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Ultraviolet radiation as a ballast water treatment strategy: Inactivation of phytoplankton measured with flow cytometry

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ABSTRACT

This study investigates different UV doses (mJ/cm^2) and the effect of dark incubation on the survival of the algae *Tetraselmis suecica*, to simulate ballast water treatment and subsequent transport.
Samples were UV irradiated and analyzed by flow cytometry and standard culturing methods. Doses of ≥400 mJ/cm^2 rendered inactivation after 1 day as measured by all analytical methods, and are recommended for ballast water treatment if immediate impairment is required. Irradiation with lower UV doses (100–200 mJ/cm^2) gave considerable differences of inactivation between experiments and analytical methods. Nevertheless, inactivation increased with increasing doses and incubation time. We argue that UV doses ≥100 mJ/cm^2 and ≤200 mJ/cm^2 can be sufficient if the water is treated at intake and left in dark ballast tanks. The variable results demonstrate the challenge of giving unambiguous recommendations on duration of dark incubation needed for inactivation when algae are treated with low UV doses.

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1. Introduction

Ships use water as ballast to ensure stability and trim during the voyage, and ambient water is pumped into ballast tanks in the hull of the ships. It is traditionally discharged without any treatment and represents a global vector for aquatic invasion. A multitude of organisms like virus, bacteria, algae and zooplankton are carried around the world in ship's ballast tanks (David et al., 2007; Drake et al., 2007; Hallegraeff and Bolch, 1991). Some organisms survive in ballast tanks and are released into new environments. If nonindigenous species adapt and establish in a new environment, they might have an impact on the native species and cause ecological change in the ocean (Gollasch et al., 2015; Ruiz et al., 1997). It is of importance to minimize and prevent dispersal of species by ballast water discharge to hinder potential harm to ecosystems, the economy, or human health (Ruiz et al., 2000).

In 2004 the International Maritime Organization (IMO) established standards for ballast water treatment through the International Convention for the Control and Management of Ship's Ballast Water and Sediments (International Maritime Organization, 2004). Regulation D-2 of the Convention sets the standard regarding category and concentration of organisms at discharge. The Convention will enter into force 12 months after being ratified by 30 States representing 35% of the merchant shipping tonnage. In August 2015 44 States, representing 32.86% of the world tonnage, have ratified the Convention. The upcoming IMO regulations have led to development of various ballast water treatment systems (BWTS) that facilitate disinfection of ballast water (David and Gollasch, 2015; Delacroix et al., 2013; Lloyd's Register Marine's, 2015a, 2015b; Stehouwer et al., 2015; Werschkun et al., 2012, 2014). All BWTS have to be approved by national authorities according to IMO regulations and/or the regulations of other national bodies (e.g. U.S. Coast Guard (USCG)).

When selecting and installing a BWTS, the shipping companies have to consider different technical and operational aspects (Lloyd's Register Marine's, 2015a, 2015b). The BWTS use a range of different treatment technologies, from processing the water with solid–liquid separation to chemical- (active substances) and/or physical disinfection (e.g. UV). The main operational cost for UV based BWTS is related to power consumption (Werschkun et al., 2014). Ship owners can reduce such costs by lowering the UV intensity, providing that the ship's discharged ballast water still complies to Regulation D-2 (International Maritime Organization, 2008a). It is therefore of interest to determine the lowest lethal UV dose and to estimate the time required for inactivation when stored in ballast tanks after irradiation.

UV irradiation is performed either by low pressure (LP) or medium pressure (MP) UV lamps (Oguma et al., 2002; Werschkun et al., 2012;

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Zimmer and Slawson, 2002). LP lamps emit UV-C radiation, primarily at 254 nm, which is most efficiently absorbed by nucleic acids and causes DNA damages (Sinha and Häder, 2002). UV induced DNA damages can be reversed by DNA repair mechanisms, referred to as photoreactivation and dark repair (Sancar and Sancar, 1988; Sinha and Häder, 2002). MP UV lamps emit radiation spanning the UV-A, -B and -C bands causing additional damage to proteins and enzymes. For instance, UV-B radiation can affect key components in photosynthesis (Fiscus and Booker, 1995; Holzinger and Lütz, 2006; Kottuparambil et al., 2012), causing energy deprivation in phytoplankton cells. Thus, it has been argued that MP UV lamps can cause a higher degree of inactivation compared to LP UV lamps (Kalisvaart, 2001; Oguma et al., 2002).

UV irradiation can leave cells in different conditions (live, dead or damaged), whereof the viability of damaged cells at discharge is uncertain (Olsen et al., 2015). Damaged cells can be unculturable, though they can be metabolic active and may pose a health risk (Oliver, 2010). Further, cellular DNA repair mechanisms can restore the genetic information (Sancar and Sancar, 1988; Sinha and Häder, 2002; Zimmer and Slawson, 2002) causing the cell to grow and replicate after discharge (Liebich et al., 2012; Martínez et al., 2012, 2013). Additionally, the terminology describing the organisms at discharge can be confusing or unclear. The IMO Convention refers to "viable" organisms (International Maritime Organization, 2004), and the Guidelines for approval of ballast water management systems (G8) define "viable organisms" as "organisms and any life stages thereof that are living" (International Maritime Organization, 2008a). USCG also uses the term "living" (United States Coast Guard, 2012).

