UV treatment of ballast water – breaking down the barrier for implementation

Report
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Approved by

17/07/2019

Signed by: Gitte Petersen
UV treatment of ballast water
– breaking down the barrier for implementation

Report

Prepared for Den Danske Maritime Fond
Represented by Mr. Peter Karlshøj, Projektleder

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in Hundested, Denmark

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<th>Martin Andersen</th>
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<td>Project manager</td>
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## FIGURES

- **Figure 1**: Outline of low pressure (LP) and medium pressure (MP) UV collimated beam systems. The emitted UV light passed through a collimating tube resulting in an even vertical UV radiation reaching the petri dish containing the test water sample. The petri dish contained a small magnetic bar and was placed on a magnetic stirrer to keep the test sample well mixed.
- **Figure 2**: Experimental timeline: A sample was collected from the test water and either A) analysed without any UV fluence (control samples), stored in dark conditions for 24 hours at ambient temperature and re-analysed or B) exposed to LP or MP UV treatments, stored in dark conditions for 24 hours at ambient temperature, second UV treatment and analysed.
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Figure 3 Class and species composition of test water used in fluence-response UV treatment experiments in Denmark (TEMP) and Singapore (TROP). Unid. = Unidentified.

Figure 4 Algal inactivation as a function of UV effect. (A) Standard Test Organism (STOs) and (B) Natural algal populations quantification evaluated by the Vital Stain method. (B) STOs and (C) Natural algal populations evaluated using the MPN method. LP = Low pressures UV, MP = Medium pressure UV, TET = Tetraselmis suecica, ODO = Odontella sp., TEMP = Temperate algal population, TROP = Tropical algal population. Punctures line signifies the discharge standards for the size group 10-50 µm which corresponds <10 org. ml⁻¹. Data from Lundgreen et al. (2018a) and Lundgreen et al. (2018b).

Table 1 Test repetitions carried out as combination of low/medium pressure UV treatment, climatic conditions and test organisms.

Table 2 Standard test organisms (STOs) or natural algal mix ranked according to the estimated fluence (F_D₂, mJ cm⁻²) needed for concentrations to meet discharge standards after UV treatments. Data from Lundgreen et al. (2018b) (Table 4 herein).

APPENDICES

APPENDIX A
Paper 1 – Lundgreen et al. 2018a

APPENDIX B
Paper 2 – Lundgreen et al. 2018b

APPENDIX C
MEPC paper (73/INF.20)
## Abbreviations

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<th>Abbreviation</th>
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<tr>
<td>BWMS</td>
<td>Ballast water management system</td>
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<tr>
<td>CMFDA</td>
<td>Chloromethylfluorescein diacetate</td>
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<td>ETV</td>
<td>US-EPA, Environmental Technology Verification Program</td>
</tr>
<tr>
<td>FDA</td>
<td>Fluorescein diacetate</td>
</tr>
<tr>
<td>IMO</td>
<td>International Maritime Organization</td>
</tr>
<tr>
<td>LED</td>
<td>Light Emitting Diode</td>
</tr>
<tr>
<td>LP</td>
<td>Low pressure UV</td>
</tr>
<tr>
<td>MP</td>
<td>Medium pressure UV</td>
</tr>
<tr>
<td>MPN</td>
<td>Most Probable Number</td>
</tr>
<tr>
<td>PSU</td>
<td>Practical salinity units</td>
</tr>
<tr>
<td>SOP</td>
<td>Standard operating procedure</td>
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<tr>
<td>SD</td>
<td>Standard deviation</td>
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<td>STO</td>
<td>Standard test organisms</td>
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<tr>
<td>USCG</td>
<td>United States Coast Guard</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>UV-T</td>
<td>Ultraviolet transmittance</td>
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<tr>
<td>VS</td>
<td>Vital Stain</td>
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1 Executive summary

2 DHI

DHI is an independent, international consulting and research organisation established in Denmark and today represented in all regions of the world with a total of more than 1,000 employees. Our objectives are to advance technological development, governance and competence in the fields of water, environment and health. DHI works with governmental agencies and authorities, contractors, consultants and numerous industries.

DHI provides independent performance evaluation of ballast water management systems (BWMS) for the type approval process. DHI has no involvement, intellectual or financial, in the mechanics, design or marketing of the products and technologies that are being evaluated. To ensure that DHI’s tests are uncompromised by any real or perceived individual or team bias relative to test outcomes, DHI’s test activities are subject to rigorous quality assurance (QA), quality control (QC) and documentation. DHI’s quality management system is certified according to ISO 9001. The certification is facilitated by the implementation of the DHI Business Management System.

DHI is conducting biological performance evaluation tests of ballast water management systems (BWMS) in accordance with the following rules, standards and guidelines:

- International Maritime Organization (IMO). International Convention for the Control and Management of Ships’ Ballast Water and Sediments
- International Maritime Organization (IMO). 2016 Guidelines for Approval of Ballast Water Management Systems (G8). Resolution MEPC.279(70)
- Marine Environment Protection Committee (MEPC). Procedure for Approval of Ballast Water Management Systems that Make Use of Active Substances (G9). Resolution MEPC.169(57)
- United States Coast Guard. Standards for Living Organisms in Ships’ Ballast Water Discharged in U.S. Waters
- United States Environmental Protection Agency, Environmental Technology Verification Program. Generic Protocol for the Verification of Ballast Water Treatment Technology

3 Objective

The objective of the current project was to confirm or disconfirm the following hypotheses and questions currently prevailing among political, scientific and commercial stakeholders:

Q1) Is it possible for UV-based systems to obtain type approval in all relevant water qualities using the Vital Stain (VS) method as outlined by the USCG?
   a. How much additional UV effect is necessary to be in line with discharge standards compared to the use of the Most Probable Number (MPN) method which is accepted by IMO
   b. Is there in connections with this a difference between UV systems using medium pressure and low pressure UV-lamps.

Q2) Does the use of standard test organisms (STOs) in test water reduce the sensitivity of the testing procedures?
a. And is this potential difference significant and quantifiable?

Q3) Is there a difference in the sensitivity between organisms from temperate and tropical climates?

- And is this potential difference significant and quantifiable?

Q1) addresses the challenge that the industry is facing after the preliminary rejection of the use of the MPN method by the US Coast Guard which many Type Approvals are based on.

Q2) and Q3) addresses two of the most debated issues in relation to the revisions of the guidelines for testing ballast water treatment systems (G8) where discussions have been carried out without any officially available unbiased data to support the outlined questions.

4 Description of the technological and biological related issues

Test procedures and methods involved in type approval of ballast water management systems (BWMS) using UV technology has been questioned. As a result, this has created uncertainty in the business and it has become unclear for shipping companies which treatment technologies to trust and to choose. Currently more than 50 % of the available treatment systems on the market are based on UV technology. Many shipping companies already have this type of treatment system installed or ordered for installation. The technology is simple to use and does not use any type of chemicals. Consequently, it is environmentally desirable, safe to use for the crew and relatively energy efficient. Thus, the overall purpose of the current project was to provide clarifying data and answers to stakeholders to address a number of presumptions and prejudices that mark the ongoing discussion regarding the use of UV technology for treatment of ballast water.

The purpose of the experimental studies was to compare the UV dose requirements for low pressure (LP) and medium pressure (MP) UV systems to meet the D-2 discharge standard for organisms in the 10-50 µm size class - comparing natural assemblages from different climatic zones (Denmark and Singapore) with possible relevant standard test organism (STOs) cultured in laboratories in the respective countries, namely *Tetraselmis suecica* and *Odontella* sp. Inactivation was evaluated using the vital stain (VS) method that assess at the immediate effects of the UV treatments and the Most Probable Number (MPN) method that evaluate the long-term effects of the UV treatments.

4.1 UV-based ballast water treatment systems

Currently there are three kinds of type approved UV-based BWMS on the market:

1. Low pressure (LP)
2. Medium pressure (MP)
3. Light Emitting Diode (LED)

Medium pressure systems dominate the market with 75 % of the systems using its polychromatic approach followed by monochromatic LP systems (21 %) and LED systems (4 %).

Monochromatic LP systems are characterized by a single high intensity energy peak. Almost all the UV energy is being focused around the wavelength 253.7 nm. The main
cellular target for LP systems is DNA which has a maximum absorption capacity around 260-265 nm. The polychromatic MP systems is characterized by having multiple energy peaks and the energy is therefore distributed over the range 185-400 nm. In addition to DNA, other vital cellular components are affected by the UV irradiation. LED systems were not part of this study and are therefore not discussed.

The present study was looking into potential differences between LP and MP UV treatments when assessing organism inactivation caused by the UV treatments.

4.2 Standard quantification methods used for validation of systems (Q1)

UV-based systems damage the DNA of organisms that hamper vital cellular functions, replication and eventually causes cell death. Compared to other types of treatment technologies the biocidal effects caused by UV treatment are delayed in time. Consequently, this delay results in very different outcomes when assessment of the UV treatment efficacy is carried out using either the Vital Stain (VS) method or the MPN method to quantify the number of organism in the size range 10-50 µm. Both methods are standard methods approved and used by the IMO to assess the efficacy of BWMS.

The VS method is based on fluorescent stains (FDA/CMFDA) that enter the cells. Enzyme activity inside the living cells will activate the stains which subsequently allow quantification of the fluorescent cells using microscopy. Dead cells have no enzyme activity and are not counted since they will not be able to activate the fluorescent stains. The VS method must be carried out within six hours of treatment at which time the decisive effects caused by the UV treatment may not yet be visible. As a result, UV treated cells still appear as living (fluorescing) and must therefore be quantified although cells are actually dying making them harmless as they pose no risk of invasion.

Instead, the delayed effects of UV treatments can be detected using the MPN method that is based on regrowth of viable organisms. The assay is evaluated after 14 days and quantification of algal cells is estimated from a series of dilutions that helps to enumerate number of viable algal cells present in the original undiluted sample.

For proper evaluation of UV treatment systems, the MPN method is suitable. If evaluated using the VS method, the UV treatment system would be more conservatively assessed as the method is not able to detect the delayed effects of UV treatments at UV doses sufficient to achieve an effect on MPN. The IMO approves the use of both methods to evaluate the efficacy of BWMS, but since 2015 the USCG only approves the use of the VS method. In contrary to the IMO, that base their evaluation on the ability of BWMS to render organisms non-viable, the USCG base their assessment on the ability of BWMS to kill organisms.

The present study investigated the additional UV effect needed to for LP and MP systems to kill organisms to the extent that concentrations satisfied discharge standards (<10 org. ml⁻¹) when evaluated with VS method related to the UV effects needed when evaluated using the MPN method.

4.3 Use of Standard Test Organisms for sound validation of UV-based treatment systems (Q2)

To properly challenge BWMS during Type Approval testing, concentrations of organisms in the size class ≥10-<50 µm must reach a minimum concentration of 1000 org. ml⁻¹ in
intake water. Often test facilities cannot realize intake concentration requirements using only natural algal populations from local waters. To be able to satisfy the intake concentration requirements the addition of cultured standard test organisms (STOs) to the natural algal population have been practiced. In addition to assist in satisfying the intake requirements, the use of STOs can also assist in fulfilling the requirements for intake water to consist of at least five species from three phyla.

The use of STOs for Type Approval to assist in fulfilling the intake concentration requirements has been questioned by experts because no supporting data exist if STOs have the same robustness towards treatments as natural algae. If STOs are less robust than natural algae their use will lower the water quality and consequently systems can potentially be Type Approved on a weak foundation. On the other hand, if STOs are more or as robust towards treatments as natural algae, systems will be properly challenged because of more conservative conditions and consequently, more environmentally safe systems will attain Type Approval.

In the current study, the UV effects needed for concentrations of STOs and natural algal populations to meet discharge standards after UV treatments were compared. The STO robustness towards UV treatments was next evaluated for their advantageous use as supplement to natural populations in Type Approval testing.

4.4 Sensitivity difference between tropical and temperate organisms towards UV-based treatment systems (Q3)

It has been hypothesized that tropical algal populations are more robust towards UV treatments than temperate algal populations because of adaptions to higher exposure to solar radiation related to temperate algal populations. Type Approval test conditions for UV-based BWMS would hence be different in tropical and temperate test facilities.

The UV energy needed for to meet discharge standards for tropical and temperate algal populations were compared to investigate if tropical algal populations were more robust towards UV treatments compared to temperate algal populations. In parallel, algal species and class composition were compared.

5 Experimental design

5.1 UV collimated beam systems and treatment procedures

The collimated beam systems consisted of a UV mercury arc lamp emitting UV light at 253.7 nm for the LP UV system and between 200-300 nm for the MP UV system. The lamps were placed in a closed chamber where UV light could only escape through a collimating tube resulting in vertical UV irradiation reaching a petri dish containing the test water sample (Figure 1).
LP and MP UV treatments were performed using the collimated beam system on standard test organisms (STOs) or natural phytoplankton compositions obtained at the DHI Ballast Water Centre - Denmark and DHI Ballast Water Centre – Singapore.

UV treatments (fluence) 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 rates (intensity) of the UV lamps. Test water containing algal cells were exposed to the UV treatments by placing 50 ml test water in a petri dish under the collimated beam system. The sample was continuously stirred without creating any vortex while stirring. After UV treatment, samples were transferred to a dark bottle and kept for 24 hours in a polystyrene box at ambient temperature (dark-hold period) to simulate ballast water tank holding time. After 24 hours, samples were gently re-suspended and transferred to a petri dish for a second UV treatment. The total desired UV fluence was obtained by adding the calculated UV fluences from the two treatments.

After the second UV treatment, samples exposed to aimed fluences of 100, 200, 500 and 1000 mJ cm\(^{-2}\) were immediately analysed for number of fluorescent organisms using the VS method. Samples exposed to 25, 50, 100 and 200 mJ cm\(^{-2}\) were analysed using the MPN method (Figure 2). Control samples were analysed for the number of living fluorescent (VS method) and viable organisms (MPN method) both before and after the 24-hours dark-hold period (Figure 2).
Figure 2  Experimental timeline: A sample was collected from the test water and either A) analysed without any UV fluence (control samples), stored in dark conditions for 24 hours at ambient temperature and re-analysed or B) exposed to LP or MP UV treatments, stored in dark conditions for 24 hours at ambient temperature, second UV treatment and analysed.

Combinations of test repetitions were carried according to Table 1.

Table 1  Test repetitions carried out as combination of low/medium pressure UV treatment, climatic conditions and test organisms.

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<th>Treatment name</th>
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<td>Temperate</td>
<td>Natural population</td>
<td>1</td>
<td>LP-TEMP-1</td>
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<td>Temperate</td>
<td>Natural population</td>
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<td>LP-TEMP-2</td>
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<td>Natural population</td>
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<td>MP-TEMP-1</td>
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<tr>
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<td>Natural population</td>
<td>2</td>
<td>MP-TEMP-2</td>
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<tr>
<td>Low pressure</td>
<td>Tropical</td>
<td>Natural population</td>
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<td>LP-TROP-1</td>
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<tr>
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<td>Tropical</td>
<td>Natural population</td>
<td>2</td>
<td>LP-TROP-2</td>
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<tr>
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<td>Natural population</td>
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<td>Tetraselmis suecica</td>
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<td>Odontella sp.</td>
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<tr>
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<td>Tropical</td>
<td>Odontella sp.</td>
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<td>Medium pressure</td>
<td>Temperate</td>
<td>Tetraselmis suecica</td>
<td>4</td>
<td>MP-TET-4</td>
<td></td>
</tr>
</tbody>
</table>

For more details see APPENDIX A and APPENDIX B.

5.2  Natural algal populations and standard test organisms

Natural temperate and tropical seawater was collected at the DHI test facility in Hundested, Denmark and Kranji Reservoir/Sembawang Park, Singapore, respectively. The water was collected approximately 20 hours prior to the start of the experiments.

