METHODS TO VERIFY ORGANISM VIABILITY IN TREATED BALLAST WATER

PhD Thesis

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Preface & acknowledgements

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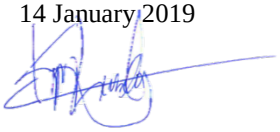
For all the technical support regarding the HCS-Platform and to assist me in which ideas to pursue and which to trash during our methods development mission I want to thank our lab technician **Annette Duus**. It has been a pleasure working with you and receiving your input.

My gratitude also goes to the **technical and scientific staff** at DHI-Denmark and DHI-Singapore for their kindness and for making me feel so welcome during my visits. It has been fantastic to experience your sincere willingness to help me with all my questions and assisting me with my experimental set-ups and sample analyses.

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And finally, thanks to my wonderful partner, **Sara Green**, for your support, inspiration, and for being the best life companion I could ever hope for. Jeg er en heldig kartoffel.

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List of abbreviations and acronyms

ATP	Adenosine triphosphate
BW	Ballast water
BWE	Ballast water exchange
BWI	Ballast Water Index
BWM	Ballast Water Management
BWTS	Ballast water treatment systems
CMFDA	5-chloromethylfluorescein diacetate
DNA	Deoxyribonucleic Acid
DWT	Dead weight tonnage
FDA	Fluorescein diacetate
HABs	Harmful algal blooms
HCS	High content screening
IMO	International Maritime Organization
LP	Low pressure
MP	Medium pressure
MPN	Most Probable Number method
PAM	Pulse-amplitude modulated fluorometry
STO	Standard test organism
US EPA	United States Environmental Protection Agency
USCG	United States Coast Guard
UV	Ultraviolet
VIDA	Vessel Incidental Discharge Act
VS	Vital Stain method

English abstract

Invasive species are considered one of the most serious threats to the balance of aquatic ecosystems worldwide. The primary source for the spread of aquatic invasive species is transport and discharge of ballast water by cargo ships which represent around 90% of the global transportation of goods. Annual costs linked to aquatic invasive species are in the order of hundreds of billions of USD. To mitigate the spread, the United Nations' International Maritime Organization (IMO) adopted the Ballast Water Convention that entered into force in 2017. The convention requires all ships with ballast water to apply a type approved Ballast Water Treatment System (BWTS) to eliminate organisms. Results from the present thesis showed that the implementation of the ballast water convention caused a growing trend in the number of ships using BWTS and that future regulatory decisions regarding type approval process and enforcement protocols may influence the adoption of certain BWTS types. Furthermore, it was found that the two most prevailing treatment types were electrolysis and UV irradiation although 80% of ships in 2017 still used ballast water exchange to reduce presence of organisms in the ballast water.

The IMO discharge standard for phytoplankton (10-50 µm size class) in treated ballast water is <10 *viable* org. ml^{-1}. The United States Coast Guard (USCG) has implemented similar rules but with a critical difference in the discharge standard where the requirement is <10 *living* org. ml^{-1}. This means that living, but *non-viable* (i.e. unable to reproduce) organisms also must be destroyed. These are allowed under IMO regulations since they pose no invasion risk. After treatment, organism concentration can be determined using two quantification approaches: Vital Stain (VS) or Most Probable Number (MPN) method. The VS method quantifies both *viable* and *non-viable* living organisms as enzyme activity in the cells activates the fluorescent stain. The MPN method is based on organisms' regrowth potential and estimates only the number of *viable* organisms. The MPN method is not approved by the USCG which has implications for UV-based BWTSs. The UV treatment does not instantly kill organisms but causes DNA damages which result in *non-viable* living organisms with delayed mortality. Results from the present thesis showed that UV-based BWTS could satisfy the USCG discharge standard for *living* organisms but 10 times more UV energy was needed to instantly kill organisms. The implementation of extended dark-hold periods after UV treatments could however reduce the extra energy needed to comply with regulations. Although requirements for <10 *living* org. ml^{-1} could be met, the results showed that the VS method was less suitable for validation of UV-BWTS because costly overtreatment was required. Quantification of *viable* organisms with the MPN method is slow (weeks) but was demonstrated to be a more robust approach because *non-viable* living organisms with not invasion potential are not quantified.

The IMO/USCG-requirement for a natural algal concentration of >1000 org. ml^{-1} in the test water to properly challenge BWTS performance cannot always be fulfilled. Results from the present thesis showed that robustness towards UV treatments was at least as high for monocultures of *Tetraselmis suecica* and *Odontella* sp. as for natural algal compositions. The two species were therefore considered good candidates as standard test organisms to validate BWTS performance to ensure more robust testing conditions and that concentration requirements in test water are met.

Current manual counting procedures are challenged by several factors such as quantification accuracy. In the present thesis a novel automated approach was developed. It combined a filter technique to fixate mobile species, a High Content Screening Platform and image analysis which significantly improved quantification accuracy, possibility for verification of results, and reduced sample counting time.

The exploration of a novel indicative analysis approach in the present thesis, showed that integration of Raman spectroscopy in future viability assessment techniques could be a promising tool for the simultaneous identification of microplankton species and their viability state. When further developed, the technique could be applied to validate BWTS performance and for monitoring harmful algal bloom events.

Danish summary

Invasive arter udgør en af de største trusler for balancen i globale akvatiske økosystemer. Omkring 90% af verdens godstransport foregår via skibe, og deres ballastvand er den primære kilde til spredning af de akvatiske invasive arter. De årlige omkostningerne relateret til skader forårsaget af akvatiske invasive arter løber op i hundrede milliarder USD. For at mindske udbredelsen af akvatiske invasive arter har FN's International Maritime Organisation (IMO) vedtaget Ballastvand-konventionen, som trådte i kraft i 2017. Konventionen kræver, at skibe med ballastvand skal anvende et typegodkendt ballastvand-behandlingssystem, som uskadeliggør tilstedeværende organismer. Vores resultater viste at implementeringen af konventionen medførte en stigende tendens i antallet af skibe der behandlede deres ballastvand og at fremtidige lovgivningsmæssige beslutninger omkring typegodkendelsesprocessen og håndhævelsesprotokoller kan påvirke valget af visse typer af behandlingssystemer. Endvidere fandt vi, at de to mest benyttede behandlingsmetoder var elektrolyse og UV-bestråling selvom 80% af skibene i 2017 stadig benyttede ballastvandudveksling-metoden til at reducere tilstedeværelsen af organismer i ballastvandet.

IMO's udledningsstandard for fytoplankton (10-50 µm) i behandlet ballastvand er <10 *levedygtige* organismer ml^{-1}. United States Coast Guard (USCG) har vedtaget lignende regler men med en væsentlig forskel i udledningsstandarden, hvor kravet er <10 *levende* organismer ml^{-1}. Det betyder at *ikke-levedygtige*, men stadig levende organismer (dvs. uden reproduktionsevne) også skal uskadeliggøres. Disse tillades ved IMO da de ikke udgør en invasionsrisiko. Efter behandling bestemmes antallet af tilbageværende organismer primært vha. to kvantificeringsmetoder: Vital Stain (VS) eller Most Probable Number (MPN) metoden. Med VS-metoden kvantificeres både *levedygtige* og *ikke-levedygtige* organismer idet enzymaktivitet i cellen aktiverer et fluorescerende farvestof. MPN-metoden er derimod baseret på organismers genvækstpotentiale og estimerer kun antallet af organismer, som er *levedygtige*. MPN-metoden er dog ikke godkendt af USCG, hvilket har store konsekvenser for UV-baserede behandlingssystemer. UV-bestråling slår ikke organismer ihjel øjeblikkeligt, men forårsager primært DNA-skader der resulterer i *ikke-levedygtige* organismer med forsinket dødelighed. Vores resultater viste, at UV-baserede systemer kunne opfylde USCG-kravet for *levende* organismer, men 10 gange højere UV-energi var nødvendig for at slå organismerne ihjel øjeblikkeligt. Den øgede energimængde kunne dog reduceres ved at indføre en forlænget mørke-perioder for det UV-behandlede ballastvand. Selvom kravet for <10 *levende* organismer ml^{-1} kunne opfyldes, viste vores resultater at VS-metoden var mindre egnet til validering af UV-systemer fordi dyr overbehandling var nødvendig. Estimering af antal *levedygtige* organismer med MPN-metoden er langsom (uger), men resultaterne viste at metoden var et bedre valg fordi *ikke-levedygtige* organismer uden invasionspotentiale ikke kvantificeres.

En naturlig algekoncentration på >1000 organismer ml^{-1} i testvandet er et IMO/USCG-krav for at sikre at behandlingssystemers effektivitet udfordres, men kravet kan ikke altid opfyldes. Vores resultater viste, at robustheden mod UV-behandling var mindst lige så høj for monokulturer af *Tetraselmis suecica* og *Odontella* sp. som for naturlige algesammensætninger. De to arter vurderes derfor som gode kandidater som standard testorganismer til validering af behandlingssystemers effektivitet for at sikre mere robuste testbetingelser, og at koncentrationskravet til testvand kan opfyldes.

Nuværende manuelle tællemetoder, er udfordret af flere faktorer bl.a. tællenøjagtighed. Vores udvikling af en ny automatiseret metode, som kombinerer en filterteknik, der fikserer mobile alger, en High Content Screening-Platform og billedanalysesoftware, var i stand til at forbedre tællenøjagtigheden væsentligt, kunne verificere tællinger, samt reducere optællingstiden.

Vores undersøgelse af en ny alternativ analysemetode viste at Raman-spektroskopi kunne være en lovende teknik til samtidig identifikation af levende mikroplanktonarter samt deres levedygtighedsstatus. En videreudvikling af teknikken vil gøre den anvendelig til validering af behandlingssystemers effektivitet og til overvågning af skadelige algeopblomstringer.

Manuscript overview

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

   Kim Lundgreen, Henrik Holbech, Knud Ladegaard Pedersen, Gitte Ingelise Petersen, Rune Røjgaard Andreasen, Christaline George, Guillaume Drillet, Martin Andersen

   Marine Pollution Bulletin 135:1190-1100 (published 20 Aug 2018).

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

   Kim Lundgreen, Henrik Holbech, Knud Ladegaard Pedersen, Gitte Ingelise Petersen, Rune Røjgaard Andreasen, Christaline George, Guillaume Drillet, Martin Andersen.

   Submitted to Biological Invasions (January 2019).

3. **Development of a novel automated analytical method for viability assessment of phytoplankton used for validation of ballast water treatment systems**

   Kim Lundgreen, Lisa Eckford-Soper, Knud Ladegaard Pedersen, Henrik Holbech

   Submitted to Journal of Applied Phycology (January 2019).

4. **Installation and use of ballast water treatment systems – implications for compliance and enforcement**

   William A. Gerhard, Kim Lundgreen, Guillaume Drillet, Raphael Baumler, Henrik Holbech, and Claudia K. Gunsch.

   Submitted to Environment International (January 2019).

5. **Raman spectroscopy as a tool to measure viability of planktonic organisms in UV-treated ballast water**

   Mikkel Andreasen, Kim Lundgreen, Henrik Holbech, Martin A. B. Hedegaard

   Manuscript in preparation

1. Introduction to the scientific field

1.1. The importance of ships' ballast water and associated risk of transporting invasive species

The growth in maritime trade caused by globalization has increased the transportation and the potential discharge of invasive species from the ships' ballast water (BW). The spreading of invasive species has had, and still has huge environmental and health related consequences and is causing tremendous socio-economic losses around the world (Ruiz et al. 1997, Ruiz et al. 2000, Seebens et al. 2017). It has been estimated that aquatic invasive species annually are responsible for global environmental damage and economic losses of more than USD100 billion (UNEP 2012). The costs include damages and repairs to fisheries, aquaculture, water supply systems, industrial infrastructure and harbors, reduced tourism and human health issues (Hallegraeff & Bolch 1991, Mccarthy & Khambaty 1994, Berdnikov et al. 1999, Molnar et al. 2008, UNEP 2012). Costs do not however include all the indirect invaluable losses caused by changes to biodiversity, ecosystem structure, and habitats.

Transportation of goods by ships is the most cost-effective method for the movement of goods all over the world. Shipping currently covers 80-90% of the global trade by volume and more than 70% by value, thereby playing a vital role in world trading (ICS 2017, UNCTAD 2018) (Figure 1).

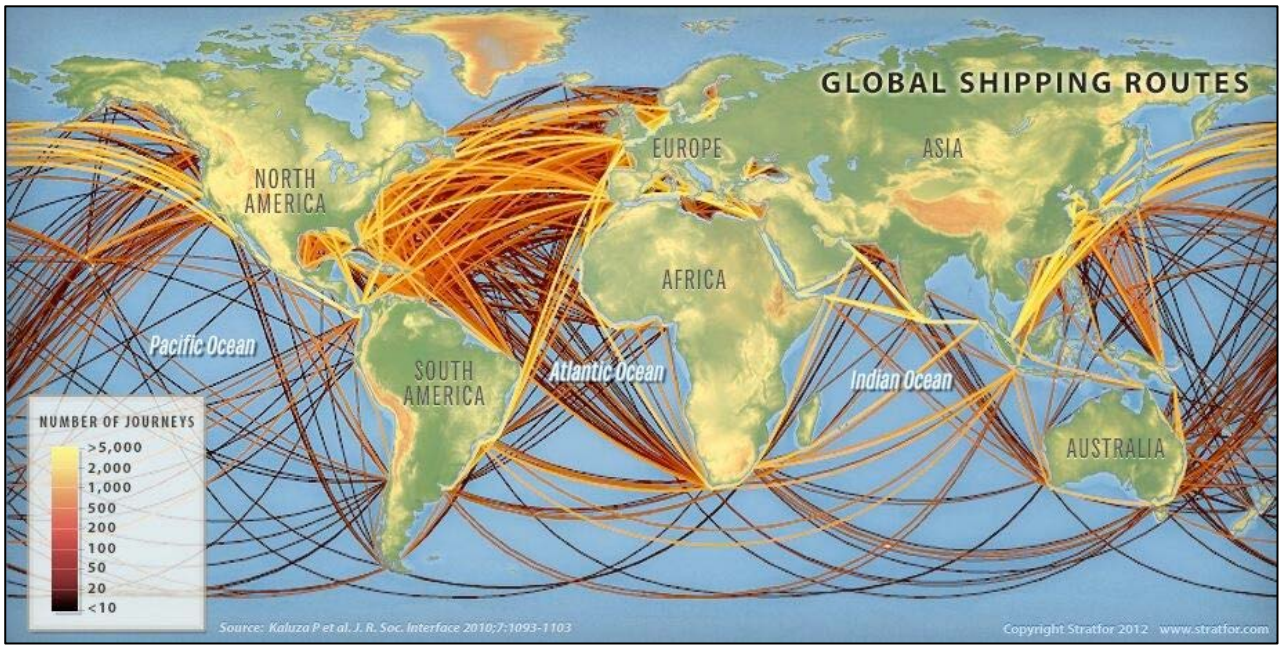


Figure 1. The annual movements of cargo ships (and thus ballast water) along the most frequent used shipping routes. From Kaluza et al. (2010).

Ballast water is a necessity for all large ships for safety and economic reasons. It is used for reinforcement of the hull and for adjusting and trimming the position of the ship during voyage in relation to cargo load for better stability and fuel consumption (Figure 2). It has been estimated that between 3 to 10 billion tons of BW water are transported around the world annually (Endresen et al. 2004, Tamelander et al. 2010). The total world-wide commercial fleet is currently estimated to consist of 94,171 vessels and is estimated to continue to grow in both number of ships and cargo-carrying

capacity (UNCTAD 2018). For general cargo carrying vessels, BW only comprises approximately 20% of dead weight tonnage (DWT = cargo mass capacity of the ship). Some of the largest bulk carriers have a DWT of 250,000 tons. In those vessels BW takes up to as much as 45% of DWT which corresponds to more than 100,000 m^3 BW (David et al. 2012, David & Gollasch 2015).

Ballast water tanks are filled with ambient water which can result in the ship acting as a global vector for transporting stowaways such as zooplankton, phytoplankton, early life-stages of invertebrate and vertebrate species, bacteria and virus. which are consequently transferred across natural barriers and released into new local ecosystems where they are not native (Molnar et al. 2008). To be acknowledged as an invasive species[1], a species must first succeed in surviving the treatment processes of the ship's ballast water treatment system (BWTS) during intake, the potentially long passage in the dark BW tanks and eventually a second treatment before discharge into a new environment (Carlton 1985). Subsequently, it must be able to survive and overcome the new conditions where different ecological factors might dominate in relation to the native natural area of the species (Carlton 1985). Over time it must be able to adapt to a degree where it succeeds in reproducing and in establishing a dominant population (Carlton 1985). In some cases, a new species will not cause any significant changes in the ecosystem where it is introduced. However, the new environment may prove to be the perfect niche for reproduction. If conditions favor the new species, the population can exhaust resources, out-compete local species and multiply into pest proportions (Hayes & Sliwa 2003). The possibility of invasion is influenced by a number of factors such as the frequency of introduction events and the number of introduced individuals per event which combined is referred to as propagule pressure (Colautti et al. 2007). However, different degrees of robustness in some species towards treatment and environmental stressor as well as their survival strategies may also play a crucial role in their invasion success when discharged (Irigoien et al. 2005, Drillet et al. 2013, Hoell et al. 2017, Briski et al. 2018).

[1] The term *invasive species* follow the definition used in Simberloff (2013 p. 3): *species that arrive with human assistance, establish populations, and spread*.

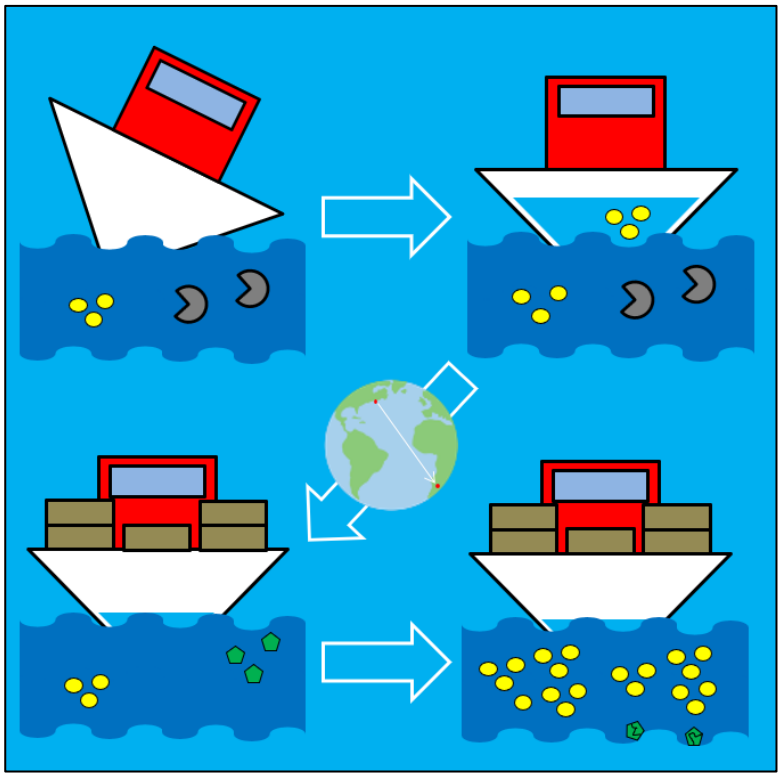


Figure 2. Ships need ballast water for stability in relation to cargo load. During ballasting conditions there is a risk that the ballast water will contain local organisms that are part of a balanced ecosystem. At the new port, the ship might have to adjust its ballast water volume according to cargo load conditions. The surviving organisms discarded with the ballast water may thrive in the new environment and can develop into pest proportions that negatively affect the new ecosystem.

The damage invasive species can exert on the environment and biodiversity is considered one of the most serious threats to aquatic ecosystems worldwide (Burgiel & Muir 2010, WWF 2016). The damage is often irreversible, and it is almost impossible to eliminate an invasive species once a population has been established (Thresher & Kuris 2004). In 2001 it was estimated that aquatic and land-based invasive species combined were responsible for losses equivalent to 5% of the, at the time, total annual world economy corresponding to USD1,400,000,000,000 (Pimentel et al. 2001). Recent predictions show no signs of saturation in the spread of invasive species (Seebens et al. 2017). Combined with the ongoing climate changes that have opened up alternative polar shipping routes as well as expansion of more temperature sensitive species, that has resulted in a need to improve efforts to mitigate invasions (Miller & Ruiz 2014, Kourantidou 2015, Hulme 2017, Lee et al. 2017, Holbech & Pedersen 2018).

1.2. Ecological consequences of invasive phytoplankton

One of the leading invasive groups of organisms are the phytoplankton which are in the top three dominant groups of organisms responsible all for recorded invasion events, the other two being crustaceans and mollusks (Molnar et al. 2008). Phytoplankton is a major and essential component of many aquatic ecosystems and plays a significant role in the primary production, the structure of food webs, as well as climate and biogeochemical processes (Sabine et al. 2004, Boyce et al. 2010). Invasive algal species can cause harmful algal blooms (HABs) that can have substantial ecological

and economic impacts in affected area. The problem is enhanced with regards to toxic or high biomass algal species. This PhD thesis will mainly focus on phytoplankton and therefore the subsequent sections of the thesis will deal with BW management issues relating to this group of organisms. The following two examples of BW related algal invasions provide insights into the direct ecological and economic impacts such invasion events can have on the affected regional areas.

One case is the large (175-500 µm) non-toxic diatom *Coscinodiscus wailesii* (Figure 3) that was originally found in the Pacific coast of North America and the North Pacific Ocean including the Sea of China and Japanese coastal waters (Gran & Angst 1931, Kokubo 1952). In 1977 it was discovered in the English Channel and occurrences soon followed in the Irish Sea, Dutch coastal waters, the Skagerrak. By 1983 it had spread to the Baltic Sea and the Atlantic coast of France (Boalch & Harbour 1977, Edwards et al. 2001). The species became so well-established that *C. wailesii* was observed to dominate phytoplankton biomasses over long time periods (Rick & Durselen 1995). This species had negative effects on the whole ecosystem by blooming to such high densities that it caused oxygen depletion which impacted areas with aquaculture production. It also outcompeted native species for limited resources and space causing a reduction in biodiversity, and altered and restricted the diet of native herbivorous consumers (Roy et al. 1989, Durselen & Rick 1999, Talgatti et al. 2010). Although *C. wailesii* is non-toxic it has had severe effects on fisheries because the species is able to produce large amounts of mucilage during blooms which clogs fishing nets and restricts trawling operations (Boalch & Harbour 1977, Mahoney & Steimle 1980). The introduction of *C. wailesii* to the north-east Atlantic was suspected to have occurred via BW transport which also attributed to the later introduction into Brazilian waters (Valente-Moreira 1987). The success of the species has been attributed to the large cell size which makes it difficult for native herbivores to consume, as well as its higher temperature and salinity tolerance compared to native phytoplankton species (Durselen & Rick 1999).

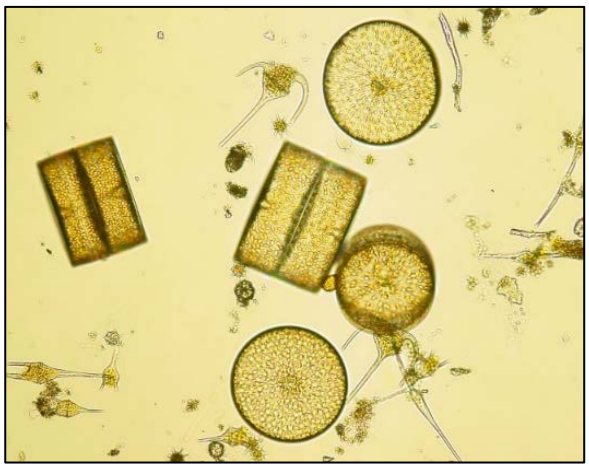


Figure 3. The diatom *Coscinodiscus wailesii* (Photo: Ann-Turi Skjevik, http:/nordicmicroalgae.org).

Another example concerns the toxic dinoflagellate *Gymnodinium catenatum* (33-45 µm) introduced into south-eastern Australian waters by BW from Japanese waters (Bolch & de Salas 2007). The species was first registered in the Gulf of California in 1939 (Graham 1943) but could have its origin from Japanese waters (Matsuoka et al. 2006). During the 1970s it was reported in Atlantic European waters (Estrada et al. 1984). The origin in European waters is still unresolved but it is suspected to

have been introduced via the Galician fishing fleet who during the 1970s were operating in Argentinian waters where the species is common (Gomez 2003). During blooms the species can produce saxitoxins which are potent neurotoxins and some of the best-known paralytic shellfish toxins (PSTs) (Oshima et al. 1987, Garate-Lizarraga et al. 2004). The toxins accumulate in shellfish such as oysters, mussels and scallops. When these are consumed by humans, it can cause paralysis, neurological and gastrointestinal problems, leading to death in 15% of cases (Band-Schmidt et al. 2010). Massive HABs of *G. catenatum* were first recorded in Tasmania in 1985 where it led to widespread closure of shellfish farms for several months (Hallegraeff et al. 1988). In the following years the HABs spread further north and during the 1990s it was also discovered at different sites in the southern part of the Australian mainland (Sonneman & Hill 1997). The resting cysts of *G. catenatum* are able to survive for long periods of time under suboptimal conditions, thereby enhancing their ability to cope with long journeys in BW or sediment of BW (Hallegraeff & Bolch 1992). Besides causing HABs affecting shellfish aquaculture production the toxin has also been reported to cause mass mortalities in shrimp farms (Alonso-Rodriguez & Paez-Osuna 2003).

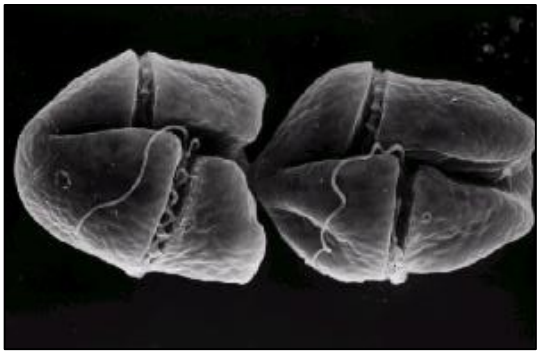


Figure 4. Scanning electron microscopy images of *Gymnodinium catenatum* (Photo: Yasuwo Fukuyo and Haryyoshi Takayama).

1.3. Legislation of ballast water treatment systems

1.1.1 The ballast water management convention for mitigation of invasions

To protect aquatic ecosystems, economical expenses and human health from the impact of invasive species *The International Convention for the Control and Management of Ships' Ballast Water and Sediments* (BWM Convention) was adopted by the International Maritime Organization (IMO) in 2004 (IMO 2004). The International Maritime Organization is a specialized agency under the United Nations, formed in 1959 with the responsibility of protecting the marine environment from harmful impacts of shipping and regulating global standards for ship safety and security. The purpose of the convention is to: *Prevent, minimize and ultimately eliminate the risks to the environment, human health, property and resources arising from the transfer of harmful aquatic organisms and pathogens* (IMO 2004). The convention entered into force 12 months after more than 30 Member States, representing 35% of world merchant shipping tonnage had ratified the convention. In September 2016, 52 Member States representing 35.14% of world merchant shipping tonnage, had ratified the treaty and therefore the convention entered into force September 2017[2]. To date, the USA has not

[2] Status of the BWM Convention at 31 July 2018: Ratified by 75 Member States representing 75.34% of world's tonnage. (UNCTAD 2018 p. 95).

acceded the IMO Convention but instead implemented their own regulatory standards which to a large degree are similar to those of IMO (US EPA 2010, US Coast Guard 2012).

1.3.1. Current IMO/USCG regulations for ballast water treatment systems

The BWM Convention includes two regulations for management of BW: Regulation D-1 addresses ballast water exchange (BWE) and Regulation D-2 addresses the performance of the ship's Type Approved BWTS that must reduce the number of organisms to meet stated discharge standards (IMO 2004) (Table 1).

In Regulation D-1 on BWE standard, ships are required to renew at least 95% of the BW volume contained in the tanks (IMO 2005). The BWE process must take place at least 200 nautical miles from nearest land (in some cases 50 nautical miles are allowed) and in water at least 200 m in depth (IMO 2005). Ballast water exchange is founded on the principle that the organisms obtained from coastal water cannot survive the discharge into open ocean water because of differences in salinity, temperature, chemical composition. And vice versa, the deep ocean or open sea waters contain much fewer organisms and these are less likely to adapt and survive in coastal water after discharge. Ballast water exchange is only a temporary measure to mitigate the spread of aquatic invasive species. Ultimately, the BWM Convention requires all ships to be installed with a BWTS that is able to comply with discharge standards in Regulation D-2 no later than 2024 (IMO 2017).

The costs of the Type Approval process and subsequent installation of BWTSs are in the order of USD 1-5 million per ship and can take up 1.5 years or more (David & Gollasch 2015, UNCTAD 2015). The BWTS must be approved in accordance with the current guidelines to meet certain discharge standards related to the number of viable or living organisms in defined size classes, as outlined in IMO Regulation D-2 or US federal regulations (IMO 2004, US Coast Guard 2012) (Table 1). The evaluation of BWTSs in relation to the terms viable or living organisms is depending on the desire to either meet criteria set by the IMO or USCG (addressed in section 1.3.3). The regulations cover a range of organism types including virus, bacteria (<10 µm), plankton ≥10-<50 µm[3] (hereafter 10-50 µm, consisting mainly of phytoplankton) and plankton >50 µm which mainly consist of zooplankton.

Table 1. Regulation D-2. Criteria of ballast water discharge standards in terms of organism type, size class and number of viable (IMO) or live (USCG) organisms present in the treated ballast water (IMO 2004, US EPA 2010).

Size class (µm)	Organism type	[Viable/live organisms]
<10	Toxicogenic *Vibrio cholera*	<1 cfu 100 ml^{-1}
	Enterococci coli	<250 cfu 100 ml^{-1}
	Intestinal *Enterococci coli*	<100 cfu 100 ml^{-1}
≥10-<50	Plankton	<10 cells ml^{-1}
>50	Plankton	<10 cells m^{-3}

[3] Those organisms that theoretically cannot pass through a 10 µm grid are evaluated to be ≥10 µm in minimum dimension and include any tentacles, spikes or appendages on organisms that prevent them from passing through a 10 µm grid (DHI 2018).

The present PhD thesis' primary emphasis is on BW management issues related to phytoplankton and therefore the following sections will mainly focus on the size class 10-50 µm.

The D-2 standards ensure that only a considerable smaller fraction of organisms is discharged into the environment compared to non-treated BW. However, even when a ship follows the regulations a high number of organisms may still be discharged into the environment. For example, the discharge of 10,000 m^3 treated BW that is in compliance with regulations could potentially contain as many as 9×10^{10} algal cells (9 org. $ml^{-1}\times1000$ ml $l^{-1}\times1000$ l $m^{-3}\times10{,}000$ m^3) in the size class 10-50 µm. This is a considerable number despite the effort of treatment and satisfying discharge standards. It is largely unknown how many organisms are needed to establish a population in a new environments but frequent inoculations of this quantity (although the number represents a mix of species) may not completely eliminate the risk of an invasion of some species (David & Gollasch 2015).

A weak point of the current D-2 standards is that they do not consider algal species <10 µm in minimum dimensions (David & Gollasch 2015). The exclusion of this size fraction of organisms in counts could result in unregulated risks which could develop into serious issues as smaller organisms are not necessarily less robust than larger organisms (Liu et al. 2016). A number of studies have addressed the issue based on results which revealed that >90% of the total phytoplankton in natural populations were <10 µm and furthermore that the size fraction included numerous toxic species such as *Phaeocystis* spp., *Pfiesteria* spp. and *Chrysochromulina* spp. (van der Star et al. 2011, Gollasch et al. 2012, David & Gollasch 2015).

Various treatments techniques are used for the removal of organisms in the BW. As a primary action almost all BWTS use a solid-liquid separation step (filtration, hydrocyclone, coagulation) to remove the large size class (>50 µm) primarily consisting of macroalgae, large protist, zooplankton, and fish larvae. The initial action is followed by a second disinfection step where different treatment techniques target organisms slipping through the filters, mainly organisms <50 µm (phytoplankton, bacteria and virus) (Figure 5). These secondary techniques are divided into two categories: (1) systems using *active* substances (chemical treatment) such as electrolysis, ozone or advanced oxidation, and (2) those using *non-active* substances (mechanical treatment) which include ultraviolet (UV) irradiation, heat, or deoxygenation (Gregg et al. 2009, Tsolaki & Diamadopoulos 2010).

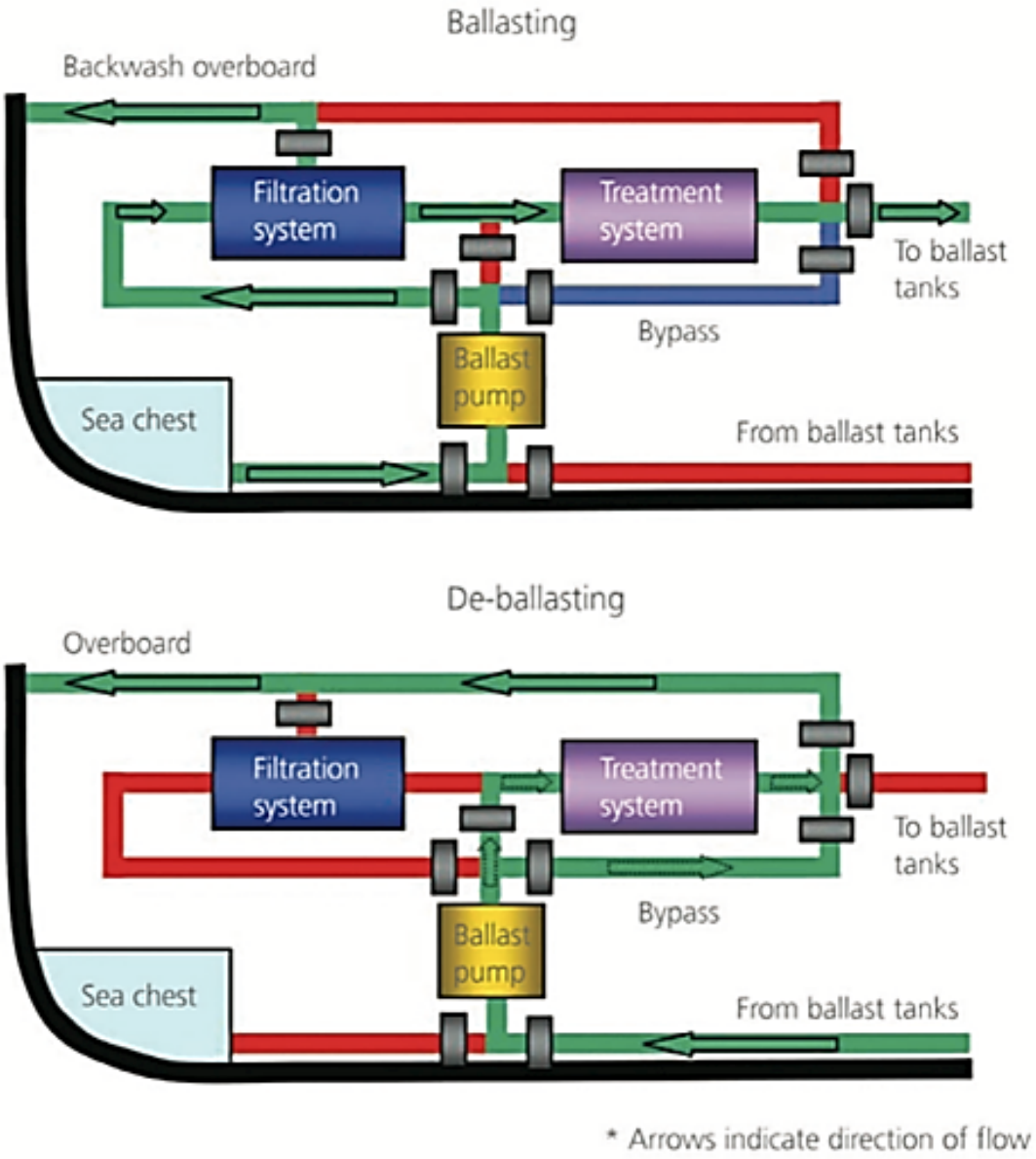


Figure 5. Diagrams showing the typical treatment process during ballasting and deballasting conditions. During deballasting conditions ballast water can either bypass the treatment system or receive a second treatment before discharge (Lloyd's Register 2012).

Type Approval certificates of BWTS are obtained through stated validation procedures (G8/G9 Guidelines) that involves land-based and shipboard test cycles at IMO/USCG approved test facilities (US EPA 2010, US Coast Guard 2012, IMO 2016c) (Figure 6). Evaluation of BWTSs are performed in relation to their use of active substances or non-active substances as treatment strategy. The essence of the validation procedures is that BWTSs must demonstrate that they consistently meet discharge standards requirements in five consecutive test cycles for each water salinity tested: freshwater (<1 psu), brackish water (10-20 psu) and marine water (28-36 psu).

