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UV fluences required for compliance with ballast water discharge standards using two approved methods for algal viability assessment

Kim Lundgreen[a,*], Henrik Holbech[a], Knud Ladegaard Pedersen[a], Gitte Ingelise Petersen[b], Rune Røjgaard Andreasen[c], Christaline George[d], Guillaume Drillet[d,e], Martin Andersen[f]

[a] Department of Biology, University of Southern Denmark, 5230 Odense M, Denmark
[b] DHI, Agern Allé 5, 2970 Hørsholm, Denmark
[c] Danish Technological Institute, Kongsvang Allé 29, 8000 Aarhus, Denmark
[d] DHI-NTU Research Centre and Education Hub, 1 CleanTech Loop, #03-05 CleanTech1, 637141, Singapore
[e] SGS-Singapore, 3 Toh Tuck Link, 596228, Singapore[1]
[f] BW-Scaling ApS, Ystadvej 2, 8600 Silkeborg, Denmark

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ABSTRACT

This study investigates the extra UV fluence needed to meet the International Maritime Organisation's ballast water discharge standards for the 10–50 µm size-class using the approved vital stain (VS) method compared to the Most Probable Number (MPN) method for organism viability assessment. Low- and medium pressure UV collimated beam treatments were applied to natural algae collected in temperate and tropical water environments and analysed using both methods. About 10 times higher UV fluence was required to meet discharge standards when using VS compared to MPN. Implementing a dark-hold period after UV treatments decreased algal viability. Length of dark-hold period to meet discharge standards decreased with increasing UV fluence. No significant differences between temperate and tropical samples were observed. The results showed that UV treated algae assessed using the VS method could meet discharge standards by increasing fluence and/or introducing a dark-hold period.

1. Introduction

To protect aquatic ecosystems and human health and to reduce economical expenses from the impact of invasive species, international guidelines on ballast water management were developed in the 1990s eventually resulting in *The International Convention for the Control and Management of Ships' Ballast Water and Sediments* (BWM Convention) adopted by the International Maritime Organisation (IMO) in 2004 (IMO, 2004) and which entered into force in 2017. The convention states that all ships must manage their ballast water using ballast water management systems (BWMS). The BWMS must be type approved in accordance with the guidelines G-8, and guidelines G-9 if the BWMS is using active substances (IMO, 2008, IMO, 2016a, 2016b). The performance of BWMS must comply with set discharge standards related to the number of viable organisms in defined size-classes. For the 10–50 µm size-class, which mainly consists of phytoplankton, the discharge standard is < 10 organisms ml^{-1}. BWMS use different methods to kill organisms in ballast water: Some systems use biocidal compounds and others treat water using electrolysis or UV irradiation combined with a physical solid-liquid separation process such as filtration (Echardt and Kornmueller, 2009; www.imo.org, 2017). UV treatment systems have already been installed in large numbers and it is estimated that > 50% of the BWMS will be based on this technology in the future (mpnballastwaterfacts.com, 2017; www.imo.org, 2017). Two types of UV treatment can be applied: A monochromatic system approach with low pressure (LP) mercury lamps emitting UV irradiation within the UVC (germicidal) range (100–280 nm). Approximately 85% of the power output is concentrated at 253.7 nm where it specifically affects the integrity of DNA and RNA in the cells that show maximum absorption at around 260 nm (Ben Said et al., 2015; Sun and Blatchley 3rd, 2017). In a polychromatic system approach, medium pressure (MP) lamps emit UV light at a higher total intensity with the energy distributed at a broader spectrum (Kalisvaart, 2004). DNA is still affected at 253.7 nm, but at a lesser degree compared to LP UV systems (about 2.7% of the total energy between 200 and 300 nm was emitted at 253.7 nm in our system). However, a broader energy spectrum in MP

* Corresponding author.
E-mail address: klundgreen@biology.sdu.dk (K. Lundgreen).
[1] (new affiliation)

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UV systems facilitate damage on other essential components in the organism. Enzyme-based repair systems are affected at around 280 nm (about 5.7% of total energy in our system) resulting in a higher inactivation impact as shown in *E. coli* (Bowker et al., 2011), adenovirus (Eischeid and Linden, 2011) and phytoplankton (Buma et al., 1996; Liu et al., 2016; Hull et al., 2017; Sun and Blatchley 3rd, 2017). Compared to other types of BWMS, the biocidal effects of UV-based systems are typically delayed in time. The damage on DNA and RNA inhibits polymerases thereby hampering vital cellular functions, replication and eventually causes cell death (Goodsell, 2001, Oguma et al., 2002, Kalisvaart, 2004, reviewed by Rastogi et al., 2010). When assessing the efficiency of UV treatments, damage limited to DNA is hard to document using the vital stain (VS) method approved by IMO and US Coast Guard (USCG). The method is based on fluorescence of CMFDA/FDA-stains activated by unspecific enzyme activity in cells allowing quantification of fluorescent organisms (i.e. organisms with esterase activity and intact cell membrane). The VS method is the standard method to count live organisms (Steinberg et al., 2011) and the assessment must be carried out within 6 h of the discharge, at which time the UV treated cells may still be fluorescent, though eventually dying. Instead, the delayed effects of UV treatments can be assessed using the Most Probable Number (MPN) method. This method is based on 14 days regrowth of viable organisms where a serial dilution approach helps to provide a quantitative estimation of viable cells present in the original undiluted, treated ballast water sample (Throndsen, 1978; Cullen and MacIntyre, 2016; MacIntyre et al., 2017). In 2015, the USCG rejected the use of MPN assays (Professionals, 2016) because, according to their interpretation, the regulations specifically require BWMS to be evaluated based on their ability to *kill* organisms which is not directly assessed by the MPN method. This paper addresses the problem aroused by the USCG interpretation of viability by investigating a simple solution: Can LP and MP UV systems meet discharge standards when assessed using the VS method by increasing the UV fluence (dose)? And if so, how much additional fluence is needed for compliance? To answer this, natural sea water samples of temperate and tropical origin were treated with different UV fluences delivered by LP and MP collimated beam systems. The acute and long-term effects of the treatments were assessed by the VS method and the MPN method.