Two algal species were selected to represent two typically used Standard Test Organisms (STOs): Tetraselmis suecica and Odontella sp. Both monocultures were grown as semi-continuous cultures at the test facilities.
In all experiments with use of natural organisms, a large variety of organisms were observed (Figure 3). Dinoflagellates were the dominating organisms in temperate waters while the tropical phytoplankton composition was dominated by diatoms (Bacillariophyceae).

![Class and species composition of test water used in fluence-response UV treatment experiments in Denmark (TEMP) and Singapore (TROP). Unid. = Unidentified.](image)

For more details see the published papers in APPENDIX A and APPENDIX B.

5.3 Vital Stain (VS) and Most Probable Number (MPN) method

Algal cells were quantified by using either VS method or the MPN method. Both methods are standard assessment methods approved by the IMO for organism quantification.

The VS is based on the combination of the two stains fluorescein diacetate (FDA) and 5-chloromethylfluorescein diacetate (CMFDA) which are able to freely diffuse across the algal cell membrane. If cells are alive enzyme activity inside the cells will activate the otherwise quenched stains and cells will fluoresce green. If the cells are dead the stains are not activated, and no fluorescent light is produced. It should be mentioned that the fluorescent signal differs between algal species. Despite being alive some species will not show any fluorescence (false negatives) and likewise, some dead cells of certain species are able to exhibit fluorescence (false positives).

The MPN method is based on a 14 days regrowth assay. Quantification of algal cells is estimated from a series of dilutions that helps to enumerate the number of viable algal cells in the original undiluted sample.

6 Results & Discussion

6.1 Q1) Obtaining Type Approval using Vital Stain method

The UV effect needed for concentrations to meet discharge standards (<10 org. ml⁻¹) was reduced significantly when inactivation of STOs and natural algal populations after LP
and MP UV treatments was evaluated using the MPN method compared to the VS method (Figure 4A-D).

LP UV treatments evaluated with the VS method were able to meet discharge standards for most experiments on natural algal populations at the highest effect of around 1350 mJ cm\(^{-2}\) (Figure 4B), but discharge standards could not be achieved when UV exposure was performed on STOs (Figure 4A).

For both STOs and natural algal populations, MP UV treatments were able to fulfil the discharge standards at UV effects $\geq 1000$ mJ cm\(^{-2}\) and consequently seemed more efficient compared to LP UV treatments when evaluated using the VS method (Figure 4A, B).

For more detailed discussions on the supplied data see APPENDIX A and APPENDIX B.

Figure 4   Algal inactivation as a function of UV effect. (A) Standard Test Organism (STOs) and (B) Natural algal populations quantification evaluated by the Vital Stain method. (C) STOs and (D) Natural algal populations evaluated using the MPN method. LP = Low pressures UV, MP = Medium pressure UV, TET = Tetraselmis suecica, ODO = Odontella sp., TEMP = Temperate algal population, TROP = Tropical algal population. Punctures line signifies the discharge standards for the size group $\geq 10\rightarrow50$ µm which corresponds $<10$ org. ml\(^{-1}\). Data from Lundgreen et al. (2018a) and Lundgreen et al. (2018b).

6.2 Q2) Sensitivity of standard test organisms

The UV effect (fluence) needed for \(T.\) suecica and Odontella sp. concentrations to fulfil the discharge standards ($<10$ org. ml\(^{-1}\)) was evaluated and related to that of natural algal populations. Results from the present study were in addition compared to similar studies on other STOs and are ranked in Table 2.

The most robust algal species towards UV treatments were \(T.\) suecica and Microcystis aeruginosa. Odontella sp. did not rank as high as the other STOs but was still within the same range as natural algal populations exposed to LP UV treatments.

Consequently, the STOs examined in the present study as well as from similar other studies were more or at least as robust as natural algal populations towards UV treatments. The addition of STOs to intake water can therefore be considered as an
advantage to help fulfil intake water requirements, control discharge requirements and finally provide more challenging or as robust testing procedures that will result in environmentally safer BWMS.

For more details see published papers in APPENDIX A and APPENDIX B.

Table 2  Standard test organisms (STOs) or natural algal mix ranked according to the estimated fluence (F_{D2}, mJ cm^{-2}) needed for concentrations to meet discharge standards after UV treatments. Data from Lundgreen et al. (2018b) (Table 4 herein).

<table>
<thead>
<tr>
<th>Algal species or mix</th>
<th>UV Type</th>
<th>UV range</th>
<th>UVT</th>
<th>F_{D2}</th>
<th>Reference</th>
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<tr>
<td>Tetraselmis suecica - exp3</td>
<td>MP</td>
<td>0-500</td>
<td>80</td>
<td>320</td>
<td>Present study</td>
</tr>
<tr>
<td>Tetraselmis suecica - exp1</td>
<td>MP</td>
<td>0-200</td>
<td>80</td>
<td>292</td>
<td>Present study</td>
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<tr>
<td>Tetraselmis suecica - exp4</td>
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<td>0-500</td>
<td>80</td>
<td>281</td>
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</tr>
<tr>
<td>Microcystis aeruginosa PCC7806</td>
<td>LP</td>
<td>0-180</td>
<td>NA</td>
<td>266</td>
<td>Sakai et al. (2011)</td>
</tr>
<tr>
<td>Tetraselmis suecica</td>
<td>LP</td>
<td>0-180</td>
<td>91</td>
<td>263</td>
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</tr>
<tr>
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<td>NA</td>
<td>257</td>
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</tr>
<tr>
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<td>80</td>
<td>244</td>
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</tr>
<tr>
<td>Tetraselmis suecica - exp1</td>
<td>LP</td>
<td>0-270</td>
<td>80</td>
<td>241</td>
<td>Present study</td>
</tr>
<tr>
<td>Tetraselmis suecica - exp2</td>
<td>LP</td>
<td>0-270</td>
<td>80</td>
<td>202</td>
<td>Present study</td>
</tr>
<tr>
<td>Tetraselmis suecica - run2</td>
<td>LP</td>
<td>0-200</td>
<td>NA</td>
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</tr>
<tr>
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<td>LP</td>
<td>0-400</td>
<td>NA</td>
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<tr>
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<td>80</td>
<td>156</td>
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</tr>
<tr>
<td>Odontella sp. - exp2</td>
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<td>96</td>
<td>117</td>
<td>Present study</td>
</tr>
<tr>
<td>Natural algae, temperate - exp2</td>
<td>LP</td>
<td>0-130</td>
<td>68</td>
<td>107</td>
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</tr>
<tr>
<td>Tetraselmis sp.</td>
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<td>89</td>
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<td>Sun and Blatchley (2017)</td>
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<td>0-130</td>
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<td>99</td>
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<tr>
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<td>85</td>
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</tr>
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<td>LP</td>
<td>0-150</td>
<td>80</td>
<td>62</td>
<td>Lundgreen et al. (2018a)</td>
</tr>
</tbody>
</table>
6.3 Q3) Sensitivity of temperate and tropical algae

When evaluated with the VS method there were no significant differences between tropical and temperate algal populations after LP UV treatments (Figure 4B).

On the other hand, tropical algal populations seemed less robust towards LP UV treatments compared to temperate algal populations when evaluated using the MPN method (Table 2).

Table 3  Standard test organisms (STOs) or natural algal mix ranked according to the estimated fluence ($F_{D,2}$, mJ cm$^{-2}$) needed for concentrations to meet discharge standards after UV treatments. Data from Lundgreen et al. (2018b) (Table 4 herein).

<table>
<thead>
<tr>
<th>Algal species or mix</th>
<th>UV Type</th>
<th>UV range</th>
<th>UVT</th>
<th>$F_{D,2}$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetraselmis suecica</td>
<td>MP</td>
<td>0-500</td>
<td>80</td>
<td>320</td>
<td>Present study</td>
</tr>
<tr>
<td>Tetraselmis suecica</td>
<td>MP</td>
<td>0-200</td>
<td>80</td>
<td>292</td>
<td>Present study</td>
</tr>
<tr>
<td>Tetraselmis suecica</td>
<td>MP</td>
<td>0-500</td>
<td>80</td>
<td>281</td>
<td>Present study</td>
</tr>
<tr>
<td>Microcystis aeruginosa PCC7806</td>
<td>LP</td>
<td>0-180</td>
<td>NA</td>
<td>266</td>
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<td>Natural algae, temperate - exp2</td>
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<td>Lundgreen et al. (2018a)</td>
</tr>
</tbody>
</table>

There was a difference in algal species and class composition between the tropical (diatom dominated) and temperate (dinoflagellate dominated) populations used in the present study (Figure 3). The composition difference could explain the difference in UV effect needed to achieve discharge standards when assessed using the MPN method. The explanation is not conclusive as one study suggested that UV sensitivity could not be predicted from taxonomy when responses were compared from different algal classes. In this study diatoms were not included, but other studies have suggested that diatoms are more robust towards UV treatments compared to other algal classes.

7 Conclusions

When assessing the efficacy of UV-based BWMS, the UV effect needed for concentrations to meet the discharge standards was 10-15 and 2-5 times higher for LP and MP UV treatments, respectively, when assessed using the VS method compared to the MPN method.

The robustness of the STOs Tetraselmis suecica and Odontella sp. towards UV treatments was higher or at the same level as natural algal populations when assessed using the MPN method. The advantageous supplemental use of robust STOs mixed with...
natural algal populations thereby support environmentally safer validation of UV-based BWMS. In addition, the use of robust STOs in test water help fulfil intake water requirements and help maintain stable concentrations in control discharge water.

There did not seem to be any difference in sensitivity between tropical and temperate algal populations towards UV treatments.

8 References


APPENDIX A

Paper 1 – Lundgreen et al. 2018a
A UV fluences required for compliance with ballast water discharge standards using two approved methods for algal viability assessment + supporting material
UV fluences required for compliance with ballast water discharge standards using two approved methods for algal viability assessment

Kim Lundgreen\textsuperscript{a,⁎}, Henrik Holbech\textsuperscript{a}, Knud Ladegaard Pedersen\textsuperscript{a}, Gitte Ingelise Petersen\textsuperscript{b}, Rune Røjgaard Andreason\textsuperscript{c}, Christaline George\textsuperscript{d}, Guillaume Drillet\textsuperscript{d,e}, Martin Andersen\textsuperscript{f}

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\textsuperscript{c} Danish Technological Institute, Kongsvang Allé 29, 8000 Aarhus, Denmark
\textsuperscript{d} DHI-NTU Research Centre and Education Hub, 1 CleanTech Loop, #03-05 CleanTech1, 637141, Singapore
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Ballast water
Invasive species
Vital stain method
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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 \textit{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...
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, Kalivasaar, 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 (Thronsden, 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 a 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 Thronsden (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 four 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
the UV lamp. For UV treatment, 50 ml test water was placed in a petri dish under the collimated beam system (SI-2, Fig. 1). The sample was continuously stirred without creating any vortex while stirring. After UV treatment, samples were transferred to a dark bottle and kept for 24 h in a polystyrene box at ambient temperature (dark-hold period) to simulate the dark conditions for 24 h at ambient temperature and re-analysed or 2) exposed to LP or MP UV treatments, stored in dark conditions for 24 h at ambient temperature, second UV treatment and analysed. $T = 0$ refers to time, $T$ (hours), since last exposure.

### 2.4. Collimated beam systems

The collimated beam systems consisted of a UV mercury arc lamp emitting UV light at 253.7 nm for the LP UV system and between 200 and 300 nm for the MP UV system. The lamps were placed in a closed chamber where UV light could only escape through a collimating tube resulting in vertical UV irradiation reaching a petri dish containing the test sample (SI-2, Fig. 1). Table 2 gives an overview of the definitions used throughout the paper based on Linden and Darby (1997) and Bolton and Linden (2003).

For LP and MP UV systems the average germicidal fluence ($F_{avg}$, J m$^{-2}$) was calculated as shown in Eqs. 12a/12b:

$$F_{avg, LP} = I_{avg} \times t$$

$$F_{avg, MP} = I_{avg} \times t$$

where $I_{avg}$ is average germicidal intensity (SI-2, Eq. 11a/11b for further details) and $t$ equal exposure time (s).

### 3. Results

#### 3.1. Algal class and species composition of test water

Dinoflagellates were the dominating organisms in temperate waters. Dinoflagellates consisted of about 90% and 75% of the algal classes present in the test water used for the LP and MP UV treatments, respectively. The tropical phytoplankton composition was dominated by diatoms (Bacillariophyceae) representing > 90% of the classes present with a large proportion of Skeletonema sp. representing 56–86% of the phytoplankton (Fig. 2).

#### 3.2. Calculation of UV fluences used in LP and MP UV treatment experiments

Average germicidal intensities, $I_{avg}$ (W m$^{-2}$) of the two collimated beam systems were estimated from Eq. (6a/6b) and (11a/11b) (SI-2). Using experiment MP-TEM-1 as example this resulted in an average germicidal intensity of:

$$I_{avg} = \frac{\Delta C \times V}{\phi \times \Delta t \times A} \times \sum_{i=1}^{n} \phi_{i} \times E_{i} \times WF$$

$$I_{avg} = 9.56 \times 10^{-6} \text{ mole l}^{-1} \times 0.051$$

where 1 Einstein = 1 mole of photons.

Subsequently, the average germicidal fluence ($F_{avg}$, mJ cm$^{-2}$) could be estimated from Eq. (12) by multiplying the total UV exposure time, $s$, which is found by adding the exposure time before and after the dark-hold period (Example provide in Table 3).

The fluences applied before and after the dark-hold period varied between 0 and 5% in all the conducted experiments.

#### 3.3. Test water stability and control samples

To validate stable test water phytoplankton concentrations throughout the experiments and to confirm that the dark-hold period did not influence concentration, three control (no treatment) samples of

---

Table 2

<table>
<thead>
<tr>
<th>Term</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incident germicidal intensity, $I_{i}$ (W m$^{-2}$)</td>
<td>“a measure of the intensity in the germicidal range upon a liquid surface” (Linden and Darby, 1997). In this paper a sub-surface technique (actinometry) was used to estimate the intensity in the germicidal range just beneath the liquid surface. As an incident beam of light in the 200–300 nm region reach the air/water interphase approximately 2.5% will be reflected and 97.5% will enter the water (Bolton and Linden, 2003). The term “incident germicidal intensity” was applied for this subsurface intensity measurement though only including 97.5% of the true incident beam.</td>
</tr>
<tr>
<td>Average germicidal intensity, $I_{avg}$ (W m$^{-2}$)</td>
<td>“the volume-averaged intensity in the germicidal range in a liquid, corrected for the absorption of the liquid and the depth of the sample” (Linden and Darby, 1997).</td>
</tr>
<tr>
<td>Average germicidal fluence, $F_{avg}$ (mJ cm$^{-2}$)</td>
<td>Average germicidal intensity × exposure time</td>
</tr>
</tbody>
</table>
test water were collected independently at three time points (0, 3 and 8 h) and counted before dark-hold was initiated. Each sample was kept under dark conditions at ambient temperature for 24 h and re-counted using the VS method (Fig. 3) and MPN method (data not shown).