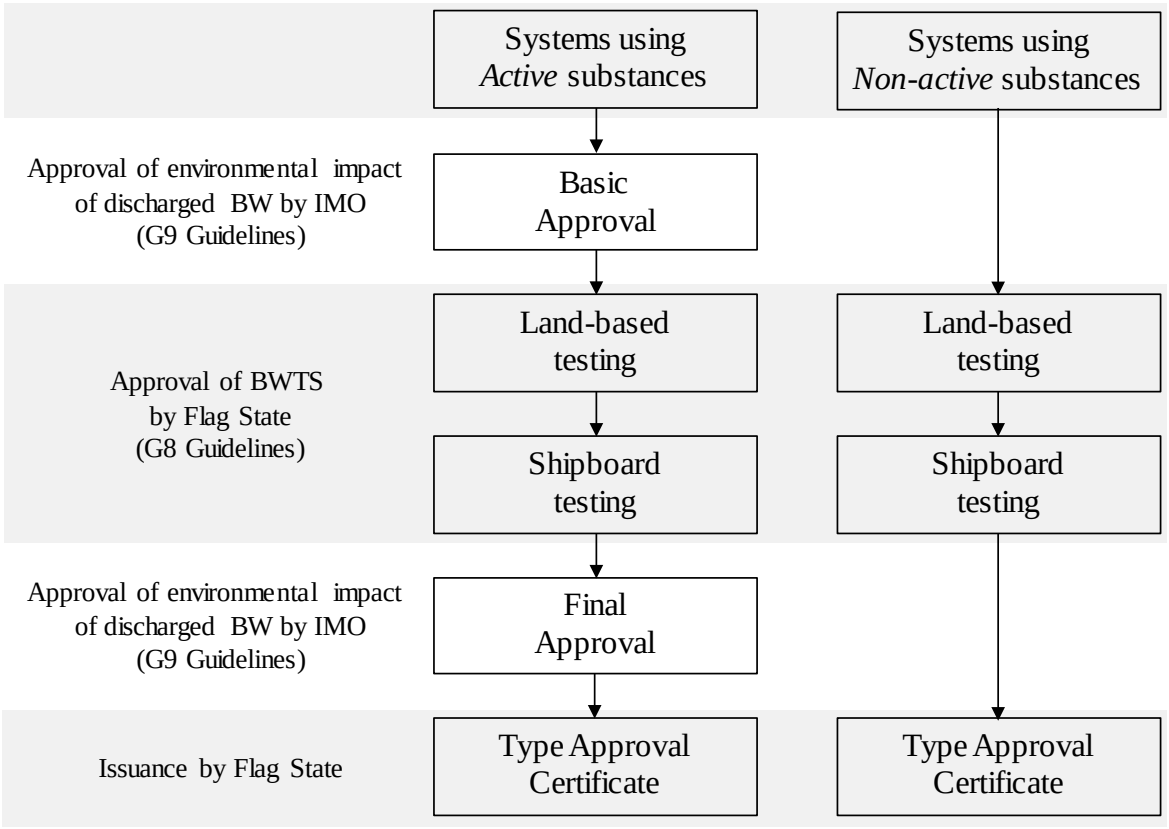


Figure 6. Schematic outline of the Type Approval process for BWTSs using active and non-active substances (based on guidelines from IMO (2008b, 2016c)).

Each test cycle consists of five steps (US EPA 2010, IMO 2016c):

1. Pumping of *intake water* to challenge tank
2. Treatment of ≥200 m^3 of intake water with BWTS
3. Pumping of ≥200 m^3 of *control water* (no treatment) to control tank
4. Holding time of treated and control water in separate tanks for:
    a. USCG: ≥24 hours for *each biological efficacy test cycle*
    b. IMO: *At least two test cycles in each salinity should be conducted in order to evaluate compliance with the D-2 standard at the minimum holding time specified by the BWMS manufacturer.*
5. Discharge of treated and control water

In addition to physicochemical requirements related to total suspended sediment (TSS), particulate organic carbon (POC) and dissolved organic carbon (DOC), specific concentration requirements of viable organisms in *intake water* and *control discharge* must be fulfilled (Table 2). To be valid, intake water in each test cycles must be initiated with high enough concentrations of viable organism to ensure that the BWTSs are properly challenged properly (US EPA 2010, IMO 2016c). Furthermore, control discharge concentration must likewise sustain a high enough concentration of viable organisms after retention time to ensure that observed elimination of organisms can be attributed to the effect of the BWTS and not environmental conditions (US EPA 2010, Drillet et al. 2013, IMO 2016c).

Table 2. Minimum concentration requirements of viable organisms in intake water and control discharge in land-based and shipboard tests for the size classes ≥10-<50 µm and ≥50 µm (US EPA 2010, IMO 2016c).

	Land-based requirements		Shipboard requirements	
Size class (µm)	≥10-<50	≥50	≥10-<50	≥50
Units	org. ml^{-1}	org. m^{-3}	org. ml^{-1}	org. m^{-3}
[Intake]	≥1,000	≥100,000	≥100	≥100
[Control discharge]	≥100	≥100	N.A.	N.A.

1.3.2. The use of standard test organisms (STOs) in test water

In land-based tests, intake requirements are 100 and 10,000 times higher than the stated D-2 discharge standards for the size classes 10-50 µm and >50 µm, respectively. For shipboard tests the minimum intake requirements are decreased to 10 times the discharge standards for both size classes. In addition, *influent water should consist of at least 5 species from at least 3 different phyla/divisions* (IMO 2016c).

Often the intake water requirements cannot be fulfilled during land-based validation procedures because natural occurring concentrations cannot be found in the environment around the respective test facilities at all times at high enough concentrations (Wright & Welschmeyer 2015). Protocols therefore suggest the use of standard test organisms (STOs) to be mixed with the natural test water, which has become standard practice at many test facilities (US EPA 2010, IMO 2016c, Global TestNet 2018). The use of STOs in validation tests has been questioned in recent international panel discussions driven by the IMO. It has been debated whether the use of STOs decrease the challenge conditions of the test water due to a potential lower robustness towards treatments (IMO 2016b, a). However, if STOs are more or at least as robust as natural occurring species, then STOs could advantageously be used as a supplement in test water to achieve minimum intake water requirements. When looking at the size class 10-50 µm, recent studies on commonly used algal STOs (*Tetraselmis suecica*, *Odontella* sp.) at test facilities showed that the species were at least as robust as natural algal compositions towards UV treatments (Olsen et al. 2016b, Lundgreen et al. *Submitted-b*). These findings suggest the potentially advantageous use of these species as test water supplements at test facilities if intake water requirements cannot be achieved using naturally occurring algal species alone.

The use of STOs as a supplement to increase organism concentration cannot be applied in shipboard tests. The set-up at land-based testing facilities allows manipulation of conditions to obtain the regulatory required testing conditions whereas the set-up in shipboard tests is restricted to the natural environment condition.

1.3.3. The viable vs. living dispute: When do organisms pose an invasion risk?

The minimum concentration requirements in intake water and control discharge for land-based and shipboard tests are similar for both IMO and USCG. Likewise are the discharge standards numerically identical, but the interpretation of requirements differs in a critical way between the two regulatory regimes. For the 10-50 µm size class, the IMO requires that BWTS …*shall discharge less than 10 viable org. ml^{-1}* (IMO 2004) whereas the USCG requires that *discharge must include fewer than 10*

living org. ml^{-1} of ballast water (US Coast Guard 2012). Living organisms are defined by showing vitality signs (intact cell membrane, metabolic activity) while viable organisms are defined by displaying both vitality signs and being reproductive (Breeuwer & Abee 2000, MacIntyre & Cullen 2016). The difference between the biological endpoints described above has shown to be problematic when evaluating efficacy of different BWTS types. For example, it has caused decisive implications for those BWTSs that are based on UV-technology since they are designed to damage cellular DNA (i.e. delayed mortality). The inflicted DNA damages hamper the cell's ability to reproduce, thereby rendering organisms harmless in relation to risk of invasion. UV-treated organisms will thus, dependent on the UV dose, for a period appear as living (show vitality signs) although they are in fact non-viable and *ecologically as good as dead* (Cullen & MacIntyre 2016). Consequently, a UV-based treatment system can obtain Type Approval when validated in accordance with IMO discharge requirements because the applied UV irradiation is sufficient to render cells non-viable and therefore in line with regulations.

The same UV system would on the other hand fail under the more conservative USCG discharge requirements since the treated water would contain too high a concentration of alive cells – even though they are rendered non-viable and pose no invasion risk (Wright & Welschmeyer 2015, Cullen & MacIntyre 2016). The USCG regulations could be attributed to be more environmentally protective, but the consequence is that the UV-based treatment systems are placed at a considerable disadvantage compared to e.g. chemical-based BWTS. To satisfy the USCG discharge standard for living organisms, a wasteful and costly overtreatment would be needed to outright kill organisms (Frazier et al. 2013, Cullen & MacIntyre 2016, Drake et al. 2016, Lundgreen et al. 2018, Lundgreen et al. *Submitted-b*)

Ballast water treatment systems that are Type Approved under IMO regulations can apply the USCG to obtain an Alternate Management System (AMS) approval. The granting of an AMS status is based on the anticipation that the manufactures of these BWTSs will conduct further testing and in addition obtain Type Approval in accordance with USCG regulations within the granted five year period (David & Gollasch 2015).

1.4. UV-based treatment systems: Functionality and viability assessment challenges

It has been predicted that the international regulations and requirements for installation of BWTSs would cause a dramatic increase in the market for treatment system orders of around USD 30-70 billion in the period 2009-2020 (Frost & Sullivan 2010, King et al. 2010, Hexa Research 2018). Although neutralization is a strict regulation requirement, chemical-based treatment systems such as the use of chlorine pose an environmental risk, from either chlorine discharge or from the formation of disinfection by-products. In addition, crew memebers are exposed to operation hazards and special precautions need to be made. On the other hand, UV irradiation combined with filtration, represents one of the prevailing treatment principles of Type Approved BWTSs as it is recognized as efficient, reliable and poses no environmental risks (Technology 2015, Gerhard et al. *Submitted*) (Figure 7).

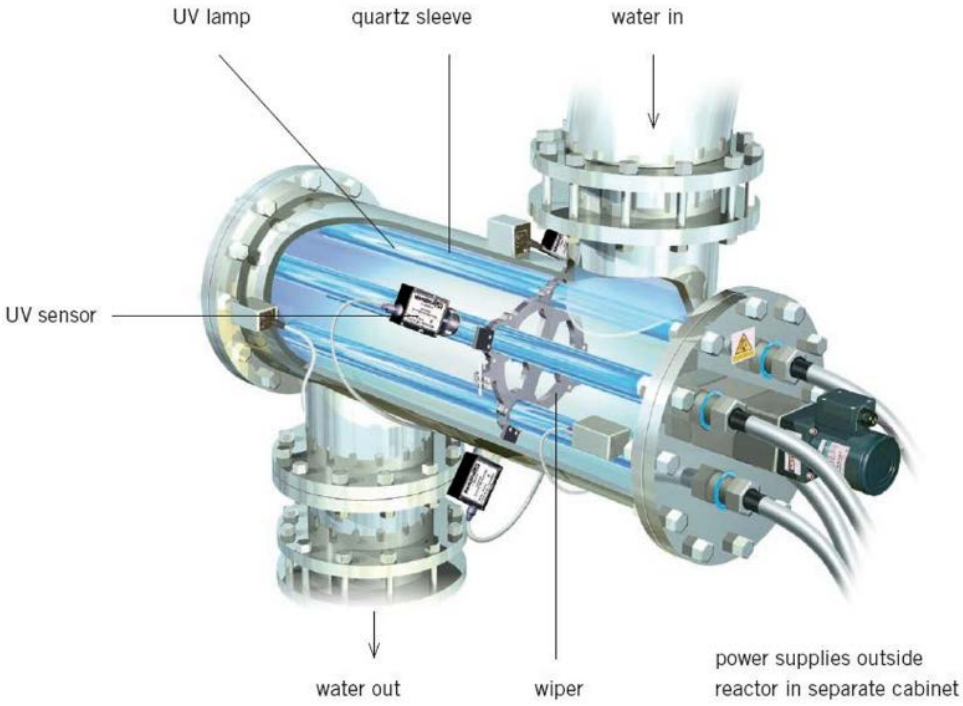


Figure 7. Diagram showing a cross flow UV-based treatment system (https://water-specialist.blogspot.com).

Currently about 46% of the BWTSs on the market are based on UV technology and of all the systems using non-active substances they account for 84% (Figure 9) (www.docs.imo.org 2018, Gerhard et al. *Submitted*). Three kinds of UV-based BWTS have been approved by the IMO: (1) Low pressure (LP), (2) medium pressure (MP), and (3) LED UV systems.

Low pressure mercury lamps emit UV light within the UV-C (germicidal) range (100-280 nm) with a high intensity peak at 253.7 nm. Approximately 85% of the power output is concentrated at this wavelength and the peak mainly targets DNA that displays a maximum absorption capacity at around 260 nm (Zimmer & Slawson 2002, Weber 2005, Ben Said et al. 2015, Sun & Blatchley 2017). The absorbed LP UV light by the nucleic acids produces abnormal C=C covalent bonds between adjacent pyrimidine bases (thymine or cytosine) on the same nucleic strand (intra-strand crosslinking). This primarily results in the formation of cyclobutane pyrimidine dimers (CPDs) that alters the structure of the DNA double helix (Figure 8) (Oguma et al. 2001, Oguma et al. 2002). The irregularity inhibits polymerase driven RNA transcription and DNA replication, thereby disrupting vital cellular functions and reproduction which eventually causes cell death (Goodsell 2001, Oguma et al. 2002, Kalisvaart 2004, Rastogi et al. 2010). Repair mechanisms (photoreactivation using visible light and dark-repair where no light is present), are present in a variety of organisms such as bacteria, plants and animals (Sinha & Hader 2002, Rastogi et al. 2010). The repair mechanisms can include removal of damaged bases by DNA glycosylase (base excision repair) and/or photoreactivation by photolyase.

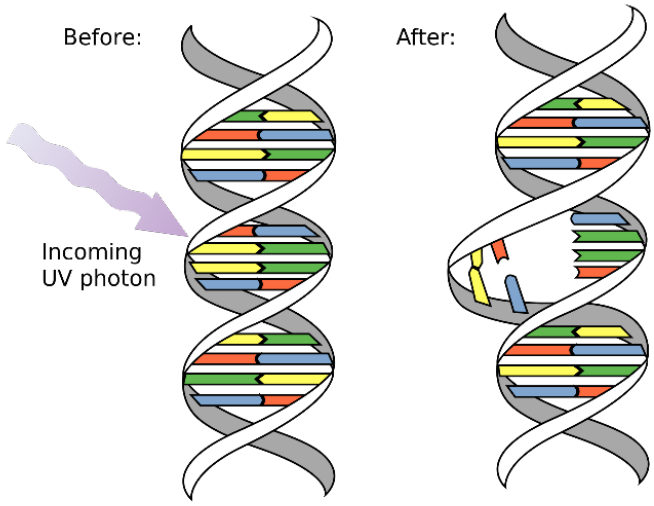


Figure 8. The UV irradiation causes pyrimidines (cytosine and thymine) to produce abnormal covalent bonds on the same DNA string. The change in structure hampers DNA replication and disrupts vital cellular functions that eventually lead to cell death.

The involved enzymes/proteins have maximum absorption capacities at around 280 nm and the enzymes are therefore potentially less affected by the LP UV irradiation (Kalisvaart 2004). Thus, LP lamps are not able to hamper functioning DNA repair systems which potentially result in higher survival rate and continuous reproduction (regrowth) of the targeted organisms (Singh 1975, Liebich et al. 2012, Martinez et al. 2012, 2013). However, several studies have shown LP UV treatments to be efficient in inactivating organisms (Sakai et al. 2011, Ben Said et al. 2015, Sun & Blatchley 2017, Lundgreen et al. 2018).

Medium pressure (MP) mercury lamps emit UV light at a higher intensity and cover a much broader spectrum including parts of UV-C, UV-B, and UV-A in the 185-400 nm range (Kalisvaart 2004). Medium pressure UV affects DNA but compared to LP UV only about 3% of the total energy between 200 and 300 nm is emitted at 254 nm, resulting in a smaller absorption of UV irradiation by DNA (Lundgreen et al. 2018). However, the higher intensity (Harm 1980) in combination with the additional targeting of repair systems within the UV-B range (280-320 nm) have shown reduced growth rates and photosynthetic activity in phytoplankton (Choi et al. 2011, Sun & Blatchley 2017) as well as reduced growth of *E. coli* bacteria (Oguma et al. 2002, Bowker et al. 2011, Quek & Hu 2013), and adenovirus (Eischeid & Linden 2011). The absorption of UV irradiation by certain amino acids at around 280 nm makes proteins and enzymes (e.g. photolyase, RNA/DNA polymerases) unstable. This results in loss of biological activity as the biomolecules denaturate (Kalisvaart 2011). About 50% of the dry weight of microorganisms consists of proteins, and damages caused by MP UV irradiation can thus have severe implications for basic cellular metabolism (Kalisvaart 2011).

Light emitting diodes (LEDs) consist of an array of small components that can be manufactured to emit UV light at multiple individual wavelengths ranging from 237 to 340 nm and in addition offer flexibility in arrangement designs (Shur & Gaska 2008). Other advantages include that UV-LEDs are not toxic upon breakage, require less energy and have longer lifespans compared to LP and MP systems (~8,000 h vs. ~100,000 h).

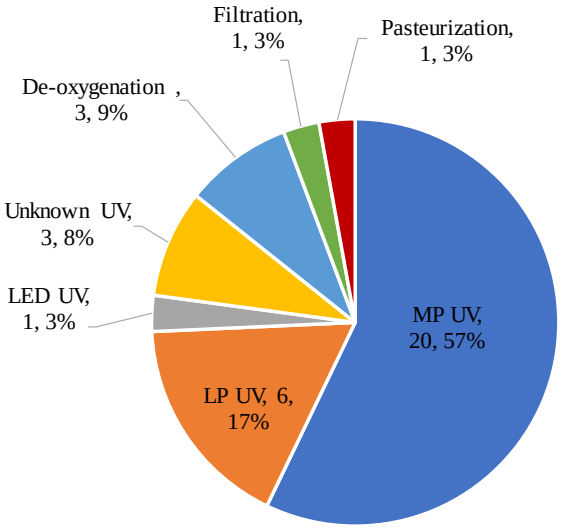


Figure 9. The distribution of Type Approved BWTS using non-active substances. The listed number after each treatment technique signifies the current IMO approved number of this type of system and its corresponding percentage wise contribution in proportion to the total number of available systems using non-active substances. Data modified from Gerhard et al. (*Submitted*).

A special challenge for monitoring effects of UV-based technologies is that the damage to cellular DNA caused by UV irradiation will either result in eventual death or in survival due to DNA repair (Sinha & Hader 2002, Rastogi et al. 2010). Currently there are no prescribed staining methods that are able to measure the direct and immediate effects of DNA damage. The currently used standard viability assessment method, which is based on staining is therefore limited when it comes to distinguishing between viable and non-viable cells. The methodologies and related issues are discussed in more detail below.

1.5. Methodologies for viability assessment: Strengths and limitations of existing methods

The BWM Convention itself does not suggest which protocols or methods can or should be used to assess efficacy of BWTS. The use of different quantification methods to evaluate the efficacy of BWTS is a central point in the dispute regarding assessments based on living (USCG) or viable (IMO) organisms.

1.5.1. Most Probable Number (MPN) and Vital Stain (VS) method

One method that is recognized by the IMO to assess viability is the Most Probable Number (MPN) method (i.e. cultivation-based method) which estimates the regrowth potential of organisms in treated BW (Figure 10). Samples of treated BW are subjected to an array of serial dilutions that after a two week incubation period in will provide a quantitative estimation of the number of viable cells present in the original undiluted sample (Throndsen 1978, Cullen & MacIntyre 2016, MacIntyre et al. 2017). This method allows the delayed biocidal effects of UV treatment to be measured because the observed living (but non-viable) cells immediately after treatment will eventually die during the regrowth period. The use of MPN method is accepted by the IMO for compliance verification of BWTS and

several studies support the use of this method for sound and robust validation of viable organisms (Madsen & Petersen 2015, Wright & Welschmeyer 2015, Cullen & MacIntyre 2016, MacIntyre et al. 2017, Blatchley III et al. 2018, Cullen 2018, MacIntyre et al. 2018). In the USA the MPN method is used for assessment of drinking water quality (US EPA 2006).

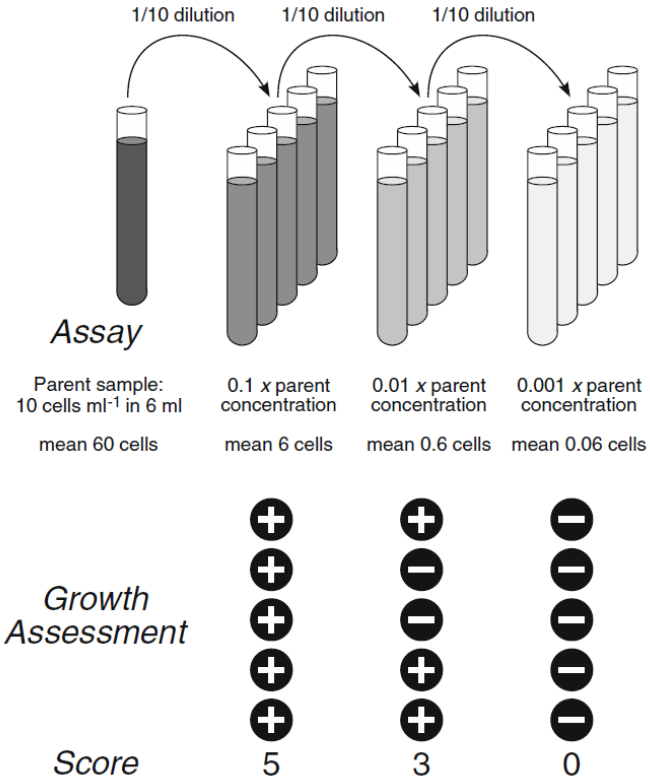


Figure 10. The principles of the Most Probable Number method. The concentration of viable cells in a sample is estimated based on incremental dilution of the sample into a series of replicated subcultures and statistical determination of the hypothetical dilution that corresponds to one viable cell per subculture tube. Initially, the chlorophyll fluorescence is estimated in all tubes and then incubated for 14 days at favorable light and nutrient conditions. If one or more viable cells are present in a tube they will reproduce and increase the chlorophyll fluorescence compared to initial measurements. Dilutions are then scored for growth or no growth which are used in the MPN calculation to estimate the number of viable cells in the original sample. In this example, the test score results in an MPN of 79 cells corresponding to 13 cells ml^{-1} (79 cells/6 ml) (Cullen & MacIntyre 2016).

In July 2016 the USCG rejected the use of the MPN method for BWTS validation procedures (Coast Guard Maritime Commons 2016). The rejection was the result of submitted appeals from four manufacturers of UV-based treatment systems where the USCG in December 2015 initially had denied their requests to use the MPN method for discharge standard verification (Coast Guard Maritime Commons 2015). The reasons were that the USCG Marine Safety Center could not approve a testing method using a different standard than that required by the USCG regulations and that the BWTSs in play therefore could not comply with the regulatory requirements of less than 10 *living* org. ml^{-1} (Coast Guard Maritime Commons 2016). The USCG regulations specifically require BWTSs to be evaluated based on their ability to *kill* certain organisms (USCG Marine Safety Center 2015) which the MPN method cannot directly assess. Furthermore, it has been argued that the MPN method is not ideal because it cannot be assumed that all species can be cultured, the method is time

consuming (lasting up to two weeks) and the obtained concentrations have large error estimates (US Coast Guard 2012, First & Drake 2013, Drake et al. 2016).

Instead, the USCG required the use of the so-called vital stain (VS) method combined with motility observations (therefore also referred to Stain-Motility method) as their benchmark test for quantification of living organism (US EPA 2010, Steinberg et al. 2011). This approach is likewise acknowledged as assessment method by the IMO where appropriate (IMO 2016d). The VS method consists of a combination of fluorescein diacetate (FDA) and 5-chloromethylfluorescein diacetate (CMFDA) that are added to samples of treated BW (Figure 11).

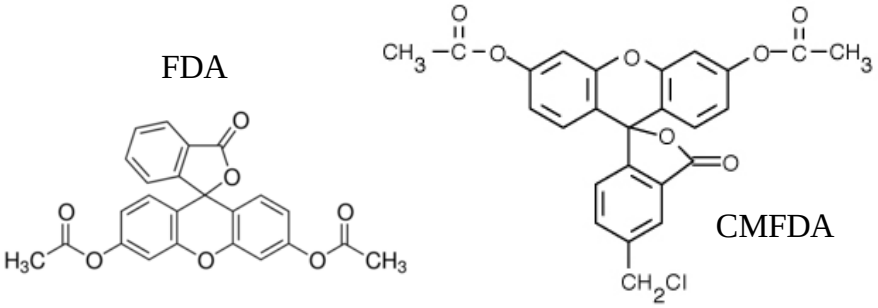


Figure 11. The chemical structure of fluorescein diacetate (FDA) and 5-chloromethylfluorescein diacetate (CMFDA) used in the Vital Stain method.

The lipophilic stains are designed to readily cross cell membrane of organisms and are initially quenched and transparent. If cells are alive, then unspecific enzyme activity will cleave the acetate groups which converts the molecule into a green fluorescent molecule that additionally becomes relatively cell impermeable. By using direct microscope counts, the number of fluorescent (living) cells can then be quantified. In addition, motility is used to assist in the quantification procedures to include non-fluorescent but living cells to correct for false-negative errors of the stains (US EPA 2010). Only non-fluorescent cells showing no motility are considered dead (US EPA 2010). The assessment of BW samples must take place within six hours after sample collection (US EPA 2010, IMO 2016d). Several studies have argued that the method is a powerful tool and robust method for evaluating the efficacy of BWTS in terms of live/dead enumeration (Peperzak & Brussaard 2011, Steinberg et al. 2011, Adams et al. 2014, Baek & Shin 2015). Critical views on the use of the VS methods include ambiguous staining of live and dead cells leading to Type I errors (false positive, i.e. overestimation) and Type II errors (false negative, i.e. underestimation) in counting (Peperzak & Brussaard 2011, Gorokhova et al. 2012, Olsen et al. 2015, MacIntyre & Cullen 2016, Cullen 2018) and variations in staining intensity related to growth stage of cells (Garvey et al. 2007). Furthermore, the inclusion of motility in the assessment procedures as back-up criterion has been criticized because it assumes that non-stained and non-motile cells are dead (MacIntyre & Cullen 2016, Blatchley III et al. 2018). Yet, several classes of phytoplankton such as diatoms are inherently non-motile and other classes stop moving after exposure to stains, exposure to fluorescent light, if stressed or under non-optimal conditions (Selvin et al. 1989).

In November 2018, the U.S. Senate passed the Vessel Incidental Discharge Act (VIDA) Bill as part of the USCG Reauthorization Act that will allow the USCG to use reproductive methods (such as the

MPN method) for testing and approval of BWTS. This means that the definition of the term *living* organisms has been redefined to ensure that non-viable organisms are not considered to be living. The bill was signed into law in December 2018 by the U.S. President and the USCG now has 180 days to draft a policy letter that describes reproductive methods based on the best available science and moreover they must consider the use of the MPN method as a Type Approval testing methodology (U.S. Senate 2018).

1.5.2. Alternative viability assessment methods

Apart from the VS and the MPN method, a number of alternative methods are used at IMO approved test facilities in the BWTS validation procedures for the size class 10-50 µm (Table 3). Some of these methods are used independently of, or in parallel with, the VS and/or MPN method. Assessment methods can be applied for a *comprehensive analysis* of BWTS for proof of compliance with stated discharge standards, but they can also be applied for an *indicative analysis* of BWTS performance (IMO 2008a, Gollasch et al. 2015a).

Detailed comprehensive analyses provide more accurate assessments, but also involve trained personnel performing exhaustive analytical procedures that are time consuming. In addition, laboratory surroundings are typically required and the use of fragile and expensive equipment. Comprehensive methods other than MPN include flow cytometry, different staining methods, and other regrowth methods.

Indicative analysis, on the other hand, in an approach based on the premise that the involved instrument must be quick and simple to operate without the need for special training. A practical advantage is that inspections interfere as little as possible with the typically tight schedule of ships. It is, however, called indicative analysis because it is often used only as a test to indicate whether there is a need for a more comprehensive analysis.

Indicative (bulk) analysis methods

A simple analysis would allow continuous autonomous monitoring of BWTS efficacy by the ship owners as well as assist Port State Control officers that are obliged to monitor compliance of ships to the BWM Convention (van Slooten et al. 2015). The application of robust indicative methods could be advantageous in advising if a ship is potentially compliant or non-compliant. If the indicative sample analysis shows gross exceedance and thus result in doubt about compliance, a more comprehensive method can be initiated. Indicative methods could save potentially compliant ships valuable time and costs, and furthermore in an early stage detect potentially non-compliant BW and thus prevent discharge of biologically contaminated BW (David & Gollasch 2015, Wright & Welschmeyer 2015).

The IMO BW sampling Guidelines G2 paragraph 6.3 addresses the need for quick screening methods and states: *Prior to testing for compliance with the D-2 standard, it is recommended that, as a first step, an indicative analysis of ballast water discharge may be undertaken to establish whether a ship is potentially compliant or non-compliant. Such a test could help the Party identify immediate mitigation measures, within their existing powers, to avoid any additional impact from a possible non-compliant ballast water discharge from the ship* (IMO 2008a). The need for first step indicative analysis is emphasized, but how the sampling and analysis should be carried out is not addressed. The

following section provides an overview of the currently most recognized/prevalent alternative assessment methods (so-called Compliance, Monitoring and Enforcement techniques) including their strengths and limitations as indicative analyses with the main focus on the 10-50 µm size class.

Table 3. Overview of quantification and viability assessment methods for the 10-50 µm size class used for land-based and shipboard tests at approved test facilities (modified from Global TestNet 2018 and DHI, personal communication). Brackets indicate country of origin. [a]used in parallel with FDA/CMFDA or [b]MPN. *Methodology not specified

Assessment method	#Users	Test facility
FDA/CMFDA	11	David Consult (SI), DHI (DK), GBF (US), GoConsult (DE), KIOST (ROK), KOMERI (ROK), MBRIJ (JP), MEA-nl (NL), MERC (US), NIVA (NO), PML (GB)
MPN	7	DHI (DK), GBF (US), KOMERI (ROK), MBRIJ (JP), MEA-nl (NL), NIVA (NO), WMR (NL)
Organisms movement	5	KE (JP), KIOST (ROK)[a], KOMERI (ROK)[a], MBRIJ (JP)[a]
Cell integrity	2	KE (JP), WMR (ML)
Growth experiments*	2	KIOST (ROK), KOMERI (ROK)[b]
FDA	2	GSI (US), KIOST (ROK)
Flow cytometry	2	GBF (US)[a], MEA-nl (NL)[a]
PAM	2	GBF (US)[a,b], MEA-nl (NL)[a,b]
Photosynthetic activity*	2	DHI (DK)[a,b], NIOZ (NL)
BWI	1	GBF (US)[b]
Neutral Red	1	MBRIJ (JP)[a]
SYTOX Green	1	NIOZ (NL)

Pulse-amplitude modulated fluorometry (PAM) has been stated as one of the most accurate methods for indicative analysis (Gollasch et al. 2015a, van Slooten et al. 2015, Casas-Monroy et al. 2016, Outinen & Lehtiniemi 2017). The method is based on the autofluorescence properties of the chlorophyll protein complex present in algal cells and measures the photochemical efficiency of photosystem II (Schreiber 2004). Results are typically available within minutes and are presented as quantitative estimation or as a qualitative bulk analysis that will indicate the viability condition of a phytoplankton sample regardless of size of the algae (Bradie 2016).

Although the method is regarded as one of the best indicative analysis methods, limitations have also been highlighted. The results can differ depending on stress, nutritional status, and cell cycle phases of organisms, and the method is vulnerable to over- or underestimation of certain organism groups (Gollasch et al. 2015a, Casas-Monroy et al. 2016). Another limitation concerns UV-based treatment systems where organisms are not instantly killed which results in false positive counts of non-viable organisms with still active photosystems (First & Drake 2014, van Slooten et al. 2015, Casas-Monroy et al. 2016). The most significant limitation is that only autotrophic organisms such as phytoplankton and some bacteria can be evaluated within the 10-50 µm size class. The regulations require all organisms within this size class to be included but since heterotrophs such as ciliates, protozoa and a

number of dinoflagellates do not contain chlorophyll, they cannot be detected. This is critical because heterotrophs often dominate coastal ecosystems where BW is generally taken up (van Slooten et al. 2015). Lastly, it has been shown that the ratio of chlorophyll *a* and cell concentration varies with growth phase and physiology, and chlorophyll *a* bulk measurements is therefore not an optimal approximation for cell concentration (Steinberg et al. 2012)

The adenosine triphosphate (ATP) assay is based on measurements of bioluminescence in the presence of luciferase enzymes (van Slooten et al. 2015). Given the central role of ATP in cellular metabolism, the presence of ATP has been regarded as a good indicator of metabolic activity in cells with and without chlorophyll. Although it has been considered a highly reliable indicative method (Outinen & Lehtiniemi 2017), it has been shown to be challenged in high salinity conditions and by varying ATP levels at different growth phase stages (van Slooten et al. 2015). In addition, high sediment loads and trace compounds can interfere with the signal (First & Drake 2014). The method fails to predict viability which means that UV-based treatment systems will give different results, compared to systems using active substances, and thus each BWTS technique should have its own set of criteria (First & Drake 2014, van Slooten et al. 2015). However, this view was challenged by Wright et al. (2015) who obtained a clear difference in ATP levels between UV-treated and control samples and argued that the method could serve as a sensitive indicator for all types of BWTSs. To ensure more accurate compliance with discharge regulations it has been proposed that ATP assay measurements should be carried out in parallel with full-scale tests of various BWTS techniques to evaluate baseline ATP levels where tests comply with regulations (First & Drake 2014, van Slooten et al. 2015).

The fluorescein diacetate (FDA) bulk viability assay can be applied as a quick indicator for biomass of living cells (Welschmeyer & Maurer 2011). The analytical process performs in a wide range of salinities (0-35 psu) and it typically takes about 1-3 hours to verify potential compliance with regulations, including staining of pre-filtered sample with FDA, incubation in the dark and final analysis with a fluorometer (Welschmeyer & Maurer 2011, Akram et al. 2015, van Slooten et al. 2015). The previously described VS method is based on cell-specific accumulation of FDA (and CMFDA) that readily diffuse through cell membranes and is hydrolyzed to fluorescein. However, unlike thiol reactive CMFDA (Poot et al. 1991), FDA is not chemically bound to specific targets within the cell and consequently there is a slow efflux of fluorescein from the cell which result in a fading of the cellular signal (Prosperi et al. 1986, Steinberg et al. 2011). Consequently, staining analysis utilizing the specific cellular signal of FDA as assessment approach must be carried out quickly after staining.

In contrast, the FDA bulk analysis method exploits the leaking properties of fluorescein from the hydrolyzed cellular FDA to produce an extracellular fluid fluorescence signal which is directly proportional to living biomass (Welschmeyer & Maurer 2011, Akram et al. 2015). The FDA bulk viability assay has been suggested by Welschmeyer & Maurer (2011) to be equal in sensitivity to ATP assay and more simple to use. However, an opposing study by van Slooten et al. (2015) found the FDA bulk viability assay to be less accurate and more time consuming that the ATP assay. In addition, there is a risk of filter damage and leakage during analysis (Akram et al. 2015, van Slooten et al. 2015) and a potential influence on fluorescens accuracy by water turbidity (Bradie 2016). The

method is only capable of assessment of living organisms and cannot identify non-viable cells. Therefore, the assay fails to evaluate UV-based treatment systems where cells can maintain intact membranes and exhibit cellular enzyme activity for many days after treatment before they die from the inflicted DNA damages (Tobiesen et al. 2011). Furthermore, several studies have shown that FDA (and CMFDA) produces inconsistent fluorescent signals because of staining of some dead species and not being able to stain some living species resulting in over- and underestimations issues, respectively (Garvey et al. 2007, Peperzak & Brussaard 2011, MacIntyre & Cullen 2016).

Vital or mortal stains can be applied on living cells without killing them. Using vital/mortal stains as a live/dead assessment approach has the advantages that visual concentration-based numeric counts can be performed selectively on the >50 µm and 10-50 µm size-classes and the results can be directly compared to published IMO D-2 and USCG standards (Wright et al. 2015). Vital stains are designed to be taken up only by living cells and therefore provide information about the number of cells showing vital signs in a sample by means of either a fluorescent (FDA, CMFDA) or non-fluorescent signal (Neutral Red) (Steinberg et al. 2011, Zetsche & Meysman 2012, Wright et al. 2015).