2. Materials & methods

2.1. Test organisms and preparation for experiments

LP and MP UV treatments were performed in a collimated beam system (section 2.4) on natural phytoplankton compositions collected at the DHI Ballast Water Centre - Denmark and DHI Ballast Water Centre - Singapore. Experimental conditions are summarised in Table 1.

Natural seawater was collected in Denmark (TEMP) or Singapore (TROP) approximately 20 h prior to experiments and transported to the laboratories where it was filtered (35 μm filter) to remove zooplankton although a small fraction of microzooplankton (*Ciliophora* sp.) still remained in a few of the samples (Fig. 2). If phytoplankton concentration was too high, test water was diluted with natural GF/C filtrated seawater from the same location or, if the concentration was too low, local seawater was added from nutrient enriched grow-out pools with high phytoplankton concentration. The final concentrations ranged from 1500 to 4000 organisms ml^{-1} in the 10–50 μm size range which was assessed using the VS method. Test water was kept on a magnetic stirrer and stirred slowly with a continuous flow of filtered air without any growth light throughout the experimental periods. Lignin sulfonate was added to the test water to obtain a desired UV Transmittance (UVT) of about 80%. Prior to experiments, a sample of test water was fixed with Lugol for taxonomic identification and species distribution (Fig. 2). For LP-TROP-2 salinity was adjusted slowly (hours) from 23.0 to 19.5 psu which did not affect the viability. For details on the sampling locations see Supporting Information (SI) 1.

2.2. Quantification of algal cells

The VS method was used to quantify the number of fluorescent organisms within the 10–50 μm size class (NSF International, 2010) using Fluorescein diacetate (FDA, Molecular Probes-Invitrogen Carlsbad, CA, USA) and 5-chloromethylfluorescein diacetate (CMFDA, Molecular Probes-Invitrogen Carlsbad, CA, USA). Organisms containing tentacles or other types of shapes that prevent them from passing through a 10 μm grid are considered ≥10 μm whereas long and slender organisms that theoretically can pass a 10 μm grid are not evaluated as ≥10 μm in minimum dimension. Stained samples of 1.0 ml were transferred to a Sedgewick Rafter Counting Chamber and counted using an inverted microscope (Olympus CKX53) with a 10× objective. An organism was classified as living if it displayed mobility and/or bright green fluorescence following the procedures described in Steinberg et al. (2011) and IMO (2016a, 2016b).

The MPN was calculated as described by Throndsen (1978) (method use reviewed by Cullen and MacIntyre, 2016). For each sample, two MPN matrices were prepared in sterilized test tubes. Each matrix consisted of three 10-fold dilutions in 19–20 ppt Keller Growth Medium (Keller et al., 1987) with five replicates per dilution. The initial fluorescence was determined in each test tube using a fluorometer (Turner TD-700 Laboratory Fluorometer). After incubation for 14 days in an incubator/rotary shaker under continuous white light (6000–10,000 lx) at 19 °C, the fluorescence was measured again. Test tubes with fluorescence measurements at least four times larger than the standard deviation of the five initial measurements were considered positive (IMO, 2016a, 2016b). The number of positive tubes from each dilution series are then used in the MPN calculation to determine the number of viable organisms present in the parent sample. For LP-TROP-1, LP-TROP-2 and LP-TROP-3 only one matrix was analysed.

2.3. Experimental design and exposure procedures

Organisms were exposed to LP or MP UV treatments. Fluences were aimed to be 0, 25, 50, 100, 200, 500 or 1000 mJ cm^{-2} by adjusting time of exposure relative to the determined fluence rate (intensity) of

Table 1
Experimental conditions of performed LP or MP UV treatment experiments on natural phytoplankton compositions from temperate or tropical climates. Test water algal concentration ($[Algal]_{TW}$) in the 10–50 μm size range was assessed using the VS method. TW = test water.

Experiment reference	UV type	Climate	$[Algal]_{TW}$ (org. ml^{-1})	T_{TW} (°C)	$T_{dark\text{-}hold}$ (°C)	Salinity (psu)	UV Transmittance (%)
LP-TEMP-1	LP	Temperate	1873 ± 89	20.3	20.1 ± 0.5	19.1	77.0
LP-TEMP-2	LP	Temperate	1667 ± 243	20.3	20.3 ± 0.3	18.6	68.2
MP-TEMP-1	MP	Temperate	3296 ± 221	18.4	18.5 ± 0.5	17.6	79.7
MP-TEMP-2	MP	Temperate	3064 ± 61	18.4	18.5 ± 0.5	18.6	79.6
LP-TROP-1	LP	Tropical	2954 ± 360	24.6	27.0 ± 0.9	18.6	78.8
LP-TROP-2	LP	Tropical	2999 ± 345	24.6	26.9 ± 0.6	19.3	80.2
LP-TROP-3	LP	Tropical	1467 ± 472	26.3	25.6 ± 0.7	19.4	79.2