### 3.4. Comparison of VS and MPN methods to evaluate UV fluence-response relationships

In all fluence-response experiments within the range of 0–300 mJ cm$^{-2}$ the number of fluorescent phytoplankton was relatively stable in all experiments and close to control sample concentrations when assed using the VS method (Fig. 4A, B, C). When assessed using the MPN method, LP UV treatments on phytoplankton from both temperate and tropical origin, showed that UV fluences from about 130 mJ cm$^{-2}$ were sufficient to meet IMO discharge standards (< 10 org. ml$^{-1}$) in all experiments except LP-TROP (MPN-3) (Fig. 4A, C). In MP UV treatments on temperate phytoplankton, IMO discharge standards were met at 200 mJ cm$^{-2}$ for both experiments and in one of the experiments (MPN-1) already at 100 mJ cm$^{-2}$ (Fig. 4B). The ratio of MPN/VS counts at about 100 mJ cm$^{-2}$ for MP UV treatments and at about 135 mJ cm$^{-2}$ for LP UV treatments was between 0.04 and 7.8%. For all experiments above 200 mJ cm$^{-2}$ the ratio was below 0.3% except in LP-TROP (MPN-3).

### 3.5. UV fluences needed for discharge compliance evaluated using the VS method

Fluence-response relationships at higher UV fluences were carried out to examine the fluences needed for concentrations to meet discharge standards (< 10 org. ml$^{-1}$) when evaluated by the VS method (Fig. 5). For temperate phytoplankton, fluences higher than 1200 and 900 mJ cm$^{-2}$ were needed for LP and MP UV treatments, respectively. For tropical phytoplankton fluences higher than 1200 mJ cm$^{-2}$ were needed to meet discharge standards in two of the experiments (LP-TROP-2, LP-TROP-3) and one experiment (LP-TROP-1) standards were not met even at the highest UV fluence (1288 mJ cm$^{-2}$) (Fig. 5).

### 3.6. Phytoplankton behaviour in relation to UV treatment

Algal cell behaviour was recorded during assessment of phytoplankton concentrations using the VS method (Table 4). There were obvious changes in mobility patterns, speed and location in the water column when UV fluence increased. In control samples, the mobile algal species typically displayed gradual and fast movements in both the horizontal and vertical plane. At 100 mJ cm$^{-2}$ movements became slower and interrupted by rotations and almost all algal cells were present on the bottom. From 200 to 1000 mJ cm$^{-2}$ the general observations were that all observed cells were present on the bottom without any movements.

### 3.7. Effect of extended dark-hold period on phytoplankton concentration in VS assessments

In three experiments (LP-TEMP-2, LP-TROP-1, and LP-TROP-2), samples were subjected to an additional dark-hold period (24 h) following the VS analysis that was carried out immediately after the second UV treatment (denoted T = 0). At the end of the additional 24-h dark-hold period (T = 24) VS counts were repeated to follow the change in the number of fluorescent organisms (i.e. dying/non-viable + viable organisms) in UV treated samples over time (Fig. 6). The phytoplankton concentration in control (no treatment) samples from temperate waters used for LP UV treatments was constant and there was no significant difference (P = 0.56) between Control (T = 0) and Control (T = 24) numbers (Fig. 6A). At 129 mJ cm$^{-2}$ the T = 24 number had decreased to 60% of corresponding T = 0 levels and between 260 and 1300 mJ cm$^{-2}$ T = 24 numbers varied between 14 and 20% of T = 0 levels. For both LP UV treatment experiments on tropical phytoplankton there was significant difference (P < 0.05) between Control (T = 24) and Control (T = 0) numbers. Control (T = 24) numbers were between 62 and 84% of Control (T = 0) levels. Between 129 to about 640 mJ cm$^{-2}$ T = 24 numbers were 2–19% of T = 0 levels and at about 1300 mJ cm$^{-2}$ numbers were 26–29% of T = 0 levels (Fig. 6B, C).

The time to wait until survival of treated phytoplankton samples reaches a concentration that meet discharge standards could be

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**Table 3**

<table>
<thead>
<tr>
<th>Target fluence (mJ cm$^{-2}$)</th>
<th>$I_{av}$ (W m$^{-2}$)</th>
<th>$s$ (sec)</th>
<th>$F_{av}$ (Ws m$^{-2}$)</th>
<th>$E_{av}$ (mJ cm$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.94</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>25</td>
<td>1.94</td>
<td>128</td>
<td>248</td>
<td>25</td>
</tr>
<tr>
<td>50</td>
<td>1.94</td>
<td>254</td>
<td>493</td>
<td>49</td>
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<tr>
<td>100</td>
<td>1.94</td>
<td>509</td>
<td>987</td>
<td>99</td>
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<tr>
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</tr>
<tr>
<td>1000</td>
<td>1.94</td>
<td>5096</td>
<td>9886</td>
<td>989</td>
</tr>
</tbody>
</table>

---

Fig. 2. Class and species composition of test water used in fluence-response UV treatment experiments in Denmark and Singapore. For each of the experiments, water was collected independently and composition was determined from preserved Lugol samples. (Unid. = unidentifiable).

---

estimated by assuming first-order kinetics. The exponential decrease in survival of organisms when exposed to UV irradiation was based on the approach used by Hijnen et al. (2006) and Stehouwer (2016) using the equation:

\[ N = N_0 e^{-zt} \]

where \( N \) is concentration of fluorescent phytoplankton, \( N_0 \) is the concentration of fluorescent phytoplankton at \( T = 0 \), \( z \) is the change in survival rate \((h^{-1})\) and \( t \) is time in hours.

By plotting the phytoplankton concentration at \( T = 0 \) and \( T = 24 \) the survival rate, \( z \) (slope of trendline), could be estimated. By using the estimated survival rates the time, \( t \), for concentrations to meet discharge standards \((N = 10 \text{ org. ml}^{-1})\) for each fluence could be estimated by re-arranging the equation:

\[ t = \ln \left( \frac{N}{N_0} \right) \times \frac{1}{z} \]

The estimated lag times for concentrations to meet discharge standards are plotted in Fig. 7. The data indicates that the higher the fluence the less time is required to meet discharge standards. Based on the MPN data (Fig. 4A, C) the lowest UV treatments for concentrations to meet discharge standards was about 130 mJ cm\(^{-2}\) (indicated by the
grey area in Fig. 7). Above this fluence (within the grey area), the time in hours to wait for VS counts to meet discharge standards can be estimated for each fluence (treatment) from the inserted dotted line. The line was based on the most conservative lag time points. Survival rates from tropical experiments were Q_{10} adjusted according to the temperature difference in the dark-hold periods between tropical (27 °C) and temperate (20 °C) experiments. The Q_{10} value was 1.7 obtained from Abughararah (1994) on UV treatment efficacy experiments.

3.8. Algal species compositions at high end UV treatments

In three experiments (LP-TEMP-2, MP-TEMP-1, MP-TEMP-2), samples exposed to the second highest treatment were preserved in Lugol for determination of the dominating algal species by counting cells with intact cell membranes (assessed using 40× magnification) (data not shown). The second highest LP and MP treatment was 647 and 500 mJ cm\(^{-2}\), respectively. Besides counting the algal species with intact membranes in the size range 10–50 μm, Lugol counts also included one algal species < 10 μm (Oocystis sp.) showing intact cell membranes. The size of one Oocystis sp. cell was determined from the size of the individual cells inside their characteristic mucilaginous envelope (SI-3/Fig. 4).

When only including the algal species in the 10–50 μm range (i.e. excluding Oocystis sp.), the algal species composition from the LP UV treatment was dominated by the dinoflagellate Gymnodinium sp. and one unidentified dinoflagellate species whereas in MP UV treatment experiments compositions were dominated by the diatom Skeletonema sp. When including algal species < 10 μm, Oocystis sp. was absent, but in MP-TEMP-1 MP-TEMP-2 it represented 18 and 67%, respectively, of algal cells with intact cell membranes after UV treatments (data not shown).
4. Discussion

The stability of the test water phytoplankton concentrations was monitored by means of control samples. For all experiments, there was no significant difference (paired t-test, P-values ranged 0.06–0.51) between control sample concentrations before and after the 24-h dark-hold period (Fig. 3). This indicated that stability between natural death rate and cell division was present during the experimental period and that changes in phytoplankton concentrations were attributable to the UV treatments. A stable test water phytoplankton concentration likewise ensured that samples collected for UV treatments during day of experiments had similar starting points making comparison between treatments possible.

Concentrations assessed by the MPN method met IMO discharge standards (< 10 org. ml$^{-1}$) for both UV systems at fluences between 200 and 260 mJ cm$^{-2}$ in all experiments except for a single one (Fig. 4). In a few experiments, compliance was achieved even around 100–130 mJ cm$^{-2}$. The failing compliance in a single experiment (LP-TROP/MPN-3) was suspected to be caused by contamination of the test tubes during the MPN measurements since unusually high densities of dinoflagellates were observed. Contrary to the results obtained using MPN it was not possible to meet discharge standards at treatments below 300 mJ cm$^{-2}$ in any experiments assessed with the VS method. In fact, there was no change in VS counts at the different treatments and counts were similar to control levels (Fig. 4).

Visual inspection of the treated samples favoured the conclusions obtained by the MPN method and not the VS method (Table 3). In all experiments, algae were affected by UV fluences of 100–130 mJ cm$^{-2}$ showing interrupted and slower movements. At 200–260 mJ cm$^{-2}$ the majority of cells were found immobile on the bottom. Also in
acCORDANCE WITH THE MPN RESULTS THERE WERE NO DIFFERENCES IN BEHAVIOUR BETWEEN LP AND MP UV TREATMENTS OR BETWEEN TEMPERATE AND TROPICAL PHYTOPLANKTON COMPOSITIONS. THE CHANGE IN BEHAVIOUR OBSERVED ALREADY AT THE LOWER FLUENCES INDICATES THAT UV TREATMENTS HAD AN IMMEDIATE EFFECT ON, NOT ONLY DNA INTENSITY (SINHA AND HADER, 2002; RASTOGI ET AL., 2010), BUT ALSO ON GENERAL PHYSIOLOGICAL FUNCTIONS OF THE TREATED ORGANISMS AS REFLECTED BY THE IMPAIRED MOBILITY. SIMILAR EFFECTS HAVE BEEN OBSERVED IN PREVIOUS STUDIES (HESSEN ET AL., 1997; MA ET AL., 2012). THE CORRESPONDING LOW MPN COUNTS AT FLUENCES IN THE RANGE 200–260 mJ cm2−2 INDICATE THAT INTERNAL DNA DAMAGE HAD OCCURRED ALONG WITH THE HAMPERED MOBILITY AS MPN COUNTS WERE SMALL FRACTIONS OF CONTROL SAMPLE PHYTOPLANKTON CONCENTRATIONS. SO, ALTHOUGH HIGH VS COUNTS WERE OBSERVED UP TO ABOUT 260 mJ cm2−2 A LARGE PROPORTION OF THE PHYTOPLANKTON CELLS WERE ALREADY DYING.


IT HAS BEEN STATED THAT THE MPN METHOD WILL PROBABLY NEVER BE ACCEPTED AS A STANDARD ASSESSMENT DUE TO TIME CONSUMPTION, THE SUSPECTED INABILITY TO CULTIVATE ALL SPECIES OF PLANKTON ORGANISMS AND INTERACTIONS BETWEEN SPECIES SUPRESSING GROWTH (STEINBERG ET AL., 2011; FIRST AND DRAKE, 2013) ALTHOUGH THIS ARGUMENT HAS BEEN CHALLENGED BY CULLEN AND MACINTYRE (2016) THAT CONCLUDED THAT THE MPN METHOD IS WELL-SUITED FOR DETERMINATION OF VIABLE PHYTOPLANKTON. WE THEREFORE INVESTIGATED TWO POSSIBILITIES OF MINIMIZING THE NUMBER OF APPARENTLY DYING ORGANISMS IN UV SYSTEMS ASSESSED AS LIVING BY THE VS METHOD. FIRST, BY SIMPLY DELAYING THE TIME FROM UV TREATMENT TO VS ASSESSMENT TO ALLOW TIME FOR THE PRESUMED DAMAGES TO PROGRESS INTO IMPAIRED MEMBRANE INTEGRITY AND ESTERASE ACTIVITY. SECOND, BY INVESTIGATING IF UV FLUENCES AT HIGHER LEVELS COULD PRODUCE DAMAGES THAT COULD IMMEDIATELY BE DETECTED BY THE VS METHOD.

THE EFFECT OF DELAYING VS MEASUREMENTS WAS INVESTIGATED BY APPLYING THE VS ASSAY REPEATEDLY IN THREE EXPERIMENTS (LP-TEMP-2, LP-TROP-1, LP-TROP-2). FIRST, IMMEDIATELY AFTER LAST UV TREATMENT (T = 0) AND AGAIN FOLLOWING AN ADDITIONAL DARK-HOLD PERIOD OF 24 h (T = 24) (Fig. 6). IN LP-TEMP-2 PHYTOPLANKTON VIABILITY WAS NOT AFFECTED BY THE DARK-HOLD PERIOD ALONE AS CONTROL (T = 24) NUMBERS WERE SIMILAR TO CONTROL (T = 0) LEVELS (NO SIGNIFICANT DIFFERENCE, P = 0.56). HOWEVER, WHEN WAITING 24 h BEFORE MEASURING CELL VIABILITY AFTER UV TREATMENTS, FLUENCES > 129 mJ cm−2 DECREASED THE NUMBER OF PHYTOPLANKTON ORGANISMS BY 40–86%. EVEN AT THE TWO LOWEST UV FLUENCES OF 33 AND 65 mJ cm−2 A DECREASE OF 17 AND 33% WAS OBSERVED, RESPECTIVELY, COMPARED TO CONTROL SAMPLE VALUES. ALSO, SIGNS OF DAMAGE WERE NOTICED AT THESE LOWER TREATMENTS AT T = 24 AS THE OBSERVED ALGAL BEHAVIOUR WAS SIMILAR TO THAT OBSERVED AT ABOUT 130 mJ cm−2 FOR T = 0. Thus, even the lowest UV treatments had a direct effect on the algal cells. THE PHYSIOLOGICAL MECHANISM BEHIND THE LOSS OF CELLS COULD BE EXPLAINED BY A MIXTURE OF CHARACTERISTIC NECROTIC AND APOPTOTIC PATHWAYS (APOPTOSIS-LIKE) FOLLOWING SIMILAR UV INDUCED DAMAGES OBSERVED AS THE FINAL RESPONSE IN THE UNCICLAL ALGAE Dunaliella viridis EXPOSED TO UV IRRADIATION (Jimenez ET AL., 2009). IN THE TROPICAL EXPERIMENTS THE ADDITIONAL DARK-HOLD PERIOD SEEMED TO AFFECT PHYTOPLANKTON CONCENTRATION AS CONTROL SAMPLES DISPLAYED SIGNIFICANT DECREASES OF 38% IN LP-TROP-1 AND 16% IN LP-TROP-2. THIS WAS PROBABLY DUE TO THE HIGHER DARK-HOLD TEMPERATURE IN TROPICAL SAMPLES (26–27 °C) COMPARED TO TEMPERATE SAMPLES (19–20 °C) (Robinson AND Williams, 1993; Drillet ET AL., 2013). IN THE TREATED TROPICAL SAMPLES THE DECREASE WAS MORE PRONOUNCED (71–98%) DISPLAYING THE DELAYED EFFECTS OF THE UV TREATMENTS COMBINED WITH THE EFFECT OF EXTENDED DARK-HOLD. TO SUM UP, TWO EXPERIMENTS MET DISCHARGE STANDARDS (< 10 org. ml−1) AT T = 0, WHEREAS AT T = 24 TWO MORE FULLFILLED THE REQUIREMENTS. THE FINDINGS SUGGEST THAT THE VS METHOD CAN INDEED BE USED AS ASSESSMENT METHOD IF UV TREATED PHYTOPLANKTON ARE SUBJECTED TO AN EXTENDED DARK-HOLD PERIOD DOWN TO
fluctuations of 130 mJ cm$^{-2}$ (lowest fluence showing MPN counts meeting discharge standards). From our two time points (T = 0 and T = 24) we estimated the length of the dark-hold periods for concentrations to meet discharge standards at different fluences. The estimations were calculated by assuming exponential decrease in survival based on inactivation of micro-organism exposed to UV disinfection following first-order kinetics described in detail by Hijnen et al. (2006) and also observed in Stehouwer (2016) in UV treated *Tetraselmis*. Survival rates were corrected for temperature by applying a Q$_{10}$ value of 1.7 to survival rates from tropical water. By following the most conservative lag time data points the minimum length of dark-hold periods to meet discharge standards could be estimated in relation to degree of treatment (Fig. 7). The higher the degree of treatment the shorter the dark-hold period needed to meet discharge standards. At the lowest fluence where MPN met discharge standards (around 130 mJ cm$^{-2}$) a dark-hold period of about 14 days was necessary for VS counts to meet discharge standards (Fig. 7). At 260 mJ cm$^{-2}$ the lag time decreased to about 3.5 days.