In contrast, mortal stains provide information about the number of dead cells and are only able to cross ruptured cell membranes. SYTOX Green and propidium iodide (red) are fluorescent molecules that stain nucleic acids inside dead cells resulting in up to a 500-fold increase in fluorescence around the nuclei (Reavie et al. 2010, Zetsche & Meysman 2012), whereas the non-fluorescent mortal stains such as Evans Blue and Trypan Blue are used by applying a more histologically approach. When using mortal stains, the typical approach for enumeration of the living cells is (1) to count the whole sample, (2) then stain, (3) count all dead cells, and finally (4) subtract this number from total counts to obtain the number of living cells (Veldhuis et al. 2001). This approach can be very time consuming and tedious.

The purpose of live/dead stains to provide a clear distinction between living and dead cells has shown to be challenging by several studies as also mentioned previously regarding FDA/CMFDA (Peperzak & Brussaard 2011, MacIntyre & Cullen 2016). For SYTOX Green ambiguous staining levels have been observed in dead and live copepods (Reavie et al. 2010, Zetsche & Meysman 2012) and phytoplankton (Peperzak & Brussaard 2011). Likewise, Maranda et al. (2013) observed that Neutral red was taken up by both live and recently dead copepods. A few older studies have similarly shown ambiguous results in the uptake of Neutral red in a variety of phytoplankton species and primarily in smaller species (Crippen & Perrier 1974, Reynolds et al. 1978), whereas successful incorporation of the stain was observed in 15 different algal species of both small and large character by Zetsche & Meysman (2012).

In flow cytometry, a stained water sample is channeled to pass a detector which can separate sub-populations of the present cells according to size and fluorescence status and the technique has therefore been regarded as an efficient technique for determination of heterogenous populations (Picot et al. 2012, Olsen et al. 2016a, Hoell et al. 2017). It can provide results within a few minutes, has a particle counting rate of around 1000 cells per second and can identify cells down to approximately 2 µm (Peperzak et al. 2018). The technique is typically used for comprehensive analyses as the system roughly weighs 20-100 kg and therefore far from easily portable (Outinen &

Lehtiniemi 2017) but has been found to be more accurate when compared to FDA, ATP, and PAM methods (Bakalar 2014). However, in *Guidance on ballast water sampling and analysis for trial use in accordance with the BWM Convention and Guidelines (G2)* flow cytometry was suggested as one of the methods for indicative analysis (IMO 2015).

Despite the highlight of quick analysis time the technique is also subjected to time consuming sample preparation and incubation or staining procedures (Outinen & Lehtiniemi 2017). Flow cytometry is an enumeration tool and is based on stains. Therefore, the technique itself cannot overcome the problem of distinguishing between live and dead cells because this limitation is rooted in the stains. For this reason flow cytometry cannot be considered as a substitute for microscopy procedures (Reavie et al. 2010). The fact that cells down to 2 µm can be detected makes the technique suitable for additional quick estimations of organism <10 µm, as it can provide a sub-population of this size class. Although outside the D-2 discharge standard size range for phytoplankton organisms <10 µm are often present at relatively high concentration in the plankton communities and more than 90% of all phytoplankton organisms fall within this category (Olenina et al. 2006, van Slooten et al. 2015). If the <10 µm size class category in the future becomes part of the assessment, then flow cytometry can provide a quick and reasonable sampling approach (Outinen & Lehtiniemi 2017).

A challenge for flow cytometry is that it cannot count single cells within multi-cell colonies but instead these are counted as one cell affecting the outcome regarding both cell count and size distribution (Peperzak et al. 2018). In addition, flow cytometry is exposed to clogging issues and sedimentation in the sample tubing (Peperzak et al. 2018).

Indicative (numeric) analysis methods

The Flow Cytometer And Microscope (FlowCAM) technique developed by Fluid Imaging Technologies, ME, USA, combines traditional flow cytometry with digital imaging microscopy and is able to record cell parameters such as length, width, diameter, and fluorescence (Sieracki et al. 1998). The technique stores recorded images for post-processing and has shown to have potential for counting and sizing microplankton as well as identifying target groups or species in mixed populations if combined with species specific probes or vital stains (See et al. 2005, Buskey & Hyatt 2006, Peterson & Nelson 2010, Camoying & Yñiguez 2016, Romero-Martínez et al. 2017, Kydd et al. 2018). A good correlation has been obtained between FlowCAM and manual microscopy counts of natural phytoplankton of various size (Sieracki et al. 1998, See et al. 2005, Camoying & Yñiguez 2016). However, other studies have reported ambiguous results of the technique either underestimating (Jakobsen & Carstensen 2011, Kydd et al. 2018) or overestimating cell counts (Álvarez et al. 2014).

Regarding analysis time, Kydd et al. (2018) reported time frames similar to that of manual counts while other studies have concluded that the technique is less tedious and faster (0.25-5 ml min^{-1}), compared to manual microscopy which allows assessment of more cells/higher volumes for improving quantification accuracy (Buskey & Hyatt 2006, Álvarez et al. 2014, Fluid Imaging Technologies 2018). Flow rates up to 60 ml min^{-1} are expected to be available in newer models (Peterson & Nelson 2010).

Depending on the setup of the technique and the objective used the FlowCAM is able to detect cells in the size range 3-1000 µm (Fluid Imaging Technologies 2018). However, colony forming algal species has been highlighted as one of the major drawbacks of the method because additional time is required to examine images for accurate counts of individuals in colonies (Reavie et al. 2010). Clogging is also a common issue and pre-filtration of the sample is recommended (Álvarez et al. 2011, Camoying & Yñiguez 2016, Romero-Martínez et al. 2017). Furthermore, contaminants (unidentified particles) of similar size as algal cells has been recorded to be present in blank samples and test water in concentrations of up to 98 contaminant particles ml^{-1} (Romero-Martínez et al. 2017). When analyzing treated BW samples where concentrations are expected to be very low (in compliance with discharge standards) this issue has shown to compromise the accuracy of the technique (Romero-Martínez et al. 2017).

The majority of studies using the FlowCAM have focused on identification of specific algal groups or species related to HABs. In general, the technique has been attributed to have potential for recognition of target cells although the sensitivity of the technique is still limited and mis-identification of both non-target and target cells have been recorded (See et al. 2005, Buskey & Hyatt 2006). In these studies, enumeration was performed based on chlorophyll fluorescence as the endpoint, which has the disadvantage of excluding heterotrophs with no or little chlorophyll but which still have the potential of being responsible for producing HABs (Garmendia et al. 2013). However, if used for BW validation where specific species identification is not needed, the system might prove useful if used in combination with vital stains (Peterson & Nelson 2010, First & Drake 2012, Romero-Martínez et al. 2017, Kydd et al. 2018).

Other indicative methods

A number of additional indicative analysis approaches exist but they are not elaborated in the present thesis. They for example include: BallastWISE (MicroWISE/DHI 2018), FDA pulse counting (Nakata et al. 2014), Flow Cytobot (Olson & Sosik 2007), molecular methods and Fast Repetition Rate fluorometry, scanning flow cytometry (FCM), imaging flow cytometer.

1.6. Quantification challenges: human variability and moving towards automated methods

Several issues have been highlighted in studies using manual microscopic quantification of phytoplankton cells as well as zooplankton. For example, when using the VS method for BWTS validation, human counting performances is affected by fatigue, identification, and subjectivity issues regarding size class classification, algal concentration levels, algal mobility, and autofluorescent debris load (Lund et al. 1958, Culverhouse et al. 2003, First & Drake 2012, Lundgreen et al. *Submitted-a*). In addition, microscopy requires a high level of taxonomic skill and the accuracy is only as accurate as the taxonomist. Different taxonomists trained in different ways can cause large person-person differences. Data Quality Indicators (DQI) are therefore required in ISO 17025 accredited laboratories performing the analysis to secure inter and intra specific bias of the analysis (International Organization for Standardization 2017). Also, each step from: collection, storage, subsampling, homogenization and counting can bring in error. Some species, e.g. *Pseudochatonella* spp. are sticky meaning that they stick to the side of containers which result in underestimation of concentration. The consequence is a high variation between replicate samples leading to inaccurate

counts by microscopists. In worst cases this can lead to approval of inefficient BWTS that are non-compliant with regulations, or vice versa, unfairly failing and fining BWTS that actually are in line with discharge standards and environmentally protective (First & Drake 2012).

The further development of automated methods along with the improvement of some existing techniques (see section 1.5.2) for quantitative viability assessment are promising candidates for addressing the highlighted issues experienced by manual microscopy (MacLeod et al. 2010). This is especially true if used in parallel with more advanced post-processing image analysis methods like machine learning (Kruk et al. 2017, Orenstein & Beijbom 2017) and other specialized software programs such as ImageJ (Igathinathane et al. 2008, Schulze et al. 2011), ZOOVIS (Bi et al. 2015), PLASA (Hense et al. 2008) and PlanktoVision (Schulze et al. 2013).

1.7. En route from life to death and resuscitation

The last part of this introduction considers the question of what it takes for an organism to be considered an invasion risk (i.e. viable) and the challenges involved in organism viability assessment.

As previously mentioned (see section 1.3.3 and 1.5) this matter has especially been a challenge regarding UV-based BWTS validation by different authorities (IMO and USCG). The MPN method provides information on the proportion of viable organism present in the original UV treated sample which means an estimated quantification of only those organisms that can reproduce. Non-viable organisms are not able to reproduce because of DNA damages but will for a while appear alive (up to days depending on fluence applied) due to the delayed mortality caused by the UV exposure. As mentioned, non-viable organisms are, despite they are still alive, not an invasion risk because they are not able to reproduce and thus ecologically as good as dead (Cullen & MacIntyre 2016). If using a vital stain approach as required by the USCG the distinction between viable and non-viable is not possible as non-viable cells will appear just as fluorescent as viable cells. Instead, this approach can only provide information on the proportion of alive and dead organisms where alive organisms that have been UV treated will be a mix of viable and non-viable cells. However, the recent VIDA Bill will amend the USCG regulations to allow the use of the MPN method as test methodology in BWTS Type Approval. This will harmonize the use of reproductive methods for organism viability assessment and will provide a much closer alignment and framework for Type Approval with IMO regulations. The MPN method, although suitable for the assessment of viable organisms in UV-treated BW, is slow and there is a demand for faster methods that are able to provide numerical viability assessments, e.g. in relation to Port State Control.

An additional issue regarding viability assessment concerns resting stages of phytoplankton species. Some species of dinoflagellates and diatoms have the ability to form resting stages (cysts) when they experience suboptimal conditions to protect themselves from hostile environments (Hallegraeff & Bolch 1991, Hamer et al. 2000). Despite the harsh conditions in BW tanks, cysts of certain species are able to stay viable for months to years while waiting for more favorable times (Gollasch et al. 2015b). Typically, cysts are more resistant than normal cells towards different BW treatment techniques, making them popular model organisms in treatment validation experiments (Gregg & Hallegraeff 2007). Furthermore, studies have shown that the fluorescent signal of cysts is weaker than that of normal cells due to their lower metabolic state and they seem relative impermeable to vital

stains (US EPA 2010, Martinez et al. 2012, First et al. 2016). In regrowth assays, the growth conditions are made as favorable as possible to induce reproduction of the organisms potentially present in in treated BW. However, reactivation of cysts is not a guarantee, and latency for reactivation might differ between species (Drake et al. 2016, Blatchley III et al. 2018). This group of organisms therefore presents an additional challenge regarding assessment of organism viability.

Future exploration of multiplex staining has potential for faster and more precise organism viability assessments (Gilbert & Boutros 2016). Often cellular fitness is based on a single staining approach which typically only acts as an indirect physiological status indicator (Hoell et al. 2017). On the other hand, multiplexing is able to provide multiparametric data which allows comparative data analysis of the same group of organisms at the same time point (Gilbert & Boutros 2016). Multiplexing that involves specific probes for DNA damages (Hwang 2014), necrosis/apoptosis characteristics (Jimenez et al. 2009, Vanden Berghe et al. 2013) or other severe cell damages (Jauzein & Erdner 2013) in combination with vital/mortal stains (Steinberg et al. 2011, Zetsche & Meysman 2012) could be potential solutions for faster organism viability assessments. Yet, so far most of the research has been done on mammalian cells. Multiplexing would allow the determination of multiple cell properties and their distribution from which subgroups of cells could be classified on the *route from life to death* (Figure 12) to provide a more detailed picture of viability status (Davey 2011).

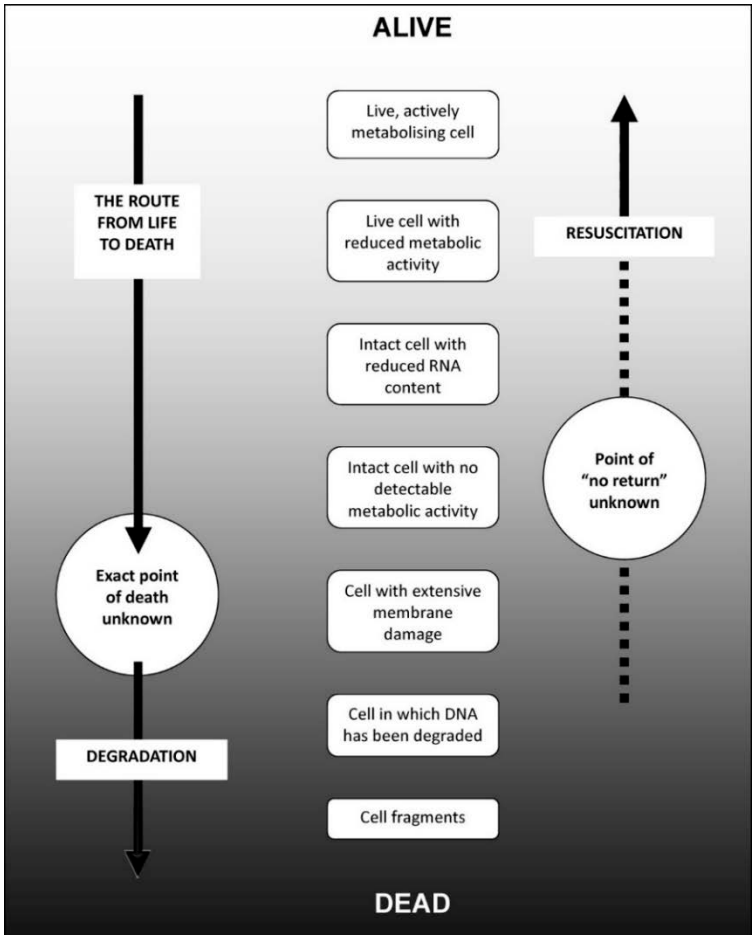


Figure 12. Cells within a population may be classified into different subgroups describing their physiological status in the route between life and death that consist of multiple steps. The exact points of reversibility of the different steps cannot be clearly defined (From Davey 2011).

2. Aims of this thesis

Both regulatory and scientific uncertainties concerning current test procedures involved in Type Approval of BWTSs have made stakeholders hesitant to ensure an effective implementation of the BWM Convention. The decision made by the USCG in December 2016 to decline the use of the MPN method for validation of UV-based treatment systems furthermore made ship-owners reluctant regarding the choice of costly treatment technologies. Consequently, the development of new reliable methods and improvements of current quantification methods used to evaluate BWTS performance is of high environmental as well as economic importance.

The thesis focusses primarily on phytoplankton in the size class 10-50 µm where the currently approved methodologies used for viability assessment are not applicable for all treatment technologies.

The main objectives are:

- To investigate the applicability of the two current standard quantification methods (MPN and VS) for viability assessment of UV treated algae.
- Furthermore, to evaluate if the methods could be applied to measure robustness among single algal species and natural algal compositions towards UV treatments.
- To develop a novel automatic approach for quantification of living algae to improve efficiency, reliability, and objectivity using a High Content Screening Platform (HCS-Platform) and image analysis tools as an alternative to manual counting.
- To elucidate and compare strengths and limitations of the MPN method, VS methods, and the novel automatic assessment method.

3. Introduction to the manuscripts

Manuscript 1 examined how much UV fluence was needed for the inactivation of natural algal compositions to comply with USCG regulations of <10 *living* org. ml^{-1} in discharged water. The concentrations were assessed using the VS method which is the only USCG approved quantification method. The UV fluences were then compared to those needed to comply with the IMO discharge standard of <10 *viable* org. ml^{-1} that allow assessment using the MPN method. The MPN method is based on a regrowth assay that permits the inflicted DNA damages and other cellular components to manifest and eventually the number of viable cells in the original undiluted sample can be quantified. According to the VS protocol analysis must be carried out immediately (<6 hours at test facilities) after treatment. Consequently, the method does not allow for a complete evaluation of any fatal DNA damages because they take time to manifest and cells will appear alive although dying. The study therefore examined the increased UV fluences needed to outright kill the organisms when applying the VS method as assessment method. It was found that approximately 10 times higher UV fluences were needed for the VS method than for the MPN method to comply with the <10 living org. ml^{-1}. (Figure 13). However, if the VS method was applied in combination with dark-hold periods the required additional UV fluence needed to comply with regulation could be reduced (Figure 13).

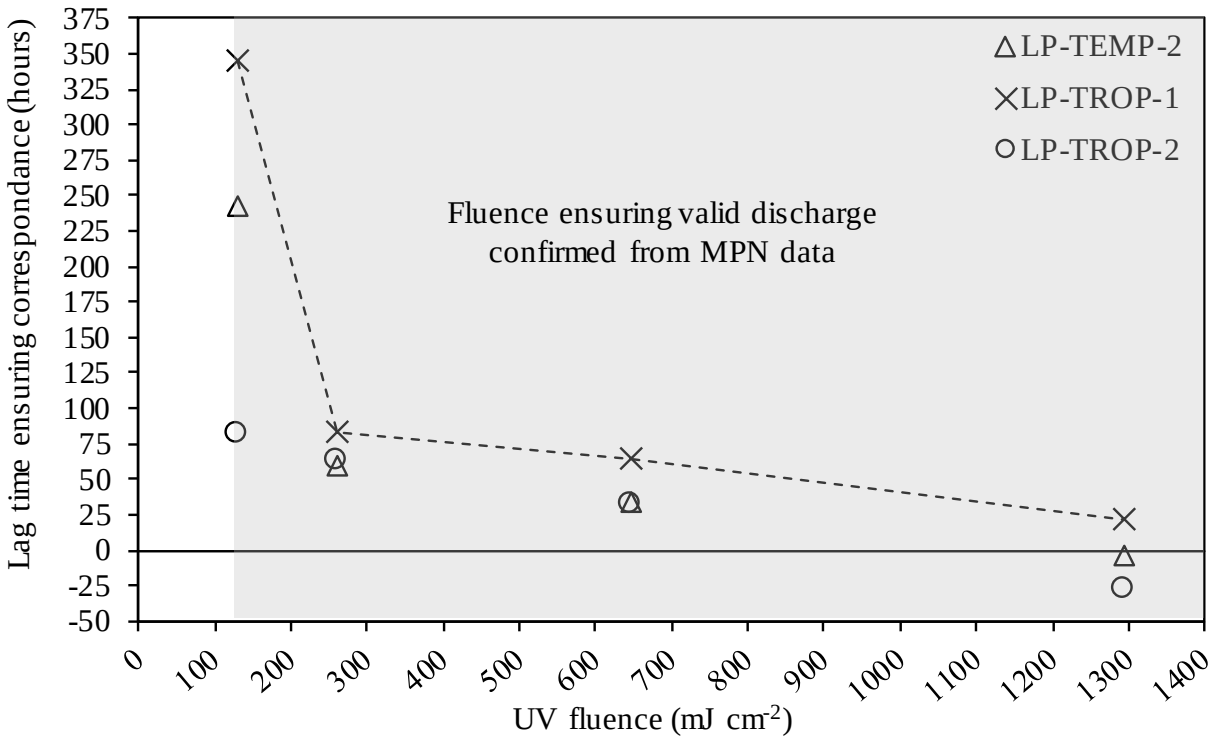


Figure 13. Estimated lag times (Q10 adjusted) to wait to achieve compliance with the discharge standard (<10 org. ml^{-1}) as a function of fluence (treatment) when assessed using the VS method. The grey area illustrates the necessary fluences to achieve compliance based on the MPN method. When using fluences higher than about 130 mJ cm^{-2}, the necessary lag time to achieve compliance can be estimated from the dotted line which has been fitted from the most conservative data points. Points below the zero line indicate that compliance is met without a lag time.

Manuscript 2 evaluated the potential use of standard test organisms (STOs) for more solid validation procedures of UV-based treatment systems (Figure 14). Test facilities use natural algal populations for BWTS validation procedures but often the IMO/USCG intake requirements of 1000 org. ml^{-1} cannot be met. The intake requirement must be fulfilled at each test cycle to make sure BWTSs are adequately challenged during validation procedures. If intake requirements in natural water cannot be met the addition of STOs could assist to fulfil the requirement and prevent delays and failed tests. However, expert panels within IMO have debated whether the quality of testing was weakened because of a possible lower robustness of STOs compared to natural algal compositions. Therefore, this study evaluated the UV fluences needed for concentrations of two species of potential STOs (*Tetraselmis suecica* and *Odontella* sp.) to meet the discharge standard when assessed using the VS and MPN method. The obtained viability potential (robustness) towards UV treatments were compared to UV fluences obtained on natural algal compositions (**Manuscript 1**). The results suggested that these STOs are at least or even more robust towards UV treatments compared to natural algal compositions when assessed with the MPN method. A higher robustness of STOs is likewise indicated when assessed using the VS method. Furthermore, the challenges outlined in **Manuscript 1** for the VS method to assess viability of UV-treated organisms were confirmed. Due to their higher viability potential, the use of these STOs in test water can strengthen the quality of testing and therefore this qualifies them as a potential supplement when intake water concentration requirements cannot be met.

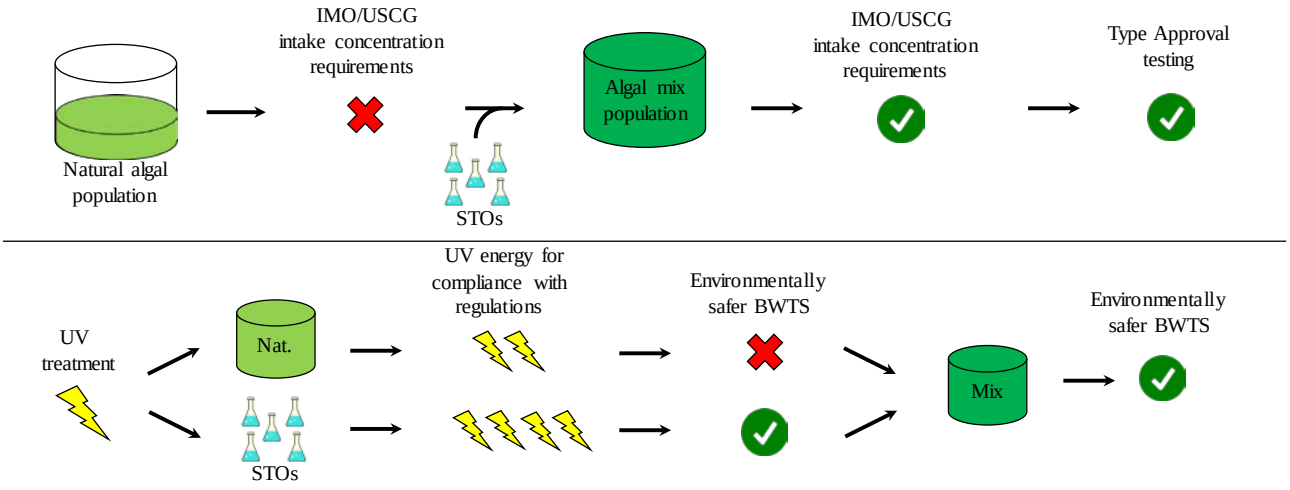


Figure 14. Schematic overview summarizing the potential advantageous use of STOs for BWTS validation.

Manuscript 3 explored the potential of applying an automatic approach for enumerating the number of fluorescent/living phytoplankton organisms during validation procedures. The automatic approach was a combination of a High Content Screening Platform (HCS-Platform) and image analysis software. The quantification of algae was based on the VS method. The standard quantification procedure for the VS method involve labor intensive direct manual microscopy counts of stained organisms. Furthermore, the procedure is challenged by being slow, vulnerable to subjectivity, fatigue and to highly mobile algae moving in the 3D environment of the standard counting chamber. Combined, these issues affect verification, quantification accuracy and overall quality. To engage and minimize these issues an automatic approach in combination with a newly developed filter technique (Figure 15) was tested on two monocultures of algae (*Tetraselmis suecica* and *Odontella* sp.) of different size and mobility behavior and results were compared to manual counts. In addition, the applicability on natural algal samples was tested. The filter approach gently immobilizes highly mobile organism which is shown to significantly improve quantification accuracy. In addition, the experiments show that the application of the HCS-Platform and subsequent image analysis could recognize and quantify algal monoculture cells to a similar level as standard manual counts but at a significantly faster counting rate. In addition, post-counting verification was made possible. Thus, sample throughput and quality can be increased while labor costs decreased.

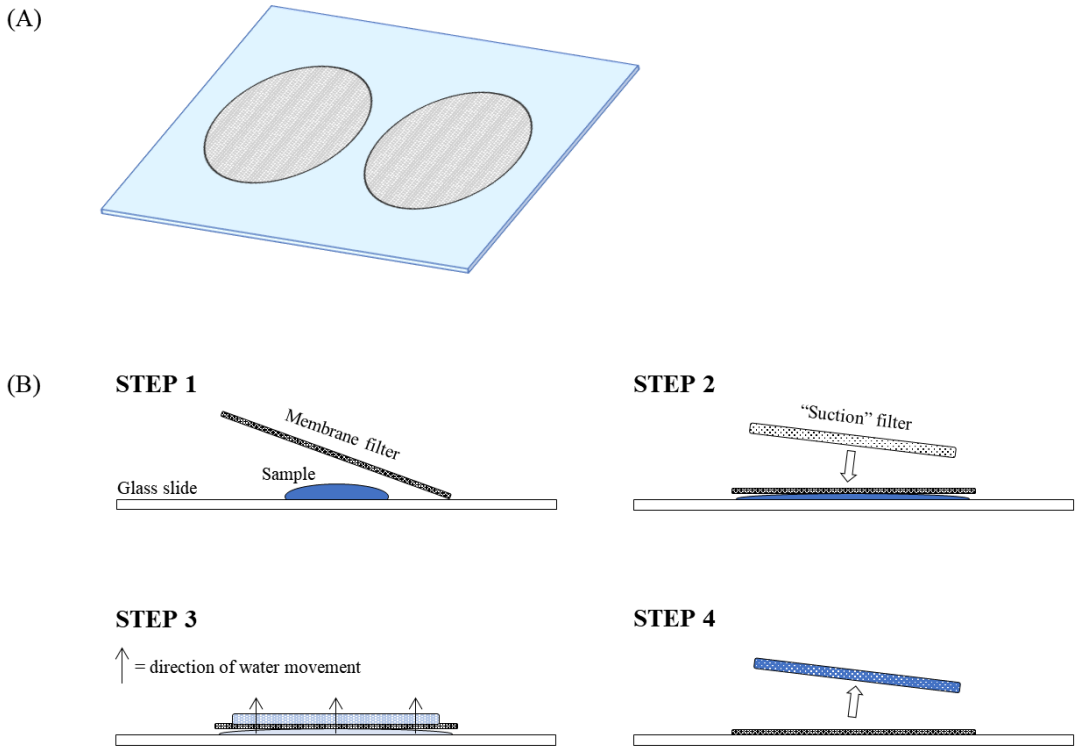


Figure 15. Outline of the different steps of the filter technique. (A) Glass slide with two outlined circles for placement of 500 µl stained sample in each circle. (B) A membrane filter (0.8 µm pore size) was carefully placed on top of each drop of stained sample (Step 1). A "suction" filter was placed on top of the membrane filter to remove water upwards through the membrane filter (Step 2 and Step 3). This caused the membrane filter to move towards the glass slide thereby trapping the stained cells in a 2D plane (Step 4). After the upwards removal of water there was still a thin layer of water left between the glass plate and membrane filter to prevent algal cells from drying out.

Manuscript 4 examined the situation of BWTSs in operation from November 2016 to October 2017 based on reports from Australia and the United States. The aim was to investigate the potential impact of the BWM convention's implementation in September 2017 on the installation and utilization rate of BWTSs. The results showed an increase the percentage of vessels fitted with a BWTS, using the system and the total discharge of treated ballast water coinciding with the entry into force of the convention (Figure 16). The study also included identification of which BWTS types were installed and used on vessels in Australia and US and the results were compared to the number of available treatment types with IMO and USCG type approval. Furthermore, the different criteria used by shipowners in the BWTS decision making process and why some seem preferred than others were presented and evaluated. The use of filtration & UV and electrolytic systems were the most dominating approved systems by the two regulatory authorities and also the preferred system types onboard vessels arriving to both of the studied countries (Figure 16). However, most vessels arriving to the studied countries were still using ballast water exchange to manage their ballast water before discharge. The determination of which BWTSs are installed and utilized is dependent on ongoing policy implementations and supervision management.

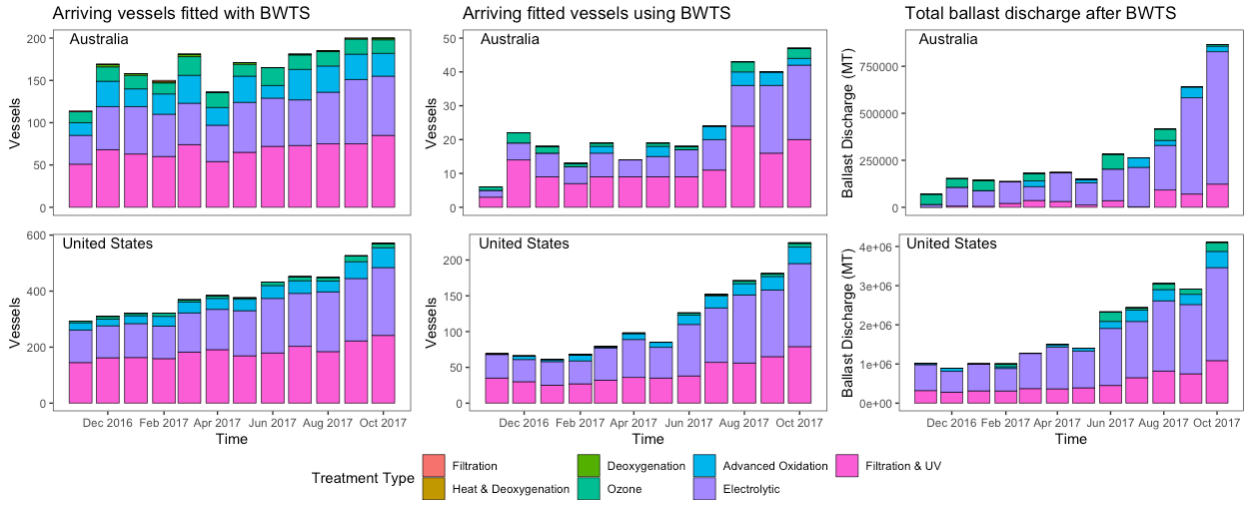
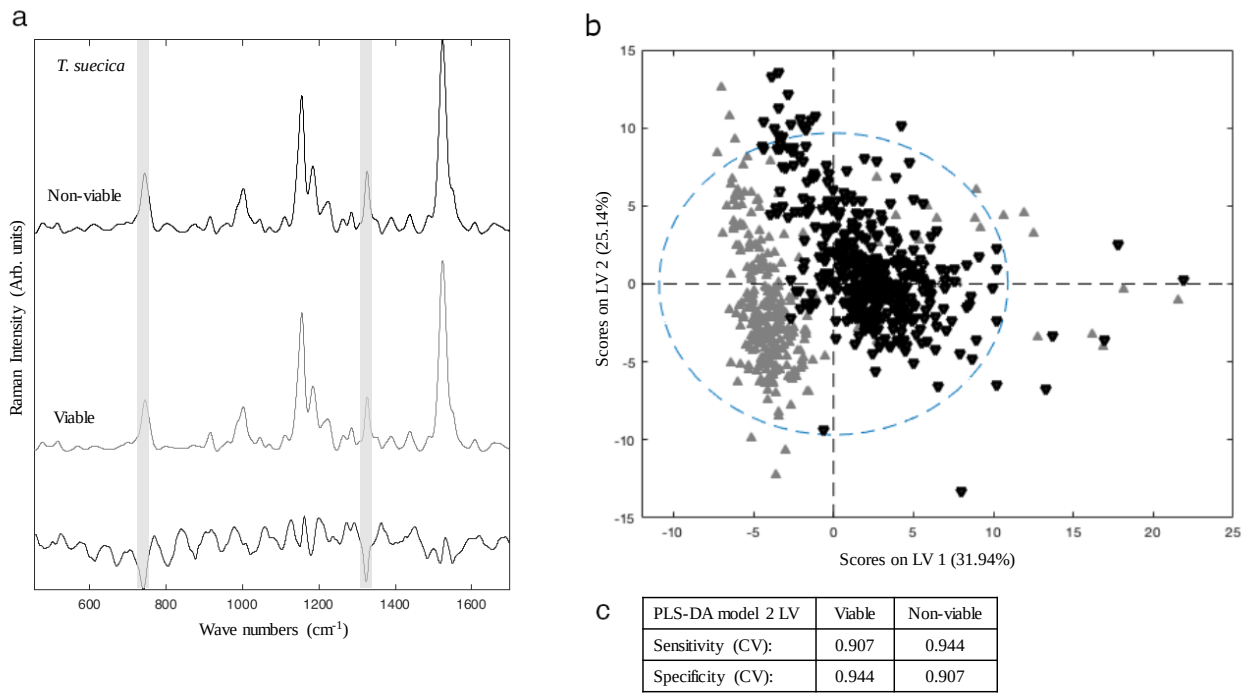


Figure 16. (Left) Unique monthly vessel arrivals fitted with ballast water treatment systems. (Center) Unique monthly vessel arrivals that use ballast water treatment systems on at least one voyage during the month. (Right) Monthly ballast water discharge treated using a ballast water treatment system. These data cover Australia (top) and the United States (bottom) from November 2016 to October 2017.

Manuscript 5 focused on the possible use of Raman spectroscopy as a tool for simultaneous algal species identification and viable/non-viable classification after UV exposure. The study was performed using monocultures of *Rhodomonas salina* and *Tetraselmis suecica*. A 100% discrimination between the two algal species could be obtained from the species specific features of the Raman spectra. The model classified 92 and 90% of the viable algae correctly for *R. salina* and *T. suecica*, respectively, as opposed to 82 and 94% for non-viable algae (Figure 17). The classification model was acceptable but still with a total classification error of 13 and 7% for *R. salina* and *T. suecica*, respectively. The UV exposure induced molecular changes and defense mechanism responses in both algal species which could be identified in their individual Raman spectra. In *R. salina*, the Raman spectrum for non-viable cells showed an upregulation of carotenoids whereas an upregulation of two saccharides were observed in non-viable *T. suecica*. Both responses have been reported to have antioxidant properties against UV induced formation of reactive oxygen species for preventing oxidative stress. In conclusion, Raman spectroscopy is a promising tool for the simultaneous identification of living microplankton species and their viability state which is applicable for validation of BWTS performance and for monitoring harmful algal blooms events.



c

PLS-DA model 2 LV	Viable	Non-viable
Sensitivity (CV):	0.907	0.944
Specificity (CV):	0.944	0.907

Figure 17. (a) Raman spectra of viable and UV exposed non-viable *T. suecica* cells from which the difference spectrum could be obtained from subtraction of the two spectra. The grey bands signify the wave numbers (740 and 1324 cm^{-1}) at which the largest differences between the two spectra were recorded. (b) score plots for LV 1 and LV 2 showing the cell sorting pattern of viable (gray) and non-viable cell (black) using the PLS ToolBox model. (c) Cross validated sensitivity and specificity of PLS-DA model using 2 Latent Variables showing high specificity and sensitivity.

4. Summary of the results obtained

In the present project, practical experience with and insight into the technical specifications and theory behind the two most prevailing organism viability assessment methods for BWTS validation were obtained. The aim was to use the obtained knowledge on the methods' strengths and limitations to explore their further potentials for more accurate viability assessment related to the IMO/USCG regulatory requirements of viable/living organisms in the size class 10-50 µm in treated BW.

The two most prevailing methods to assess algal viability are the VS method used by the USCG, and a combination of the VS and MPN method, used by the IMO (depending on which type of BWTS to be validated). The VS method is suitable for validation of BWTSs using mainly active substances, but several studies have deemed the VS method less suitable to validate UV-based treatment systems because of its limitations to distinguish between viable and non-viable cells (Section 1.5.1). The MPN method accounts for such limitations but since the use of this method was rejected by the USCG for BWTS validation, UV-based treatment systems are placed at a disadvantage for obtaining USCG approval. The decision to reject the MPN method for BWTS validation by the USCG has major implications since close to 50% of all IMO Type Approved BWTS are UV-based.

