additional 7 days verified this. All viable cells (both undamaged and repairable) were detected by day 14, confirming that cell repair and growth were conservatively modeled. These repair and growth models are supported by studies of other algae, in which growth rates after UV treatment were equal to those of control samples after an initial lag.[12,25]

By the principle of dose reciprocity,[39] equal doses of light delivered in different combinations of time and intensity cause equal effects. Algal CPD−DNA repair did not differ with repair light intensity, indicating that repair occurred more rapidly or was affected less significantly than could be detected. Alternatively, a lack of dose reciprocity suggests that R_{CPD} kinetics were driven primarily by enzymatic interactions between photolyase and CPD−DNA, where light was not the limiting factor.[8,19,40] Photolyase enzymatic reactions are fast (<1 ns) and efficient (quantum yield of ≈1),[40,41] causing photorepair to be much more rapid than dark repair mechanisms.[25] Additionally, because nutrients had no effect on R_{CPD} kinetics, these data are consistent with repair rates being determined by enzymatic reaction rates, rather than biological growth rates. This indicates that photorepair would be unaffected by environmental conditions (light or nutrients) or taxon-specific conditions (growth rate and/or phase).

Because photorepair of UV damage occurs rapidly within incubations, culture-based enumeration of treated samples is accurate, fair to UV and other BWTSs, and environmentally protective. This information supports recent efforts[15−17,42−44] to assess enumeration techniques that could be appropriate for UV and other BWTS, allowing faster approval and adoption within the U.S. to comply with increased regulatory stringency.

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Notes

The authors declare no competing financial interest.

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