Other studies have likewise suggested that a dark-hold period following UV treatments would potentially minimize or prevent regrowth (Sutherland et al., 2001; Olsen et al., 2016; Romero-Martinez et al., 2016). This could be important information for ship owners and developers of BWMS regarding installation and design of the BWMS, i.e., whether UV treatments should be applied to ballast water during uptake, discharge, both or through recycling procedures during voyage.

The efficiency of UV treatments in killing phytoplankton organisms is supported by several studies on single-organism cultures (Buma et al., 1996; Choi et al., 2011; Liltvedt et al., 2011; Tao et al., 2013; McGivney et al., 2015; Sun and Blatchley 3rd, 2017) and natural samples (Sutherland et al., 2001; Casas-Monroy et al., 2016). A recent study on *Tetraselmis* cultures found the same range of LP fluences around 120 mJ cm$^{-2}$ to be sufficient to obtain concentrations meeting discharge standards when measured using the MPN method (Sun and Blatchley 3rd, 2017).

Similar to our findings other studies also found that the MPN method was successful in meeting discharge standards after UV treatment of test water (Liebich et al., 2012; Stehouwer et al., 2015). In the present study, no regrowth was observed within the 14 days incubation period before samples were analysed. In both Stehouwer et al. (2015) and Liebich et al. (2012) regrowth of repaired cells or growth of a subpopulation of unaffected cells after UV treatments was observed. Stehouwer et al. (2015) observed regrowth in samples of natural plankton communities in both LP and MP UV treated samples between 7 and 12 days of incubation under optimal conditions and even in one of the UV experiment where fluence was increased 400% above normal. Likewise, Liebich et al. (2012) observed regrowth in discharge water subjected to two LP UV treatment rounds with 5 days holding time in between treatments after 9–12 days of incubation under simulated natural growth conditions. In both studies, actual values for treatment fluences were not provided and a simple explanation for the discrepancies could be that they were too low to render the organisms non-viable. This emphasizes the importance of measuring actual fluences in UV treatment studies to make comparisons possible. Even though no regrowth was observed in the present study, it cannot be ruled out that it could occur beyond the 14 days incubation period. In the present study, a 14 days incubation period was chosen based on standard procedures described in IMO (2016a, 2016b), recommendations by DHI-Denmark based on a large number of tests to validate the use of the MPN method (Madsen and Petersen, 2015) and lastly from arguments provided by MacIntyre et al. (2017) and Hull et al. (2017).

Further, UV detectors should be calibrated according to a uridine standard as demonstrated in the present work where a UV sensor initially used for determining fluence rates in the LP UV system was found to be underestimating by almost 25% when compared with uridine standards. Consequently, the fluences of the LP UV experiments had to be adjusted, which is the reason why LP UV fluences differ and are higher than the initially aimed fluences of 25, 50, 100, 200, 500 and 1000 mJ cm$^{-2}$ (Fig. 4, Fig. 5).

In our final experiments, we investigated if UV treatments at high fluences could inflict sufficient damage to algae to be immediately considered dead using the VS method. In both UV systems, about 10 times the energy was needed for VS counts to meet discharge standards compared to the energy needed when using the MPN method. When assessed using the MPN method around 130 and 200 mJ cm$^{-2}$ was required to meet discharge standards for LP and MP UV treatments, respectively. The VS method required about 1300 mJ cm$^{-2}$ and 1000 mJ cm$^{-2}$ for LP and MP UV treatments, respectively, to meet discharge standards (Fig. 5). As previously discussed, an exact comparison of the relative efficiency of the two UV systems was not possible due to the need for post-experimental adjustments of the actual fluences delivered by the LP UV system. However, other studies on i.e. *Escherichia coli* have suggested MP UV systems to be more efficient than LP UV systems (Oguma et al., 2002; Zimmer and Slawson, 2002).

The temperate test water was dominated by dinoflagellates and tropical test water by diatoms, in particular *Skeletoonema* sp. (Fig. 2). It has been suggested that tropical species in general are more robust than temperate species towards UV treatments due to adaptations to the natural higher intensities of UV light from solar irradiation (Helbling et al., 1992; Rastogi et al., 2014). In the present study, we could not find clear evidence to support this hypothesis. There was no significant difference (t-test, P = 0.26) between temperate and tropical VS counts at the highest LP fluences (Fig. 5) and MPN data showed that tropical algal compositions decreased faster (more affected by UV treatments) compared to temperate algae compositions (Fig. 4). Any differences in response to UV treatments could alternatively be caused by differences in species composition. In three temperate phytoplankton samples, the species composition of algae with intact membranes was determined in Lugol fixed samples made immediately after the second highest LP and MP UV treatments (647 and 500 mJ cm$^{-2}$, respectively) (data not shown). In control samples of MP-TEMP-1 and 2, *Skeletoonema* sp. consisted of 13–20% of the total observed number of cells. When re-counted after the UV treatment it was the dominating species and consisted of 80–93% of the remaining phytoplankton organisms. This is in accordance with earlier findings where the diatoms *Skeletonema*, *Thalassiosira*, *Pseudo-nitzschia* and *Chaetoceros* were the dominating genera after UV treatments (Sutherland et al., 2001; Liebich et al., 2012; Martinez et al., 2013; Stehouwer et al., 2013; Stehouwer et al., 2015). Although it seems that diatoms are the dominating algal class after UV treatments our findings do not suggest that diatoms necessarily are more robust towards UV treatments compared to other classes of algae. A study by MacIntyre et al. (2017) suggested that sensitivity to UV irradiation cannot be predicted from taxonomy when they compared the response of different algal classes even though their study did not include diatoms. More data is needed on the effect of UV treatments of different algal classes/species to be able to evaluate if any differences in composition, geographical location of test facility and/or the seasonal variation in phytoplankton composition could affect the treatment intensity needed for concentrations to meet discharge standards.

Interestingly, Lugol counts revealed that *Oocystis* sp. was found to constitute about 20–70% of the intact phytoplankton species in MP-TEMP-1 and 2. The species is < 10 µm and consequently should not be counted according to the IMO protocol (D-2 discharge standards). Discharge standards requirements could therefore be fulfilled even if ballast water contained high numbers of this species. *Oocystis* sp. was also observed in a few of the VS counts at the highest UV treatments, but the algae were disregarded in counts due to the size range restriction. *Oocystis* sp. was not quantified in the VS counts, but if included, the additional numbers would without doubt change the initial compliance with discharge standards to a non-compliance status in a few of the experiments. On the other hand, MPN data showed that *Oocystis* sp. eventually did not survive the UV treatments as the MPN counts met discharge standards already at the lower UV treatments (Fig. 4). The MPN method does not discriminate size, and consequently the growth
potential of all organisms in a sample, regardless of the size is evaluated. MPN analysis would therefore be able to detect exceptionally robust organisms < 10 μm that would otherwise be excluded in VS counts due to size restrictions. Despite the protocol cut-off limits when using the VS method, little attention to solve this issue has been given to the invasive potential of organisms < 10 μm. Smaller organisms are not necessarily less robust than larger species (Liu et al., 2016) and several-land-based tests have revealed that > 90% of the total phytoplankton present was in the size fraction < 10 μm that is known to include numerous toxic species (van der Star et al., 2011). The issue could pose an unregulated risk, especially if some organisms < 10 μm appear to be less sensitive to UV treatments which has been addressed in previous studies (Gollasch et al., 2007; van der Star et al., 2011; Gollasch et al., 2012; Liebich, 2013; Casas-Monroy et al., 2016; Stehouwer, 2016).

5. Conclusions

We have shown that compliance with IMO/USCG discharge standards using the VS method in UV based treatment systems is feasible. However, the consequence is that about 10 times higher fluences must be applied to meet discharge standards compared to assessments using the MPN method. Alternatively, an extended dark-hold period after treatment can be implemented. The lag time to meet discharge standards will decrease the higher the fluence applied. An estimated lag time of 14 days was necessary to meet discharge standards using the VS method at the same fluence where the MPN method fulfilled the discharge requirements. No obvious differences were observed between LP and MP treatments on natural phytoplankton from temperate waters. Our data did not support the suggested hypothesis that tropical algae are more robust towards UV treatments because of an evolutionary adaption to higher solar radiation conditions. Nor did our findings suggest any differences in UV treatments on different algal classes/species.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpollbul.2018.08.043.

References


Ben Said, M., Ben Mustapha, M., Hassen, A., 2015. The impact of power supply frequency on the MPN method at the same time of 14 days was necessary to meet discharge standards using the VS method. Alternatively, an extended dark-hold period after termination in bench-scale UV experiments. J. Environ. Eng. ASCE 129 (3), 209–215.


Supporting information (SI)

SI-1

Water sampling details

Test water from Denmark representing natural phytoplankton from brackish temperate waters was prepared by mixing natural seawater from local waters (55°57'34.56"N, 11°50'29.41"E) and seawater from grow out pools at the facility. Test water from Singapore representing natural phytoplankton from brackish tropical surface waters was collected using a 5 L water sampler at Kranji Loop Bridge (1°26'9.58"N, 103°45'10.26"E)/Kranji Reservoir (1°26'18.19"N, 103°44'12.38"E) for LP-TROP-1 and at Sembawang Park (1°27'51.32"N, 103°50'14.00"E) for LP-TROP-2 and LP-TROP-3.

SI-2

Collimated beam system

Figure SI.1. Outline of low pressure (LP) and medium pressure (MP) UV collimated beam systems. The emitted UV light passed through a collimating tube resulting in an even vertical UV radiation reaching the petri dish containing the test sample. The petri dish contained a small magnetic bar and was placed on a magnetic stirrer to keep the test sample well mixed.
Uridine actinometry, preliminary parameter estimation

Uridine actinometry is a chemical method to estimate the average germicidal intensity, based on the UV facilitated decay of dissolved uridine. Prior to uridine actinometry several sub-parameters were determined including: quantum yield of uridine, uridine absorption spectra, uridine molar extinction coefficient spectrum and lamp output spectrum. A brief description of these procedures is given below.

**Uridine concentration and absorption spectrum**

UV (200-300 nm) absorption spectra of uridine were measured for four different uridine concentrations: 0.01, 0.25, 0.05 and 0.10 mM using a spectrophotometer (Shimadzu UV-1800) and a quartz cuvette (Hellma Analytics). The four absorption spectra were converted into a uridine molar extinction coefficient ($\varepsilon$) spectrum based on least square method for each wavelength (1 nm). The obtained spectrum was used to estimate uridine concentrations ($\varepsilon$ at 262 nm) and uridine UV absorption of low pressure (253.7 nm) and medium pressure (200-300 nm) UV irradiation based on Beer’s Law. The obtained molar extinction coefficient spectrum for uridine is shown below in Figure 2.

![Figure SI.2. Uridine extinction coefficient.](image-url)
Lamp output and photon distribution

For the low pressure (LP) lamp all irradiation was assumed emitted at a wavelength of 253.7 nm corresponding to 471.5 kJ Einstein\(^{-1}\) (1 Einstein = 1 mole of photons), Eq. 1.

\[
E_{E,253.7} = N_A \times h \times c / \lambda \quad \text{(Eq. 1)}
\]

Where \(E_{E,254}\) is energy (J Einstein\(^{-1}\)) at 253.7 nm, \(N_A\) is Avogadro’s number (6.022×10\(^{23}\) photons mole\(^{-1}\)), \(h\) is Plank’s constant (6.626069×10\(^{-34}\) J s), \(c\) is the speed of light (2.998×10\(^8\) m s\(^{-1}\)) and \(\lambda\) is the wavelength (253.7 nm).

For the medium pressure (MP) lamp, the relative irradiation spectrum (Figure 3) was measured using an Ocean Optics USB 2000+ spectrophotometer with a 400 µm Premium fiber sensor with a 0.113 nm resolution. The relative irradiation at each wavelength \(I_{r,\lambda}\) was converted to a fraction of emitted photons, \(P_{f,\lambda}\), spectrum by Eq. 1a and 1b.

\[
E_{E,\lambda} = N_A \times h \times c / \lambda \quad \text{(1a)}
\]

\[
P_{f,\lambda} = \frac{I_{r,\lambda}}{E_{E,\lambda}} \sum_{200}^{300} I_{r,\lambda} \quad \text{(1b)}
\]

Where \(E_{E,\lambda}\) is energy (J Einstein\(^{-1}\)), \(N_A\) is Avogadro’s number (6.022×10\(^{23}\) photons mole\(^{-1}\)), \(h\) is Plank’s constant (6.626069×10\(^{-34}\) J s), \(c\) is the speed of light (2.998×10\(^8\) m s\(^{-1}\)) and \(\lambda\) is the wavelength (nm).
Quantum yield estimation

Uridine is a water-soluble UV absorbing compound which upon UV irradiation is decomposed at a rate proportional to the absorbed photons. At incident germicidal intensity >10 W m\(^{-2}\) the ratio between molecule decomposition and photon absorption (quantum yield) are constant at \(\approx 0.022\) moles Einstein\(^{-1}\) (1 Einstein = 1 mole of photons), while at lower intensities the quantum yield is reduced (Linden and Darby 1997). In order to estimate the quantum yield in the lower range (1-10 W m\(^{-2}\)) a second order polynomial was fitted to the quantum yield vs. intrinsic germicidal intensity plot as seen in (Linden and Darby 1997). The found polynomial is given below (Eq. 2):

\[
\varphi = -0.1362 \times I_i^2 + 0.1238 \times I_i -0.006 \quad \text{(Eq. 2)}
\]

Where \(\varphi\) = quantum yield (moles Einstein\(^{-1}\)) and \(I_i\) is incident germicidal intensity (W m\(^{-2}\)).
**Uridine UV exposure procedures**

Applying exactly the same setup as for phytoplankton sample exposure, 50 ml 0.05 mM uridine was exposed to the collimated UV beam for 45-60 min. The exact uridine concentration \(C\) prior to and after UV exposure was estimated by spectroscopy at 262 nm.

**Fraction of absorbed photons**

As UV passes through the sample, a given fraction is absorbed by uridine. The fraction of absorbed irradiation \(f(\lambda)\) through a given sample depth \(l\) at a given wavelength \(\lambda\) can be estimated by combining the “–log” relationship between absorbance and transmittance with Beer’s Law (Eq. 4).

\[
f(\lambda) = 1 - T(\lambda) = 1 - 10^{-\varepsilon(\lambda)Cxl} \quad \text{(Eq. 4)}
\]

Where \(C\) is the uridine concentration (M).

In LP UV systems photons are emitted at 253.7 nm. \(f_{253.7}\) was estimated using Eq. 4 with \(\varepsilon_{254} = 8690.3 \text{ M}^{-1} \text{ cm}^{-1}\) (see 0), and \(C\) = to the average uridine concentration observed prior/upon UV exposure (differed by less than 20%).