Two of our studies (Manuscript 1 and Manuscript 2) on natural algal populations and monocultures of *Tetraselmis suecica* and *Odontella* sp. exposed to UV irradiation demonstrated that the VS method was less suitable for viability assessment of UV exposed organisms. The results showed that for natural algae it was possible to achieve compliance with the stated discharge standard of living organisms using the VS method. However, about a 10-fold increase in UV fluence was required compared to that needed using the MPN method to achieve compliance with the USCG regulation of <10 living org. ml^{-1} in the size class 10-50 µm. The additional energy needed to meet the discharge standard could however be reduced by implementing extended dark-hold periods after UV treatments depending of fluence applied. Both studies contribute with new data and knowledge that quantitatively show that if the VS method is used for BWTS validation, UV-based treatment systems are placed at a considerable disadvantage because costly overtreatment is required to meet the discharge standard for living organisms as required by the USCG. The results from the two studies do however demonstrate a solution to reconcile the USCG and IMO regulations. A disadvantage is, however, that the increased fluences needed, result in higher capital expenditures, power consumption, and greenhouse gas emission. The findings also showed the more suitable use of the MPN method for viability assessment of UV-based treatment systems and support motions for the USCG to reconsider the use of the MPN method for validation of UV-based treatment system performance. The MPN method is suitable for viability assessment but it takes weeks to obtain results. Therefore, there is a need for faster and more simple methods for indicative analysis of treated BW by for example Port State Control. Part of the studies in the present thesis, investigated Raman spectroscopy as such an assessment technique. Promising results presented in Manuscript 5 showed the integration of Raman spectroscopy possessed the potential to accurately classify the number of viable and non-viable organisms in treated BW as well as for species identification. The technique should be further explored for the development of a faster, more reliable and simpler method for indicative analysis.

The results from Manuscript 1 and Manuscript 2 show that small differences in policy-making and regulatory requirements can have relatively big impacts on the associated biological and environmental issues that the regulations address. The important role of policy and regulations following the entry into force of the BWM Convention was shown in Manuscript 4 where a coinciding increase in the installation and usage of BWTSs was observed together with an increase in the total discharge of treated BW in the USA and Australia. Future policy decisions on BW management and BWTS related to the full implementation of the BWM Convention will likely have impact on the type of BWTSs installed and will probably also affect the shaping and use of current and new viability assessment methods (Manuscript 4).

The UV fluence-response relationships on monocultures of *Tetraselmis suecica* and *Odontella* sp. (Manuscript 2) showed that laboratory cultivated algal monocultures were at least, or even more, robust towards UV treatment than natural algal when evaluated using the MPN method. When evaluation was performed using the VS method, LP UV system did not perform within the fluence range applied in the study. Medium Pressure UV systems were required to increase fluences significantly to be able to comply with discharge standard regulations as also addressed in Manuscript 1. The findings suggest that the two species are good candidates as STOs in relation to BWTS validation to ensure more robust testing conditions and that concentration requirements for intake water more often can be met. The influence of STOs on test water quality has been widely debated and the results of the study therefore provide relevant and timely contributions within the scientific field of BW regarding Type Approval testing and policy development.

A novel automated approach based on the VS method for improvement of quantification accuracy and efficiency was developed and tested (Manuscript 3). The VS method has its limitations as addressed in Manuscript 1 and Manuscript 2, but the method is still generally considered one of the most robust staining methods for BWTS validation. Several publications have addressed issues present in current quantification approaches. Although numerous commercial BWTSs exist, no appropriate quick and robust quantification approach to assess their efficacy have yet been presented. The results from Manuscript 3 demonstrated that a developed filter technique in combination with a HCS-Platform and image analysis produced faster and more accurate counts of algal monocultures compared to standard manual counts. Although the automated approach was challenged in quantification accuracy of natural algae, the automated approach provides promising results that contribute to address challenges such as fatigue, counting time, and subjectivity regarding size class and live/dead classification. Especially, the implementation of the filter technique showed to be very efficient in immobilizing highly mobile algal species such as *Tetraselmis suecica*. The 2D counting conditions provided by the filter technique significantly contribute to increased quantification accuracy compared to standard 3D volume counts.

One issue that has been consistent throughout the manuscripts is how to deal with organisms <10 µm. The regulatory decided size class classification of 10-50 µm for the smallest sized planktonic organisms has been an experimental and technical challenge but also the biological reasoning behind the decision has been challenging to understand and manage. Several studies have addressed the issue about the large invasive potential of <10 µm planktonic organism, but little attention to solve the issue has been given. The presence of autofluorescent debris of sizes close to the minimum size requirement

of 10 µm in natural algal samples considerably interfered with accuracy of manual counts of natural algal samples in both Manuscript 1 and Manuscript 3. Typically, autofluorescent debris is just below 10 µm. By applying an automated approach that is efficient in size class classification due to preprogrammed cell recognition criteria, a higher quantification accuracy within the size class 10-50 µm is possible (Manuscript 3). However, that the automated approach contributes to attain a higher quantification accuracy does not solve the potential biological implication of these undocumented organisms. Conservative manual counts of water samples would thus be more environmental protective, although less accurate according to regulations, because counts will include a larger proportion of organisms just <10 µm. The manual counts would also potentially include a large proportion of autofluorescent debris which on the other hand could result in unfairly disqualifying BWTS under validation. Currently, the MPN method is the only suitable and IMO approved method that incorporates organisms <10 µm. The MPN method does not discriminate size and is unaffected by autofluorescent debris as this is taken into consideration in control samples. Consequently, the growth potential of all organisms is assessed regardless of size.

The experience obtained from the experimental studies in this thesis provided insight into the strength and limitations of the different methods applied. A list of relevant factors involved in quantification methods are presented and compared in Table 4. The automated approach has several advantages over MPN and the manual VS method, but there are still some crucial challenges that need attention. Differentiation between viable and non-viable organisms is not possible with the stains (FDA/CMFDA) used in the VS method which is the staining procedure currently integrated in the automated approach. The development of alternative stains with more reliable cell staining properties, and which can identify only viable cells, would improve the automated approach considerably. If such stains were applied in combination with more sophisticated image analysis procedures for more accurate quantification of natural algal samples the automated approach in combination with the filter technique would become a powerful alternative to existing quantification approaches.

Table 4. Summary table comparing strengths and limitations of two of the prevailing methods (VS and MPM) used in BWTS validation and the novel developed automated approach in combination with the filter technique. A "+" signifies that the method is well suited for dealing with the faced issue and a "–" signifies that the method is challenged.

Issue	MPN	Vital stain (manual)	Automated approach
Accuracy (immobile organisms)	+	+	+
Accuracy (mobile organisms)	+	–	+
Viable/non-viable distinction	+	–	–
Efficient staining	+*	–	–
Counting rate/analysis time	–	–	+
Labor friendly	–	–	+
Fatigue prevention	–	–	+
Objectivity	+	–	+
10-50 µm size class identification	–	–	+
Identification of heterotrophs	–	+	+
Considering plankton <10 µm	+	–	–
Documentation/data storage	–	–	+

*not applicable/relevant

5. Perspectives and future thoughts

The studies of this thesis have taken the development and improvement of methods for viability assessment of planktonic organisms in BWTS validation a few steps further. A universal enumeration method for viability assessment that could work in all types of water and take into account all the different biological diversities, physiologies/morphologies, and size distributions among plankton species, classes and communities is needed. The implementation of such a method would be ideal but is obviously a major challenge.

One of the largest concerns is the presentation of a solution that offers fast and accurate assessment of organism viability in treated BW regardless of treatment type. The MPN method is currently the most accurate method for viability assessment and recently (4 Dec. 2018) the U.S. Senate passed the Vessel Incidental Discharge Act (VIDA) Bill that will allow the USCG to use reproductive methods (such as the MPN method) for testing and approval of BWTSs. This is good news for manufacturers of UV-based treatment systems and at the same time the bill harmonizes USCG and IMO regulatory requirements. However, the MPN method is considered too slow by shipowners and Port State Control that demand faster assessment methods to deliver results within hours. Based on arguments from the currently available literature on quantification approaches the attitude is that at present trained microscopists are the only option for accurate detailed BW analysis that follow regulatory requirements. As addressed in this thesis, there are several challenging issues involved with manual counting approaches and the continuous focus on the development of automated approaches is therefore highly relevant. Especially, regarding image analysis procedures and machine learning implementation for perfecting quantification accuracy on natural plankton samples of high composition complexity is important. The use of complex natural plankton samples is essential to improve techniques and is one of the most important components to include in validation of techniques. However, it is not surprising that current viability quantification approaches produce ambiguous results for different types of organisms when considering the high biodiversity that exists in the phytoplankton community. Therefore, the promising first steps of the automated HCS method as well as Raman spectroscopy as improved viability assessment methods should be further investigated in future studies.

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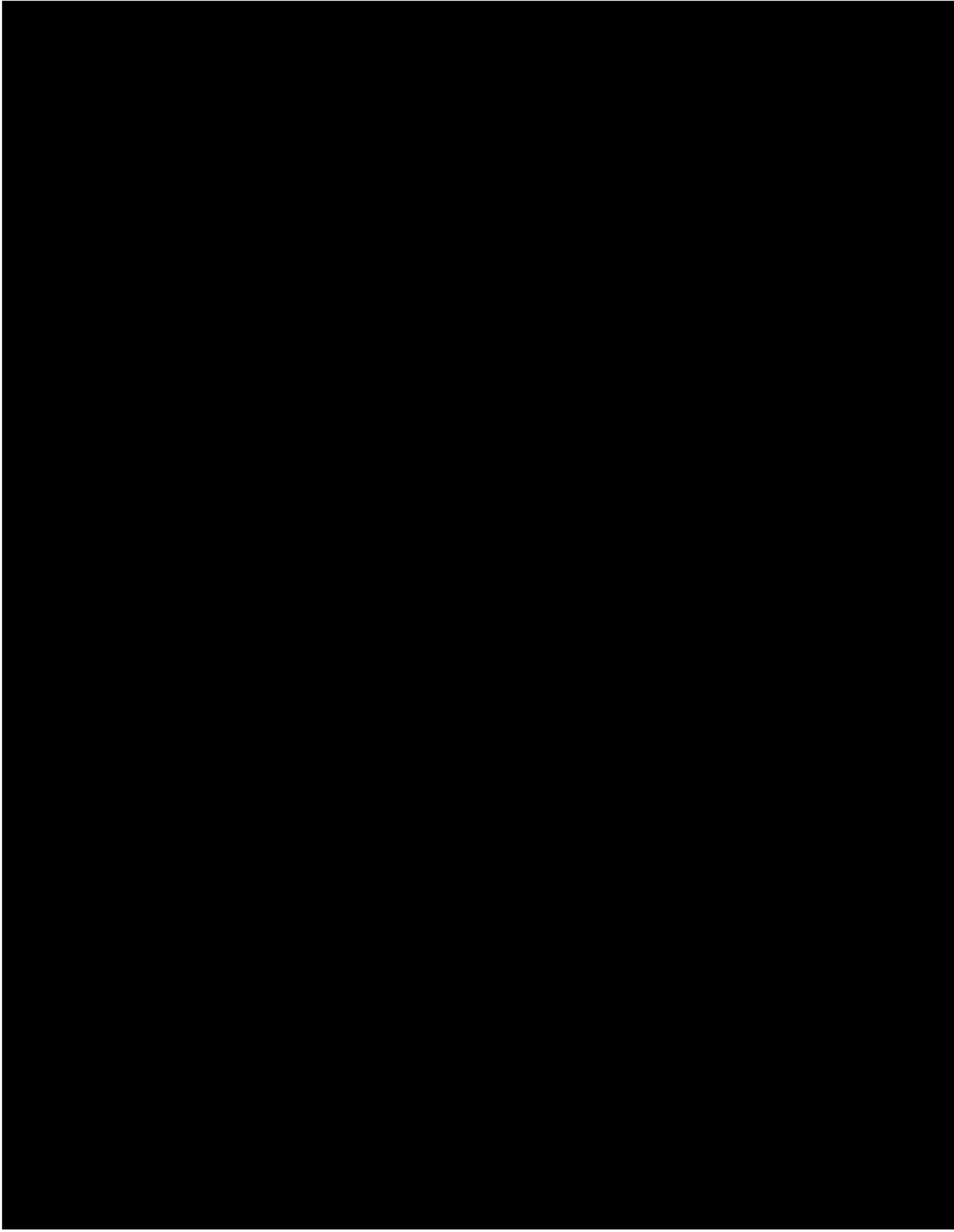
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7. Manuscripts



Manuscript 1

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

Kim Lundgreen, Henrik Holbech, Knud Ladegaard Pedersen, Gitte Ingelise Petersen, Rune Røjgaard Andreasen, Christaline George, Guillaume Drillet, Martin Andersen

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

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ABSTRACT

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

1. Introduction

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

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

2. Materials & methods

2.1. Test organisms and preparation for experiments

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

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

2.2. Quantification of algal cells

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

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

2.3. Experimental design and exposure procedures

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

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

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

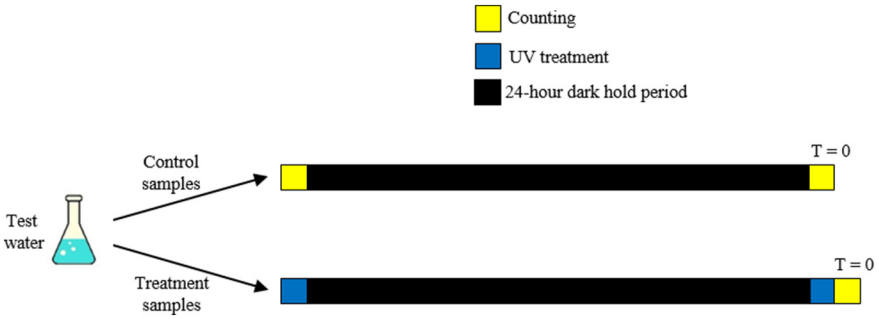


Fig. 1. Experimental timeline: A sample was collected from the test water and either 1) analysed without any UV fluence (control samples), stored in dark conditions for 24 h at ambient temperature and re-analysed or 2) exposed to LP or MP UV treatments, stored in dark conditions for 24 h at ambient temperature, second UV treatment and analysed. $T = 0$ refers to time, T (hours), since last exposure.

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 ballast water tank holding time. After 24 h, 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 (Fig. 1).

Control samples were analysed for the number of fluorescent (VS method) and viable organisms (MPN method) both before and after the 24-h dark-hold period (Fig. 1).

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:

$$F_{avg,LP} = I_{avg} \times t \quad (12a)$$

$$F_{avg,MP} = I_{avg} \times t \quad (12b)$$

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-TEMP-1 as example this resulted in an average germicidal intensity of:

$$I_{avg.} = \frac{\Delta C \times V}{\varphi \times \Delta t \times f_{MP} \times A} \times \Sigma_{300}^{200} P_{f,\lambda} E_{E,\lambda} \times WF \rightarrow$$

$$I_{avg.} = \frac{9.56 \times 10^{-6} \text{ mole l}^{-1} \times 0.05 \text{ l}}{0.0176 \text{ mole Einstein}^{-1} \times 3600 \text{ sec} \times 0.5861 \times 0.0022 \text{ m}^2} \times 459336 \text{ J Einstein}^{-1} \times 0.725 = 1.94 \text{ W m}^2$$

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
Definitions UV intensities and fluences used throughout the paper.

Term	Description
Incident germicidal intensity, I_i (W m^{-2})	"*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.
Average germicidal intensity, I_{avg} (W m^{-2})	"*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).
Average germicidal fluence, F_{avg} (mJ cm^{-2})	Average germicidal intensity × exposure time

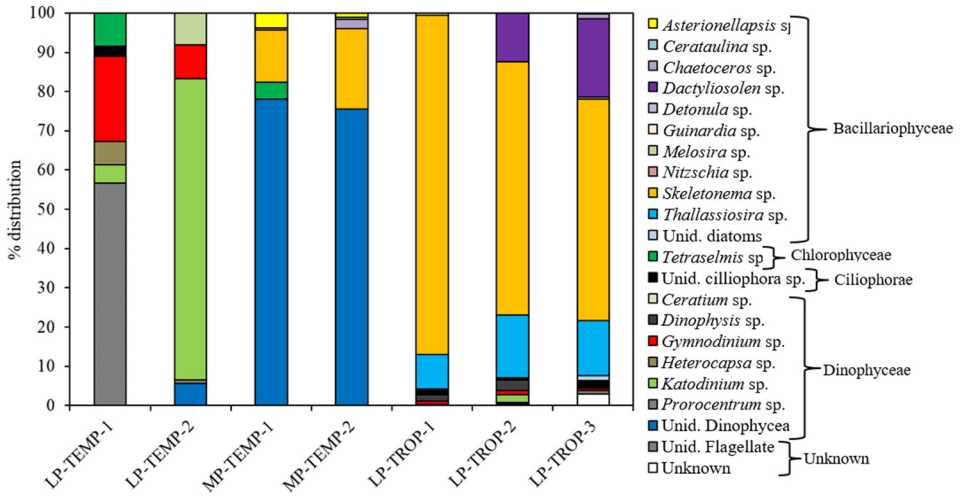


Fig. 2. Class and species composition of test water used in fluence-response UV treatment experiments in Denmark and Singapore. For each of the experiments, water was collected independently and composition was determined from preserved Lugol samples. (Unid. = unidentified).

Table 3
Calculation procedure for average germicidal fluence ($F_{avg.}$, mJ cm^{-2}) using experiment MP-TEMP-1 as example.

Target fluence (mJ cm^{-2})	$I_{avg.}$ (W m^{-2})	s (sec)	F_{avg} (Ws m^{-2})	F_{avg} (mJ cm^{-2})
0	1.94	0	0	0
25	1.94	128	248	25
50	1.94	254	493	49
100	1.94	509	987	99
200	1.94	1020	1979	198
500	1.94	2550	4947	495
1000	1.94	5096	9886	989

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

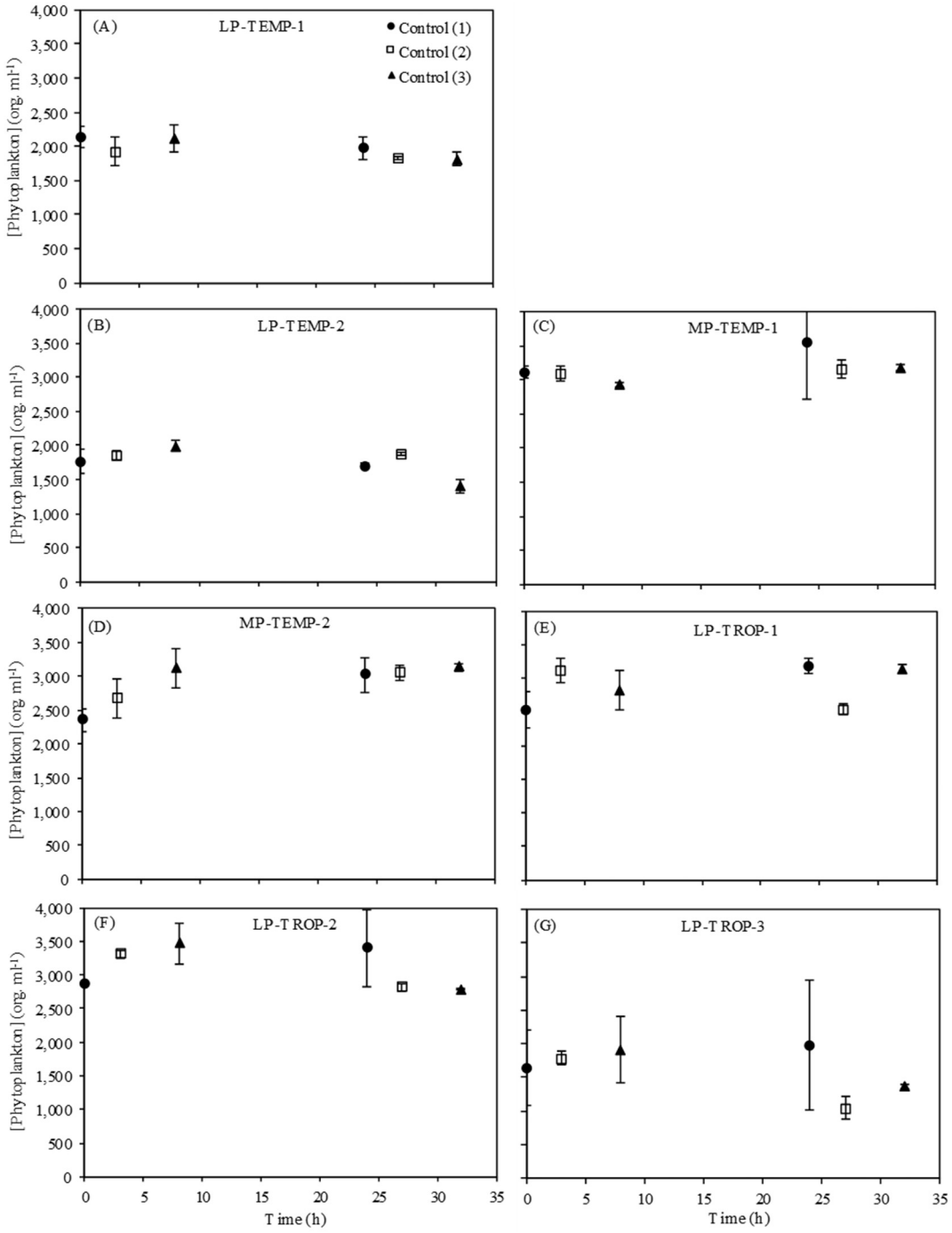


Fig. 3. Test water phytoplankton concentrations (mean ± SD, $n = 2$) used for (A) LP-TEMP-1, (B) LP-TEMP-2, (C) MP-TEMP-1, (D) MP-TEMP-2, (E) LP-TROP-1, (F) LP-TROP-2, and (G) LP-TROP-3. Phytoplankton concentrations was determined using the VS method before and after a 24-h dark-hold period in three independent control (no treatment) samples: Control (1), Control (2) and Control (3). The three pre-dark-hold samples were collected at three time points (0, 3, and 8 h) and each sample was re-counted after 24-h dark-hold.

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 ($N = 10$ org. ml^{-1}) for each fluence could be estimated by re-arranging the equation:

$$t = \ln(N/N_0) \times 1/-z$$

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

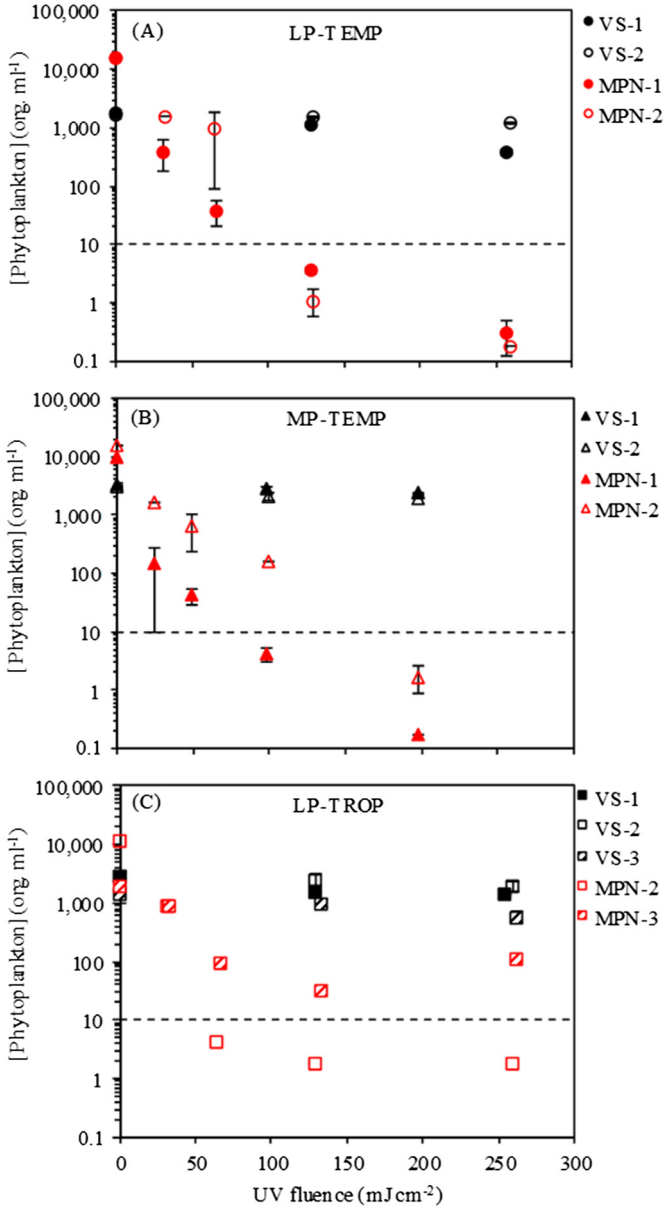


Fig. 4. Fluence-response relationships showing number of fluorescent (VS method) and viable (MPN method) organisms (log_{10} scale) per ml in samples exposed to different total UV fluences. (A) LP-TEMP-1 and LP-TEMP-2, (B) MP-TEMP-1 and MP-TEMP-2, and (C) LP-TROP-1, LP-TROP-2 and LP-TROP-3. In LP-TROP the MPN-1 analysis failed and no data was obtained. Data points are mean ± SD, $n = 2$ except MPN data from LP-TROP where $n = 1$.

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 dominating in LP-TEMP-1 samples exposed to about 640 and 1290 mJ cm^{-2} in VS counts, although they were not quantified. In LP-TEMP-2 *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).

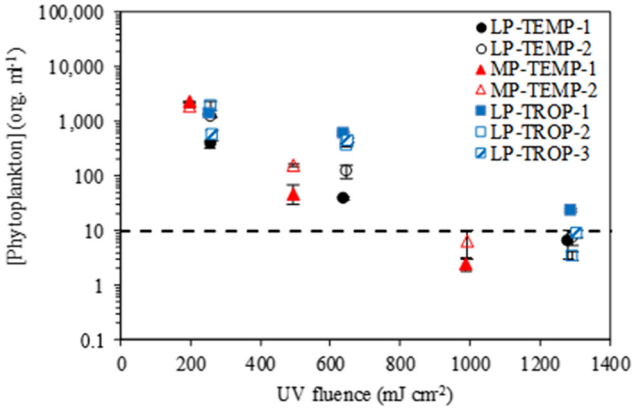


Fig. 5. Fluence-response relationship experiments showing number (mean ± SD, n = 2) of fluorescent organisms (log_{10} scale) per ml exposed to different UV fluences. Experiments were performed on temperate phytoplankton compositions exposed to either LP (LP-TEMP) or MP (MP-TEMP) UV treatments and tropical phytoplankton compositions exposed to LP UV treatments (LP-TROP). All samples were assessed using the VS method.

Table 4
Observed general behaviour of fluorescent algal cells from VS counts present in control samples (no treatment) and in different UV treated samples.

Fluence (mJ cm^{-2})	Experiment	Observed behaviour
0	LP-TEMP	High swimming activity, most on bottom, some in upper part
0	MP-TEMP	90% of cells mobile, present in whole water column
0	LP-TROP	High mobility, of mobile cells: 50% on bottom, 50% in the whole column
130	LP-TEMP	Slower movements, rotating in small circles, almost all cells on bottom
100	MP-TEMP	Little mobility (small bursts of slow rotation), all on bottom
130	LP-TROP	No mobility, all on bottom except very few
260	LP-TEMP	Very slow when moving, rotating around themselves, all on bottom
200	MP-TEMP	No mobility, all on bottom
260	LP-TROP	No mobility, all on bottom except very few
640	LP-TEMP	No mobility, all on bottom
500	MP-TEMP	No mobility, all on bottom
640	LP-TROP	No mobility, cell membrane seems damaged in some of the few left
1290	LP-TEMP	No mobility, all on bottom
1000	MP-TEMP	No mobility, all on bottom
1290	LP-TROP	No mobility, all on bottom

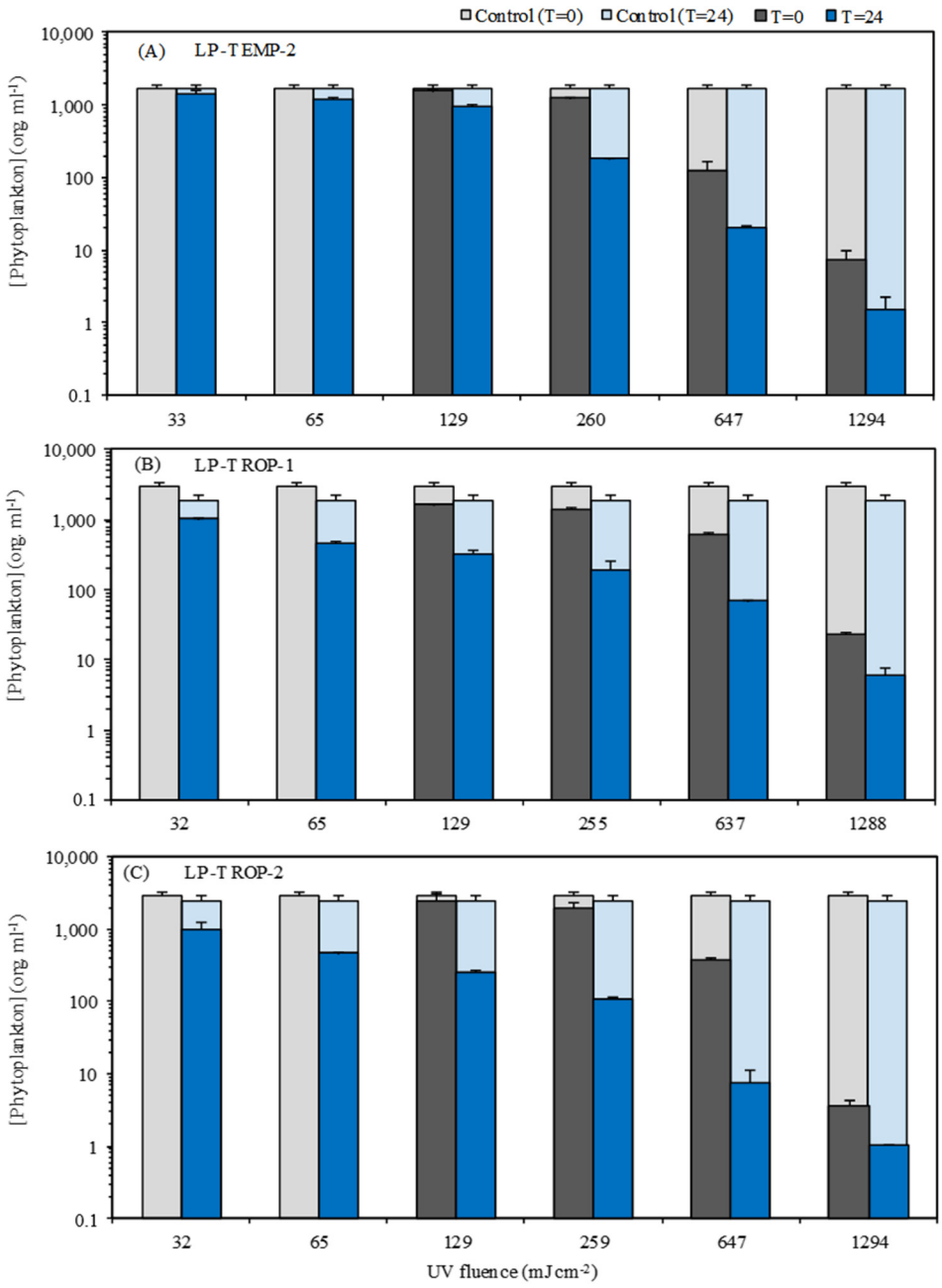


Fig. 6. Fluence-response experiments showing VS counts (mean ± SD, $n = 2$) immediately after last exposure (T = 0) in relation to VS counts after a 24-h extended dark-hold period (T = 24) on natural phytoplankton compositions from (A) LP-TEMP-2, (B) LP-TROP-1 and (C) LP-TROP-2. For reference, all Control (T = 0) and Control (T = 24) counts are included in the figures in the "background". For T = 0 no counts were performed at the two lowest fluences.

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

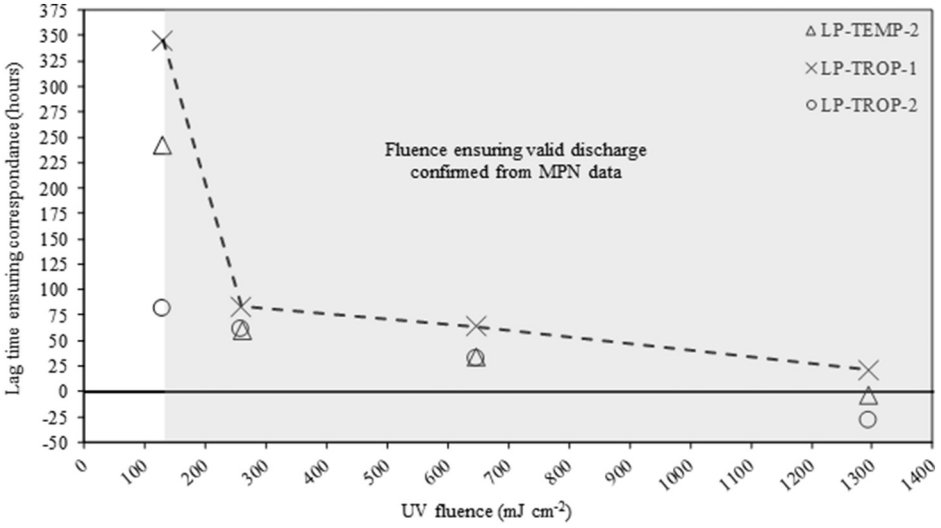


Fig. 7. Estimated lag times (Q_{10} adjusted) to wait for concentrations to meet discharge standards (< 10 org. ml^{-1}) as a function of fluence (treatment) when assessed using the VS method. The grey area illustrates the necessary fluences for concentrations to meet discharge standards based on the MPN method. When using fluences higher than about 130 mJ cm^{-2} (within the grey area), the necessary lag time for concentrations to meet discharge standards can be estimated from the dotted line which has been fitted from the most conservative data points. Points below the zero line indicate that discharge standards are met without a lag time.

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 integrity (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 cm^{-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 cm^{-2} a large proportion of the phytoplankton cells were already dying.

The inability of the VS method to correctly detect effects of different kinds of treatments (UV, heat) has been indicated in several previous studies (Tobiesen et al., 2011; MacIntyre and Cullen, 2016). The reason probably lies in the way cell viability is estimated. As long as a dying plankton organism is capable of performing esterase reactions on CMFDA/FDA and of retaining membrane integrity it will be counted as viable in the VS method. This has created an acute problem for ship owners with UV based BWMS installed since the USCG in 2015 stipulated that only the VS method can be used for type approval (Professionals, 2016). Including non-viable organisms in ballast water viability assessments makes compliance with discharge standards more conservative and stringent. On the other hand, the inclusion could unfairly fail systems that meet discharge standards as the organisms are non-viable and “ecologically as good as dead” (Cullen and MacIntyre, 2016; MacIntyre and Cullen, 2016). There is an increased risk of false positives and false negatives when using the VS method. It has been shown that certain dead cells can express fluorescence intensity 1–2 orders of magnitude higher than that of living cells (false positives), and moreover, certain stained living cells fail to fluoresce (false negatives) (MacIntyre and Cullen, 2016). A high proportion of false positives will for example lead to an overestimation of concentration of living cells and exaggerate the difference between the VS and MPN method and vice versa if a high proportion of false negatives are present. Despite of the mentioned limitations, the present study did not compensate for the risks of including false positives or negatives. The reasoning behind this was that a potential over- or underestimation using the VS method would likewise be an issue that is not compensated for when used during land-based or shipboard tests. We wanted to obtain quantifications as close to “real life” conditions as possible and therefore followed the standard procedures described in IMO (2016a, 2016b).

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 (aponecrosis-like) following similar UV induced damages observed as the final response in the unicellular 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 fulfilled 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

fluences 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 was 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; Liltved 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 against 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 *Skeletonema 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, *Skeletonema* sp. consisted of 13–20% of the total observed number of cells. When recounted 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.marpolbul.2018.08.043.

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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

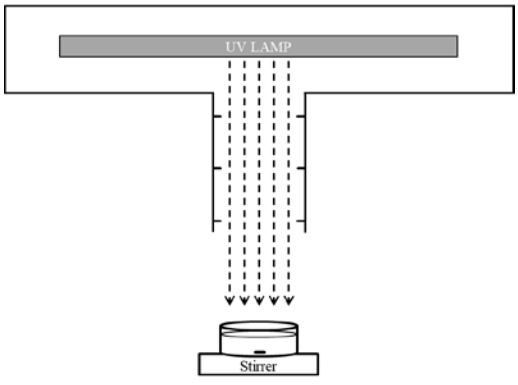


Figure SI.1. Outline of low pressure (LP) and medium pressure (MP) UV collimated beam systems. The emitted UV light passed through a collimating tube resulting in an even vertical UV radiation reaching the petri dish containing the test sample. The petri dish contained a small magnetic bar and was placed 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 (ε) spectrum based on least square method for each wavelength (1 nm). The obtained spectrum was used to estimate uridine concentrations (ε 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.