Determining the condition of UV irradiated cells is a complex task. On the other hand, cheap, fast and reliable methods to analyze ballast water are necessary for approval of BWTS technologies and for compliance testing of ballast water discharge (International Maritime Organization, 2013). Testing for compliance can be performed in two steps; an indicative and a detailed analyses. An indicative analysis is a relatively simple and quick measurement that gives a rough estimate of the number of viable organisms in the ballast water at discharge. Examples of indicative analysis methods are e.g. BallastCAM and various fluorescence or ATP detections (Drake et al., 2014; First and Drake, 2013, 2014; Gollasch and David, 2012, 2015; van Slooten et al., 2015). If an indicative analysis shows compliance to Regulation D-2, there is no need for a detailed analysis. Should the indicative analyses be non-compliant, however, a detailed analysis must be undertaken to give robust and direct measurements determining the concentration of viable organism in ballast water discharge according to Regulation D-2. Quantification of live bacteria traditionally relies on cultivation methods, which is time-consuming and may give false negatives as several species are uncultivable although viable (Roszak and Colwell, 1987; Staley and Konopka, 1985). Flow cytometry (FCM) has been suggested as a promising method for detailed analysis (International Maritime Organization, 2013; Peperzak and Gollasch, 2013). FCM facilitates rapid detection, enumeration and characterization of organisms in combination with fluorescent dyes, and enables to study populations and communities indirectly (Peperzak and Brussaard, 2011; Shapiro, 2000).

Previously a FCM protocol was developed to distinguish between live and dead *Tetraselmis suecica* cells (Olsen et al., 2015). For UV irradiated samples the FCM protocol could not distinguish between live and damaged cells, as the latter contain both dying and repairable cells. The current study uses the FCM protocol to elaborate on different UV doses and the effect of dark incubation on inactivation of the algae *T. suecica*, to simulate a ballast water treatment and subsequent transport. Our specific objectives were to:

1) Determine the minimum UV dose that permanently inactivates the algae.
2) Quantify effects of different UV doses on *T. suecica*.
3) Estimate the time of dark incubation required to permanently inactivate the algae treated with UV doses lower than minimum permanently inactivation dose.
4) Provide recommendations for ballast water management.

2. Material and methods

The phytoplankter specie *T. suecica* (Strain K-0297, Scandinavian Culture Collection of Alga and Protozoa, University of Copenhagen, Denmark) was selected as a test organism. It was cultured in 24 PPT artificial sea water (Marine SeaSalt, Tetra, Melle, Germany) added 0.12% Substral (The Scotts Company (Nordic) A/S, Naverland, Glostrup, Denmark), at 15 °C, 100 rpm, 14:10 light:dark cycle and 90 lx light intensity (Flora-Glo, T8, 20 W). The culture was diluted in growth medium to a density of 10^4 live cells ml^{-1} prior to irradiation, monitored by FCM.

Irradiation was performed using a collimated beam MP UV lamp (800 W) (BestUV, Hazerswoude, The Netherlands) (Olsen et al., 2015). For each experiment three samples of 15 ml diluted *T. suecica* culture were irradiated with the same UV dose in a petri dish (inner diameter 6 cm, culture depth 7 mm) while mixed with a 1 × 0.4 cm magnetic stir bar at 200 rpm in room temperature (RT). The intensity (mW/cm^2) of the UV lamp was fixed and the exposure times used were 155, 233, 311, 622 and 1244 s for UV doses 100, 150, 200, 400 and 800 mJ/cm^2, respectively. The irradiated samples were transferred to sterile 50 ml polypropylene tubes (Fisher Scientific), so was the control samples, including 2 × 15 ml non-irradiated cells and 10 ml dead cells. The dead cells were killed by fixation with formaldehyde at 5% final concentration (36.5–38% formaldehyde, Sigma-Aldrich). All tubes were wrapped in aluminum foil and incubated in the dark with loosened lids at 15 °C.

First, a pre-study over 5 days was performed to observe the inactivation effect of different UV doses and dark incubation, and to test whether this effect was interpretable with FCM. This was followed by two complete experiments, denoted as exp-I and exp-II, and an overview of the set-up for these experiments is given in Fig. 1.

For FCM analysis, the samples were stained with 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) and analyzed with an Attune Acoustic Focusing Cytometer (Olsen et al., 2015). The samples in the pre-study were analyzed at days 1, 3 and 5 after treatment. In exp-I samples were analyzed at days 1, 3, 6, 9, 13 and 22, and in exp-II samples were analyzed at days 1, 3, 6, 10, 15 and 22 after treatment (Fig. 1). The samples in exp-I and -II were analyzed at different intervals due to logistics. A previously defined gate (i.e. a collection of single cell FCM-signals) in the FCM dot plots was used for analysis. The gate was

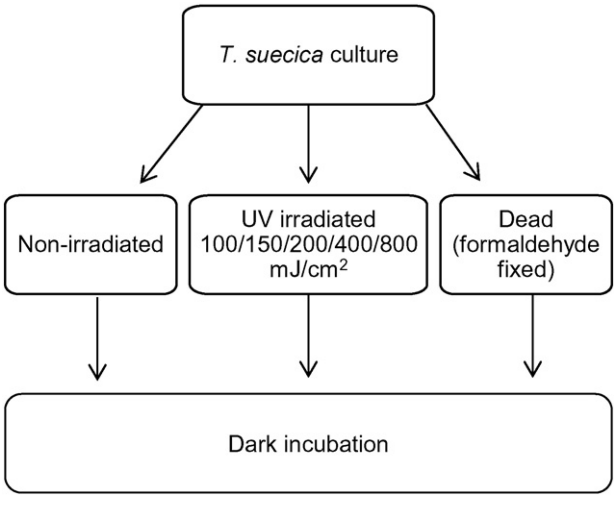

Fig. 1. Experimental set-up showed by a flow diagram. This set-up was followed in the pre-study, exp-I and exp-II.

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