For MP UV systems, the emitted UV is not restricted to a single wavelength. The fraction of absorbed irradiation for medium pressure \(f_{MP}\) was calculated as a lamp spectrum weighted average of \(f_{200-300}\) as given by Eq. 5.

\[
f_{MP} = \frac{\sum_{200}^{300} (f(\lambda)I(\lambda))}{\sum_{200}^{300} I(\lambda)} \quad \text{(Eq. 5)}
\]

Where \(I(\lambda)\) is the relative photon contribution at the given wavelength.
Photon flux

The found $f_{253.7}/f_{MP}$ was added to Eq. 6a/Eq. 6b to calculate the total photon flux ($J_P$, Einstein s$^{-1}$ m$^{-2}$) through the sample.

\[
J_P^{254} = \Delta C \times V / (\phi \Delta t f_{253.7} A) \quad \text{(Eq. 6a)}
\]

\[
J_p^{MP} = \Delta C \times V / (\phi \Delta t f_{MP} A) \quad \text{(Eq. 6b)}
\]

Where $V$ is the sample volume (litres), $\Delta C$ is the uridine concentration decline (M), $\Delta t$ is the UV exposure time (s), $f_{253.7}/f_{MP}$ is the fraction of absorbed irradiation, $A$ is the surface area of the sample (m$^2$) and $\phi$ is initially set to 0.022 mole Einstein$^{-1}$.

Incident germicidal intensity

For LP UV systems, the incident germicidal intensity ($I_i$, W m$^{-2}$) was calculated multiplying the obtained photon flux, $J_P^{253.7}$, by the found photon energy, $E_{E,254}$ (Eq. 8a)

\[
I_i = J_P^{253.7} \times E_{E,253.7} \quad \text{(Eq. 8a)}
\]

For MP UV systems the obtained photon flux, $J_p^{MP}$, was applied in Equation 8b together with $P_{f,\lambda}$ and $E_{E,\lambda}$ (Eq. 1a and Eq. 1b) to estimate the total incident germicidal intensity ($I_i$).

\[
I_i = \sum_{200}^{200} J_P^{MP} \cdot P_{f,\lambda} \cdot E_{E,\lambda} \quad \text{(Eq. 8b)}
\]
As $I_i$ was lower than 10 W m$^{-2}$, $\varphi$ was less than 0.022 moles Einstein$^{-1}$ (see 0). The correct $\varphi$ was therefore calculated iterative by repeating Eq. 2, 6 and 8 as described below. To ease understanding, the firstly applied $\varphi$ is added subscript 1 ($\varphi_1$). The initially $\varphi, \varphi_1,$ was set to 0.022 moles Einstein$^{-1}$. The described procedure (Eq. 1-8) was used to find $I_{i-1}$. $I_{i-1}$ was next inserted in Eq. 2 to calculate a corrected temporary quantum yield value, $\varphi_1^*$. A new quantum yield value, $\varphi_2$, was then calculated as the average value of $\varphi_1$ and $\varphi_1^*$ as shown in Eq. 9

$$\varphi_2 = (\varphi_1 + \varphi_1^*)/2 \quad \text{(Eq. 9)}$$

The found $\varphi_2$ was used in Eq. 6 and 8 to calculate a new $I_i$, ($I_{i-2}$) This iteration process was repeated until the $\varphi$ error between $\varphi$ and $\varphi^*$ was less than 1 %.

**Water factor**

As photons penetrate the sample some will be absorbed by the sample medium why the intensity of light decreases as it passes through the sample. The relationship between the intrinsic intensity and the volume averaged intensity is described by the water factor ($WF$), Eq. 10 (Bolton and Linden 2003)

$$WF_{\lambda} = \frac{1 - 10^{-a_\lambda l}}{a_\lambda l \ln(10)} \quad \text{(10)}$$

Where $a_\lambda$ is the decadic absorbance coefficient for a given wavelength (absorbance for 1 cm path length) and $l$ is the sample depth (cm).
**Average germicidal intensity**

For LP and MP UV systems average germicidal intensity, $I_{avg}$, was calculated by combining Eq. 8a/8b and Eq. 10 as shown in Eq. 11a/11b:

$$I_{avg,LP} = J_P^{254} \times E_{E,253.7} \times WF_{f,253.7} \quad (11a)$$

Where $GF_{f,253.7}$ was set to 1 and $WF_{f,254}$ was calculated using Eq. 10 with $a_\lambda = a_{253.7}$ and measured for each sample using a spectrophotometer and a quartz cuvette.

$$I_{avg,MP} = \sum_{200}^{300} J_P^{MP} \times P_{f,\lambda} \times E_{E,\lambda} \times WF_{f,\lambda} \quad (11b)$$

Where $WF_{f,\lambda}$ was calculated using Eq. 10 with $a_\lambda$ measured with 1 nm resolution from 200-300 nm for each batch using a spectrophotometer and a quartz cuvette.

**SI-3**

*Size determination of Oocystis sp.*

The size of one *Oocystis* sp. cell was determined from the size of the individual cells inside their characteristic mucilaginous envelope (Figure 4).
Figure SI.4. *Oocystis* sp. belongs to the green algae class Trebouxiophyceae. Typically, between 2-8 cells are surrounded by a mucilaginous envelope or expanded mother cell wall. Predominantly *Oocystis* are found in freshwater, but they are also present in marine environments (Photo: [http://protist.i.hosei.ac.jp/PDB/Images/Chlorophyta/Oocystis/sp_6c.html](http://protist.i.hosei.ac.jp/PDB/Images/Chlorophyta/Oocystis/sp_6c.html)).

**References**


Use of standard test organisms for sound validation of ballast water UV treatment systems
Use of standard test organisms for sound validation of UV-based ballast water treatment systems

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ABSTRACT

To challenge ballast water treatment system (BWTS) efficacy for organisms in the size-class 10–50 μm, intake concentration during tests must reach certain minimum requirements. Often, natural concentrations are too low to meet intake requirements and standard test organisms (STOs) are added. We tested the robustness of Tetraselmis suecica and Odontella sp. to a range of UV-treatments to explore fluences needed to meet the IMO discharge standard (< 10 org. ml⁻¹) evaluated using two viability assessment methods. To meet discharge standards, fluences of > 1000 mJ cm⁻² were required using vital stain whereas 135–500 mJ cm⁻² were needed using regrowth assays. Besides, results suggest that T. suecica and Odontella sp. were at least as robust as natural algae towards UV-treatments. We suggest the advantageous use of these species as STOs in test water to support intake water requirements and to obtain more conservative validation of UV-based BWTS to ensure more environmental protective procedures.

1. Introduction

The anthropogenic spreading of invasive species via ship's ballast water (and other vectors) has been addressed as one of the biggest threats to the environment, biodiversity and diseases (Burgiel and Muir, 2010; Drillet, 2016; WWF, 2016). Currently no saturation in the spreading of invasive species has been observed (Seebens et al., 2017) and predictions show that the combination of potential biological invasions facilitated by the increasing globalization to more remote corners of the planet and the ongoing changing climate conditions will boost the spreading of invasive species (Hulme, 2017). Especially polar regions are in the danger zone for new invasions as the cold climate conditions that previously acted as a barrier for the spreading of more temperature sensitive invasive species have been weakened (Kourantidou et al., 2015; Lee et al., 2017; Holbech and Pedersen, 2018). To prevent further spreading of non-indigenous species via ballast water, the International Maritime Organization (IMO) ratified the International Convention for the Control and Management of Ship’s Ballast Water and Sediments (IMO, 2004) in 2016 and the convention entered into force September 2017. The United States Coast Guard (USCG) introduced similar regulations in 2012. The implementation of these global regulations generates a dramatic increase in the demand for approval of ballast water treatment systems (BWTSs) in accordance with the requirements set up in the regulations.

The BWTSs are required to pass a series of tests at an IMO/USCG approved test facility to achieve a BWTS Type Approval. Test water containing organisms (phyto- and zooplankton) of different size classes is prepared and used by test facilities for the testing of BWTSs. Concentrations of organisms are assessed in intake water and treated discharge water which has passed through the BWTS and been stored in a holding tank for at least one day to simulate ballast water tank conditions (IMO, 2016c). In addition, the organism concentration is assessed in control discharge water, which is kept like the test water but has bypassed the BWTS. The quality of the control discharge water provides information on the effect of the holding period on organism concentration to ensure that elimination of organisms is caused by the BWTS and not environmental conditions (Drillet et al., 2013; IMO, 2016c).

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Under the IMO, BWTS efficacy for the size class $\geq 10^{-}<50 \mu m$ (hereafter $10^{-50} \mu m$), mainly comprising of phytoplankton is assessed based on number of viable organisms in the test water which means that organisms can be alive but have lost their ability to reproduce and pose no invasion risk (IMO, 2016b). The USCG bases their assessments on the number of living organisms (US Coast Guard, 2012) which has created implications for ships using UV-based treatment systems. Exposure to UV irradiation mainly targets DNA thereby destroying the cells’ ability to reproduce. Such cells will, at least for a period, show signs of life and be classified as living although dying (Reviewed by Rastogi et al., 2010). The damage to vital cell components will eventually lead to cell death which can be detected using the Most Probable Number (MPN) method – one of two recognized methodologies applied for the enumeration of potential invasive species in the 10–50 μm size class (IMO, 2016d). The MPN method is based on the regrowth potential of the treated organisms (Wright and Welschmeyer, 2015; Cullen and MacIntyre, 2016; IMO, 2016d; Cullen, 2018) whereas the vital stain (VS) method is based on the activation of fluorescent stains (FDA/CMFDA) by unspecific enzyme activity inside living (but not necessarily viable) cells (US EPA, 2010; Steinberg et al., 2011; IMO, 2016d). Thus, the VS method includes both viable and living, but non-viable organisms in the enumeration procedures, but they cannot be distinct from each other. Both methodologies are accepted within the IMO but the use of the MPN method was rejected by the USCG in 2016 (Coast Guard Maritime Commons, 2016). This has implications for the UV-based treatment systems comprising of about 46% of the BWTS market (www.docimo.org, 2018, Gerhard et al., submitted) because only the MPN method is suitable for assessing the number of viable organisms of UV-based treatment systems. Therefore, the UV-based treatment systems cannot be properly tested by the USCG and they will fail type approvals that otherwise would succeed if assessed by the MPN method (For a more detailed description of the debate see Blatchley et al. 2018). However, UV-based treatment systems can comply with the discharge standard using the VS method if about 10 times more UV energy is applied compared to the energy levels required for compliance when assessed using the MPN method (Drake et al., 2016; Lundgreen et al., 2018).

Test water requirements dictated by both IMO and the USCG are similar when it comes to the minimum concentration of organisms needed at intake and control discharge during land-based testing (Table 1). To be valid, a land-based test must be initiated with $>1000$ org. $\cdot$ ml$^{-1}$ in the 10–50 μm size class and $>100,000$ org. $\cdot$ m$^{-3}$ in the > 50 μm size class (US EPA, 2010; IMO, 2016c). The minimum requirement for shipboard testing is set at a lower limit for both size classes, equal to 10 times the discharge standards (Table 1) (IMO, 2016c).

Naturally occurring organism concentration requirements in intake water in land-based testing cannot be found everywhere and at all times (D’Agostino et al., 2015). Therefore, protocols suggest the use of standard test organisms (STOs) (US EPA, 2010, IMO, 2016c). The revision of the G8 Guidelines stipulates in the paragraph on the use of STOs (2.4.23) that “the use of STOs should not be considered standard practice and the Administration should in every case review that the selection, number and use of supplementary STOs ensures that the challenge posed to the BWTS provides an adequately robust test. The use of STOs should not bias a test for or against any specific treatment process”. The last sentence of this quote aims at ensuring that a particular STO is not favoring a treatment technology at a test facility but does not directly request that STOs should have the same robustness as naturally occurring populations (where robustness varies). The position of the regulatory bodies in this respect seems clear: STOs may be used to reach the minimum number of organisms during land-based testing. They should be at least as robust as the majority of the organisms at a test site and should not generate a positive or negative bias to any technology. During recent international panel discussions driven by the IMO, experts agreed that the amount of data available to provide solid recommendations in terms of the use of STOs during testing was limited. It was agreed that STOs could have the advantage to increase comparability between tests, could support the requirement to reach a minimum concentration of organisms for testing, and provide safer tests if STOs are found to be at least as robust as naturally occurring species (IMO, 2016a, 2016b).

To provide more insight on STO robustness and their potential use in test water for more solid BWTS validation, we investigated the robustness of two algal species towards low pressure (LP) and medium pressure (MP) UV treatments for the potential use as STOs. Algal robustness was evaluated using both the VS and MPN method to investigate strengths and limitations of applying a vitality versus a viability assessment method. The two algal species, Tetraselmis suecica and Odontella sp., are customary practice to use as supplement to natural seawater for validation of BWMS at test facilities (Global TestNet, 2018). Odontella sp. belongs to the diatoms whereas T. suecica belongs to the small prasiophytes. Both groups are part of the dominating groups of coastal and neritic marine habitats (Norris et al., 1980; Not et al., 2012). The two species are thus representatives of relevant and ecologically significant taxa. Likewise, the algae are easily cultivated under both temperate and tropical conditions. The obtained fluorescence-response relationships of the two species were finally related to studies on natural algal compositions exposed to similar UV treatments.

### 2. Materials and methods

#### 2.1. Test organisms and preparation for experiments

Low- or medium pressure UV treatments were performed on *Tetraselmis suecica* (SCCAP, K-0297) and *Odontella* sp. monocultures (fluence range: 0–1500 mJ cm$^{-2}$). *Odontella* sp. was isolated from Singapore coastal waters. Experimental conditions are presented in Table 2. Monocultures of *T. suecica* were initially grown as semi-continuous cultures in Keller’s Culture Medium at 20 psu (Keller et al., 1987) in a temperature-controlled room at 19°C and 16:8 hour light/dark cycle. In one experiment (LP-TET run 2) salinity was by a mistake 27 psu which was not detected until the day of the experiment. Due to a human error, the culture medium had not been slowly adjusted down to 20 psu from the benchmark salinity of 27–30 psu normally used in the algal culture room. To avoid osmotic shock on the algal cells by instant adjustment to 20 psu it was decided to continue using the 27 psu test water solution. Monocultures of *Odontella* sp. were grown as semi-continuous cultures in F/2 Culture Medium at 20 psu (Guillard and Ryther, 1962; Guillard, 1975) in a temperature-controlled room at 25°C and 24-hour light conditions. The final test water solutions for both

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1 This is not considering the microbiological requirements (organisms < 10 μm).

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### Table 1

Minimum requirements for test water organism concentrations for the size classes $10^{-}<50$ μm and $>50$ μm during land-based and shipboard testing in *intake* and *control discharge*, as well as maximum requirements (D-2 discharge standards) for organism concentration in *treated discharge* water which has passed through the BWTS (IMO, 2004; US EPA, 2010; IMO, 2016c).