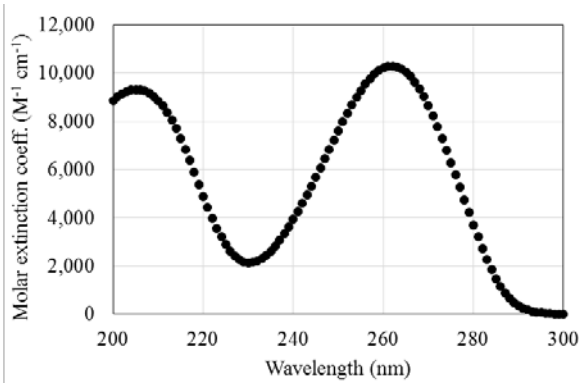


Figure SI.2. Uridine extinction coefficient.

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.

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

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 λ 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.

$$E_{E,\lambda} = N_A \times h \times c / \lambda \qquad \text{(1a)}$$

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

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 λ is the wavelength (nm).

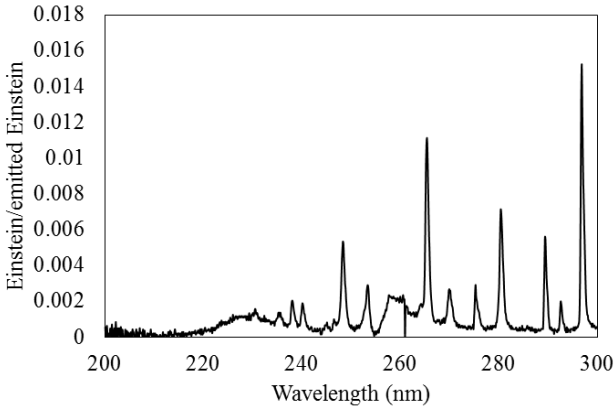


Figure SI.3. Relative photon distribution for MP lamp.

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 ≈ 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):

$$\varphi = -0.1362 \times I_i^2 + 0.1238 \times I_i - 0.006 \qquad \text{(Eq. 2)}$$

Where φ = 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_λ) through a given sample depth (l) at a given wavelength (λ) can be estimated by combining the "–log" relationship between absorbance and transmittance with Beer's Law (Eq. 4).

$$f_\lambda = 1 - T_\lambda = 1 - 10^{\varepsilon_\lambda \times C \times l} \quad \text{(Eq. 4)}$$

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 ε_{254} = 8690.3 M^{-1} 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}-f_{300} as given by Eq. 5.

$$f_{MP} = \frac{\sum_{200}^{300}(f_\lambda I_{r,\lambda})}{\sum_{200}^{300} I_{r,\lambda}} \quad \text{(Eq. 5)}$$

Where I_λ 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^{λ}, Einstein s^{-1} m^{-2}) through the sample.

$$J_P^{254} = \Delta C \times V / (\varphi \, \Delta t \, f_{253.7} \, A) \qquad \text{(Eq. 6a)}$$

$$J_P^{MP} = \Delta C \times V / (\varphi \, \Delta t \, f_{MP} \, A) \qquad \text{(Eq. 6b)}$$

Where V is the sample volume (litres), ΔC is the uridine concentration decline (M), Δt 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 φ 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)

$$I_i = J_P^{253.7} \times E_{E,253.7} \qquad \text{(Eq. 8a)}$$

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).

$$I_i = \sum_{200}^{300} J_P^{MP} \cdot P_{f,\lambda} \cdot E_{E,\lambda} \qquad \text{(Eq. 8b)}$$

As I_i was lower than 10 W m^{-2}, φ was less than 0.022 moles $Einstein^{-1}$ (see 0). The correct φ was therefore calculated iterative by repeating Eq. 2, 6 and 8 as described below. To ease understanding, the firstly applied φ is added subscript 1 (φ_1). The initially $\varphi_{,}$ φ_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, φ_2, was then calculated as the average value of φ_1 and $\varphi_1{}^*$ as shown in Eq. 9

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

The found φ_2 was used in Eq. 6 and 8 to calculate a new I_i, (I_{i-2}) This iteration process was repeated until the φ error between φ and φ* 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)

$$WF_{f,\lambda} = \frac{1-10^{-a_\lambda l}}{a_\lambda l \ln(10)} \qquad (10)$$

Where a_λ 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:

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

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.

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

Where $WF_{f,\lambda}$ was calculated using Eq. 10 with a_λ 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).

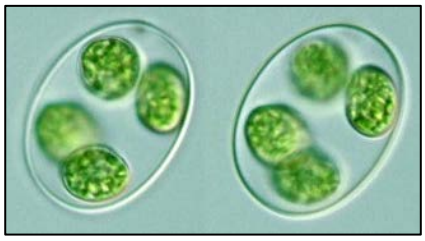


Figure SI.4. *Oocystis* sp. belongs to the green algae class Trebouxiophyceae. Typically, between 2-8 cells are surrounded by a mucilaginous envelope or expanded mother cell wall. Predominantly *Oocystis* are found in freshwater, but they are also present in marine environments (Photo: http://protist.i.hosei.ac.jp/PDB/Images/Chlorophyta/Oocystis/sp_6c.html).

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Manuscript 2

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

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

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Abstract

The International Maritime Organization (IMO) ballast water convention requires ships carrying ballast water to have a type approved ballast water treatment system (BWTS) installed to kill or render harmless potential invasive organisms. To challenge BWTS efficacy for organisms in the size-class 10-50 µm (mainly phytoplankton), the intake concentration during tests must reach certain minimum requirement. Often, natural phytoplankton concentrations at testing facilities are too low to meet the intake requirement and standard test organisms (STOs) are added. It has been debated whether the quality of testing is weakened due to lower robustness of STOs towards UV treatments compared to natural algae. The hypothesis was tested by exposing two algal species, *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 the two current approved viability assessment methods: vital stain (VS) and Most Probable Number (MPN). Results showed, that to meet the discharge standard, fluences of >1000 mJ cm^{-2} was required when using VS whereas 135-500 mJ cm^{-2} was needed using MPN. Besides, the results suggest that *T. suecica* and *Odontella* sp. were more or 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, maintain stable concentrations in control discharge and lastly to create more conservative validation of UV-based BWTS performance to ensure more environmental protective procedures.

Keywords: Ultraviolet irradiation, Ballast Water, Most Probable Number, Vital Stain, Standard Test Organisms, Phytoplankton

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 & 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 2015, Lee et al. 2017, Holbech & 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).

Under the IMO, BWTS efficacy for the size class ≥10-<50 µm (hereafter 10-50 µm), 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 posing no invasion risk (IMO 2016b). The USCG base 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 & Welschmeyer 2015, Cullen & 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.docs.imo.org 2018, Gerhard et al. *In prep.*) 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 III 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[1] (Table 1). To be valid, a land-based test must be initiated with more than 1,000 org. ml^{-1} in the 10-50 µm size class and more than 100,000 org. 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).

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). For both size groups 5 species from 3 phyla must be present in *intake* water. For shipboard testing control discharge is not required and therefore not applicable (N.A.)

	Land-based requirements		Shipboard requirements	
Size class (µm)	≥10-<50	≥50	≥10-<50	≥50
Units	org. ml^{-1}	org. m^{-3}	org. ml^{-1}	org. m^{-3}
[Intake]	≥1,000	≥100,000	≥100	≥100
[Control discharge]	≥100	≥100	N.A.	N.A.
[Treated discharge] (D-2)	<10	<10	<10	<10

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 STO 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

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

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, b).

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. 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 prasinophytes. 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 fluence-response relationships of the two species were finally related to studies on natural algal compositions exposed to UV treatments.

Materials and Methods

Test organisms and preparation for experiments

Low- or medium pressure UV treatments were performed on *Tetraselmis suecica* (SCCAP, K-0297) and *Odontella* sp. monocultures. *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. Monocultures of *Odontella* sp. were grown as semi-continuous cultures in F/2 Culture Medium at 20 psu (Guillard & Ryther 1962, Guillard 1975) in a temperature-controlled room at 25°C and 24-hour light conditions. The final test water solutions for both algal species were prepared 16-20 hours 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 Transmittance (UVT, 1 cm path length) of about 80%. In LP-ODO run 2 the UVT was unadjusted due to a broken down fluorometer on day of experiments.

Table 2. Experimental conditions for each of the eight performed experiments. LP = Low Pressure UV, MP = Medium Pressure UV, TET = *Tetraselmis suecica*, ODO = *Odontella* sp., number of experimental runs, standard test organism (STO) species, climate of experimental location, test water (TW) algal concentration based on the VS method, temperature, dark-hold temperature, salinity and UV transmittance (UVT).

Experiment ID	Run	STO	Climate	$[Algal]_{TW}$ (org. ml^{-1})	T_{TW} (°C)	$T_{dark-hold}$ (°C)	Salinity (psu)	UVT (%)
LP-TET	1	*T. suecica*	Temperate	2622±85	21.4	21.4±0.2	20	80
	2			3095±356	21.4	21.4±0.2	27	80
LP-ODO	1	*Odontella* sp.	Tropical	3536±137	23.6	26.5±0.7	20	81
	2			3976±219	24.3	24.6±1.4	20	96
MP-TET	1	*T. suecica*	Temperate	2735±88	19.0	18.1±0.1	18	80
	2			4171±333	18.9	18.1±0.1	19	81
	3			4000±141	17.2	17.0±0.2	20	80
	4			4588±168	18.0	17.5±0.1	20	80

Quantification of algal cells

The vital stain (VS) method was used to determine the total number of live organisms within the 10-50 µm size class and followed the method described by Lundgreen et al. (2018). The method uses a combination of two fluorescein based vital stains: Fluorescein diacetate (FDA, Molecular Probes-Invitrogen Carlsbad, CA, USA) and 5-chloromethylfluorescein 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-specific esterase activity within living cells and converted into a green fluorescent cell-impermeant molecule that can be quantified from direct microscope counting by using a Sedgewick Rafter Counting Chamber (Steinberg et al. 2011, IMO 2016d). During VS counts the behaviour of the algal cells at the different UV treatments was also recorded with focus on mobility changes and location of cells in the water column of the counting chamber. Two samples of 200 µl test water was counted for all control (no treatment) samples. A more "confined" drop of 200 µl of highly mobile *Tetraselmis suecica* resulted in significantly more accurate counts compared to 1 ml. If mobility was high and/or concentration was still >50% initial concentration after treatment two samples of 200 µl test water were counted. If mobility was low and/or concentration had decreased below about 50% two samples of 1 ml was counted.

The Most Probable Number (MPN) method was used to determine the regrowth potential of *T. suecica* and *Odontella sp*. in control samples and after different UV treatments. For control samples, three dilution series were prepared consisting of three 10-fold dilutions each consisting of five replicates. For treated samples two dilution series were prepared. For each tube the initial fluorescence was determined using a fluorometer (Turner TD-700 Laboratory Fluorometer). Next, samples were incubated in Keller's Growth Medium (Keller et al. 1987) added to filtrated local seawater for 14 days in temperature controlled room on a rotary shaker (Heidolph UNIMAX 2010, 100 rpm) under continuous white light conditions (6,000-10,000 lux) and at 19/25°C

(temperate/tropical conditions). After incubation the fluorescence was determined for each tube and by following the procedures described in IMO (2016d) the number of viable organisms in the undiluted sample could be estimated. For LP-ODO-TROP-1 and LP-ODO-TROP-2 only one dilution series was analysed in treated samples.

Collimated beam set-up

The UV irradiation energy that potentially reaches and affects organisms is defined as the *average germicidal intensity*, I_{avg} (W m^{-2}). It was calculated from the direct energy output of the UV lamps defined as the *incidental germicidal intensity*, I_i (W m^{-2}) by correcting for factors (water absorbance, reflection, divergence, transmittance) that will decrease I_i and hence the irradiating reaching the volume of water (i.e. volume-averaged) (Linden & Darby 1997, Bolton & Linden 2003) (Figure 1). The relative irradiation spectrum was measured using an Ocean Optics USB 2000+ spectrophotometer with a 400 µm Premium fibre sensor 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_i from the MP mercury lamp (Heraeus) at each wavelength was irregularly spread out over the whole 200-300 nm range (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 (Figure 1). When integrated the total I_i 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 (Figure 1). The total I_i 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_i 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_{avg} (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.

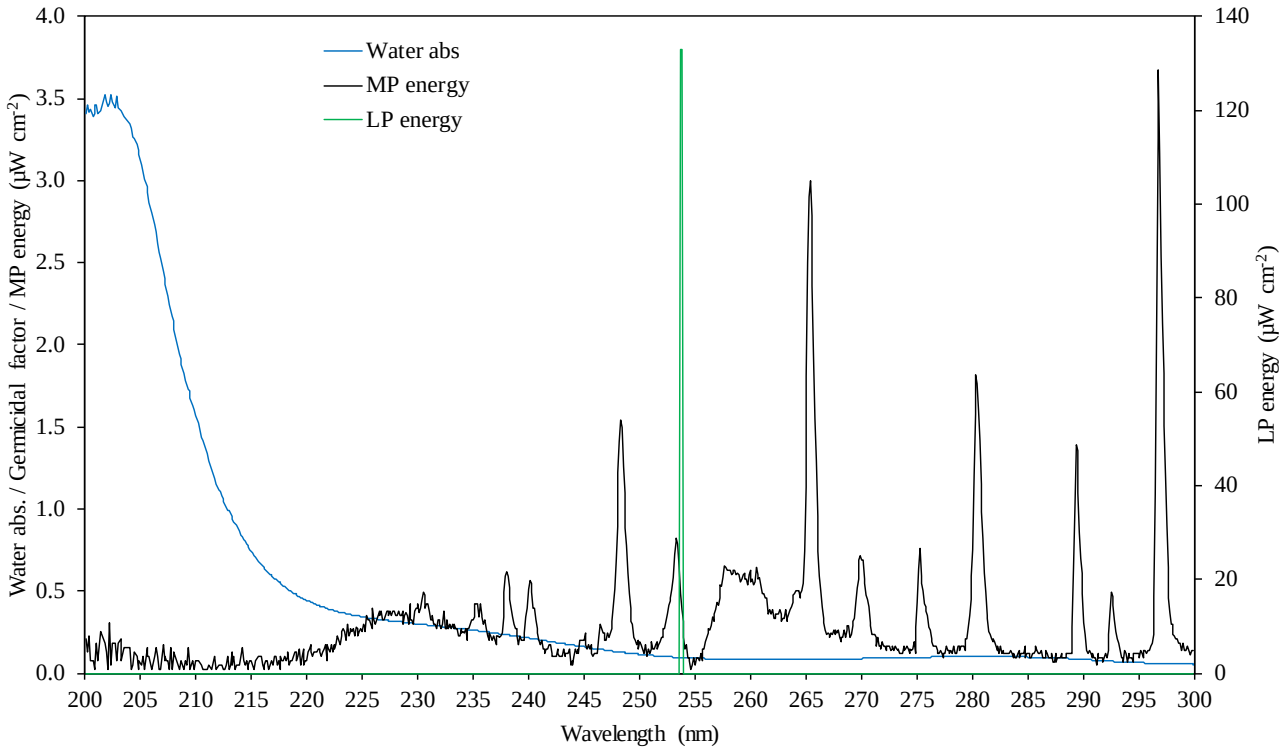


Figure 1. Collimated beam UV spectra as a function of irradiation energy (*incidental germicidal intensity* – i.e. not water factor adjusted). The black line shows the recorded UV spectrum between 200 and 300 nm of the polychromatic medium pressure (MP) system. The green line shows the UV spectrum of 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.

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 hours 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 (Figure 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.

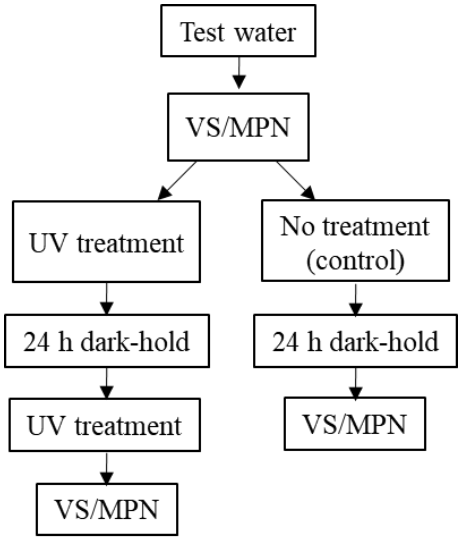


Figure 2. Experimental flow chart: A 50 ml sample was collected from the test water and either 1) quantified as control sample (no treatment), stored in dark conditions for 24 hours at ambient temperature and re-analyzed or 2) exposed to LP or MP UV treatments, stored in dark conditions for 24 hours at ambient temperature followed by a second UV treatment and lastly quantified.

Samples for UV treatments were collected from the test water at different time points during the day of experimentation. To confirm that test water algal concentrations were stable, control samples (no treatment) were collected and evaluated at three time points (0, 3, and 8 hours). Each sample was then kept in a dark box for 24 hours and re-assessed to determine if the dark-hold period affected algal concentration.

Regression analysis of MPN data

First-order kinetics regressions could be fitted after log-transformation of the MPN data using Equation 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 ($2\log_{10}$ 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:

$$MPN_F = MPN_{F=0} \text{ for } F \leq F_{Th} \quad (1a)$$

$$MPN_F = MPN_{F=0} \cdot e(-k[F-F_{Th}]) \text{ for } F > F_{Th} \quad (1b),$$

where 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 $MPN_{F=0}$ 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 $MPN_F = 0.01 \times MPN_{F=0}$. 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 used for. 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.

Results

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, 5 degrees of freedom) and three (LP-TET-2, LP-ODO-2, MP-TET-2) showed a significant increase (P < 0.05, 5 degrees of freedom) (Figure 3) after the dark-hold period.

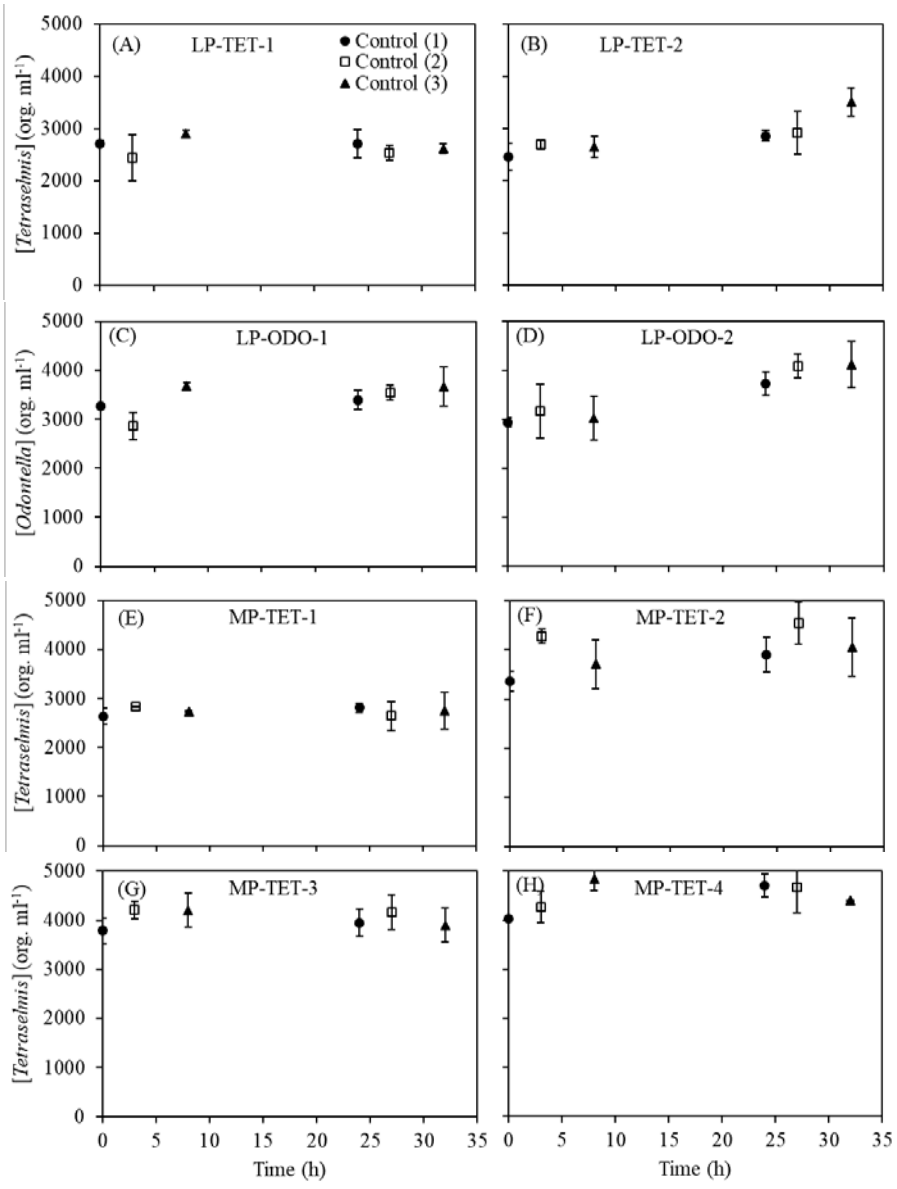


Figure 3. Test water concentrations (mean±SD, org. ml^{-1}) of *T. suecica* or *Odontella sp*. was determined using the vital stain method before and after a 24-hour dark-hold period in three independent control samples (no treatment). Control samples were collected at three time points (0, 3, and 8 hours) and each sample was re-assessed after a 24-hours dark-hold period (24, 27 and 32 hours). (A) and (B) LP-TET-1 and LP-TET-2 (C) and (D) LP-ODO-1 and LP-ODO-2, and (E), (F), (G) and (H) MP-TET-1, MP-TET-2, MP-TET-3 and MP-TET-4. For each data point n=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 1279-1505 mJ cm^{-2} (Figure 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 (Figure 4).

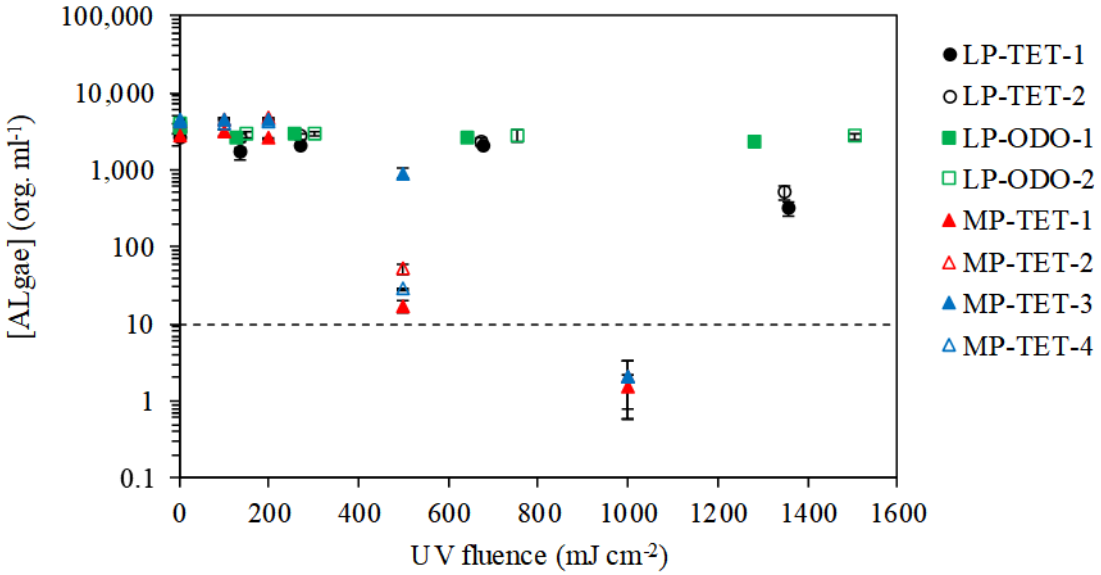


Figure 4. The fluence-response relationships show effects of LP and MP UV treatments on *T. suecica* and *Odontella* sp. concentrations (log_{10} scale, mean±SD, org. ml^{-1}) evaluated using the VS method. The punctured line signifies the upper concentration limit for the discharge standard (<10 org. ml^{-1}). For each data point n=2.

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 (Figure 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 Figure 5 (red circles).

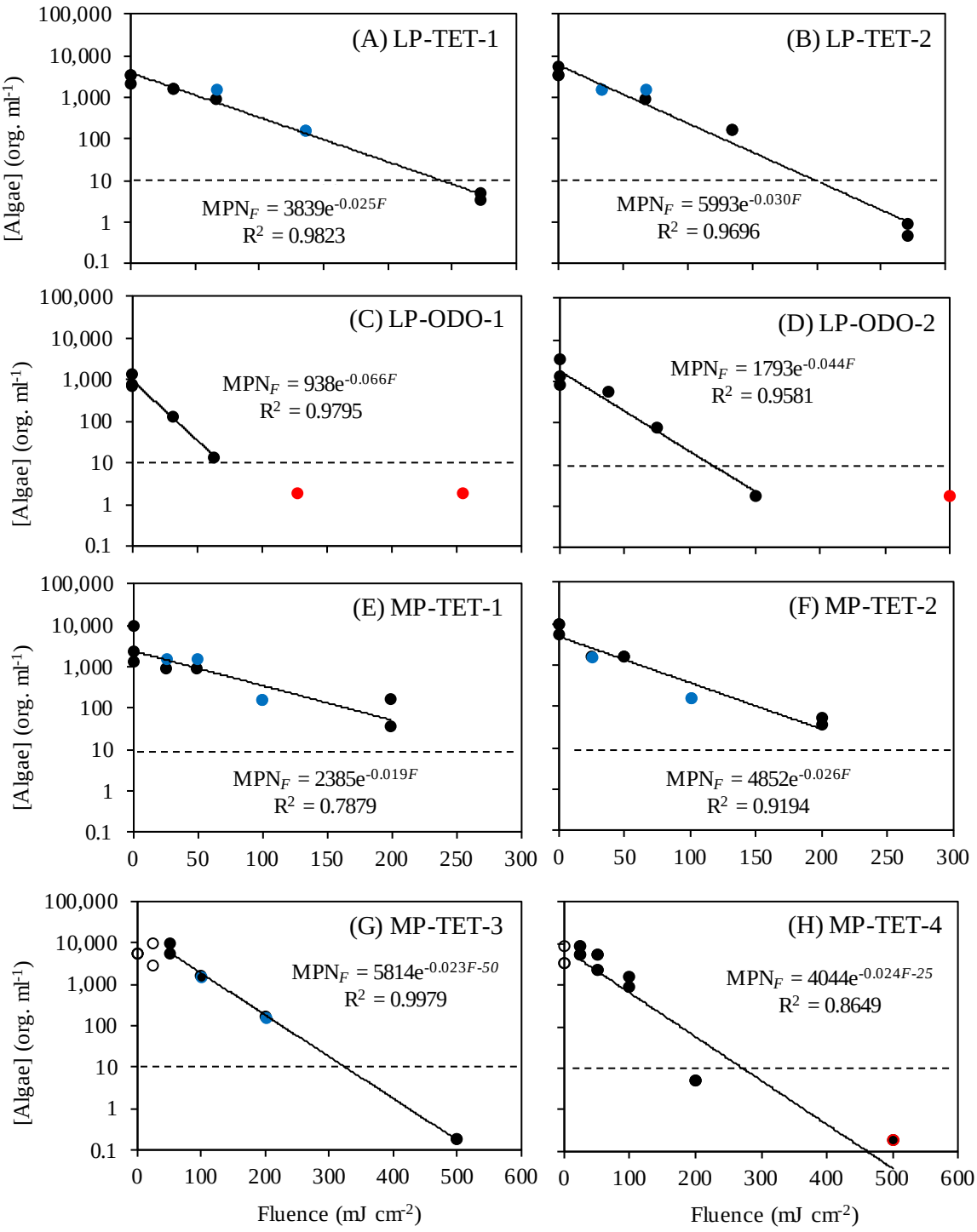


Figure 5. The fluence-response relationships show the effect of LP and MP UV treatments on *T. suecica* and *Odontella* sp. concentration (log_{10} scale, org. ml^{-1}) evaluated using the MPN method. The trendline equations follow the definition in Eq. 1a and Eq. 1b. The punctured line signifies the upper concentration limit for the discharge standard (<10 org. ml^{-1}). Open symbols in (G) and (H) signify data points below the threshold fluence, F_{Th}. All-positive values (MPN scores of 5-5-5) are marked as blue circles and are included in trendline estimations. Non-detect values (MPN scores of 0-0-0) are marked as red circles and are not included in trendline estimations. Note the different fluence range on the x-axis in (G) and (H).

The fluence required to reduce the number of viable organisms to 1% of the initial concentration ($2\log_{10}$ reduction) was calculated by adapting the sensitivity term $F[0.01]$ (mJ cm^{-2}) from MacIntyre et al. (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. The ranking 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 $2\log_{10}$ 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 70-105 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.

Table 3. The calculated fluence ($F[0.01]$) needed for reducing initial concentration ($MPN_{F=0}$) 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 have been included.

UV type and Exp ID	#data points of regression	k (cm^2 mJ^{-1})	Shoulder F_{Th} (mJ cm^{-2})	Intercept $MPN_{F=0}$ (org. ml^{-1})	$F[0.01)$ (mJ cm^{-2})
MP-TET-3	8	0.023	50	5814	250
MP-TET-1	11	0.019	0	2385	242
MP-TET-4	9	0.024	25	4044	217
LP-TET-1	11	0.025	0	3839	184
MP-TET-2	11	0.026	0	4852	177
LP-TET-2	11	0.032	0	5993	144
LP-NAT-TEMP-1	11	0.041	0	2765	112
LP-ODO-2	6	0.044	0	1793	105
MP-NAT-TEMP-2	11	0.044	0	9060	105
LP-NAT-TEMP-2	10	0.050	0	9235	92
LP-ODO-1	5	0.066	0	938	70
MP-NAT-TEMP-1	9	0.074	0	3040	62
LP-NAT-TROP-1	5	0.113	0	11562	41

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 (Figure 4 and Figure 6, circular symbols). Counts performed 24 hours, T(24), and 96 hours, T(96), after T(0) resulted in

concentrations in line with the discharge standard at 500 mJ cm^{-2} (Figure 6). At T(96), the concentration at 200 mJ cm^{-2} had decreased to about 30% (1200 cells ml^{-1}) of control levels (Figure 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 (Figure 3G, H).

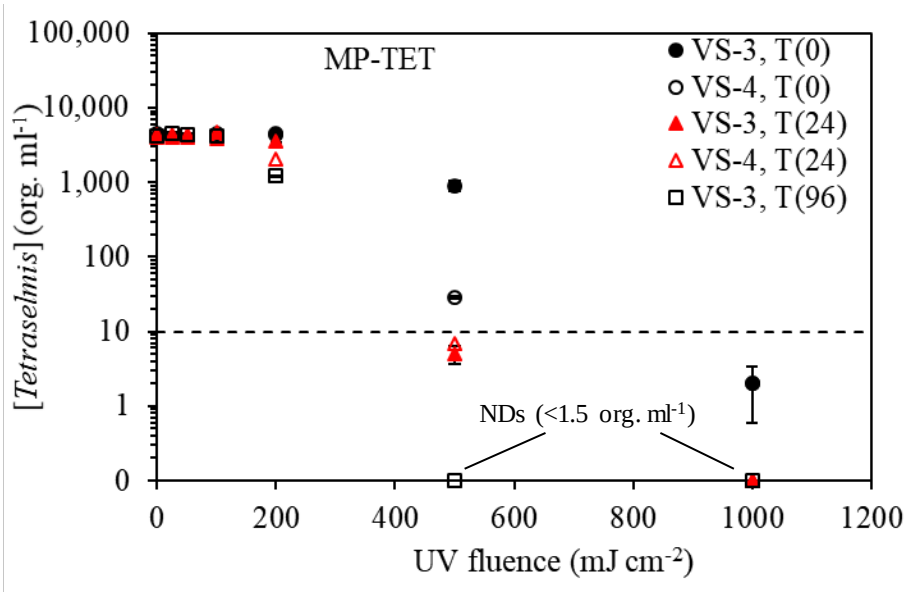


Figure 6. Fluence-response relationship of MP UV treatments on *T. suecica* concentrations (log_{10} scale, mean±SD, org. ml^{-1}) showing VS counts of algal cells at 24 hours (T(24)) and 96 hours (T(96)) after last MP UV treatment in relation to counts immediately after last exposure at T(0). T(0) values are from Figure 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.

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 (Figure 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 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.

Table 4. Observed behaviours of *T. suecica* cells exposed to a range of LP and MP UV treatments. In MP, T(0) observations were based on all four MP-TET experiments and carried out immediately after last UV treatment. In MP, T(24) observations were based on MP-TET-3 and MP-TET4 and carried out 24 hours after last exposure. In MP, T(96) observations was based on MP-TET-3 and carried out 96 hours after last exposure.

Fluence (mJ cm^{-2})	**Experiment**	**Observed behaviour**
0	LP, T(0)	90% in whole water column, high mobility, few non-moving on bottom
	MP, T(0)	90% of cells mobile, present in whole water column
	MP, T(24)	90% of cells mobile, present in whole water column
	MP, T(96)	90% of cells mobile, present in whole water column
25	MP, T(24)	90% of cells mobile, present in whole water column
	MP, T(96)	90% of cells mobile, present in whole water column
50	MP, T(24)	Slower, large proportion on bottom
	MP, T(96)	Majority on bottom, little mobility
135	LP, T(0)	90% in whole water column, high mobility, few non-moving on bottom
100	MP, T(0)	Little mobility, small bursts of slow rotation, all on bottom
100	MP, T(24)	Very few moving slowly, all on bottom
100	MP, T(96)	No mobility, all on bottom
270	LP, T(0)	Majority on bottom, few swimming slowly
200	MP, T(0)	No mobility, all on bottom
200	MP, T(24)	No mobility, all on bottom
200	MP, T(96)	No mobility, all on bottom
660	LP, T(0)	No mobility, all on bottom
500	MP, T(0)	No mobility, all on bottom
500	MP, T(24)	No mobility, all on bottom
500	MP, T(96)	No cells present
1350	LP, T(0)	No mobility, cell wall fractured/irregular
1000	MP, T(0)	No mobility
1000	MP, T(24)	No cells present
1000	MP, T(96)	No cells present

Discussion

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 (Figure 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 (Figure 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 (Liltved et al. 2011). Fluences about 41 and between 92-112 mJ cm^{-2} were needed for a $2log_{10}$ reduction of tropical and temperate natural algae composition, respectively, in LP UV treatments assessed using the MPN method (Lundgreen et al. 2018). For a $2log_{10}$ 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. LC_{50} 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-$5log_{10}$ inactivation of *Tetraselmis* sp. assessed using the MPN method (Sun & 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} (Figure 5E-H). For comparison with other MP UV fluence-response studies Sakai et al. (2011) found a $2log_{10}$ 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 III et al. 2018). This theory was investigated in a UV treatment study by Sun & 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 254-280 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 & 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 & 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 Figure 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. Figure 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) – more than 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 (Figure 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 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 ($2\log_{10}$ 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 & 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 Figure 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 & 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 & 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 & 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 $2\log_{10}$ 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 & Waite 2004). A recent study

have 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 & 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 µm, 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 µm 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). 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 ($F_{D\text{-}2}$, mJ cm^{-2}) needed for algal concentrations to meet the discharge standard ($MPN_D = 10$ org. ml^{-1}) could be estimated over range of $MPN_{D=0}$ values (Figure 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 ($MPN_{D=0}$). The higher fluences are required because increases in cell density will affect test water UVT due to the shading effects of the cells.

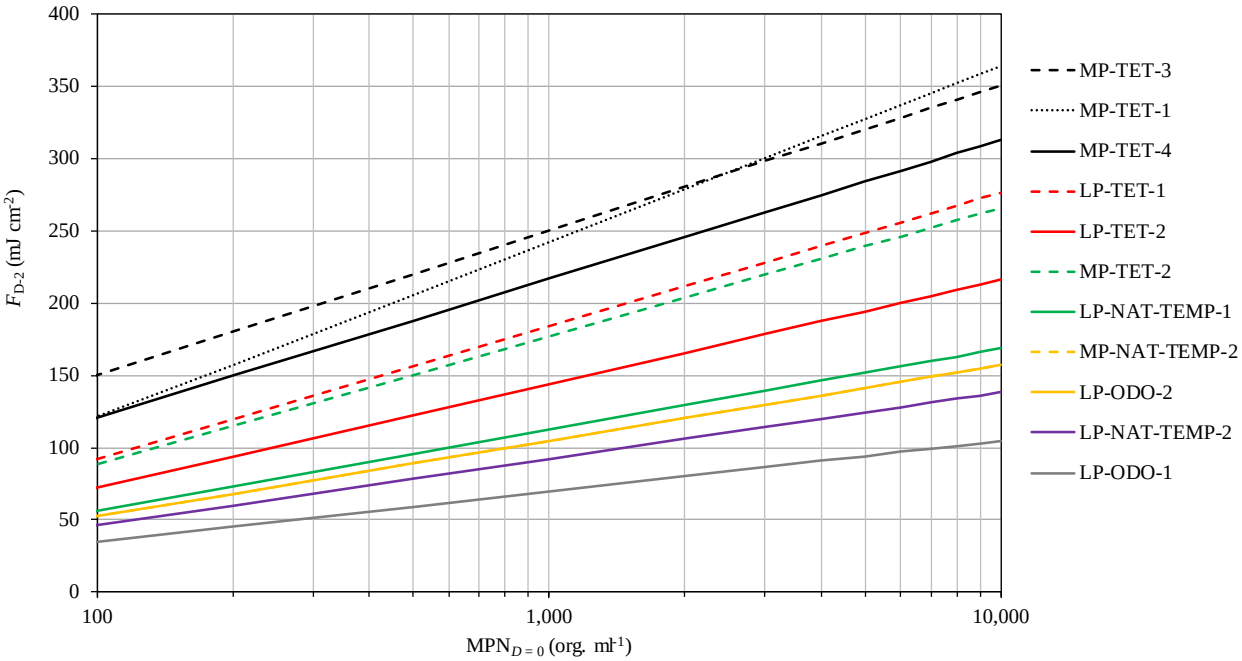


Figure 7. Estimated fluences ($F_{D\text{-}2}$, mJ cm^{-2}) required for different standard test organism (STO) and natural algal concentrations to meet the discharge standard (<10 org. ml^{-1}) over a range of initial algal concentrations (MPN$_{D=0}$, org. ml^{-1}) when assessed using the MPN method. Data on natural algae compositions were obtained from Lundgreen et al. (2018).

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 MEPC279/70 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 phytoplankton 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 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 suggest 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 harvesting of naturally occurring organisms) (IMO 2016a, Annex 2). 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 hydrocarbonoclasticus;* phytoplankton (10-50 µm): *Tetraselmis suecica*; and zooplankton (>50 µm) *Artemia salina*, *Branchionus plicatilis L.*) as supplement to challenge water to fulfil 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 is 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.

Conclusions

Tetraselmis suecica and *Odontella* sp., were evaluated as potential standard test organisms (STOs) in type approval of BWTS. 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*. The results suggest these 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.

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Manuscript 3

Development of a novel automated analytical method for viability assessment of phytoplankton used for validation of ballast water treatment systems

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Development of a novel automated analytical method for viability assessment of phytoplankton used for validation of ballast water treatment systems

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Abstract

To limit the spreading of aquatic invasive species, regulations require ships' ballast water to be treated before discharge. To validate ballast water treatment system (BWTS) performance, treated water is analyzed for living organisms in different size classes. Quantitative assessment of the size-class 10-50 µm (mainly phytoplankton) is carried out using the vital stain method, which requires labor intensive manual microscope counts of fluorescent (*i.e.* living) cells. The method is slow, demands specialized personnel and challenged by subjectivity and mobile organisms. Using a High Content Screening Platform (HCS-Platform) and image analysis, we developed an automated, objective and faster quantification method. The automated method neutralized subjectivity by using fixed cell recognition parameters for image analysis. The implementation of membrane filters gently manipulated the organisms into a 2D-plane that reduced mobility. Quantifications were performed at different concentrations using monocultures of slow-moving *Rhodomonas salina*, highly-mobile *Tetraselmis suecica* and natural algae. Results were compared to the standard manual counting procedure. Automated counts of monocultures were comparable to manual counts at low and medium concentration levels. Manual counts of *T. suecica* at high concentration levels were significantly lower compared to automated counts stressing the challenge to count mobile cells in 3D. Natural algal counts were similar for both counting approaches, but accuracy was challenged by colony forming species and high number of algal species ~10 µm. Automated counts were significantly faster than manual counts. In conclusion, the HCS-Platform showed promising results as an alternative quantitative phytoplankton assessment method for BWTS validation.

Keywords Advanced Microscopy; Algae; Invasive Species, Ballast Water; Image Analysis; Monitoring; Ballast Water Treatment Systems

Introduction

The Ballast Water Management Convention (IMO 2004) entered into force in 2017 which created an increase in the demand for the installation of ballast water treatment systems (BWTSs) in ships. The convention states that to prevent the spread of aquatic invasive species all ships carrying ballast water (with few exceptions) are required to have a Type Approved BWTS installed that removes, kills or renders organisms harmless (IMO 2004). In the Type Approval certification process, the performance of BWTSs are evaluated at certified land-based testing facilities through the collection of treated ballast water (BW) samples and subsequent quantification of viable organisms in different size classes. The size classes consist of plankton ≥50 µm, plankton ≥10 to <50 µm (hereafter 10-50 µm) as well as some indicator microbes (Toxigenic *Vibrio cholerae*, *Escherichia coli*, and intestinal *Enterococci*) (IMO 2004).

The validation procedures for Type Approval require that a BWTS must consistently demonstrate that certain discharge standards are met in five consecutive land-based tests cycles for each of three test water salinities: freshwater (<1 psu), brackish (10-20 psu) and marine water (28-36 psu) (IMO 2016a). The land-based test cycles are followed up with a ship-board test which also consists of five consecutive test cycles. The purpose of the shipboard test is to validate the BWTS performance after installment on the ship. The discharge standards are outlined in IMO Regulation D-2 and state that treated BW must contain <10 org. m^3 for plankton ≥50 µm and <10 org. ml^{-1} for plankton 10-50 µm (IMO 2016a).

Test intake water and control discharge water must fulfill certain concentration requirements. To ensure that BWTS are properly challenged in the treatment process the concentration requirement for intake water for the size class 10-50 µm during land-based testing is ≥1,000 org. ml^{-1} (IMO 2016a). Control discharge water is a certain volume of the intake water that has bypassed the treatment system and is stored in a holding tank to simulate BW storage conditions. To confirm that an observed elimination of organisms in treated discharge water is a result of the BWTS and not environmental conditions, the concentration requirement for control discharge water for the size class 10-50 µm is ≥100 org. ml^{-1} (IMO 2016a). The IMO G8 Guidelines for approval of ballast water management systems state that for intake, control discharge and treated discharge water, the number of 1 ml subsamples for the size class 10-50 µm that need to be analyzed for each test cycle is 3, 6 and 6, respectively (IMO 2016a). In land-based validation procedures a total volume of 15 ml per test cycle is therefore required to be analyzed.

For quantification of organisms in the size-class 10-50 µm (mainly phytoplankton), the most widely accepted standard method is the Vital Stain (VS) method (Steinberg et al. 2011; IMO 2016b). When using the VS method, regulations state that BW samples must be analyzed within six hours (IMO 2016b). The analysis involves staining of the BW samples with the fluorescent dyes: fluorescein diacetate (FDA) and 5-chloromethylfluorescein diacetate (CMFDA) which both freely cross the cell membrane. If cells are alive, the initially quenched stains are activated by unspecific enzyme activity. The resultant cell-impermeable green fluorescent molecule allows the cells with enzyme activity and intact cell membrane to be quantified as living through direct microscopic counts (IMO 2016b; Steinberg et al. 2011; Rotman and Papermaster 1966).

The VS method is suitable for evaluation of BWTSs targeting cell membrane integrity but is inadequate for evaluating UV-based treatment systems because UV irradiation inflict DNA damage

which result in living, but non-viable organisms with delayed mortality. Consequently, if the VS method is used non-viable organism are quantified because the organisms may still be alive and exhibit enzyme activity although the cells are dying and pose no invasion risk. To avoid an overestimation of cell counts and the unfair failing of UV-based treatment systems in validation procedures, then the Most Probable Number (MPN) method (regrowth assay) could be applied as an effective alternative method. Unlike the VS method, the MPN method assesses the number of viable organisms. To be in compliance with the IMO discharge standard regulations there should be <10 *viable* org. ml^{-1} the (IMO 2016b).

However, in 2016 the USCG rejected the use of the MPN method and instead insisted on the use of the VS method in validation procedures for BWTSs (Coast Guard Maritime Commons 2016). To comply with the USCG regulations of <10 *living* org ml^{-1} using the VS method, then UV-based treatment systems must increase energy outputs to a degree where the cell membrane integrity is compromised in order for the VS method to accurately verify the cell damages (Lundgreen et al. 2018; Drake et al. 2016).

The accuracy of the VS method has been questioned in a number of studies which have highlighted the problem with ambiguous staining of live and dead cells, as well as the variability in staining intensities among different algal species and growth stages (Peperzak and Brussaard 2011; Garvey et al. 2007; MacIntyre and Cullen 2016). Furthermore, the VS method involves labor intensive direct microscope counts by specialized personnel that are challenged by fatigue and subjectivity regarding size class and live/dead classification. The VS method is typically carried out using a standard Sedgewick Rafter counting chamber holding a volume of 1 ml. The vertical water column of the counting chamber allows highly mobile algal species to move in 3D, further complicating counting performance. All together the described issues limit the performance of the *human counting machine* and significantly reduce quantification accuracy (Steinberg et al. 2012). Despite the concerns raised about accuracy and sensitivity, several studies have described the method as a powerful and robust method for evaluating treatment performances of BWTSs (Steinberg et al. 2011; Adams et al. 2014; Baek and Shin 2015).

To address the outlined issues with manual counts, the aim of the current study was to develop an automated, faster and more objective counting approach based on the VS method which could be used as an alternative approach for BWTS validation. Here we describe an approach involving a High Content Screening (HCS) Platform that scans test water samples containing phytoplankton where VS have been applied. Subsequently, samples underwent image analysis for quantification of fluorescent algal cells. The automated approach was performed in combination with a filter technique that limited the mobility of highly mobile organisms. The approach was tested on fluorescent beads, monocultures of two algal species: (1) low mobility *Rhodomonas salina* and (2) high mobility *Tetraselmis suecica*, and on natural algal compositions. The results obtained from automated counts were compared to standard direct manual counts using the VS method to evaluate the effects of concentration levels, mobility, cell size and algal species composition (complexity) on quantification accuracy.

Materials and Methods

Experimental design

The test water (20 psu, Marine SeaSalt, Tetra, Germany) was prepared to represent three composition complexity levels: (A) low complexity with fluorescent beads, (B) medium complexity with algal monocultures and (C) high complexity with natural algae. At each composition complexity level different algal concentrations were evaluated using manual microscope and automated counts for comparison.

(A) Fluorescent beads

Fluorescent beads (6 µm FocalCheck microspheres, F14807, Invitrogen) were initially used to test the manual and automated performance standard on non-mobile objects of a similar shape and size and fluorescence intensity as algal cells. To challenge the size recognition properties of manual and automated counts, beads with a diameter below the minimum value of the size class 10-50 µm were chosen.

(B) Algal monocultures

Tetraselmis suecica and *Rhodomonas salina* were grown as semi-continuous cultures at 20°C in 500 ml Erlenmeyer bottles containing 20 psu artificial seawater (Marine SeaSalt, Tetra, Germany) enriched with f/2-medium (Guillard and Ryther 1962) under 12:12 h light/dark conditions. Three concentration levels were evaluated: (1) low level at 10-30 org. ml^{-1}, (2) medium level at 100-140 org. ml^{-1} and (3) high level at 1100-1300 org. ml^{-1}. For each concentration level a total of ten counting rounds were carried out consisting of one manual and one automated count. One round typically took 45-60 minutes including preparation of equipment/microscopes, rinsing and evaluation of counts.

(C) Natural algal populations

Natural seawater was collected from Kerteminde Fjord, Denmark in November 2018 (17.6 psu, 10.2°C). Approximately 10 liters of seawater were subsequently filtered through a 45 µm filter device (polyamide, Ø47 mm) to remove large particles and zooplankton. The water was then transported to the laboratory and slowly (~2 h) acclimatized to room temperature (18°C). For experiments, the water container homogenized by gentle inversion before transferring 100 ml to an Erlenmeyer flask that was placed on a magnetic stirrer (300 rpm). For evaluation of living organisms eight manual and ten automated counts were carried out. In addition, a sample of 50 ml was preserved with 1% acidified Lugol's for later determination of algal species and phyla composition.

Quantification procedures of algal cells

The number of living cells in the test water was quantified using the VS method. A combination of Fluorescein diacetate (FDA) and 5-chloromethylfluorescein diacetate (CMFDA, both from Molecular Probes-Invitrogen Carlsbad, CA, USA) was used. Samples were prepared by adding 5.5 µl of 500 µM CMFDA and 5.5 µl of 1000 µM FDA to 1.1 ml test water in an Eppendorf tube that was kept in a dark container and counted after 10-15 minutes. Classification of living and dead cells during quantification followed the criteria described in Steinberg et al. (2011) and IMO (2016b).

Manual microscope counts

Manual counts were performed using a standard 1 ml Sedgewick Rafter counting chamber (Pyser – SGI Limited, UK). To quantify the algal concentrations (org. ml^{-1}), the whole stained sample was counted on a fluorescence microscope (Leica DMR) using a 10× objective (10×/25 PH1, HC PL FLUOTAS) (Table 1). All manual samples were counted by the same person to keep variations in subjectivity and individual visual criteria at a minimum.

Automated counts

Automated counts of algal cells were performed on a High Content Screening Platform (HCS-Platform) (Nikon Eclipse T*i*-E inverted microscope, Zyla sCMOS camera) with integrated image analysis software (NIS-elements imaging software). On a glass slide, 2×500 µl drops of stained sample were placed within two outlined circles (Fig. 1a). Two membrane filters (diameter = 47 mm, pore size = 0.8 µm) were carefully placed on top of the drops which spreads out the stained sample under the membrane filters. On top of each membrane filters a "suction" filter was placed to produce an upwards removal of water through the membrane filter. Consequently, the membrane filters were gently forced towards the glass slide and at the same time algal cells were trapped in a 2D plane as they are unable to move through the membrane filter. The "suction" filters were then removed, and the glass slide was placed in the stage of the HCS-Platform and covered with a black box to prevent external light from interfering with the analysis (Fig. 1b).

The glass slide was scanned using a 4× objective to obtain multiple images of each 3×3 mm that in combination covered the whole area of the two circles (Table 1). The images were afterwards subjected to an *object count analysis* using the NIS-Elements imaging software with pre-set algal cell recognition parameters to estimate the number of algal cells per ml.

Table 1. The calculated number of "frozen" images needed to scan a full sample in a standard Sedgewick Rafter counting chamber compared to using the filter technique.

	Manual microscope	Filter technique
Objective magnification	10×	4×
Eyepiece magnification/total magnification	10×/100×	-
Field of view diameter/square (mm)	2.5	3×3
Field of view area (mm^2)	4.9	9
Scan area of Sedgewick Rafter/filters (mm^2)	1000	3470
Number of images to ideally cover whole scan area*	204	475[a]
*including "horizontal slices" factor (×70/×1)	14,490	475

[a]In theory 385 images was needed to cover the whole area but in practice 475 images were needed to make certain circle edges were covered completely

For the two monocultures, the algal cell recognition parameters were based on *Area*, *Width*, *MinFeret* (the minimum caliper diameter), *Circularity*, *Mean intensity* and an *Intensity threshold* (Table 2). Recognition criteria were based on the range of recognition parameter measurements of 1195 individuals of *T. suecica* and 1140 individuals of *R. salina*. The minimum and maximum values of the ranges when combining both species were selected as recognition criteria to ensure the inclusion of both species in the object count analyses. The natural algal cells were a mix of species, shapes and

sizes and the recognition criteria were based on the range of recognition parameter measurements of 140 individuals.

Table 2. Range of cell recognition parameters based on *n* individuals of *Tetraselmis suecica*, *Rhodomonas salina* and natural algae and the selected recognition criteria for running Object Count analysis in NIS-Elements.

	Tetraselmis suecica	*Rhodomonas salina*	Recognition criteria monocultures	Natural algae	Recognition criteria natural algae
n	1195	1140	1195+1140	140	
Area (µm)	85.3-320	56.0-291	56-320	74.7-1309	74-2000
Width (µm)	6.16-14.9	5.5-13.4	5.5-	6.5-26.4	6.5-
MinFeret (µm)	8.12-18.0	8.16-18.0	8.1-50	9.7-41.8	9-50
Circularity	0.55-0.98	0.62-0.98	0.54-1.00	0.53-0.97	0.53-1.00
Roughness				0.84-0.96	0.84-1.00
Mean intensity	2966-3910	2854-3787	2854-	2846-3808	2846-
Intensity threshold	1800	1600	1800	1800	1800

To evaluate the accuracy of the cell recognition criteria used for the automated approach, every image from a randomly selected high concentration level scan of monocultures (*R. salina*: Run#5 and *T. suecica*: Run#5) and natural seawater (Run#5) were thoroughly evaluated manually and compared to the automated counts.

Automated counts of autofluorescent beads were performed using a Sedgewick Rafter counting chamber. An aliquot (1 ml) of sample containing beads was transferred to the counting chamber and left for 10 minutes to allow sedimentation of the beads. The sample was scanned using two different magnification objectives (4× and 10×) on the HCS-Platform and analyzed with the image analysis software.

Counting times

Counting time was defined as the time from when the Sedgewick Rafter counting chamber/glass plate with filters was placed in the microscope/HCS-Platform until a final concentration was estimated by tally counter for the manual counting approach or through object count analysis software for the automated approach. Counting time is presented in min ml^{-1}. In addition, counting times (min ml^{-1}) were extracted from a number of randomly selected land-based tests of BWTSs performed at DHI, Denmark (certified by IMO and USCG to test and approve BWTS).

Identification and quantification of natural algal populations

Samples were analyzed with an inverted microscope according to the Utermöhl method (Utermöhl 1931). An aliquot (50 ml) of the Lugol's preserved natural seawater sample was gently mixed by inversion before settling in a sedimentation chamber for 24 h. Microalgae where then identified down to species level (where possible) using a Nikon inverted microscope (Eclipse TS2-FL).

Immobilization experiments using acetic acid

The highly mobile algal species, *T. suecica* is pH sensitive and can be immobilized at pH of approximately 5.0 for an extended period of time (hours) without any effect on the cell membrane

(data not shown). A modified procedure from Steinberg et al. (2012) was used to test immobilization efficiency of acetic acid on *Tetraselmis suecica* and *Rhodomonas salina* (Appendix A). A volume of 500 µl stained sample with algal cells was mixed with 600 µl of 5 mM acetic acid+test water (AA water). The final mix thus contained 2.73 mM AA water and stained test water. Both automated and manual counts were performed using a Sedgewick Rafter counting chamber. In addition, a Sedgewick Rafter counting chamber modified from 1 ml to 250 µl was used.

Statistics

Statistical analysis was carried out using IBM SPSS Statistics 24. Comparison of means were done using a one-way ANOVA and a significance level of 0.05. Data was also tested for normality (Shapiro-Wilk) and homogeneity of variances. Where appropriate tests were followed up with Tukey's post hoc test for testing multiple means in a dataset against each other. Comparison of regression lines and interaction analysis were performed using ANCOVA (Statgraphics 18) and a significance level of 0.05.

Results

Fluorescent beads

There were no significant differences between manual and automated counts (n=5) of 6 µm fluorescent beads at the three different overall mean concentration levels of 1292±45, 69±3 and 3.8±0.2 beads ml^{-1} (Fig. 2). Overall mean concentration levels were defined as the mean of the counted concentrations for each approach at each level.

Comparison of manual and automated counting performances

Counts were carried out over a period of approximately 8-10 hours. Time did not have any effect on algal concentrations in the test water (Fig. 3 and Fig. 4). Counts for both approaches and for both monoculture species only deviated slightly above or below the mean of all count lines except forFig 3e, where the automated counts were consistently lower than the manual counts.

For natural algal species counts the variations around the overall mean were larger compared to monoculture counts, but there was no significant effect of time on the variation in the test water concentration (Fig. 4).

At the low and medium algal concentrations, no significant differences were observed for *R. salina* and *T. suecica* (Fig. 5 a and b). At the highest concentration, automated counts for *R. salina* were significantly lower than manual counts and vice versa for *T. suecica* (Fig. 5c).

The mean concentration for natural algal samples were around 165 cells ml^{-1}. There was no significant difference between the manual and automated quantification approach when a 4× magnification objective was used, whereas a significantly smaller concentration was counted when using a 10× magnification objective (Fig. 6).

Counting times

For both monoculture algal species, manual counting times were significantly higher than automated counting times (Fig. 7). Manual counting times at the low and medium concentrations were around 8

minutes and typically about 3 minutes slower than automated counts. At the high concentration levels, manual counting times were around 12-14 minutes and between about 7 and 9 minutes slower than automated counting times. While algal concentration level had no effect on automated counting times, there was a significant increase in counting times for both monoculture species when algal concentrations were <1000 cells ml^{-1} (Fig. 7). Counting times were not recorded for *T. suecica* at medium concentration levels.

Based on the results obtained on counting times of monocultures the total time to count 15 ml (required by IMO for land-based validation) using the manual approach would approximately be (3 ml intake subsamples×12.5 min + 6 ml control discharge subsamples×8 min + 6 ml treated discharge subsamples×8 min) 133.5 min (2 h 14 min) (Table 3). The total time to count 15 ml using the automated approach will be 75 min (1 h 15 min) corresponding to 44% less time per test cycle when compared to the manual approach. Adding all the test cycles together from each of the three salinities this becomes a total volume of (15 ml×5 test cycles×3 salinities) 225 ml (Table 3). Consequently, for land-based tests the total time to count the volume required by the guidelines using the manual and automated approach will be 33 h 23 min and 18 h 45 min, respectively.

Table 3. The number of 1 ml subsamples of each test water type required by the regulations. From the number of test cycles per salinity and the number of salinities (freshwater, brackish water and marine water) the total volume of all subsamples to be counted can be calculated for a land-based test. The counting times (min ml^{-1}) based on the results obtained on monocultures at the different concentration levels are used to calculate the total time needed to count the total volume of a land-based test.

Test water type	Intake water	Control discharge	Treated discharge	Total
Subsamples of 1 ml	3	6	6	
#test cycles per salinity	5	5	5	
# of salinities	3	3	3	
Total volume (ml)	45	90	90	225
Manual counting time (min ml^{-1})	12.5	8	8	
Manual total time (min)	562.5	720	720	2002.5
Automated counting time (min ml^{-1})	5	5	5	
Automated total time (min)	225	450	450	1125

Automated counts of natural algae compositions using a 4× magnification objective took around 6 min ml^{-1} which was significantly lower than manual counting times of around 11 min ml^{-1} (Fig. 8). Automated counting using a 10× magnification objective took around 17 min ml^{-1} and was significantly higher than both manual and automated 4× counting times (Fig. 8).

Counting times (min ml^{-1}) obtained from DHI, Denmark, included mean organism concentrations and total counting times from inlet (3 ml), control discharge (6 ml) and treated discharge (6 ml) water samples (n=10 for each) collected from freshwater (FW), brackish water (BrW) and seawater (SW) tests. Statistical analysis showed significant higher counting times with increasing concentration at all salinities (P_{FW} = 0.0004; P_{BrW} = 0.026, P_{SW} = 0.025). There was effect of salinity on counting times with increasing concentration between BW and SW samples whereas counting times of FW samples were significantly different from both BrW (P=0.04) and SW (P=0.017).

Natural algal composition

For algal species identification, 652 individuals were identified in a total volume of 5.1 ml which was equivalent to 128 org. ml^{-1}. The Lugol's counts showed that the natural seawater samples comprised of 31 species divided between nine phyla (Fig. 10). The five most dominating species were *Pseudo-nitzschia* spp. (colony forming diatom, 14%), unidentified pennate diatom (12%), unidentified cryptomonad (cryptophyte, 8%), *Chattonella* sp. (raphidophyte, 6%) and euglenoid spp. (euglenoids, 5%) which all together represented 45% of the total number of identified algal cells (Fig. 9). Diatoms and dinoflagellates were the two most dominating phyla representing 40 and 25%, respectively, of the total phytoplankton concentration.

Accuracy of the automated approach

To evaluate the accuracy of the automated approach a "manual review" of the image analysis process was performed (Table 4, Fig. 11). In total 475 images were produced from each scan that when combined covered the surface area of both circles in the filter technique. Each image was then manually evaluated and compared to the data from the automated image analysis.
For *R. salina*, there were 56 cases where there was disagreement between the manual review and the automated approach. The cases include cells identified by the manual review but not the automated approach and vice versa. The overall result was a net difference of 20 cells corresponding to 1.8% deviation from the total automated count. For *T. suecica*, there were 16 cases of disagreement between the manual review and the automated approach which resulted in a net difference of 5 cells corresponding to 0.4% (Table 4).

In natural algal samples, the automated approach was not able to identify and count all cells of colony forming algal units. Therefore, there were 64 cases of disagreement between manual review counts and automated counts. The proportion of cases of disagreements is 43% when related to the automated counts of 150 identified cells. The cases of disagreements resulted in a net difference of 67 cells corresponding to a 45% higher manual review count compared to the automated count (Table 4). If excluding colony forming individuals the net difference was reduced to 22 cells corresponding to 15% higher manual review counts. Furthermore, cases of disagreement decreased to 29%.

Table 4. Comparison of "manual review" counts and automated counts of *Rhodomonas salina* and *Tetraselmis suecica* monocultures (high concentration level scan) and natural algae (presented with and without colony forming algae) and their corresponding percentage-wise deviations from the automated approach.

	"Manual review"	Automated approach	% deviation
Rhodomonas salina	1118	1138	1.8
Tetraselmis suecica	1214	1219	0.4
Nat. algae (+ colony forming species)	217	150	45
Nat. algae (– colony forming species)	172	150	15

An example of identification of algal cells based on the recognition criteria fed into the image analysis software is presented in Fig. 11. In the example with *R. salina*, Fig. 11a shows 11 potential fluorescent algal cells. Based on the recognition criteria the image analysis software identified eight of these to be algal cells and three did not fulfil the criteria and were thereby not categorized as algae (Fig. 11b).

During the “manual review”, eight cells were identified and thereby in agreement with the recognition criteria. In the example with *T. suecica*, Fig. 11c shows 11 potential algal cells. In the “manual review”, nine cells were identified whereas the image analysis software identified 10 cells (Fig. 11d).

Effects of acetic acid on immobilization of algal cells

Acetic acid treated *T. suecica* showed no significant differences between the two automated counts and the manual count (Fig. 12). Moreover, similar counts were obtained in non-treated samples when using the modified version of a Sedgewick Rafter counting chamber (250 µl volume). Significantly lower ($P<0.05$) manual counts of non-treated *T. suecica* cells were obtained using a standard Sedgewick Rafter counting chamber.

Manual counts of acetic acid treated *R. salina* were not significantly different from any of the non-treated counts. Automated counts using 4× and 10× magnification objectives were both significantly lower than the other counts.

Discussion

A number of automated analysis methods for the estimation of living phytoplankton are presently available. These methods include among others pulse-amplitude modulated fluorometry (PAM), ATP assays, FDA bulk analysis assays and flow cytometry (Casas-Monroy et al. 2016; van Slooten et al. 2015; Wright et al. 2015; Peperzak et al. 2018; Outinen and Lehtiniemi 2017). The instruments for these methods are often simple to use and are designed to be handled by untrained personnel to obtain quick indicative analyses of treated BW. However, these methods only provide bulk estimates of organism concentrations. They are not directly applicable to estimate compliance with BW discharge standards regulations that are specifically related to numerical concentrations below a specified limit in defined size classes. Other automated approaches have been suggested but most of the methods provide bulk analysis results and not a numeric outcome as required by the regulations. To our knowledge only two automated methods are able to provide numeric outcomes: flow cytometry and FlowCAM (combination of flow cytometer and imaging microscopy). Studies performed on the use of these methods have highlighted challenges for the size class 10-50 µm which include accurate counting of colony forming algal species as well as clogging and sedimentation issues in the flow tube (Reavie et al. 2010; Peperzak et al. 2018; Romero-Martínez et al. 2017; Kydd et al. 2018). In flow cytometry no images are recorded for potential post-analysis and it only provides a numeric outcome. This feature is possible in FlowCAM, but often this process is time consuming and increases overall analysis time (Reavie et al. 2010).

Therefore, to fulfill the numerical and size class requirements as well as more accurately quantify colony forming individuals the VS method is currently the most suitable method available. It is not as fast as indicative analyses but is nevertheless able to provide results within hours which is significantly faster than regrowth assays as the MPN assay which typically take weeks. In addition, for ships to be allowed to enter US waters, the VS method is currently the only assessment method approved by the USCG for evaluation of BWTS performance. In the present study we have addressed some of the above-mentioned quantification quality issues (fatigue, subjectivity, size identification, high algal mobility, low counting rates) linked to the VS method by applying an automated cell quantification approach in combination with a filter technique for preparing the sample.

Performance of manual and automated counting approaches

Experiments were initially performed on 6 µm autofluorescent beads (Fig. 2). The aim was to explore if a 4× magnification objective could perform as well as a 10× magnification objective in identifying and quantifying beads of a size which was just below the lower limit of the 10-50 µm size class. The field of view is larger when using a 4× compared to a 10× magnification objective which reduces the number of images needed to scan a full sample thus reducing counting rate as well as digital storage space. The results showed equal accuracy at all concentration levels as well as between manual and automated counts (Fig. 2). This implied that the automated approach using a 4× magnification objective was equally accurate at low and high bead concentration levels and was also able to identify fluorescent beads down to 6 µm (Fig. 2).

The choice to use small sized *R. salina* was to explore the effect of cell size on quantification quality. The data showed that algal species in the 8-9 µm range became more difficult to count using the manual approach because the algal cells could be confused with autofluorescent debris which typically was in the 6-7 µm size range (Fig. 5). Consequently, quantification accuracy was lowered. This was especially significant at concentration levels of >1000 org. ml^{-1}, where a deviation of 17% was observed. The likely explanation was that autofluorescent debris was also included in counts, resulting in more conservative (ecologically safer) counts. The opposite – and ecologically worse – situation could also occur depending on the criteria set by the microscopist counting the sample. If the cognitive visual criteria set by the microscopist vary or drift during the counting process, combined with the time pressure for efficient counting, results will suffer from inaccuracy and thus lower quantification quality. The presence of a high detrital load in samples has previously been shown to decrease counting accuracy as well as counting rates during a study where the recovery success of 50 µm microbeads added to natural plankton water samples was investigated (First and Drake 2012).

The definition of the size class 10-50 µm in the regulations has been addressed and criticized in several studies (van der Star et al. 2011; Gollasch et al. 2007; Gollasch et al. 2012; Liebich 2013; Casas-Monroy et al. 2016; Lundgreen et al. 2018; Liu et al. 2016). No studies have, to the authors knowledge, shown that species <10 µm are less robust towards treatments and should therefore be considered potential invasion risks. A large proportion of all known algal species are <10 µm which includes several toxic or nuisance species such as *Pseudochattonella verruculosa*, *Microcystis viridis*, *Skeletonema* sp., *Thalassiosira* sp. and *Phaeocystis* sp.. To better protect aquatic environment from invasions by phytoplankton it would be rational to include the species <10 µm in the validation procedures of BWTSs. However, the results obtained on *R. salina* suggest that manual counts of natural test water which is a mix of all cell sizes will become more inaccurate when <10 µm cells are included. Consequently, there is a risk of overestimating cell numbers due to the inclusion of autofluorescent debris (false positives) which could result in BWTSs unfairly failing in type approval tests. Similar observations were encountered by Peperzak et al. (2018) who experienced difficulties in distinguishing between 2 µm *Micromonas pusilla* cells and other small particles using brightfield microscopy. The automated approach examined in this study was more accurate in enumerating *R. salina* and separating them from autofluorescent debris compared to the manual approach (Table 4 and Fig. 5). Even though *R. salina* cells were only slightly larger than fluorescent debris the difference was still large enough for the recognition parameters to efficiently distinguish *R. salina* cells from the

debris. Correct identification by the automated approach was confirmed by a manual review of one of the automated analyses where only a 1.8% deviation between counts was observed (Table 4). If smaller algal species compared to *R. salina* had been examined (less than around 8 µm) using the manual approach, then the accuracy of the results would decrease as the distinction between living algal cells and debris would become too blurred and too difficult to identify. However, Peperzak et al. (2018) reported in their study that flow cytometry is capable of accurately enumerating cultured cells down to 2 µm. To efficiently exclude autofluorescent debris in the analyses, the MPN method is the most suitable method available as the regrowth assay does not distinguish between cell sizes and will effectively include phytoplankton species <10 µm. Another approach to minimize the impact of autofluorescent debris could be to investigate other fluorescent stains than FDA/CMFDA.

The equal deviations observed for both manual and automated counts indicate that the identification criteria remained constant in all counts for both counting approaches and suggest that the deviations could be explained by variations in sample concentrations. Furthermore, the relatively small variation between results suggest that both approaches were reliable counting methods for *R. salina*.

For *T. suecica* the biggest challenge was the mobility which significantly affects the accuracy of the manual method. The highly mobile *T. suecica* cells were difficult to locate in the water column of the Sedgewick Rafter counting chamber when counted manually. This was especially evident at the high concentration level because several cells simultaneously moved around in the X and Y plane of the field of view as well as in and out of focus (Z plane) (Fig. 5). Consequently, cells were overlooked resulting in a lower quantification quality. The filter technique used in the automated approach gently forces the highly mobile *T. suecica* cells into a 2D plane which significantly improved the quantification accuracy. Using the filter technique to immobilize the *T. suecica* cells most likely explains the significantly higher *T. suecica* cell counts observed in the automated approach compared to the manual approach (Fig. 5). This hypothesis was further supported by the results obtained from the lowered Sedgewick Rafter counting chamber as well as the cells immobilized with acetic acid (Fig. 12). Thus, by using the filter technique a more accurate estimation of the number of cells present in the sample was obtained. As *T. suecica* is slightly bigger than *R. salina*, judgement of cell size and separation from autofluorescent debris became less of a challenge (Table 2). The results from the manual review of the automated approach on *T. suecica* likewise support the clearer distinction as there was little disagreement in the comparative analysis (0.4%, Table 4). The larger the algal cells the more accurately they can be distinguished from autofluorescent debris improving quantification accuracy. That larger cells increase enumeration accuracy is supported by a study by First and Drake (2012) where manual counts of 150 µm microbeads had a higher recovery than 50 µm microbeads with and without debris.

The larger deviations (17% for *R. salina* and 13% for *T. suecica*) between the mean manual and automated counts observed at the high concentration level (Fig. 5c) were caused by the manual counts suffering from a mix of identification issues, maintaining efficient counting rates and issues related to counting a volume (3D) compared to a surface (2D).

The analysis of organism composition of the natural samples showed high diversity in number of phyla (8) comprising of 29 algal and 2 ciliate species (Fig. 10). The natural samples were thus considered to be of high composition complexity to further challenge the performance of the

automated approach. The use of complex natural plankton samples is central to perfect a single technique and has been highlighted as one of the most important components to include in validation of techniques (Steinberg et al. 2012). For the natural seawater samples, good agreement between manual and automated (4×) counts was observed (Fig. 6). However, there was a 45% difference in cell numbers when performing the manual review of the automated counts (Table 4). This was largely caused by the automated approach not being able to correctly recognize and quantify individual cells of colony forming units which resulted in an underestimation of cell concentration in the automated approach. Furthermore, correct judgement of cell size in the manual approach was difficult because the dominating cell size for a large number of the species were close to the lower limit of the 10 µm. The underestimation of colony forming individuals by the automated approach was therefore probably counteracted by the conservative inclusion of too small cells in the manual approach which resulted in similar counts by the two counting approaches. The study by Peperzak et al. (2018) likewise highlighted that in living samples judgement of living (fluorescent intensity) cells as well as sizing are key issues encountered by microscopists. To overcome the challenge with colony forming individuals for the automatic counting, advanced machine learning should be investigated and implemented in future studies.

For all the comparative counting experiments, data was collected over a period of 8-10 hours (Fig. 3 and Fig. 4). The plots showed that time did not have an impact on the concentration level of the organisms throughout the sampling period. Any differences between manual and automated counts could thereby be attributed to other variables such as cell concentration level, composition complexity, cell size, and/or mobility behavior.

The colony forming species encountered in the natural sample consisted of *Pseudo-nitzschia* spp. (14.4%), *Skeletonema costatuum* (3.8%), *Leptocylindrus danicus* (2.5%) and *Thalassiosira nordenskioldii* (0.2%) (Fig. 10). In the manual approach it was possible to determine vitality and count the individuals of these colony forming species. The variations in staining intensity of the individual cells did however challenge correct identification of the vital individuals. In the automated approach, the recognition criteria used in the image analysis process could in most cases not distinguish individual cells within colonies. Particularly recognizing and separating close individual cells and handling varying staining intensities of colony forming individuals were the key issues. Variations in staining intensity have previously been reported as an issue when applying the VS method on natural phytoplankton populations and different algal cultures (Peperzak and Brussaard 2011; MacIntyre and Cullen 2016; Garvey et al. 2007). The diatoms *Pseudu-nitzschia* spp. and *Leptocylindrus danicus* were especially problematic, which resulted in an underestimation of cell concentrations in the automated counts. To be able to accurately count natural samples which regularly contain colony forming species then the image analysis procedure needs to be refined and could be solved by using a more advanced approaches such as other automatized quantification and species identification programs (PlanktoVision, ImageJ) (Schulze et al. 2011; Schulze et al. 2013). Similar issues are also encountered when using flow cytometry (Peperzak et al. 2018; Zhou et al. 2012; Christaki et al. 2011) and FlowCAM (Reavie et al. 2010; Camoying and Yñiguez 2016; Romero-Martínez et al. 2017).

To see if it was possible to obtain more accurate counts and increase quantification quality in the automated approach a scan using a 10× magnification objective was performed (Fig. 6).