<table>
<thead>
<tr>
<th>Size class (μm)</th>
<th>Units</th>
<th>Intake</th>
<th>Control discharge</th>
<th>Treated discharge (D-2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\geq 10^{-}&lt;50$ org. $\cdot$ ml$^{-1}$</td>
<td>$\geq 1000$</td>
<td>$\geq 100,000$</td>
<td>$\geq 1000$</td>
<td>$\geq 100$</td>
</tr>
<tr>
<td>$\geq 50$ org. $\cdot$ m$^{-3}$</td>
<td>$\geq 10$</td>
<td>$\geq 10$</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>$&lt; 10$</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>
algal species were prepared 16–20 h prior to experiments. Test water was kept in a lit laboratory on a magnetic stirrer and stirred slowly with a continuous flow of filtered air without any growth light. Finally, lignin sulfonate was added to the test water to obtain a desired UV filtered air without any growth light. Finally, lignin sulfonate was added to the test water to obtain a desired UV concentration of two fluorescent based vital stains: Fluorescein diacetate (FDA, Molecular Probes-Invitrogen Carlsbad, CA, USA) and 5-chloro fluorescein diacetate (CMFDA, Molecular Probes-Invitrogen Carlsbad, CA, USA). Both stains are quenched by acetyl groups and are designed to be able to pass freely through cell membranes. The quenched stains are activated through cleavage of the acetyl groups by non-enzymatic germicidal intensity \( I_{\text{light}} \) by correcting for factors (water absorbance, reflection, divergence, transmittance) that will decrease \( I_{\text{light}} \) and hence the irradiating reaching the volume of water (i.e. volume-averaged) following the same procedures as described in the Supplementary material in Lundgreen et al. (2018). The relative irradiation spectrum was measured using an Ocean Optics USB 2000+ spectrophotometer with a 0.113 nm resolution. The relative spectrum energy values could be calculated based on the direct energy output of the UV lamps determined using uridine actinometry (UV triggered conversion of the chemical compound uridine) following the same procedures as described in the Supplementary material in Lundgreen et al. (2018). The \( I_{\text{light}} \) from the MP mercury lamp (Heraeus) at each wavelength was estimated to be around 279 nm (polychromatic) with energy peaks of 14, 8, 27, 16, 9 and 27 μW cm\(^{-2}\) around 248, 254, 265, 280, 289 and 297 nm (all sum of ± 1 nm), respectively (Fig. 1). When integrated the total \( I_{\text{light}} \) for the MP UV was estimated to be around 279 μW cm\(^{-2}\). Energy from the LP lamp (20.4 W, Trojan Technologies) is defined as a single peak (monochromatic) at 254 nm (Fig. 1). The total \( I_{\text{light}} \) at around this wavelength was estimated to be around 223 μW cm\(^{-2}\). The collimated beam set-up followed the procedures described in Lundgreen et al. (2018) and the Supplementary material herein. In short, the set-up included estimation of the LP and MP collimated beam \( I_{\text{light}} \) using uridine actinometry. Next, samples were placed under the collimated beam apparatus and exposed to UV light for a certain amount of time (exposure time) until the desired fluence (mJ cm\(^{-2}\)) was achieved. The desired fluences were calculated from \( I_{\text{light}}\) (W m\(^{-2}\)) × exposure time (s). Exposure times for LP UV experiments ranged from 111-4773 seconds and for MP UV experiments 61-2598 seconds.
2.4. Experimental design and exposure procedures

Organisms were exposed to LP or MP UV treatments at fluences (doses) aimed at 0, 25, 50, 100, 200, 500 or 1000 mJ cm\(^{-2}\). Samples of 50 ml test water was transferred to a petri dish and placed under the LP or MP collimated beam apparatus and exposed to UV treatments for a specific length of time to obtain the desired UV fluence. During exposure samples were continuously stirred using a magnetic stirrer (at a moderate speed so no vortex was created) to ensure homogeneous mixing of the samples. Next, samples were transferred to a dark bottle and stored for 24 h in a polystyrene box at ambient temperature. After 24 hours sample bottles were carefully mixed by inversion for homogenizing the sample and transferred to the petri dish for a second UV treatment. The total desired UV fluence was obtained by adding the two calculated UV fluences. After the second UV treatment, samples exposed to 100, 200, 500 and 1000 mJ cm\(^{-2}\) were immediately analysed for number of fluorescent organisms using the VS method (Fig. 2). In addition, samples exposed to 25, 50, 100 and 200 mJ cm\(^{-2}\) were analysed using the MPN method to evaluate the regrowth potential. In two experiments (MP-TET-3, MP-TET-4) samples exposed to 500 mJ cm\(^{-2}\) were also analysed using the MPN method.

Fig. 1. Collimated beam UV spectra as a function of irradiation energy (incidental germicidal intensity – i.e. not water factor adjusted). The black line shows the recorded UV spectrum between 200 and 300 nm of the polychromatic medium pressure (MP) system. The green line shows the UV spectrum of monochromatic low pressure (LP) system. The energies of both systems were based on actinometry measurements from the relative distribution of energy obtained by using an Ocean Optics spectrophotometer with Premium fibre sensor. In addition, the variation in water absorption (blue line) is shown as a function of wavelength. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.5. Regression analysis of MPN data

First-order kinetics regressions could be fitted after log-transformation of the MPN data using Eqs. (1a) and (1b) adapted from MacIntyre et al. (2017) (Eq. 8a and 8c herein). The fluence, \(F\) (termed dose, \(D\) in MacIntyre et al. (2017)) required to reduce the number of viable organisms to 1% of the initial concentration (2log\(_{10}\) reduction) was determined by adopting the sensitivity term \(F(0.01)\) (mJ cm\(^{-2}\)). In MacIntyre et al. (2017) relative values were used in the equations because fluence rates were proprietary. In the present study absolute values were used to fit the equations:

\[
\text{MPN}_F = \text{MPN}_F(0) \text{ for } F \leq F_{th} \quad \text{(1a)}
\]

\[
\text{MPN}_F = \text{MPN}_F(0)e(-k(F - F_{th})) \text{ for } F > F_{th} \quad \text{(1b)}
\]

where \(\text{MPN}_F\) is the number of viable organisms at fluence \(F\) (mJ cm\(^{-2}\)), \(F_{th}\) is threshold fluence (mJ cm\(^{-2}\)) below which there is no effect on
algal viability, $k$ is the inactivation constant (slope) (cm$^2$ mJ$^{-1}$), and $\text{MPN}_{F=0}$ is the number of viable organisms after treatment at or below $F_{Th}$.

Next, the sensitivity term $F[0.01]$ (mJ cm$^{-2}$) could be found by solving Eq. (1b) where $\text{MPN}_{F=0.01} = 0.01 \times \text{MPN}_{F=0}$. Non-detect values (NDs, resulting from MPN scores of 0-0-0 with no positive tubes in any of the dilutions) were excluded from the data sets. All-positive values (resulting from MPN scores of 5-5-5 with positive tubes in all dilutions) were included in fits. More details on NDs and all-positives are provided in the Discussion.

3. Results

3.1. Test water stability and effect of dark-hold period on algal concentration

No decline in concentrations was observed after the 24-hour dark-hold periods. Paired $t$-test revealed that five experiments showed no significant difference ($P = 0.20-0.99$) and three (LP-TET-2, LP-ODO-2, MP-TET-2) showed a significant increase ($P < 0.05$) (Fig. 3) after the dark-hold period.

3.2. Fluence-response relationship of UV treatments on T. suecica and Odontella sp. concentrations

When analysed using the VS method, neither T. suecica nor Odontella sp. concentrations satisfied the discharge standard (< 10 org. ml$^{-1}$) at the highest LP UV fluences which ranged between 1279 and 1505 mJ cm$^{-2}$ (Fig. 4). In MP UV treatment experiments, concentrations of T. suecica satisfied the discharge standard at fluences around 1000 mJ cm$^{-2}$ when analysed using the VS method (Fig. 4).

In all experiments on T. suecica and Odontella sp. fluence-response relationships of UV treatment on algal regrowth (MPN data) displayed an exponential decrease in concentration and first-order kinetics.
Regression lines could be fitted after log-transformation of the MPN data (Fig. 5). Trendline equations were adapted to Eq. (1b) that integrates the potential presence of a threshold fluence below which viability is unaffected by fluence. All regression lines showed that fluence had a significant negative effect ($P < 0.05$) on MPN concentration. At higher fluences NDs (MPN scores of 0-0-0) were observed. The NDs are included in Fig. 5 (red circles).

The fluence required to reduce the number of viable organisms to 1% of the initial concentration (2log$_{10}$ reduction) was calculated by adapting the sensitivity term $F[0.01]$ (mJ cm$^{-2}$) from MacIntyre et al.
Table 3

The calculated fluence (F[0.01]) needed for reducing initial concentration (MPN₁₀₀  ) 100 times for *T. suecica* and *Odontella* sp. based on data from the present study and for natural algal compositions based on data obtained from Lundgreen et al. (2018). The F[0.01] values have been ranked from highest to lowest fluence needed to achieve a 100 times reduction in algal concentration. The number of data points used for each regression line has been included.

<table>
<thead>
<tr>
<th>UV type and Exp ID</th>
<th>#data points of regression</th>
<th>Fluence (mJ cm⁻²)</th>
<th>Shoulder Intercept (org. ml⁻¹)</th>
<th>F[0.01] (mJ cm⁻²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LP-TET-3</td>
<td>8</td>
<td>0.023</td>
<td>50</td>
<td>5814</td>
</tr>
<tr>
<td>LP-TET-1</td>
<td>11</td>
<td>0.019</td>
<td>0</td>
<td>2385</td>
</tr>
<tr>
<td>LP-TET-4</td>
<td>9</td>
<td>0.024</td>
<td>25</td>
<td>4044</td>
</tr>
<tr>
<td>LP-TET-2</td>
<td>11</td>
<td>0.025</td>
<td>0</td>
<td>3839</td>
</tr>
<tr>
<td>LP-TET-2</td>
<td>11</td>
<td>0.026</td>
<td>0</td>
<td>4852</td>
</tr>
<tr>
<td>LP-NAT-TEMP-1</td>
<td>11</td>
<td>0.032</td>
<td>0</td>
<td>5993</td>
</tr>
<tr>
<td>LP-NAT-TEMP-2</td>
<td>11</td>
<td>0.041</td>
<td>0</td>
<td>2765</td>
</tr>
<tr>
<td>LP-ODO-1</td>
<td>6</td>
<td>0.044</td>
<td>0</td>
<td>1793</td>
</tr>
<tr>
<td>MP-NAT-TEMP-2</td>
<td>11</td>
<td>0.044</td>
<td>0</td>
<td>9060</td>
</tr>
<tr>
<td>LP-NAT-TEMP-3</td>
<td>10</td>
<td>0.050</td>
<td>0</td>
<td>9235</td>
</tr>
<tr>
<td>LP-ODO-1</td>
<td>5</td>
<td>0.066</td>
<td>0</td>
<td>938</td>
</tr>
<tr>
<td>MP-NAT-TEMP-1</td>
<td>9</td>
<td>0.074</td>
<td>0</td>
<td>3040</td>
</tr>
<tr>
<td>LP-NAT-TROP-1</td>
<td>5</td>
<td>0.113</td>
<td>0</td>
<td>11,562</td>
</tr>
</tbody>
</table>

(2017). The ranked F[0.01] values from fluence-response experiments on *T. suecica* and *Odontella* sp. are listed in Table 3 and compared with the results obtained from similar analysis on natural algal compositions previously reported in Lundgreen et al. 2018. Comparison of regression lines and interaction analysis (ANCOVA) showed that fluence overall had a significantly higher impact (P < 0.001) on *Odontella* sp. and natural algal concentrations compared to *T. suecica* concentrations. By taken the statistical analysis into consideration as well as the ranking of F[0.01] values, the data shows that monocultures of *T. suecica* in general require higher fluences for rendering non-viable compared to *Odontella* sp. and natural algal compositions. *T. suecica* required between 144 and 250 mJ cm⁻² for a 2 log₁₀ concentration reduction of viable organisms. Natural algal compositions from tropical waters were ranked as most UV-sensitive with an estimated F[0.01] of 41 mJ cm⁻². Tropical *Odontella* sp. F[0.01] values ranged between 70 and 105 mJ cm⁻² which was about 40 mJ cm⁻² below the lowest estimated F[0.01] for LP UV treated *T. suecica*. There were no statistically significant differences between LP and MP UV treatments.

3.3. Effect of extended dark-hold period after MP UV treatments on vital stain counts

Counting of *T. suecica* using the VS method immediately after last MP UV treatment at T(0) resulted in concentrations above the discharge standard at fluences up to 500 mJ cm⁻² and concentrations did not satisfy the discharge standard until fluences of 1000 mJ cm⁻² were applied (Figs. 4 and 6, circular symbols). Counts performed 24 h, T(24), and 96 h, T(96), after T(0) resulted in concentrations in line with the discharge standard at 500 mJ cm⁻² (Fig. 6). At T(96), the concentration at 200 mJ cm⁻² had decreased to about 30% (1200 cells ml⁻¹) of control levels (Fig. 6). *T. suecica* concentrations at between 25 and 100 mJ cm⁻² counted at T(0), T(24) and T(96) did not differ from control levels (Fig. 3G, H).

3.4. Effect of UV treatments on algal behaviour

There were clear immediate effects of UV treatments on *T. suecica* behaviour when observed under the microscope during VS counts for both LP and MP UV treatments (Table 4). In control samples, without UV treatment, algal cells were typically mobile and present in the whole water column of the counting chamber. In MP UV treated samples at 100 mJ cm⁻², cells became immobile and were only present on the bottom of the chamber whereas LP UV treated cells remained mobile in the whole water column up to 135 mJ cm⁻². Algal cells that became completely immobile in the lower fluence range were able to stay alive (VS method) for several days after UV treatments (Fig. 6). Above 270 mJ cm⁻², all cells were rendered immobile for both LP and MP UV treatment experiments. *Odontella* sp. is a non-mobile algal species and

![Fig. 6. Fluence-response relationship of MP UV treatments on *T. suecica* concentrations (log₁₀ scale, mean ± SD, org. ml⁻¹) showing VS counts of algal cells at 24 h (T(24)) and 96 h (T(96)) after last MP UV treatment in relation to counts immediately after last exposure at T(0). T(0) values are from Fig. 4. For each data point n = 2. Data points (NDs) on the x-axis line (y = 0.1 org. ml⁻¹) signify quantification analyses where no cells were encountered in the counted volume of 2 ml.](image-url)
hence no behavioural differences in relation to UV treatment were observed. All algal cells were immobile and located on the bottom of the counting chamber in control samples and at all UV treatments.

4. Discussion

The main findings of the current experiments will be discussed in the following paragraphs. This will include the robustness of *T. suecica* and *Odontella* sp. exposed to either LP or MP UV-treatments as well as the observed different fluence levels required to meet stated discharge standards when evaluated using the two viability assessment methods – the VS and MPN method. The potential advantageous use of *T. suecica* and *Odontella* sp. as STOs will be further discussed.

In the present experiments, test water fulfilled the required algal concentration of ≥1000 org. ml⁻¹ (Table 1) set for intake test water used at IMO approved land-based test facilities during BWTS Type Approval testing. The time-series showed either stable or increased algal concentrations in control samples (no treatment) measured before and after dark-hold periods for each individual UV treatment experiment. This implies that test water conditions throughout the experimental period or dark-hold period caused no decline in algal concentration. Declines in algal concentrations in the experiments are therefore being attributed to UV treatments.

In LP UV treatment experiments, neither *T. suecica* nor *Odontella* sp. concentrations satisfied the discharge standard at the highest tested fluences of about 1400–1500 mJ cm⁻² when assessed using the VS method (Fig. 4). In addition, similar results were obtained in fluence-response relationships of LP UV treatments on concentrations of another strain of *T. suecica* (CSIRO) carried out under tropical conditions (27 °C) (data not shown). Using the same experimental setup, it was previously shown that concentrations of natural phytoplankton from both temperate and tropical conditions satisfied the discharge standard at fluences around 1300 mJ cm⁻² in LP UV treatments (Lundgreen et al., 2018). In MP UV treatments, concentrations of *T. suecica* assessed using the VS method met the discharge standard at 1000 mJ cm⁻² in all four experiments (Fig. 4) which were similar to results obtained on natural algal populations at this fluence by Lundgreen et al. (2018).