However, the results were significantly lower than the both manual and automated (4×) counts. When using a 10× objective the depth of focus was reduced (see section 5.2 and Berek (1927)) which resulted in some algal cells being out of focus and being consequently missed by the image analysis software. This suggests that the filter technique does not force cells into a perfectly flat 2D plane. Instead, the distance between glass plate and membrane filter is sufficiently small for the 4× objective to capture all cells within its field of focus. On the other hand, the depth of focus for a 10× objective is too narrow and not able to cover the whole vertical column which results in some cells being out of focus. A solution could be to include image stacking in the z-plane, but such operation would increase data and analysis time dramatically.

Acetic acid was very efficient in immobilizing *T. suecica* cells (Fig. 12). Similar counts of non-treated cells were obtained using the lowered Sedgewick Rafter counting chamber, which suggests that cell membrane structure was not affected by the acetic acid. The observed difference in counts between non-treated *T. suecica* cells using a standard vs. a lowered Sedgewick Rafter counting chamber suggest that reducing the height of the water column significantly improves counting accuracy of highly mobile algal species. The pH sensitivity of *R. salina* was different from *T. suecica*. Acetic acid treated *R. salina* clearly showed changes in membrane structure and decreased fluorescent intensity which resulted in cells being more difficult to identify. Here, the automated approach could not accurately recognize acetic acid treated cells using any of the magnifications. Even though manual counts of acetic acid treated cells were not significantly different from non-treated counts they had become more difficult to visually identify. Using acetic acid as an immobilization agent seems to be highly efficient for quantification of some algal species but others. However, our results also show that the pH sensitivity of algal species differ and the use of acetic acid compromises counting accuracy. The potential use of acetic acid to immobilize highly mobile species in natural algal populations and to improve counting performance is therefore not a recommended approach.

Counting times

For both monoculture species the automated counting times at all concentration levels were similar with rates around 5 min ml^{-1} (Fig. 7). Since the HCS-Platform performs the same scanning and quantification procedure for each sample no variation was expected. The small observed variation was caused by internal data storage limitations in the software that after a number of analyses had to be restarted to clear the data storage memory. At all concentration levels and for both species, manual counting times were significantly slower than automated counting times (Fig. 7). The slower counting times in the manual approach can mainly be explained by two factors. First, a 10× objective must be used for more reliable identification of cells. The field of view area is thereby smaller compared to the automated approach (4.9 vs. 9 mm^2) where a 4× objective is sufficient for the software to accurately identify cells (Table 1). Second, a Sedgewick Rafter counting chamber has a water column height of 1000 µm. Consequently, a vertical scan at each field of view must be performed to be certain to locate all the 10-50 µm cells that are potentially present anywhere in the water column. The microscope settings for the manual approach gives a depth of focus of 14.4 µm (calculated from the formula in Berek 1927) which means that at any given time only a small horizontal slice of the vertical column is in focus. In theory this means that about 70 horizontal slices must be checked in each field of view to cover 1000 µm. However, the vertical range for each field of view can typically be covered

in a few seconds by moving the stage up or down. It does not seem as a major time-consuming process but overall it adds up when scanning the whole volume of the counting chamber. In the automated approach, the filter technique produced a much larger surface area ($A_{filter} = \pi \times r^2 = \pi \times (½ \times 47\ mm)^2 = 1735\ mm^2$, hence 3470 mm^2 for the two scanned filters) which needed to be scanned (Table 1). On the other hand, no vertical scans were needed. By applying the filter technique in parallel with the use of a 4× objective a larger surface area could be scanned faster compared to the manual approach. To obtain faster counting times the smallest possible magnification for identifying target organisms must be used to obtain as large a field of view as possible and to increase depth of focus as also highlighted by First and Drake (2012).

Manual counting times at the highest concentration level for both species were significantly slower than at low and medium concentration levels (Fig. 7). The reason is that for both algal species, more cells had to be identified and counted in the whole water column of the field of view before scanning onwards. In addition, for the samples containing *R. salina* it became more difficult to distinguish between fluorescent algal cells and autofluorescent debris (abiotic material) of similar size and more time was required for correct identification. As *T. suecica* was slightly bigger than *R. salina*, algal cells were easier to distinguish from autofluorescent debris and therefore this was less of an issue during manual counts of *T. suecica*. On the other hand, it was more difficult to keep track of the increased number of highly mobile 3D moving *T. suecica* which caused an increase in the time used for more accurate quantification. The observed slower counting times at the high concentration levels in the present study was likewise observed by First and Drake (2012) in their experiment using microbeads. High concentration levels can thus affect counting performance because there is a risk of underestimating (or in our case also overestimating) concentrations of cells (First and Drake 2012).

Based on the counting times obtained on monocultures from the present study, the estimated time needed to count the total volume of VS samples for the size group 10-50 µm in a full land-based test (Table 3) could be calculated. Our data showed that the automated approach potentially will save a testing facility approximately 15 hours of counting time per land-based test. The counting times obtained from DHI for different concentration levels and salinities were comparable to the manual counting times of monocultures and natural algae in the present study. Using the data from DHI it was estimated that a full land-based test would take 31.2 hours versus 33.4 hours in this study (Table 3). In addition, the statistical analysis suggests that FW samples takes more time to count with increasing concentration compared to BrW and SW samples which is supported by statements from trained personnel on their experiences with sample counts (personal communication).

For natural seawater samples, significantly higher counting times were likewise recorded for manual counts compared to the automated counts using a 4× magnification objective (Fig. 8). Manual counting times were about three minutes slower when counting natural seawater compared to monocultures. The slower counting times are partly explained by the presence of more autofluorescent debris. In addition, counting times were affected by the large variety in size and shape of the numerous species present in the natural seawater sample. It takes more time to identify if a cell fulfills the criteria or not.

For the natural seawater samples, an automated scan using a 10× magnification objective was additionally performed (Fig. 8). The field of view area for this approach was only 1 $µm^2$ and 1131 images were needed to cover the scanning area of just one circle on the glass plate (Fig. 1).

Therefore, the scanning time for 1 ml was >3 times higher compared to using a 4× magnification objective. If using a larger magnification (i.e. 10×) had improved quantification accuracy considerably then the tradeoff of a slower counting time may be considered as an overall improvement of quantification quality.

Other methods tested to examine fixation of mobile algal species

Immobilization or fixation of highly mobile algal species can significantly reduce fatigue of the microscopist, decrease counting time, and increase identification accuracy that overall can improve quality of the quantification procedures. In the present study, different approaches were tested for immobilization of algal cells without directly or indirectly decreasing the health status of the cells which would interfere with the evaluation of BWTS performances. Flocculating agents have the ability to bind to organic matter (cells) to induce sedimentation of cells which for example have been used in harvesting procedures of microalgae rich in lipids used for biofuel production (Hu et al. 2008). The technique could likewise be useful to sediment BW samples although flocculation has been reported not to be 100% efficient and that efficiency differs among species (Ummalyma et al. 2017; Lertsutthiwong et al. 2009; Gerchman et al. 2017). Apart from flocculation, the present study also examined other different immobilization techniques such as gel entrapment and coating surfaces with cell-binding agents to find the most suitable method for algal immobilization (Appendix B). The performed pilot experiments on immobilization techniques in Appendix B showed that all the investigated techniques were unsuitable for algal immobilization. However, the filter technique which was developed proved to be the most suitable and reliable technique for algal viability assessment and so this technique was applied in the present study (3.2.2).

Conclusions

The described automated method could be a remedy to overcome the main challenges experienced in microscopy and flow cytometry. In the present study the automated method was tested using the VS method and adapted to the strengths and limitations of the FDA/CMFDA stains. Manual microscopy is slow which can affect both the vitality and fluorescent signal, making it vulnerable to subjectivity and fatigue among microscopists. The counting times of the automated approach were up to three times faster than the manual approach which significantly reduces the overall time of land-based tests. The automated approach provides fast and objective counts with the possibility of *post hoc* examination of the images, while flow cytometry has no visual checks which can lead to the inclusion of inanimate green auto-fluorescent particles in the counts (Tang and Dobbs 2007). Furthermore, the automated approach could, like the FlowCAM be adapted for algal monitoring in connection to HABs by applying specific probes of different colored fluorophores for toxic/non-toxic species identification.

The automated approach in combination with the filter technique was able to immobilize highly mobile species such as *T. suecica* to accurately identify samples at concentration levels from around 10 to more than 1000 cells ml^{-1}. Estimations of natural algal concentrations were similar for the automated and manual approach of cells within the 10-50 µm range, but the presence of colony forming algal species reduced the quantification accuracy of the automated approach because the image analysis was challenged by identification of individual colony units. The quantification

accuracy of both approaches were reduced when counting cultured algal cells as well as natural phytoplankton species around or below 10 µm because the presence of autofluorescent debris interfered with accurate identification of cells.

The future development of more efficient stains – that might even be able to target and include DNA damaged cells – to replace or supplement FDA/CMFDA would further improve the automated method. In combination with the rapid developments within image analyses and machine learning, the post-analysis option could be improved further by applying machine learning for more accurate quantification (Kruk et al. 2017; Orenstein and Beijbom 2017). Therefore, we believe that this method could potentially become a strong and reliable tool for viability assessment of phytoplankton used for validation of BWTS.

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Supporting Information

Supporting Information A

Conversion of Heinz vinegar to acetic acid

For immobilizing *Tetraselmis* cells, Steinberg et al. (2012) added "…*two drops of acetic acid (Heinz vinegar) to 10 ml aliquots of the sample*". We converted this into a more "laboratory friendly" procedure based on a mix of test water (20 psu) and 0.5 M acetic acid.

We determined the pH to be ~5.0 after adding two drops of vinegar to 10 ml test water which resulted in efficient immobilization of *Tetraselmis suecica*. To convert this finding into a procedure using 0.5 M acetic acid, we calculated the volume of 0.5 M acetic acid needed to be added to 10 ml test water to obtain a similar pH of 5.0. The calculation was based on a titration experiment where the pH continuously was recorded while stepwise adding 0.5 M acetic acid to 50 ml test water (Fig. 13). From the equation of the polynomial trendline obtained in Fig. 13 the exact volume to obtain a pH of 5.0 could be calculated for 50 ml and 10 ml test water. A volume of 57 µl of 0.5 M acetic acid had to be added to 10 ml test water to obtain a pH of 5.0 which corresponds to 2.85 mM acetic acid + test water (2.85 mM AA water).

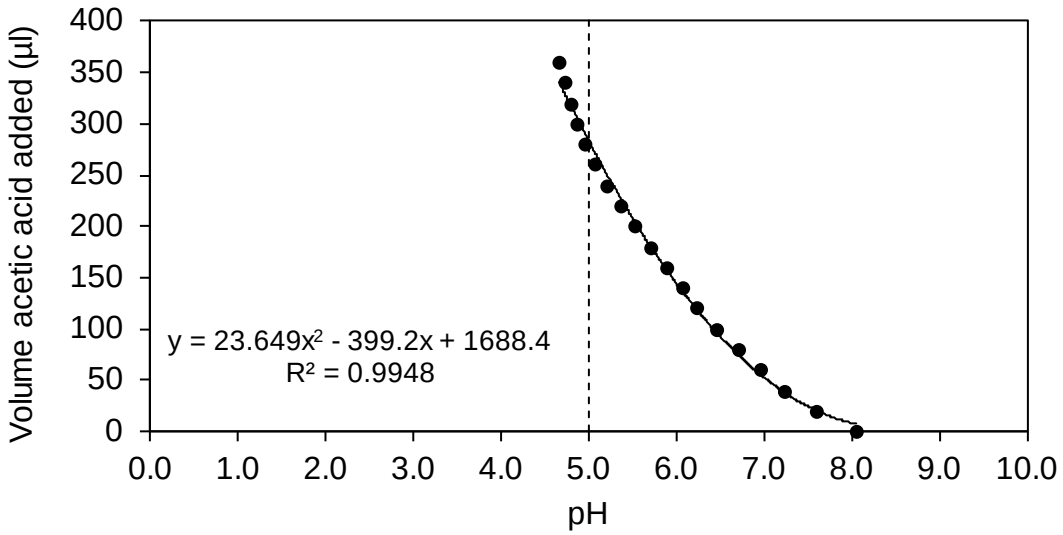


Fig. 13. Titration experiment showing the decrease in pH in 50 ml test water while adding 0.5 M acetic acid.

Supporting Information B

Cell immobilization techniques

A number of pilot experiments examined different cell immobilization techniques based on the available literature. The purpose was to find the best suitable approach for immobilization of algal cells to increase quantification accuracy of viability assessment methods used for validation of ballast water treatment systems.

Flocculant agent: Polydiallyldimethylammonium chloride (PDADMAC)

The addition of the cationic polymer polydiallyldimethylammonium chloride (PDADMAC, 20% w/v solution in H_2O, Sigma–Aldrich, CAS#26062-79-3) is a common procedure for the harvesting of oil-accumulating microalgal species to be used as feedstock for biodiesel production (Hu et al. 2008). A study by Gerchman et al. (2017) investigated the flocculation properties of PDADMAC, chitosan and Superfloc® and found PDADMAC to be the most efficient flocculant for sedimentation of two algal species. The green microalgae *Chlorella vulgaris* showed a 90% flocculation success within 20 min while *Nannochloropsis salina* showed a 25% flocculation success within 4 h. Based on this study a pilot experiment was performed to examine the flocculation properties of PDADMAC on *Tetraselmis suecica*. A vital stained sample of *T. suecica* was added PDADMAC to a final concentration of 10 mg l^{-1} which was within the range used by Gerchman et al. (2017). Stained samples±PDADMAC were added to a Sedgewick Rafter counting chamber to observe if the number of algal cells on the bottom of the counting chamber increased over time due to flocculation. In addition, if bottom counts differed from control samples without PDADMAC. No effects of PDADMAC on the sedimentation rate of *T. suecica* was observed and cells were present in the whole water column over a time period of 15-20 min. The flocculation agent was concluded not to be applicable for immobilization of highly mobile algal species. Based on these preliminary results, the results of Gerchman et al. (2017) as well as other inconclusive studies using chitosan as flocculation agent (Lertsutthiwong et al. 2009; Moreno-Garrido 2008) further experiments with chitosan and Superfloc® were not carried out.

Flocculant agent: KlarVit

The flocculation properties of KlarVit (VinoFerm) on algal cells was tested. KlarVit is a two-compound product consisting of Kieselsol (A) and Gelatine (B) commonly used in in the clarification process of wine, beer and juices to sediment solids present in the liquids. KlarVit was tested at recommended dose (ratio: 1/4000) for flocculation efficiency on *T. suecica* and compared to non-treated samples. The experiment was next repeated at 8, 20 and 100 times recommended dose. Experiments were performed in 96 well plates. 1 ml of test water with *T. suecica* was added the 2 components of KlarVit according to the prescribed procedure and next stained with CMFDA/FDA. Samples were kept in the dark for 10 min and then 100 µl were transferred to 96 well plate for analysis. The water column height in the well was approximately 3 mm. Location of algae cells in the water column was analyzed for every 5 minutes up to 20 min by screening the water column from bottom until highest occurring algae. In all experiments *T. suecica* cells were observed 2000 µm above the bottom of the well and were observed to be evenly distributed in the water column

throughout the observation period (15-20 min). Thus, KlarVit showed no effect on the sedimentation/flocculation of *T. suecica* cells.

Gel entrapment methods: alginate

One of the most widely used natural polysaccharide gels for entrapment of living cells is alginate which can be extracted from different species of brown algae (Moreno-Garrido 2008). The use of alginate for immobilization is advantageous as it provides a neutral physical-chemical and transparent environment for the cells (De Araújo and Santana 1996). The polymer is soluble in cold water and is able to quickly form a thermostable gel (Moreno-Garrido 2008). Alginate does not dissolve properly in seawater. However, Hertzberg and Jensen (1989) developed a protocol which enabled the mixing of alginate in seawater to immobilize seven marine microalgal species. A 2.3% alginate solution (Pane et al. 1998) was prepared in 10 ml test tubes by adding 228 mg alginate acid sodium salt (Sigma Aldrich, CAS#9005-38-3) to 10 ml salt water solution (5 psu). The alginate solution was kept at 1 rpm on a rotation device to mix and remove air bubbles from the solution for at least 24 h. The immobilization property of the alginate solution was tested by mixing 900 µl alginate solution with 100 µl vital stained *Rhodomonas salina* or *Tetraselmis suecica* which was then transferred to a Sedgewick rafter counting chamber. The alginate solution proved unsuitable for immobilization of *T. suecica* as the highly mobile *T. suecica* cells were able to find narrow water channels within the alginate gel which affected quantification accuracy. Furthermore, apart from X-Y scans of the Sedgewick Rafter counting chamber a high number a Z-stacking images were needed to cover the whole water column of the counting chamber. Based on the pilot experiments alginate did not seem suitable to use as immobilization approach for improving quantification accuracy.

Chemical attachment: Polymer coated surfaces for enhancing cell-binding capacity

Polymer covered well plate bottoms have cell-binding properties (Thermo Scientific 2010) and therefore Nunc™ MicroWell™ 96-Well Optical-Bottom Plates with Polymer Base (ThermoFisher, CAS#152037) were tested. The well plates were designed for fluorescence experiments. The bottom of the wells was covered with a polymer base (Poly-D-Lysine) which facilitates the binding of cells to the surface. For testing the ability of the polymer base to capture mobile algal cells, 50 µl of algal suspension with approximately 2000 cells ml^{-1} of *Rhodomonas salina* or *Tetraselmis suecica* were added to wells. The final height of the water column was 1.5 mm. There was no enhanced attachment to the polymer base covered bottom in neither of the two species. Cells continued to move around in the whole water column and time did not have any effect on attachment. Even cells at the bottom in direct contact with the polymer base could freely move around.

Other potential immobilization techniques (not yet tested)

- Corning® Cell-Tak Cell and Tissue Adhesive, 1mg (Product #354240)
- Poly-L-Lysine, 0.1% w/v in water (Sigma-Aldrich, CAS#25988-63-0)

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Manuscript 4

Installation and use of ballast water treatment systems – implications for compliance and enforcement

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Installation and use of ballast water treatment systems – implications for compliance and enforcement

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Abstract

When the International Maritime Organization (IMO) adopted the International Ballast Water Management (BWM) Standards in 2004, the organization proposed two management options: ballast water exchange (BWE) and ballast water performance standard (D-2) which require the use of ballast water treatment systems (BWTSs). Non-existent when the convention was adopted, the BWTS manufacturers developed diverse technologies to achieve the outlined standards (D-2 standard). The BWM Convention (MEPC 69/4/4) entered into force in September 2017, and required the use of BWTSs within an outlined timetable.

This study explores the spread of BWTSs by examining IMO Type Approval records from 2007 to 2018 and country-level databases in the United States and Australia from November 2016 through October 2017 with the goal of understanding the real-world impact of policy on the installation and utilization of BWTSs. The IMO Type Approval records revealed that 65 BWTSs using seven different treatment processes were approved. Notably, only 13 BWTSs using two treatment processes (filtration & UV; electrolytic) are currently approved by the USCG. Electrolytic and filtration & UV treatment processes are used to treat the vast majority of ballast water treated with BWTS arriving to both countries examined in this study (Australia, 84%; USA, 89%). In addition, the implementation of the BWM Convention in September 2017 appears to have increased utilization of BWTSs in both countries. From November 2016 to October 2017, there was an increase in the percentage of vessels fitted with BWTS, vessels fitted with a BWTS utilizing the system, and total ballast water discharge treated with BWTS in both Australia and the United States.

Keywords

Ballast water, ballast water treatment system, ballast water management, maritime regulation, maritime policy

1. Introduction

1.1. Evolution of ballast water management

Despite being evoked during the conference to adopt the MARPOL (1973), the risk of introduction of non-indigenous species through ballast water and sediments was introduced at the International Maritime Organization (IMO) for the first time in 1988 (GEF-UNDP-IMO GloBallast Partnerships and IOI, 2009). Within the same periods, countries such as Canada (1989) and the United States of America (1990) developed voluntary ballast water management (BWM) requirements which became mandatory for vessels entering their waters. In 1993, the United States developed mandatory regulations requiring ballast water exchange (BWE) for vessels destined for Great Lakes and, in 1997, Vancouver Port Authority imposed BWE (Scriven et al., 2015). Among other countries developing national legislations (e.g. Chile, Israel, New-Zealand), Australia introduced voluntary ballast water management arrangement in 1991 to face increasing bio-invasions. To strengthen its control on ballast water discharge, Australia developed the same year as Brazil (2001) mandatory requirements enforceable under the Quarantine Act 1908 (Bax et al., 2003; Department of Agriculture and Water Resources, 2017).

To avoid the multiplication of uncoordinated national requirements, the IMO developed *International Guidelines for Preventing the Introduction of Unwanted Aquatic Organisms and Pathogens from Ships' Ballast Water and Sediment Discharges* (1991) which in 1997 became the *Guidelines for control and management of ships' ballast water to minimize the transfer of harmful aquatic organisms and pathogens (*IMO Assembly Resolution A.868(20)). Insufficient to respond to the growing threat on marine ecosystems and pushed by member States affected by Harmful Aquatic Organism and Pathogens (HAOP), the IMO prepared a binding agreement (David and Gollasch, 2014). Adopted as a risk mitigation instrument in 2004, the *International Convention for the Control and Management of Ships' Ballast Water and Sediments* (Ballast Water Management Convention or BWM Convention) recognized two management standards:

1) standard D-1 on ballast water exchange (considered as an interim solution to phase-out when technology options became available) requires vessels to exchange their ballast water uploaded in coastal area for ballast water taken in open ocean ("whenever possible […] 200 nautical miles from the nearest land and in water at least 200 meters in depth […]" Regulation B-4); and

2) standard D-2 on ballast water performance establishes water quality standards which can be reached after processing the water trough approved ballast water treatment systems (BWTSs) (Lloyd's Register, 2010).

From the entry into force of the Convention to 08 September 2024, the vessels have to comply to D-2 Standard as D-1 standard will phase out. After a difficult journey towards ratification, the BWM Convention entered into force in September 2017.

Indeed, urged by the IMO Secretary General (2012) and member states pressure to develop national requirements, the International Chamber of Shipping (main shipowners' trade organization) finally decided in 2014 to stop discouraging the ratification of the BWM Convention.

For shipowners, compliance with the BWM Convention standards D-1 or D-2 signifies additional operation and capital expenses (International Chamber of Shipping, 2014; Sekimizu, 2012). In this respect, D-2 will eventually require the use of BWTSs, which imply costs for installation and maintenance and operation.

Shipowner trade organizations primarily justified their opposition by pointing towards the lack of robustness in the equipment and approval process. However, the IMO final report on the implementation of the ballast water performance standard described in regulation D-2 of the BWM Convention (MEPC 69/4/4) highlighted that only a limited number of BWTSs were operated at the time of the study in 2015 (prior to the Convention entering into force) and there was no evidence to support shipping industry claims. However, facing skeptic comments from industry lobbies about treatment technology reliability, the Maritime Environment Protection Committee resolution MEPC.290(71) (2017) introduced "the experience-building phase associated with the BWM Convention" with intention of collecting information about various equipment related to D-2 and their functionality in real conditions. It will be an invaluable tool to improve, assess, and rank the various technologies available on the market (Gallagher, 2018a).

1.2. Ballast water treatment systems

Ballast water treatment systems (BWTS) are a possible solution to help mitigate the spread of aquatic invasive species transported via vessel ballast water. The BWM Convention states that all vessels discharging ballast water must apply a BWTS that has been Type Approved in accordance with current regulations (IMO, 2004). The performance of BWTSs must comply with certain discharge standards related to the number of viable organisms in defined size classes as outlined in IMO Regulation D-2 (IMO, 2004) (Table 1).

Table 1. Overview of IMO discharge standards (Regulation D-2) for organism in defined size classes (IMO, 2004).

Size class	Organism type	Discharge standard
<10 µm	Toxicogenic *Vibrio cholera*	<1 cfu 100 ml^{-1}
	Enterococci coli	<250 cfu 100 ml^{-1}
	Intestinal *Enterococci coli*	<100 cfu 100 ml^{-1}
≥10-<50 µm	Plankton	<10 org. ml^{-1}
>50 µm	Plankton	<10 org. m^{-3}

Ballast water treatment systems are designed to reduce the concentration of organisms in ballast water to considerable smaller fractions and are promising in significantly reducing the risk of spreading potential invasive species.

Different treatment techniques are used for the removal of organisms in ballast water. The Table 2 lists the different technology approved and currently available on the market (December 2018). Additional details on treatment processes (except filtration) are presented in Appendix A.

Table 2. Ballast water treatment system modes of operation and the number of each treatment type approved (US Coast Guard 2018, www.docs.imo.org 2018). Updated 07 December 2018

Treatment type	Mode of operation	IMO approved	USCG approved
Non-active substances systems		*35*	*3*
Filtration & UV	Pre-treatment using filtration followed by UV during ballasting and UV only during de-ballasting.	30	3
Filtration	Initial coarse filter followed by membrane filter at both ballasting and de-ballasting.	1	
Deoxygenation	Creation of a hypoxic environment by displacing oxygen with inert nitrogen through bubbling into the BW.	3	
Heat+deoxygenation	The combined use of deoxygenation and a pasteurization unit based on waste heat (<40°C) from the ship.	1	
Active substances systems		*30*	*10*
Electrolytic	An electrical generated current produces a disinfection solution of sodium hypochlorite based on the NaCl present in the seawater.	15	10
Advanced oxidation	Biocides that are strong oxidizing agents are added to the ballast water to produce free radicals.	11	
Ozone	A strong oxidizing agent that is added to the ballast water generated onboard from the ambient air.	4	
TOTAL		**65**	**13**

1.3. Ballast water treatment system efficacy

Manufacturers of BWTSs are increasingly reticent to freely share data with scientists and regulators because of an uncertain regulatory environment and variability of testing protocols. Some manufacturers are concerned that different agencies, such as the United States Coast Guard (USCG) or individual State Lands Commissions, will impose increasingly stringent standards in the future, and they open themselves to problems by sharing data openly today (LoBiondo, 2011).

1.4. Ballast water exchange? Still widely used by vessels included in this study

Ballast Water Exchange (BWE) with mid-ocean waters during voyage and according to Guidelines G6 (Resolution MEPC.124(53)) has historically been used to decrease the likelihood of introducing HAOP from ballast water discharges because organisms from mid-ocean waters are less likely to colonize coastal environments (Moen, 2011; Saint Lawrence Seaway Development Corportation et al., 2012). Regulation D-1 on BWE standard requires a renewal of at least 95% of the water contained in the tank. The mid-ocean water has to be loaded according to regulation B-4 (at least 200 nautical miles - in all cases at least 50 nm from nearest land - and in water at least 200 m in depth). As recalled in regulation B-4.4 and G6, the conduct of BWE may jeopardize

vessel safety (loss of stability, overstress, sloshing loads, torsional loads, reduction in maneuverability, reduction in draughts, impacts on visibility, icing, occupational hazards and fatigue of crew, excessive forces on securing arrangements, etc.) (GEF-UNDP-IMO GloBallast Partnerships and IOI, 2009). While the biological efficiency of BWE has proven results when there are high salinity differences (e.g. discharge of mid-ocean water in fresh water environment such as in Great Lakes area), numerous limitations and uncertainties remain, including the impacts of a remaining 5% of coastal water (Isbester and Chao, 1993), non-homogeneous distribution of species in tanks (Gollasch et al., 2007), the risk of regrowth when uploading mid-ocean water, harmful algal blooms, cysts and proliferation of organism in sediments (Hallegraeff, 1998), uncertainties related to discharge of coastal water in open ocean, and risk related to discharge of mid-ocean water in coastal areas. Furthermore, BWE remains a time-consuming procedure, especially for large vessel types. It also mobilizes manpower and equipment and involves high operational costs in terms of energy consumption compared to BWTSs (GEF-UNDP-IMO GloBallast Partnerships and IOI, 2009; Vorkapic et al., 2018).Therefore, BWTSs provides solutions to overcome such limitations. Finally, it seems that in case in D-2 failure, BWE may be considered (on *ad hoc* basis) as a contingency measure by national administrations.

1.5. Type Approval procedures

Two main regulatory regimes are driving Type Approval of BWTSs used onboard vessels. The BWM Convention regulation D-3 stipulates that BWTSs must be tested according to the Guidelines developed by IMO (G8, now a Code, and G9). All systems must be tested under the G8 guidelines for approval of BWTSs. The Guidelines were recently revised (2016), have been promulgated as a Code, and are now mandatory in their entirety. Code for approval of ballast water management systems MEPC.300(72)-2018, which outlines land-based and ship-based testing protocols, takes effect on October 2019.

In addition to the BWMS Code (G8), the G9 Guidelines for approval of BWTSs that use active substances (e.g. electrolytic, advanced oxidation, ozonation) are to be applied in addition to normal testing procedures. The process of testing for active substances requires manufacturers to submit a report to the IMO requesting a basic approval prior to land-based and ship-board tests. The Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection (GESAMP) – Ballast Water Working Group on Active Substances or WG34 - supports the IMO to grant basic approval. The GESAMP WG34 assesses the risks created by the use of the active substances (to the crew, the vessel, and the environment) (GESAMP, 2019). Finally, the manufacturer provides a second dossier to the IMO after land-based and ship-based testing for Final Approval by GESAMP. Only after, the administration may deliver a Type Approval certification.

Though it is not common, some nations have their own approval process. For example, the United States regulatory framework of testing BWTSs is guided by the application of the *Generic Protocol for the Verification of Ballast Water Treatment Technology* (ETV protocol). Unlike for

IMO, the United States ETV protocol includes testing guidance for BWTSs using active substances and those which do not (NSF International, 2010).

Following the revision of the G8 Guidelines from IMO, both regimes are now relatively similar and can be considered compatible. The approval under the previous version of the G8 Guidelines is no longer permitted and the installation of systems approved under the old testing guidelines is soon to be forbidden. Yet, there is a difference between the regulatory regimes in the definition of viability. Viability under the United States rules refers to organisms that are alive at the time biological testing is performed. Viability under the IMO rules refers to organisms capable of reproducing. This has been especially difficult for BWTS manufacturers of UV-based treatment systems because the treatment is intended to stop the organisms from reproducing (Lundgreen et al., 2018). A recent bill passed through the United States Congress ("Commercial Vessel Incidental Discharge Act") may result in the acceptance of methods assessing regrowth of organisms for Type Approval thereby further aligning both regulatory regimes for the Type Approval of BWTS (House of Representatives, 2018).

1.6. Global adoption of different ballast water treatment systems

Previous research has explored the spread of BWTSs in the United States, but little is known about their global use (Gerhard and Gunsch, 2018). This study examines Australian ballast water reporting forms and the United States National Ballast Information Clearinghouse (NBIC) from November 2016 through October 2017. To our knowledge, the present study is the first to examine the situation of BWTSs operating in different global regions. Not engaged in discussing the robustness and efficiency of BWTSs, the paper intends to identify which systems are installed and used on vessels as well as to explain why some systems seem preferred than others with the goal of understanding the real-world impacts of ballast water management policies.

2. Methods

2.1. BWTS data acquisition and analysis

Information about the different BWTSs were obtained from official Type Approval Certificates of each BWTS accessed from the IMO website (https://docs.imo.org). From each Type Approval Certificate, the following information was obtained: name of system, use of active or non-active substances, IMO approval date, name of administration and country, treatment type, manufacturer, power consumption (if available), and country of origin. This study includes 65 systems with type approval after screening criteria were applied. Upgrades to existing systems were not included in this analysis. For example, Alfa Laval PureBallast 1.0, PureBallast 2.0, and PureBallast 3.0 were all included, but PureBallast 3.1 and PureBallast 3.2 were not included.

Supplementary power consumption estimates of the different treatment types were obtained from a report authored by Lloyd's Register (2012).

2.2. BWTS installation and usage data acquisition

Ballast water data for the United States and Australia was gathered for the time period from 1 November 2016 to 31 October 2017.

United States information was gathered via online accession of the National Ballast Information Clearinghouse (NBIC) (National Ballast Information Clearinghouse, 2017). All vessels arriving to the United States from outside United States Exclusive Economic Zone (EEZ) must submit a ballast water report. These forms are eventually made available to the public through the NBIC.

Australian data was acquired via a request to officials within the Australian Department of Agriculture & Water Resources. Any vessel-identifying details were removed from the Australian arrivals database by the Australian government prior to sharing the database.

2.3. BWTS installation and usage data analysis

The prevalence of BWTSs is reported by converting the records to unique arrivals during each segment of time (whether month or year). For the purpose of this study, unique arrivals were defined in the United States database by recording the number of unique IMO numbers within each time period of examination. In the Australian database, the deidentified data had arbitrary vessel IDs assigned during deidentification. Each ID was unique to a vessel, so multiple visits from a unique ID were removed, which left the number of unique vessel arrivals. This decreased the influence of vessels that made multiple arrivals in the statistics regarding BWTS installation and usage rates. Repeat arrivals were included in the analysis of ballast water discharge volume.

Several BWTS types did not have high installation or usage rates; therefore, they were combined to a category “Other”. These systems are: 1) Filtration, 2) Heat & Deoxygenation, 3) Deoxygenation, 4) Ozone, and 5) Advanced Oxidation.

Analysis of the ballast water reporting forms from Australia and the United States was performed in R using custom code. All visualizations were performed using the reshape2 and ggplot2 R packages (Wickham, 2018, 2009). All analyses were performed for each country except analysis regarding vessel types, which was not possible with the anonymized Australian database. Classical statistical tests are not appropriate on these databases, because the data is theoretically a complete record of activity in both countries during the study time period (November 2016 to October 2017). Statistical analyses are intended for analyzing samples; whereas, this study examines the entire population of vessels arriving during the given dates.

3. Results

3.1. BWTS types with IMO Final and Type Approval

From 2007 to 2017, a total of 65 treatment systems received Type Approval and were reported to the IMO. Our data show that the predominant treatment system was filtration & UV comprising 46% of approved treatment systems, followed by electrolytic and advanced oxidation treatment systems consisted of 25% and 15%, respectively (Figure 1, Left). The remaining systems including

filtration, heat & deoxygenation, deoxygenation, and ozone, which altogether made up 14% of approved systems (Figure 1, Left).

The first system was approved in 2007 followed by an increase in number of approved systems until 2013 with up to ten approvals per year (Figure 1, Right). From 2015 to 2017, only nine total systems were approved showing a decrease in number of approvals from five in 2015 to one in 2017 (Figure 1, Right). The approval of filtration & UV treatment systems increased gradually and dominated the number of approvals until 2013 (Figure 1, Right).

The approval of electrolytic, advanced oxidation, ozone, and deoxygenation treatment systems were spread almost evenly over the time period dominated by electrolytic treatment systems followed by advanced oxidation, ozone, and deoxygenation (Figure 1, Right). One filtration treatment system was approved in 2013 and likewise one heat & deoxygenation in 2015 (Figure 1, Right).

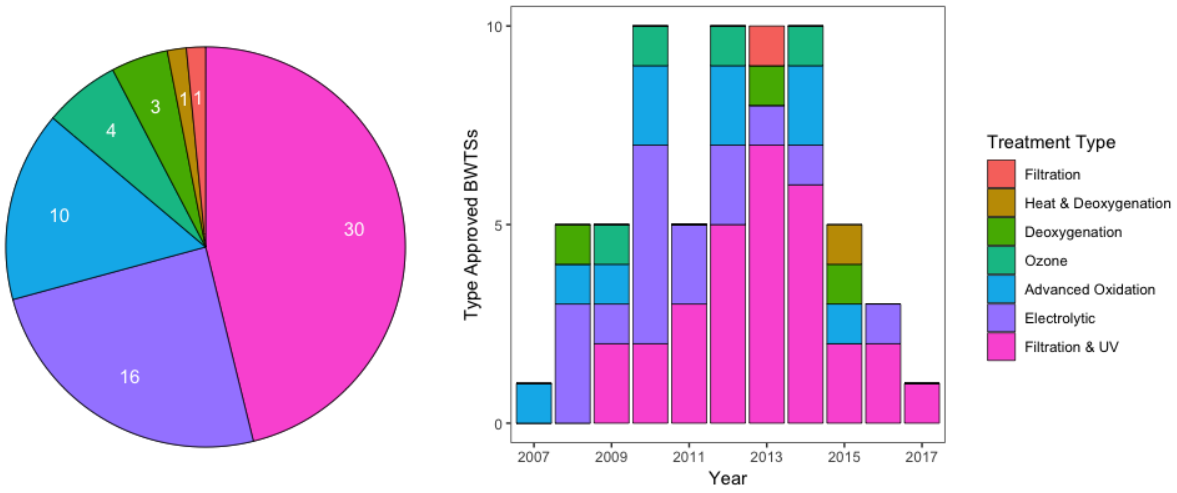


Figure 1. (Left) Pie chart showing the number of the different treatment types currently available on the BWTS market. (Right) Number of Type Approved BWTSs and treatment type distribution of each year from 2007 to 2017.