In LP UV treatments assessed using the MPN method, concentrations of *T. suecica* and *Odontella* sp. satisfied the discharge standard at about 270 and 130 mJ cm⁻², respectively. This is in line with other fluence-response studies using LP UV systems where it was found that fluences in the range 100–150 mJ cm⁻² were needed for a 90% reduction of *Tetraselmis suecica* assessed using a cell count approach after 120 h of incubation (Littved et al., 2011). Fluences about 41 and between 92 and 112 mJ cm⁻² were needed for a 2log₁₀ reduction of tropical and temperate natural algae composition, respectively, in LP UV treatments assessed using the MPN method (Lundgreen et al., 2018). For a 2log₁₀ reduction of *Microcystis aeruginosa* fluences of about 180 mJ cm⁻² were reported by Sakai et al. (2011) using a 14 days incubation period and fluorescence microscopy for counting of cell numbers. Lₐₐ values for *T. suecica* and *Pseudokirchneriella subcapitata* were 353 and 236 mJ cm⁻², respectively, assessed by fluorescence microscopy based on an approach using TO-PRO-1 iodide (McGivney et al., 2015). And finally, a fluence of about 120 mJ cm⁻² was reported for a 4.5–5log₁₀ inactivation of *Tetraselmis* sp. assessed using the MPN method (Sun and Blatchley, 2017).

In the MP UV treatment assessed using the MPN method, concentrations met the discharge standard at fluences around 500 mJ cm⁻² and in one of the four performed experiments, the discharge standard was met at 200 mJ cm⁻² (Fig. 5E–H). For comparison with other MP UV fluence-response studies Sakai et al. (2011) found a 2log₁₀ reduction of *Microcystis aeruginosa* exposed to fluences of about 180 mJ cm⁻² using a 14 days incubation period and fluorescence microscopy for counting of cell number. Furthermore, Olsen et al. (2016) reported that the minimum fluences for permanent inactivation of *T. suecica* were in the range 200–400 mJ cm⁻² using the MPN method as assessment and by introducing a one day dark incubation period. The authors recommended a minimum fluence of 400 mJ cm⁻² for treating ballast water to permanently inactivate organisms which is in line with the findings of 200–500 mJ cm⁻² in the present study.

The observed difference between the two assessment methods in the present study can be explained by the slow manifestation of damages inflicted by the UV irradiation. Monochromatic UV irradiation mainly targets DNA, where damages will hamper vital cellular functions and cell division eventually leading to cell death (Jimenez et al., 2009; Rastogi et al., 2010). However, up to a certain UV fluence the cell membrane remains intact and some enzymes continue to function. The cell’s enzyme activity will thereby still be able to activate the fluorescent CMFDA/FDA stains resulting in the immediate higher VS counts compared to MPN counts where damages have had time to manifest and cause mortality or non-viability.

Overall, higher fluences were needed in MP UV treatments when assessed using the MPN method compared to LP UV treatments but there was no statistical difference between the two UV types. An explanation could be that in a polychromatic approach a higher total fluence is necessary to apply enough energy at certain wavelengths responsible for inflicting sufficient damage of vital cellular components. In a monochromatic approach, the energy is concentrated around 254 nm, whereas in a polychromatic approach it is only about 3% of the total applied energy at this wavelength (Lundgreen et al., 2018). Consequently, in a monochromatic approach, already at lower fluences a high amount of energy is applied at 254 nm affecting DNA and RNA.

The manifestation of these damages takes time which explains why the use of the VS method at T(0) does not satisfy the discharge standard whereas it is met using the MPN method. At low fluences in a polychromatic approach the energy at the wavelengths responsible for inflicting damage is probably not sufficient to cause vital impairment. If fluence is increased, the corresponding increase in the energy at various wavelengths will be able to inflict damage on other vital cell components in addition to DNA (Blatchley et al., 2018). This theory was investigated in a UV treatment study by Sun and Blatchley (2017) looking at the effects of different wavelengths on inactivation of *Tetraselmis* sp. by means of narrow bandpass optical filters. The authors found that ideal inactivation occurred between 254 and 280 nm and concluded that damage to other biomolecules besides DNA must play an important role in inactivation of organisms exposed to MP UV treatments (Sun and Blatchley, 2017). Similar results were obtained by Choi et al. (2011) that investigated the inactivation of *Tetraselmis* sp. using UV LED irradiation and found highest inactivation in the 260–270 nm range. The results in the present study suggest that the MP UV treatments at >500 mJ cm⁻² facilitated damage to other essential cellular components related to membrane integrity. This caused an additional inactivation of cells as observed at 1000 mJ cm⁻² when quantified with the VS method supporting the findings of Sun and Blatchley (2017). In LP UV treatments, damages are focused merely on DNA, thus leaving the cell membrane intact which could explain that inactivation was not profound at the highest fluences compared to MP UV treatments when assessed using the VS method. On the other hand, at fluences <500 mJ cm⁻² the energy in MP UV treatments seems insufficient in facilitating enough damage to DNA and other biomolecules to satisfy the discharge standard assessed by the MPN method. Behavioural observation in MP UV treatments indicated some immediate effects of the UV treatments as algae showed signs of immobilization at around 100 mJ cm⁻². The behavioural observations were thus similar to those observed in LP UV treatments in the present study as well as on natural algal populations (Lundgreen et al., 2018) but did not cause non-viability.

As mentioned by Olsen et al. (2016), it is important to keep in mind that current fluence-response relationships are based on controlled collimated beam experiments performed in the laboratory. Such results cannot directly be compared to that of commercial flow-through UV chamber BWTSs that typically includes two (or more) treatment types.
(e.g., filtration + UV) to reinforce the efficacy of the system (Olsen et al., 2016).

Dark-hold periods did not affect VS counts in control (no treatment) samples as Pairwise t-tests showed no significant differences in algal concentrations at T(0), T(24), and T(96). The fact that no change in concentration was observed for T. suecica after 96 hours extended dark-hold period (≈ 4 days dark-hold in total) suggest that T. suecica could be used as STO in intake test water to realize control discharge requirements. Various STOs have been used successfully in most test facilities, supporting that their survival in control tanks is acceptable (Global TestNet, 2018). As shown in Fig. 4, fluences in the magnitude of 1000 mJ cm$^{-2}$ for MP UV treatments resulted in concentrations below the discharge standard when immediately assessed after last treatment at T(0) using the VS method. Fig. 6 shows that already in T(24) samples no fluorescent organisms were detected from VS counts suggesting that this magnitude of MP UV treatment was lethal to T. suecica. The immediate effects of the UV damages on cells at 500 mJ cm$^{-2}$ were less profound since the discharge standard was not achieved at T(0). However, the inflicted UV damages were fatal as concentrations satisfied the discharge standard in T(24) samples and further, no fluorescent cells were detected in T(96) samples. The fact that UV damages were fatal at 500 mJ cm$^{-2}$ was supported by the MPN results where algal concentrations were predicted to be 0.18 org. ml$^{-1}$ (or below) – > 50 times below the discharge standard. At 200 mJ cm$^{-2}$ no immediate changes were seen in T(0) VS counts but a 73% reduction was observed in T(96) samples compared to T(0) which suggests that some damaging effects of UV treatments had occurred at this fluence. This can be compared to MPN data at 200 mJ cm$^{-2}$, where two samples showed an average decrease of > 97% compared to control sample concentrations and in one sample concentrations were below the discharge standard (Fig. 5E-H). The observed decrease in concentrations over time from T(0) to T(96) (supported by MPN data) suggests a slow manifestation of the MP UV damages in cells at 200 mJ cm$^{-2}$, which could lead to cell death if extended dark-hold periods are implemented. The effect of extended dark-hold periods following UV treatments is supported by other studies (Sutherland et al., 2001; Olsen et al., 2016; Romero-Martinez et al., 2016; Lundgreen et al., 2018).

One-way ANOVA analysis revealed no immediate effects of MP UV treatment on concentrations within the range of 25–100 mJ cm$^{-2}$ combined with dark-hold periods in samples analysed using the VS method since counts at T(0), T(24) and T(96) were not different from control sample concentrations. Although VS counts remained constant, behavioural observations showed a decreased mobility as the dark-hold period increased suggesting that UV damages were nevertheless inflicted on the treated cells in the range 25–100 mJ cm$^{-2}$ as also reported in natural algal populations in Lundgreen et al. (2018).

By following the approach presented in MacIntyre et al. (2017) it was possible to determine the fluence (termed dose in MacIntyre et al. (2017)) required to reduce the number of viable organisms to 1% of the initial concentration (2log$_{10}$ reduction) by adapting the sensitivity term $F(0.01)$ (mJ cm$^{-2}$). The approach considers the existence of shoulders and serves as an objective procedure for comparing fluence-response data between different algae and algal compositions independent of initial concentration. The dilutions chosen for the MPN experiments were based on ‘educated guesses’ which in several cases resulted in data points below the limit of detection (NDs) or in saturated responses (all-positives). For best practices to ensure that appropriate dilutions are chosen when working with cultures the approach described in MacIntyre et al. (2017) should be taken into consideration. To deal with the NDs we applied the approach by Sun and Blatchley (2017) and based on arguments presented in Cullen (2018), Helsel (2010) and Jarvis et al. (2010) these values were excluded from regression data. It is argued that in a ND score, MPN = 0 with a two-tailed ($P = 0.025$) upper confidence limit that could be considered as a lower limit of detection of the method (Jarvis et al., 2010). Thus, the observed NDs are below the limit of detection and not included in trendline estimations. NDs are nevertheless included in Fig. 5 because they inform that no regrowth was detected beyond these fluences. The all-positives are the result of MPN scores of 5-5-5 which means that positive growth was observed in all five tubes of each dilution. The calculation of MPN estimates in the present study were based on the table presented in Garthright and Blodgett (2003) where all-positive scores provide a minimum assumed value (e.g. > 1600 org. ml$^{-1}$, when using a dilution series of 0.1; 0.01; 0.001) without confidence limits. Based on the same dilution series, a different interpretation is provided in Jarvis et al. (2010) where an all-positives MPN score is categorised as infinity (≈) with a 95% confidence interval [650; ∞]. An exclusion of the all-positive values in the present data sets would decrease the robustness of the analysis because the number of data points used for regression would be reduced. However, including the all-positives could bias the analysis because all-positive MPN scores with no confidence limits (Garthright and Blodgett, 2003) or the lower limit of the 95% confidence interval (Jarvis et al., 2010) are included in the analysis. To explore bias, the impact of all-positive scores on the obtained fluence-response trendlines was investigated. The interaction analysis (ANCOVA) was run on data sets excluding all-positives and including all-positives using tables from both Garthright and Blodgett (2003) and Jarvis et al. (2010). All runs resulted in similar outcomes: The impact of fluence on MPN was significantly lower ($P < 0.001$) for T. suecica compared to Odontella sp. and natural algae. It is therefore concluded that inclusion of all-positive MPN scores did not bias the data in the present study.

Despite the challenges with the data set described above, the ranking clearly shows that monocultures of T. suecica in general are more robust towards UV treatments compared to Odontella sp. and natural algal compositions. T. suecica required between 144 and 250 mJ cm$^{-2}$ for a 2log$_{10}$ concentration reduction of viable organisms. The data also showed that MP UV treatments on T. suecica seemed less efficient compared to LP UV treatments although not statistically significant. Natural algal compositions from tropical waters were ranked as most UV-sensitive which might be explained by either the algal mix containing the weakest composition of algal species, or by more challenging test water and MPN analysis incubation conditions (e.g. higher temperatures). The higher incubation temperatures could likewise explain the lower robustness of tropical Odontella sp. compared to T. suecica. Consequently, F(0.01) is probably underestimated and Odontella sp. and tropical natural algae will most likely be ranked higher if incubated at similar temperatures as temperate experiments. The fact that no change in Odontella sp. concentration could be recorded using VS counts even at the highest fluence applied (about 1500 mJ cm$^{-2}$) clearly shows that the VS method also underestimated the effects of UV treatment in this species. The difference in robustness towards UV treatments between T. suecica and Odontella sp. can also be affected by their structural differences. T. suecica is rich in intracellular polysaccharides and the cell wall consist of complex carbohydrates (Becker et al., 1998; Kermanshahi-Pour et al., 2014) whereas a defining feature of diatoms as Odontella sp. is the silica cell wall called frustule (Raven and Waite, 2004). A recent study has suggested diatoms to be protected against UV exposure by their frustules (Aguirre et al., 2018) but other physiological features could possibly influence the robustness towards UV treatment of Odontella sp. and T. suecica.

A fluence-response study on Tetraselmis sp. by Sun and Blatchley (2017) found $k = 0.0792$ cm$^{-2}$ (about 50% below that of the present study) which might be explained by variances in the two different strains of Tetraselmis used in the two experiments. The applied sensitivity approach towards UV treatment adopted from MacIntyre et al. (2017) showed a 6-fold difference between species in the present study. This was slightly less than the 10-fold difference observed in 12 phytoplankton species from seven divisions using relative fluences (absolute fluences were proprietary) within the range of commercial applications (MacIntyre et al., 2017). In parallel, the two studies show that there seems to be large variations in sensitivity between algal species.
towards UV treatments which potentially could affect the outcome of validation tests if performed on phytoplankton in either end of the scale.

The ranked $F(0.01)$ values suggest that monocultures of *T. suecica* could be a promising candidate as STO for validation of UV treatment systems. The species can be cultivated in a wide salinity range (Fabregas et al., 1984) and would therefore be suitable for both brackish water and seawater validation tests. Individual cells of *Tetraselmis* sp. are occasionally below the minimum size cut-off of $10\mu m$, but for practical reasons and for more safe assessments, borderline species are included in counts (IMO, 2016d). The inclusion of smaller organism in validations of BWTSs is presently being debated because by following current regulations algal species $< 10\mu m$ are unregulated risks which are not accounted for and this higher risk of bio-invasions of small species has been addressed in several studies (Gollasch et al., 2007; van der Star et al., 2011; Gollasch et al., 2012; Liebich, 2013; Casas-Monroy et al., 2016; Lundgreen et al., 2018; Trindade de Castro and Veldhuis, 2019). Even though the tropically cultured algae, *Odontella* sp. was less robust towards LP UV treatments compared to *T. suecica* it was ranked higher than $F(0.01)$ values from tropical LP UV treated natural algal compositions. Therefore, this algal species could likewise be considered as a potential tropical STO. To further support this view, VS counts were much higher for *Odontella* sp. when compared to LP UV treated natural algal compositions (Lundgreen et al., 2018) and thus, seemed far more robust towards LP UV treatments.

Based on the obtained $K$ values the fluence ($F_{D-2}$, $mJ cm^{-2}$) needed for algal concentrations to meet the discharge standard (MPN$_x = 10$ org. ml$^{-1}$) could be estimated over range of MPN$_{x-0}$ values (Fig. 7). The figure shows that the fluences needed for meeting the discharge standard must be adjusted according to algal type or composition and initial concentration (MPN$_{x-0}$). The higher fluences are required because increases in cell density will affect test water UVT due to the shading effects of the cells.

These results outline that the VS method is less suitable for validation of UV-based treatment systems compared to the MPN method. When assessed by the VS method, UV-based treatment systems would fail despite applying fluences 5–10 times higher than that needed for the MPN method to comply with the discharge standard. For natural algal compositions exposed to UV treatments it was recently shown that it was feasible to meet the discharge standard using the VS method but about 10 times higher UV fluences were required compared to assessments using the MPN method (Lundgreen et al., 2018). The higher UV fluences needed for *T. suecica* and *Odontella* sp. compared to natural algal compositions suggest that these potential STO candidates are more robust towards UV treatments when assessed using the VS method. Immediate effects of UV treatments were nevertheless observed on algal behaviour during the VS counts where mobile *T. suecica* cells were immobilized at fluences above $270\ mJ \ cm^{-2}$. The observed functional response support that immediate effects caused by the UV irradiation had occurred despite no change in VS counts was observed.

Besides the requirements listed in Table 1 it is additionally stated in MEP/279/70 that influent water should consist of at least 5 species from at least 3 different phyla/divisions (IMO, 2016c). There is probably no facility that can ensure that there is a mix of organisms used in every single test, all year round which represent the “more robust populations”. The robustness of one species against a technology can be evaluated and compared to one population or assemblage at any point of time, but this cannot be considered the “more robust population” at a facility. It probably changes every day and certainly during the year. The ETV protocol states that: “It is critical to evaluate the effectiveness of a treatment system under water quality conditions that are challenging to the technology being tested (US EPA, 2010 - 5.2.1).” Not all groups of organisms can ensure that a minimum robustness is achieved in a comparable way between test facilities around the world. Technically, only standard test organisms may ensure this, as mentioned in the final report to NSF International by Woods Hole Oceanographic Institution: the surrogate species would, if inactivated or killed by a treatment, provide assurance that a broad range of other organisms also would be inactivated or killed (Anderson et al., 2008). The supplement of STOs with at least the same robustness as natural phytoplankon assemblages as shown for *T. suecica* and *Odontella* sp. could therefore assist in realizing minimum number of species in intake water with the objective to ensure more robust test populations and provide a more protective foundation for conservative BWTS testing.

The organism requirements for intake water during shipboard testing raise an interesting question about the objective of the requirements set for land-based testing. The objective of Type Approval testing is to ensure that a system’s biological efficacy will be evaluated...
K. Lundgreen, et al.

as a function of its ability to kill or remove organisms that are naturally occurring and represent the more robust ambient populations at a test site (US EPA, 2010 - section 5.2.2). It could be questioned why the concentration requirement at intake differs between land-based and shipboard testing (Table 1). This statement from the ETV protocol also suggests that more robust STOs should be used because the requirements for organism density of intake water in land-based testing cannot be met everywhere and all the time. Furthermore, there are no scientific data supporting the idea that assemblages present in highly productive areas would be more robust than those encountered in less productive areas. Therefore, it is possible that testing against 100 robust organisms is more conservative (safe) than testing against 1000 weak ones. Previous studies have for example shown diatoms to be dominating in discharge water samples and therefore could be considered more robust than other algal classes against UV treatments (Kang et al., 2010; Liebich et al., 2012; Martinez et al., 2013; Stehouwer et al., 2013; Stehouwer et al., 2015). A study by Liebich et al. (2012) showed high regrowth potential after UV treatments in three species of diatoms and suggested them to be suitable indicator organisms for testing the biological efficacy of UV-based treatment systems. If locally isolated STOs are considered inappropriate for testing (e.g. too weak), obtaining the minimum organism concentration in intake water will remain a challenge for which practical propositions should be made. It would be unreasonable to believe that testing of BWTSs could only be carried out when the naturally occurring organism concentration is adequate for both size classes (≥50 μm and 10–50 μm) simultaneously. This is apparently taken into account by the standard set for shipboard testing where the minimum organism concentration is lower compared to land-based testing standards. Therefore, in land-based testing experts have recommended to simply ignore the minimum concentration of cells required; aligning with the approach used during shipboard testing. This proposition was, however, not accepted by the IMO and the minimum concentration required under the IMO (and USCG) regulations must therefore be attained by other means (STO, river cultures or minimum concentration required under the IMO (and USCG) regulations). This proposition was, however, not accepted by the IMO and the minimum concentration required under the IMO (and USCG) regulations must therefore be attained by other means (STO, river cultures or minimum concentration required under the IMO (and USCG) regulations). This proposition was, however, not accepted by the IMO and the minimum concentration required under the IMO (and USCG) regulations must therefore be attained by other means (STO, river cultures or minimum concentration required under the IMO (and USCG) regulations). This proposition was, however, not accepted by the IMO and the minimum concentration required under the IMO (and USCG) regulations must therefore be attained by other means (STO, river cultures or minimum concentration required under the IMO (and USCG) regulations). The results from the present study suggests that testing facilities that are challenged in realizing intake water requirements would benefit from using *T. suecica* and *Odontella* sp. as STO-representatives as supplement in test water for BWTS Type Approval testing. A study by D’Agostino et al. (2015) likewise showed the advantageous use of cultured organisms (Bacteria (< 10 μm): *Alcanivorax borkumensis*, *Marinobacter hydrocarbofacicacious*; phytoplankton (10–50 μm): *Tetraselmis suecica*; and zooplankton (> 50 μm): *Artemia salina, Brachionus plicatilis* L.) as supplement to challenge water to fulfill intake concentration requirements and to overcome problems arising from natural conditions such as season and weather.

It can be argued that the existing requirements for survival of organisms in the control discharge water are very low (Table 1). The motivation for installation of BWTSs is that organisms are expected to survive in the ballast water tanks during voyage thereby posing a bio-invasion risk which need to be neutralized by the BWTS. The consequence at test facilities with low survival of organisms in control discharge water is that BWTSs only need to eliminate a small proportion of the organisms originally present in intake water to pass validation compared to the greater challenge faced by BWTSs at a test facility with high survival concentrations in control discharge. The supplementation STOs such as *T. suecica* and *Odontella* sp. to intake water could potentially increase survival in control discharge water. Consequently, this will result in more conservative and aligned testing conditions because BWTSs are more challenged. We suggest that test water supplementation of thoroughly tested STOs would be a sound solution to meet intake water and control discharge concentration requirements.

5. Conclusions

*Tetraselmis suecica* and *Odontella* sp., were evaluated as potential standard test organisms (STOs) in type approval of BWTSs. Both species were shown to be more or at least as robust as naturally occurring organisms when exposed to LP and MP UV treatments and assessed using the MPN method. A 24-hours dark-hold period simulating short-term ballast water holding time did not affect *T. suecica* or *Odontella* sp. concentrations in control samples and neither did 96 hours extended dark-hold periods for *T. suecica*. This suggests that species to be good candidates for type approval testing across facilities. The use of STOs gives a repeatable conservative assessment of the required fluence – for LP as well as MP UV systems – when the MPN method is used for efficacy evaluation. When the two proposed STOs are used in combination with the VS method, LP UV systems are disqualified, and for MP UV systems, the required fluence increases significantly resulting in wasteful overtreatment, increased capital expenditures, power consumption and greenhouse gas emissions.

Acknowledgements

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microalgae Tetraselmis suecica in batch cultures with different salinities and nutrient concentrations. Aquaculture 42 (3), 207–215.


APPENDIX C

MEPC report
D MEPC paper (73/INF.20)
HARMFUL AQUATIC ORGANISMS IN BALLAST WATER

On the use of standard test organisms as surrogate for the robust testing of ballast water management systems in view of their type approval

Submitted by Denmark

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Strategic direction, if applicable: 1

Output: Not applicable

Action to be taken: Paragraph 7

Related documents: None

Introduction

1 The International Convention for the Control and Management of Ship’ Ballast Water and Sediments was prepared in 2004 and entered into force in September 2017.

2 The Code for Approval of Ballast Water Management Systems (resolution MEPC.300(72)) set forth a series of requirements for the testing of ballast water management systems (BWMS) in view of their type approval by Administrations. These requirements include a minimum density of viable organisms that must be included, across the three size classes referred to in the D-2 performance standard (regulation D-2), in the inlet water of land-based test cycles.
3 The requirements from the Code are $10^4$ living bacteria/mL; $> 1,000$ organisms/mL for the 10 to 50 µm class size; and $> 100,000$ organisms/m³ in the size class $> 50$ µm during land-based testing.

4 The use of standard test organisms (STOs) to meet the requirements of the Guidelines (G8) (now Code) during land-based testing has been evaluated by the members of the correspondence group, and a sub-group assessing the pros and cons was set up. The conclusions of the sub-group working on STOs was reported to the correspondence group, which in turn reported the information to the IMO in document MEPC 69/4/6. At the time, the amount of data available to provide solid recommendation to the correspondence was found limited and therefore the group could not set forth a set of quantitative limits (minima or maxima) for the use of STO during land-based testing of BWMS. Yet, the group agreed that the minimum requirement in terms of living organisms to be present in the inlet water could be very difficult to achieve without the addition of STO during some seasons or under certain climatic conditions.

5 The recommendations pertaining to the use of STO are described in paragraph 2.32 of the annex to the Code for Approval of Ballast Water Management Systems. The Administrations are to review the selection of the STO to ensure that the tests can be considered valid and the use of STOs should not impair the robustness of the testing. As such, test organizations using STOs during the type approval testing in laboratory-based setup, should perform validation of the STOs used at their facility. There is an existing list of STOs used across test facilities and published by the Global TestNet (http://www.globaltestnet.org/Discussions).

6 The information included in the annex to this document reports information on the validation of two STOs used in test facilities. The data support the use of two tested STOs; *Tetraselmis suecica* and *Odontella* sp. in temperate and tropical test facilities, respectively. The results presented here suggest that the use of STOs is not impairing the robustness of tests, inversely, the results support the view that STOs are somehow increasing the robustness of tests and therefore may be recommended as a good procedure to ensure comparability between test cycles.

**Action requested of the Committee**

7 The Committee is invited to take note of the information contained in this document.
ANNEX

Introduction

The purpose of the study was to compare the UV dose (fluence) requirements for low pressure (LP) and medium pressure (MP) UV systems to meet the D-2 discharge standard for organisms in the 10 to 50 µm size class – comparing natural assemblages from different climatic zones (Denmark and Singapore) with possible relevant standard test organisms (STOs) cultured in laboratories in the respective countries, namely *Tetraselmis suecica* and *Odontella* sp. A list of STOs used in test facilities has been published by the Global TestNet on its website (http://www.globaltestnet.org/Discussions).

Materials and methods

**Preparation of test organisms and test water**

Natural seawater was collected in Denmark (TEMP) and Singapore (TROP) and coarsely filtered through a 35 µm mesh to remove zooplankton from the samples. The concentration of organisms was adjusted to the range of 1,500–4,000 organisms per mL either by dilution or addition from a nutrient enriched pool of natural organisms.

Monocultures of *Tetraselmis suecica* were grown in Keller’s Culture Medium at 19°C and diluted to the desired concentration. *Odontella* sp. were isolated from Singapore natural waters and a monoculture grown in batch cultures with F/2 Culture Medium at 25°C and diluted to the desired concentration.

The salinity of all samples was adjusted to 20±2 ppt of salinity and the UV-transmittance adjusted to 80% using lignin-sulfonate as per normal type approval testing.

**Quantification of algal cells**

Quantification of algal cells was carried out using FDA/CMFDA dual staining method as approved by IMO through submission at PPR 4 and the evaluation of cell robustness was assessed using the MPN dilution culture method (BWM.2/Circ.61). Analytical procedures used were approved by relevant Administrations and class societies for use in type approval testing.

**Collimated beam apparatus for UV exposure**

A collimated beam apparatus was used to expose the algae samples to different doses of UV light. The collimated beam apparatus consisted of a UV mercury arc lamp emitting UV light at 253.7 nm for the low-pressure UV system and between 200 to 300 nm for the medium-pressure UV system. The lamps were placed in a closed chamber where UV light could only escape through a collimating tube resulting in vertical UV irradiation reaching a petri dish containing the test sample. Exposure times were adjusted to reach the desired UV dose.

**Experimental design**

Organisms were exposed to low pressure or medium pressure UV treatments. Fluences were adjusted by the time of exposure relative to the determined fluence rate (intensity) of the UV lamp and aimed to be 0, 25, 50, 100 and 200 mJ/cm² for samples analysed using the MPN procedure and 0, 100, 200, 500 and 1000 mJ/cm² when analysed using the dual staining method.
For UV treatment, 50 mL test water was placed in a petri dish with continuous gentle stirring under the collimated beam system. After exposure, samples were transferred to a dark bottle and kept for 24 hours in a polystyrene box at ambient temperature (dark-hold period) to simulate ballast water tank holding time. After 24 hours, samples were gently re-suspended and transferred to a petri dish for a second UV treatment. The total desired UV fluence was obtained by adding the calculated UV fluences from the two treatments. After the second UV treatment, samples were immediately analysed for number of living/viable organisms.

Control samples were analysed for the number of living/viable organisms both before and after the 24-hours dark-hold period.

Combinations of test repetitions were carried according to Table 1.

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Results

In all experiments with use of natural organisms, a large variety of organisms were observed (Figure 1). Dinoflagellates were the dominating organisms in temperate waters while the tropical phytoplankton composition was dominated by diatoms (Bacillariophyceae).
Figure 1: Class and species composition of test water used in fluence-response UV treatment experiments in Denmark (TEMP) and Singapore (TROP). Unid. = Unidentified.

Dose-response data of the different treatment schemes using natural assemblages are presented in Figures 2 and 3.

Figure 2: Dose-response curves of natural algae assemblages analysed using the MPN method.

Figure 3: Dose-response curves of natural algae assemblages analysed using the dual staining method.

For natural organisms, low-pressure UV treatments on phytoplankton from both temperate and tropical origin, showed that UV fluences from about 130 mJ/cm² were sufficient to meet the D-2 discharge standards (<10 organisms per mL) in all experiments except LP-TROP (MPN-3) (Figure 2). When using the dual staining method, a fluence 10 times larger was required (Figure 3).

In medium-pressure UV treatments on temperate phytoplankton, IMO discharge standards were met at 200 mJ/cm² for both experiments and in one of the experiments (MPN-1) already at 100 mJ/cm² (Figure 2). When using the dual staining method a fluence of 1000 mJ/cm² was required (Figure 3).

Dose-response data of the different treatment schemes using STOs are presented in Figures 4, 5 and 6.

Figure 4: Dose-response curves of *Tetraselmis suecica* exposed to low-pressure UV-treatment.

Figure 5: Dose-response curves of *Tetraselmis suecica* exposed to medium-pressure UV-treatment.
Figure 6: Dose-response curves of *Odontella* sp. exposed to low-pressure UV-treatment.

For the STO *Tetraselmis suecica*, a fluence of around 270 mJ/cm$^2$ was sufficient to meet the D-2 discharge standard using low-pressure UV treatment by use of the MPN method, while almost no effect was observed using the dual staining method even at a dose of 1,350 mJ/cm$^2$ (Figure 4).

For medium-pressure UV treatment experiments, the highest fluence where the MPN method was applied for viability assessment was about 200 mJ/cm$^2$. Since concentrations did not satisfy discharge standards at this fluence level in MP-TET-1 and MP-TET-2, additional experiments were conducted (MP-TET-3 and MP-TET-4, including MPN assessments at treatments of about 500 mJ/cm$^2$. Concentrations satisfied discharge standards at 500 mJ/cm$^2$ and in one experiment also at around 200 mJ/cm$^2$ (Figure 5).

For the STO *Odontella* sp., a fluence of approximately 130 mJ/cm$^2$ was sufficient to meet the D-2 standard using low-pressure UV treatment. No effect was observed using the dual-staining method even at a fluence of 1,500 mJ/cm$^2$ (Figure 6).

For both *Tetraselmis suecica* and *Odontella* sp. they show as strong or stronger robustness towards UV-treatment compared to natural assemblages when using the MPN method - and significantly higher robustness when using the dual-staining method - especially with regards to low-pressure treatment.

**Conclusions**

The validation carried out on natural plankton assemblages and cultured STOs and presented here support that the STOs are as robust to UV treatment as natural organisms and should therefore be considered equal to natural plankton assemblages when testing for type approval using the MPN method. However, when using the dual-staining method it was not possible to detect any treatment effect on *Odontella* sp., and only a marginal effect on *Tetraselmis suecica* when applying low-pressure UV-treatment, even at the highest fluences applied.