3.2. Fittings and usage for United States and Australia

In the period from November 2016 to October 2017, the United States and Australia both observed an increase in the percent of unique arriving vessels that were fitted with BWTSs (Figure 2, Left). The percent of unique vessels with a BWTS arriving in the United States increased from 9.2% to 17% and Australia increased from 11% to 14%. Both countries reported increases in the percent of fitted vessels using their installed BWTS on the voyage prior to arrival during this time period, with the United States increasing from 24% to 39% and Australia increasing from 5.3% to 24% (Figure 2, Center). Finally, both countries reported an increase in the percent of total ballast water discharge that was treated using a BWTS, with the percentage in the United States increasing from 3.7% to 12% and Australia increasing from 0.2% to 1.9% (Figure 2, Right).

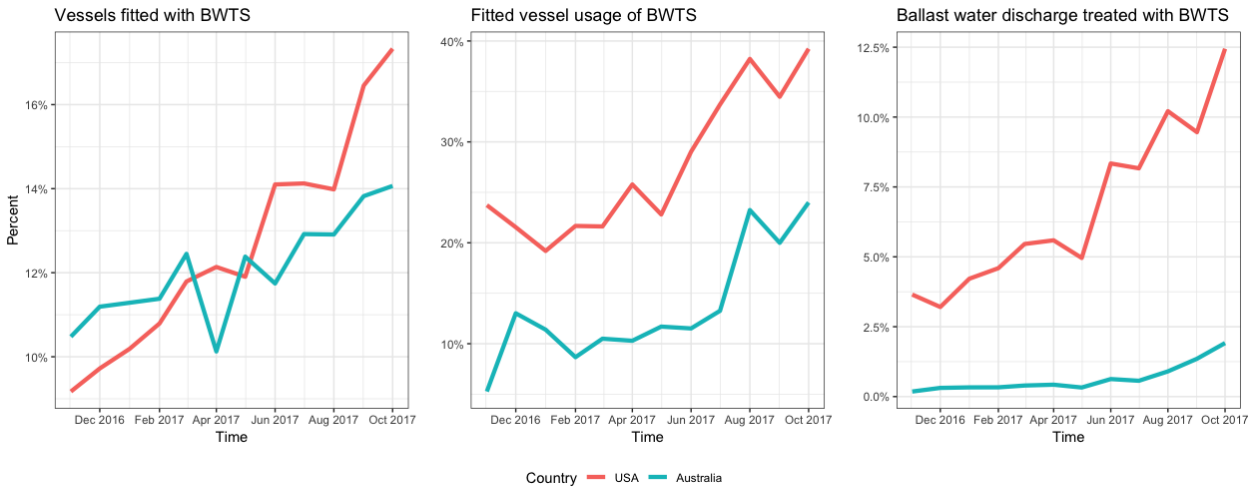


Figure 2. (Left) The monthly percent of vessel arrivals fitted with ballast water treatment systems. (Center) The monthly percent of vessels fitted with ballast water treatment systems that are using the systems to treat ballast. (Right) The monthly percent of total ballast water discharge treated using ballast water treatment systems. These data cover Australia and the United States from November 2016 to October 2017.

3.3. BWTS types used in USA and Australia

The total number of unique monthly arrivals with a BWTS installed increased in each country from November 2016 to October 2017 with Australia increasing from 114 to 200 and the United States increasing from 293 to 571 (Figure 3, Left). The BWTS treatment type in both countries was dominated by two systems: 1) Filtration & UV; and 2) Electrolytic. The remaining systems will be referred to as "other" BWTSs.

Australia experienced an increase in the number of unique monthly arrivals fitted with BWTSs utilizing filtration & UV (51 to 85), electrolytic (34 to 70), and all others (29 to 45). The percentage of each BWTS treatment type did not markedly change. The United States experienced a similar increase in BWTSs using filtration & UV (145 to 242), electrolytic (116 to 242), and all others (32 to 87). The percentage of each BWTS treatment type changed slightly with the largest decrease observed in filtration & UV systems (50% to 42%) and the largest increase observed in other treatment types (11% to 15%).

The number of unique monthly arrivals using BWTSs grew dramatically for both countries (Australia, 6 to 49; United States, 69 to 224) (Figure 3, Center). The growth was dominated by an increase in electrolytic (Australia, 2 to 22; United States, 33 to 116) and filtration & UV systems (Australia, 3 to 20; United States, 35 to 79). The composition of BWTSs used to treat ballast water changed from November 2016 to October 2017. Among treatment types arriving to the United States, the largest increase was observed in advanced oxidation systems (1.4% to 10%) and the largest decrease was observed in filtration & UV systems (51% to 35%). The percent changes in Australian systems was calculated starting in Dec 2016 (n = 19) because of a small sample size in November 2016 (n = 6). Among Australian unique monthly arrivals using BWTSs, the largest increase was observed in electrolytic systems (26% to 45%) and the largest decrease was observed in filtration & UV systems (74% to 41%).

Finally, the total volume of arriving ballast water designated for discharge that was treated using BWTSs was predominantly processed by either electrolytic or filtration & UV systems in both countries (Australia, 84%; USA, 89%). In addition, the amount of ballast water designated for discharge treated using BWTSs grew dramatically in both countries with an increase of over 790 thousand metric tons per month in Australia and an increase of over 3 million metric tons per month in the United States (Figure 3, Right). Despite this large increase, the composition of ballast water treatment types remained largely the same throughout the observed time period.

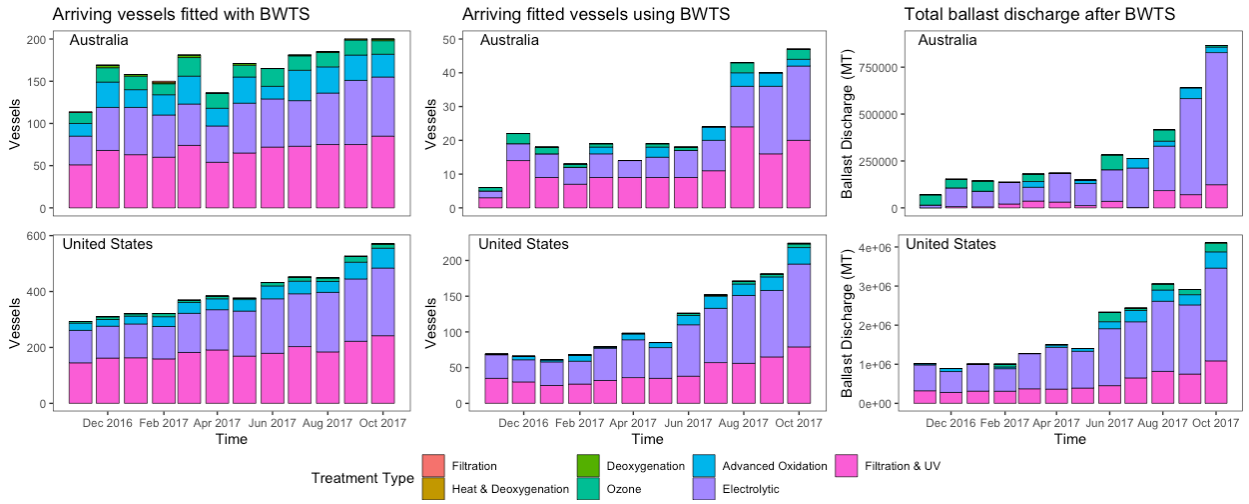


Figure 3. (Left) Unique monthly vessel arrivals fitted with ballast water treatment systems. (Center) Unique monthly vessel arrivals that use ballast water treatment systems on at least one voyage during the month. (Right) Monthly ballast water discharge treated using a ballast water treatment system. These data include Australia (top) and the United States (bottom) from November 2016 to October 2017.

3.4. Number of unique vessel types arrivals using BWTS

Analyzing vessel types was only possible using the United States database because information regarding vessel types was removed from the anonymized Australian database. The number of unique vessels with BWTSs to arrive in the United States from Nov 2016 to Dec 2017 was 626.

The percent of vessels using each BWTS type during the study time period was electrolytic (338 representing 54% of the total), filtration & UV (201, 32%), advanced oxidation (72, 12%), ozone (13, 2.1%), and deoxygenation (2, 0.3%). Bulkers and tankers (n = 560) comprised 90% of the unique vessel arrivals with a BWTS installed in the United States from Nov 2016 to Dec 2017 (Figure 4, Left).

The primary BWTS types for bulkers (n = 292) and tankers (n = 268) were electrolytic treatment (319, 57%) and filtration & UV treatment (159, 28%) (Figure 4, Right).

General cargo vessels (n = 24) present a similar proportion of BWTS types to tankers (Figure 4, Right). In container (n = 18), passenger (n = 7), and other vessel types (n = 14), the dominant treatment type was filtration & UV treatment (33, 85%) (Figure 4, Right).

For RoRo vessels (n = 3), treatment was split equally between filtration & UV, electrolytic, and advanced oxidation (Figure 4, Left). Unlike Australia, none of the vessels arriving in the

United States during the defined time period were fitted with a BWTS that relied on only filtration or deoxygenation & heat.

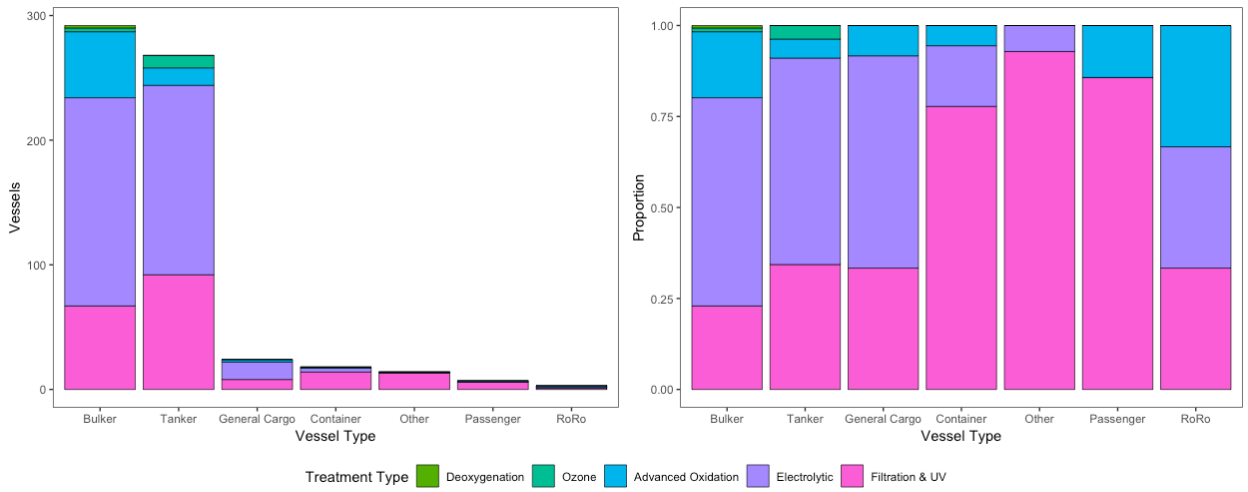


Figure 4. (Left) Number of unique vessel arrivals of each vessel type stratified by treatment type. (Right) Proportion of each vessel type outfitted with each treatment type. These data cover the United States from 1 November 2016 to 31 October 2017.

4. Discussion

4.1. BWTS choice determinants

The selection of BWTSs remains a difficult decision for shipowners. Many factors are considered such as capital and operating expenditures, IMO/USCG type approval status, installation complexity, system expected reliability, reputation of BWTS manufacturer, type of vessel and system integration, environmental variables of the operating environment (salinity, turbidity, or temperature). The factors influencing these decisions can vary in weight depending on shipowner, vessel size, operating region, and cargo requirements.

Factors influencing the selection of BWTS were gathered in October 2018 with input from a major ballast water consultant (Mouawad Consulting AS) and validated by interviews conducted with two prominent companies (a global container liner operating 65 ships equipped with BWTS and a major European ferry operator operating ships with BWTS) (Table 3). The order on the list does not indicate importance, because each shipowner weights the criteria according to their needs.

Operation and maintenance costs are among the most important factors involved in decision making (American Bureau of Shipping, 2017, 2014; Vorkapic et al., 2018). The costs are comprised of: 1) electrical power and fuel required to generate treatment materials (UV, biocides, inert gas, ozone); 2) consumables such as UV lamps, chemicals, filters, etc.; and 3) crew resources required for training, operation and maintenance. The required power for operating large power-consuming systems such as electrolytic and filtration & UV may be of concern in the decision making process regarding both treatment costs and availability of shipboard power (David & Gollasch 2015).

Table 3. Factors considered by shipowners when selecting ballast water treatment systems.

Cost related to equipment	• Capital expenditure (equipment, financing, long standing agreements with supplier and yards) • Operational expenditures (spare parts, supply, energy, crew, onboard maintenance favored)
Approval	• IMO approval • USCG approval • Additional country-specific requirements/testing
Installation	• Expenses to 'ensure it will work' represent 1.5 to 4 times the price of the equipment • Requires extensive planning (8 to 12 months) • Availability of equipment by manufacturer – waiting time between 12 to 20 weeks • Preparation of work to reduce time in yard (delivery of the equipment and some initial job prior yard) • Consideration for space, volume, weight, compatibility and integration with vessel systems and material • Location of the system close to ballast water pumps • Selection of yard and teams with experience in retrofitting and/or new-building • Completion between 12 and 18 days in yards • Three step approval by class: design + equipment testing + final commissioning
Technology	• Determined by vessel trade and volume to treat • Preference for established technology • Focus on a single technology for the fleet • Some technology using certain active substances are disregarded • Energy consumption • Technical capacities and limitations • Automation level, alarms and self-monitoring • Complexity of systems (integration of numerous subsystems) and reliability (i.e. total number of components) • Redundancy of treatment processes • Contingency plan • Risks free system including with corrosion • Safety of the system in operation and explosion proof systems • Holding time reduced or suppressed • Footprint
Manufacturer	• Continuous survey of the market • Reputation of the brand • Good service and long-lasting links • Global service for spare part, supplies and assistance • Long term relationship • Warranty periods • Training solutions for crew (onboard, computer based-training, manual, etc)
Other	• Environmental load • Simplicity of the system • Easy to operate • Easy maintenance and repair • Type and age of vessel • Type of seas encountered during voyages of the vessel
Some issues	• Design issue (e.g. moisture on control systems for valves) • Defective installation (e.g. piping) • Electrical and automation failures • Improper integration of ballast water treatment control systems with the ship's systems • Difficult interaction with yards • Many subcontractors involved in installation • Additional cost for adequate integration and automation compatible with existing systems • No additional test after installation to verify biological efficiency

A study by Vorkapic et al. (2018) investigated the operational costs based on the unit energy consumption of a number of commercially available BWM solutions. The investigation compared BWE, electrolytic, and filtration & UV as treatment approaches. In the study, ballast volume of 17,740 m^3 and flow of 800 m^3 h^{-1} were assumed. Estimated power consumptions for each complete treatment cycle were 35, 23, and 5 kWh 100 m^{-3} for BWE, filtration & UV (loading and unloading), and electrolytic systems, respectively. The estimated mean power consumption for electrolytic systems based on numbers obtained from Lloyd's Register (2012) were similar to those calculated by Vorkapic et al. (2018) with 5.3 kWh 100 m^{-3}. Filtration & UV differed by a factor two with an estimated power consumption of 10.5 kWh 100 m^{-3}, but this could be explained by not including treatment during both loading and unloading in the report by Lloyd's Register (2012). Based on information from IMO Type Approval Certificates of UV-based treatment systems power consumptions were in the range of 7.6-12.7 kWh 100 m^{-3} (www.docs.imo.org; 2018) and thus comparable to the report from Lloyd's Register. For a filtration & UV systems to obtain USCG Type Approval, 10 times more UV energy is required to comply with the USCG regulations of <10 living org. ml^{-1}, which is due to a critical differences in the viability assessment methods between IMO and USCG (Lundgreen et al. 2018). Consequently, power consumption becomes 230 kWh 100 m^{-3} for a full treatment cycle. Power consumption estimates from treatment systems such as ozone, advanced oxygenation, and deoxygenation were 8.7, 2.7, and 2.0 kWh 100 m^{-3}, respectively (Lloyd's Register 2012). The numbers do not include additional costs for chemicals or the neutralization process. Interestingly, the power consumption data for the most prevailing method (BWE) is the costliest procedure being 1.5 and 7 times higher than filtration & UV and electrolytic, respectively. In addition, BWE has to take place offshore which means that costly manpower time is used to exchange the ballast water before the vessel can enter the port. Despite regulation B-4 paragraph 3 recalling that vessels shall not deviate for exchange, operational constraints may affect route and time to destination which implies additional costs. The other treatment types are able to treat ballast water during the journey or at the port in parallel with off and onloading activities which will save valuable time.

Between the two most commonly used BWTS types, electrolytic systems involve fewer energy related expenditures that filtration & UV; however, lack of long-term data on system in real operation do not allow definitive cost effectiveness comparison. In addition, cost effectiveness on the long-run should consider other operational expenditure such as supplies, spare parts, manpower for operation and maintenance, and reliability. For electrolytic treatment, additional costs for the neutralization process must still be considered. However, these costs are minimal when compared to the overall purchase and installation price of a BWTS and will only have a small influence in the decision-making process. Instead, other factors such as installation costs and availability of equipment, safety and simplicity of operation and maintenance, reliability and availability of supplies, reputation and support by manufacturers, maximum treated flow rate, and environmental load are of higher importance.

In our interviews, companies we interviewed highlighted that the crew is expected to maintain and repair the systems, which confirms the IMO survey MEPC 69/4/4. Therefore,

prioritizing simple and reliable BWTSs is of high relevance in the decision-making process. This was supported by results in a 2017 questionnaire (including 27 shipowners and 220 vessels) performed by the American Bureau of Shipping on *Best Practices for Operation of Ballast Water Management Systems* where it was reported that one of the biggest challenges shipowners faced was maintaining an effective training program for crew to minimize human errors and ensure safe, reliable operation of equipment and BWTSs (American Bureau of Shipping, 2017).

Furthermore, reports of extensive software updates, hardware maintenance, and system malfunctions were highlighted as typical issues experienced by crew operating the BWTSs (American Bureau of Shipping, 2017). The survey also reported more incidents of hardware failure, maintenance events, and logging issues for electrolytic systems compared to filtration & UV (American Bureau of Shipping, 2017). On the other hand with filtration & UV systems, shipowners reported that UV lamps often did not meet their expected life-times and frequently needed replacement (American Bureau of Shipping, 2017). The survey outcomes confirmed the failures and malfunctions already identified in MEPC 69/4/4.

With limited data from real operation and diversity of ship operation, it is premature to advocate for any system. Extensive onboard data collection is needed to draw a definitive picture of systems' performance. This lack of visibility facilitated the reticence of the industry to integrate such equipment and justified the Experience Building Phase prompted by the IMO (BWM.2/Circ.67 – 2018).

4.2. Installation of BWTSs on vessels in the United States and Australia

Electrolytic and filtration & UV systems dominate (with greater than 80% of total vessel arrivals with an installed BWTS). Electrolytic systems are likely widespread because many vessels operate in a marine environment where there is enough salinity to use the system and the chlorine can be generated ship-board without the need to carry additional chemicals. Furthermore, stakeholders inherently understand the technology of chlorination in light of its universal application to water treatment. Similarly, filtration & UV systems do not require additional chemical carriage and the technology has seen increasing applications to water treatment around the world (e.g. drinking water, tertiary wastewater treatment). Notably, filtration & UV systems are widespread despite uncertainties that followed the USCG rejection of MPN as a validation method (Coast Guard Maritime Commons 2016).

Interestingly, heat-deoxygenation and filtration are BWTS types that are rarely observed in Australia (four total arrivals) and are not encountered in the United States. The rarity of these systems may lie with specific challenges such as retrofitting vessel tanks and resting stages of certain organisms able to survive anoxia (Holmstrup et al., 2006).

In the coming years, the BWTS types other than electrolytic and filtration & UV installed may increase; however, the timeline for this change is not clear given the current regulatory climate, especially as extensions are offered to shipowners by the USCG.

More than 80% of vessels arriving to the United States and Australia were not fitted with a BWTS as of November 2017. Therefore, the prevailing method of compliance with BWM

requirements remains BWE. However, the growing number of vessels equipped with a BWTS in the study period shows that shipowners are preparing for D-2 compliance.

4.3. Usage of BWTSs in the United States and Australia

Adoption of BWTSs among vessels during the examined time window does not differ meaningfully between the United States and Australia. There was a similar increase in the percentage of vessels with a BWTS installed and actually utilizing the system in both countries (USA increased by 15%; AUS increased by 19%). Though vessels arriving to Australia slightly outpaced their United States counterparts with regards to uptick in utilization of installed systems, the percentage of vessels with an installed BWTS that actually used the system prior to arrival remained much higher in the United States than Australia (USA, 39%; Australia, 24%). This disproportionate difference in utilization rate may be explained by several factors, including: 1) different rules regarding usage of installed BWTS under IMO and USCG; 2) Vessel General Permits in the United States encourage the use of installed BWTSs; 3) longer voyage times for vessels coming to the United States allow more time for BWM; and 4) despite stringent enforcement regime in Australia and the United States, it seems that heavy penalties in the United States support compliance (Gallagher, 2018b, 2017).

In addition, there was a marked difference in the amount of total ballast discharge treated with BWTSs (USA, 12%; Australia, 1.9%). These findings are especially insightful with regards to the percentage of total vessel arrivals that utilized a BWTS in each country (USA, 6.7%; Australia, 3.4%). When the percentage of total ballast water treated with BWTSs is higher than the percentage of total vessels utilizing the systems, this indicates that vessels utilizing BWTSs are moving more ballast per vessel than the average arrival. Possible explanations for this difference are: 1) larger vessels may be adopting BWTS earlier in the United States (because of concerns about regulation and penalties); 2) new builds tend to be fitted with a BWTS, and new builds may compose a larger proportion of arrivals in the United States; 3) large companies may be prioritizing BWTS installation for their vessels in the United States over those in Australia.

4.4. Implications for the future global use of BWTS types – Compliance Monitoring and Enforcement

While the Type Approval process ensures that BWTSs are robustly tested against standard conditions described in the guidance documentation (BWMS Code/G8, G9, and ETV), it does not assure that systems will work at any time and for any types of water being loaded in the ballast water tanks. Moreover, current performance standards only include selected indicator microbes, which may be not be sufficient to fully characterize the risk of ballast to the surrounding aquatic environment (Drillet et al., 2016).

The revision of the G8 Guidelines (now included in BWMS Code) has promulgated the notion of operational limitation (salinity and temperature) and systems design limitations (dependent of treatment technologies) which is to be reported on the Type Approval certificate for each new system approved. Most (if not all) systems have been approved with no holding time.

Class societies may approve a BWTS and report no minimum holding time for treatment efficacy. This lack of information creates a difficult position for shipowners who need to make an informed decision. Other limitations may be reported on the Type Approval (e.g. minimum salinity, temperature, UV-T).

4.4.1. Compliance Monitoring and Enforcement (CME)

The regime under BWM Convention is made of three layers of duties: Flag State, Port State, and Coastal State. Flag State inspectors or Recognized Organization surveyors verify the compliance of vessel before issuing an International Ballast Water Management Certificate. While the certificate is considered a *prima facie* evidence of compliance, Port State Control (PSC) officers assess on-going compliance by checking BWTS functioning in operation. However, considering the diversity of the BWTSs, PSC officers may have difficulties in assessing each system. Finally, the Coastal State monitors the marine environment to assess efficiency of other layers and implements contingency measures (GEF-UNDP-IMO GloBallast Partnerships and WMU, 2013).

During inspections, the crew's ability to conduct ballast water activities and the condition of equipment may provide indications on the functionality of an installed BWTS, but the only way to ensure compliance is to physically sample and analyze ballast water. Unfortunately, current testing protocols against D-2 standards are not easy to implement and may impact the success of the convention by reducing opportunities to assess on-going biological compliance (First et al., 2018). This creates a bit of a conundrum because most of the organizations having experience in testing ballast water against D-2 discharge are also those involved in the type approval testing, thus generating a potential conflict of interest if required to test for compliance (whether it is systems tested by themselves or their competitors). Yet, enforcement by testing is a key to the success of such goal-based regulation (Pomeroy et al., 2015) and therefore there is a need to define simple protocol to be able to enforce the convention; eventually using a higher concentration of organisms in water as a enforcement limit to ensure higher legal defensibility.

Whatever administrative controls are, the overall responsibility for compliance remains in shipowners' hands. Indeed, shipowners decide which BWTS to install and how allocate human and financial resources for operation (quantity and quality of crew and maintenance/repair budgets).

Experience from thousands of sampling event by SGS globally (>120 ports) shows that vessels are generally compliant with the Vessel General Permit Guidelines for ballast water, though the regulatory limits exist solely for two bacteria and a few chemical measures. Full compliance tests reported by the Singapore Government at MEPC (MEPC 68/2/13) also supported that ships installed with BWMS were mostly meeting the D-2 discharge standards and only one ship out of six did not meet the standard. However, during ad hoc discussions with crew members, the crew raised concerns about BWTS maintenance and limited staff to monitor BWMS alarms when in operation (Guillaume Drillet, Personal Observations). For example, a low UV-I alarm may be overlooked during ballasting operations because of multiple other activities taking place simultaneously (cargo operation, bunkering, arrival of supply, special maintenance on main

engine, etc.). This emphasizes the need for improved data regarding the implementation and functionality of BWTSs but also to consider the reduced manning onboard ships.

4.4.2. Vessel General Permit – an on-going monitoring requirement

The United States requirements to sample ballast water for issuance of a Vessel General Permit (VGP) open the possibility to elaborate continuous monitoring program. The process of testing surrounding a VGP is restricted in terms of biological analysis (*E. coli, Enterrococci,* and total heterotrophic bacteria) and chemical analysis (TRO and residual chemical).

Despite these limitations, acquiring a VGP obligates stakeholders to commit resources dedicated to compliance assessment and regularly assess BWTS functionality. Additionally, it prompts shipowners and BWTS manufacturers to work together to collect samples and provide training for the crew prior to future testing. As such this mandatory sampling scheme should be seen as a step toward the successful implementation of ballast water management globally.

4.4.3. Experience Building Phase

Recently launched by the IMO (MEPC.290(71)-2017 and BWM.2/Cir.67 –2018), the "data gathering and analysis plan for the Experience Building Phase (EBP) associated with the BWM Convention" intends to collate data during the initial phase of implementation of the BWM Convention, particularly on BWTS functionality and the practice of PSC. The program aims to monitor and improve the BWM Convention.

From Spring 2019, Flag States, Port States, and stakeholders such as shipowners are invited to populate the IMO database on BWM Convention. From 2021 to 2022, the final report and amendments will be presented to IMO Member States. A non-penalization clause will be enforced during this time period as is required by the industry.

5. Conclusion

This analysis highlights the important role of regulations and policy in shaping practice. Entry into force of the IMO BWM Convention in September 2017 coincides with an increase in the percentage of vessels fitted with BWTS, vessels fitted with a BWTS utilizing the system, and total ballast water discharge treated with BWTS in both Australia and the United States. Though IMO Type Approval records revealed that 65 BWTSs using seven different treatment processes were approved, only 13 BWTSs using two treatment processes (filtration & UV; electrolytic) are currently approved by the USCG. There may be a higher proportion of these systems installed and utilized worldwide as a result of USCG approval. Indeed, these two processes are used on the vast majority of ballast water treated with BWTSs arriving to both countries (Australia, 84%; USA, 89%).

Despite the increasing utilization of BWTSs, the majority of vessels arriving to the studied countries are still using BWE to manage ballast water prior to discharge. As more vessels become compliant with the BWM Convention in the coming years, ongoing policy implementation and oversight at various levels of government will play an important role in determining which BWTSs are installed and utilized worldwide. Policy surrounding BWM and BWTSs enacted in the next

few years while the BWM Convention is implemented in its entirety will likely impact the types of BWTSs installed and used by shipowners. Like any regulation, the development of Compliance Monitoring and Enforcement programs are necessary to ensure on-going compliance. In this respect, the requirements of the VGP provides a good opportunity for shipowners to develop self-monitoring procedures.

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7. Abbreviations

BWE – Ballast Water Exchange
BWM – Ballast Water Management
BWTS – Ballast Water Treatment System
CME – Compliance Monitoring and Enforcement
DBP – Disinfection By-Product
EEZ – Exclusive Economic Zone
IMO – International Maritime Organization
NBIC – National Ballast Information Clearinghouse
PSC – Port State Control
USCG – United States Coast Guard
VGP – Vessel General Permit

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9. Appendix A

9.1. Ballast water treatment systems

Power consumption values are based on data obtained from Lloyd's Register (2012) and IMO Type Approval Certificates (www.docs.imo.org 2018). Number of approval from the BWTS described are obtained from www.docs.imo.org (2018) and US Coast Guard (2018) (Updated 07 December 2018).

9.1.1. Non-active substance systems

9.1.1.1. Ultraviolet based treatment systems

IMO/USCG approvals: 30/3

Disinfection strategy: DNA damage and protein/enzyme denaturation.

Pros: Environmentally friendly, no DBPs, extensively used in municipal and industrial water treatment applications, immediate discharge possible.

Cons: Relies on high UVT for efficient penetration, relatively high power consumption rates, moderate number of reported maintenance and operational incidents.

Power consumption (Lloyd's/IMO – no overlapping data): 10.5±5.6 kWh 100 m^{-3} (n=9) / 7.6-12.7 kWh 100 m^{-3} (n=10)

Description: Treatment systems using UV irradiation to inactivate organisms compose 90% of all treatment systems using non-active substances and 46% of all IMO Type Approved treatment systems (www.docs.imo.org 2018). Three kinds of UV BWTSs are currently approved by the IMO: 1) low pressure (LP), 2) medium pressure (MP), and 3) LED systems. In general, the UV-based treatment systems damage the DNA of the organism thereby disrupting vital cellular functions and reproduction which eventually leads to cell death (Goodsell 2001, Oguma et al. 2002, Kalisvaart 2004, Rastogi et al. 2010). The LP UV-based treatment systems are based on monochromatic UV irradiation with a high energy intensity peak at 254 nm which mainly affects cellular DNA with a maximum absorption capacity around 260 nm (Zimmer & Slawson 2002, Weber 2005, Ben Said et al. 2015, Sun & Blatchley 2017). DNA damage can be reversed through repair mechanisms that involve certain enzymes (Sinha & Hader 2002, Rastogi et al. 2010). Enzymes and proteins have maximum absorption capacities around 280 nm, so LP UV irradiation potentially only has a small effect on their functionality (Kalisvaart 2004). However, several studies have shown LP UV treatments to be efficient in inactivation of organisms (Sakai et al. 2011, Ben Said et al. 2015, Sun & Blatchley 2017, Lundgreen et al. 2018). The MP UV-based treatment systems are based on polychromatic UV irradiation, which affects a much broader spectrum than LP UV. Compared to LP UV, less energy is emitted at 254 nm. On the other hand, MP UV treatment systems have higher energy output which helps destroy the enzymes and

proteins involved in DNA repair or other vital cellular functions. Consequently, MP UV systems have shown to be efficient in reducing growth and photosynthetic activity in phytoplankton (Choi et al. 2011, Sun & Blatchley 2017).

9.1.1.2. Deoxygenation

IMO/USCG approvals: 3/0

Disinfection strategy: Asphyxiation of organisms which obstructs metabolic activity/respiration.

Pros: Environmentally friendly, limits corrosion, low maintenance costs

Cons: Limited effects on organisms with resting stages, long treatment times (days) necessary, not suitable for short voyages

Power consumption: 2.0±1.4 kWh 100 m^{-3} (n=2)

Description: Three BWTS using deoxygenation have obtained IMO Type Approval (Table 1). The treatment can be based on a variety of techniques for stripping the water from oxygen such as purging of nitrogen gas (Tamburri et al. 2002), a mix of high nitrogen and carbon dioxide (Husain et al. 2003), vacuum chambers (Browning Jr. et al. 2004), or application of a Venturi Oxygen Stripping system (Tamburri et al. 2004). One advantage of using deoxygenation is that shipowners benefit from reduction in corrosion because the deoxygenated conditions limit the oxidation process of metallic structures (Tamburri et al. 2002). Thus, the treatment approach has benefits for the both the shipping industry and environment (Tamburri et al. 2004). Yet, deoxygenation may present a limitation in that resting stages of plankton organisms may be able to survive long period of anoxia and yet germinate/hatch and initiate new populations. (Holmstrup et al 2006).

9.1.1.3. Heat and deoxygenation

IMO/USCG approvals: 1/0

Disinfection strategy: Cell wall destruction, denaturation of proteins, asphyxiation

Pros: Environmentally friendly, exploits onboard waste energy, limits corrosion

Cons: High maintenance, obtaining and maintaining high temperatures in cold waters, potential discharge of water at temperatures above ambient, corrosion

Power consumption: N.A.

Description: One system using a mix of heat (pasteurization) and deoxygenation (Nitrogen injection) has obtained IMO Type Approval. Based on data from a number of studies, heat has been suggested to effectively eliminate organisms in ballast water at temperatures above 35°C which must be maintained for at least 20 h (Quilez-Badia et al. 2008). If treating at higher temperatures a larger impact is achieved even at shorter exposure times and in addition, treatments are more efficient in eliminating temperature tolerant organisms such as vegetative bacteria, fungi and viruses (Gregg et al. 2009). The treatment type is regarded as cost efficient compared to other

treatment types (Gregg et al. 2009). Disadvantages of this treatment approach is the potential increased corrosion caused by the higher temperatures and the possible higher maintenance of the heat exchangers (Mackey et al. 2000). However, corrosion has also been highlighted to be reduced due to the combined deoxygenation step (Bawat 2018, ORtech Marine 2018). Furthermore, concerns have been raised about reaching and maintaining temperatures high enough for disinfection when operating in colder regions as well as impacts on the environment when discharging large amounts of heated water (Gregg et al. 2009).

9.1.2. Active substance systems

9.1.2.1. Electrolytic

IMO/USCG approvals: 15/10

Disinfection strategy: Cell wall destruction, increased cell permeability, enzyme and nucleic acid breakdown.

Pros: No storage of toxic chemicals needed, extensively used in municipal and industrial water treatment applications, relatively low operational costs

Cons: Only works in saltwater (or with addition of salt), high concentrations necessary for effective elimination of cysts and organisms with resting stages, production of DBPs, corrosion, less efficient in low temperatures (increase in power output needed), production of highly flammable hydrogen, special ventilation required, high number of reported maintenance and operational incidents.

Power consumption: 5.3±2.1 kWh 100 m^{-3} (n=11)

Description: Electrolytic BWTSs comprise almost 25% of all IMO Type Approved treatment systems (Table 1) (Gregg et al. 2009, www.docs.imo.org 2018). The electrolysis of NaCl results in dissociation into Na^+ and Cl^- which further reacts to produce Cl_2 (free chlorine) (Tsolaki & Diamadopoulos 2010). The highly flammable H_2 must be vented to decrease any risk of explosion. Upon ballast water discharge, excess chlorine must be neutralized by adding sulphur dioxide or sodium sulfite to produce non-toxic chloride ions (Cl^-) (Matousek et al. 2006, Tsolaki & Diamadopoulos 2010). Concerns about using electrolytic BWTSs include the formation of disinfection by-products (DBPs) such as trihalomethanes (THM) and haloacetic acids (HAA), which are formed when naturally occurring organic and inorganic matter reacts with chlorine. DBPs have endocrine disrupting properties and can persist in the marine environment where they are able to bioaccumulate in the food chain (Jenner et al. 1997). Furthermore, chlorine has also been suggested to increase corrosion rate of the vessel (Zhang et al. 2004).

9.1.2.2. Advanced oxidation

IMO/USCG approvals: 11/0

Disinfection strategy: Cell wall destruction, increased cell permeability, enzyme and nucleic acid breakdown.

Pros: Extensively used in municipal and industrial water treatment applications, low number of reported maintenance and operational incidents.

Cons: Storage of toxic chemicals onboard, high concentrations necessary for effective elimination of cysts and organisms with resting stages, production of DBPs, corrosion, involves a neutralization process before discharge, special ventilation rooms required.

Power consumption: 2.7±3.1 kWh 100 m^{-3} (n=4)

Description: Instead of using electrolytic-generated chloride as disinfectant, chloride (and chloride dioxide ClO_2) can be added directly to ballast water. Typically, this approach is applied to freshwater systems, because electrolysis is not possible without a minimum concentration of salt. The impact of the oxidizing biocides is dependent on their concentration, pH, exposure time, and the type and quantity of compounds formed (Gregg et al. 2009). Overall the treatment method is estimated to be more costly than generating the biocide through electrolysis (Gregg et al. 2009). In addition, biocidal effects are active within a larger pH range and are less affected by organic material (Gregg et al. 2009). The concerns regarding inefficient post-neutralization of the biocide and the formation of toxic DBPs are similar to those of BWTSs using electrolysis.

9.1.2.3. Ozone

IMO/USCG approvals: 4/0

Disinfection strategy: Cell wall destruction, increased cell permeability, enzyme and nucleic acid breakdown.

Pros: Environmentally friendly, very efficient in eliminating bacteria and virus

Cons: High concentrations needed to eliminate larger organisms, corrosion, production of DBPs, moderate number of reported maintenance and operational incidents.

Power consumption: 8.7±1.5 kWh 100 m^{-3} (n=3)

Description: Ozone is a powerful but unstable oxidant that can be generated directly from air. Ozone reacts with molecules within or on the surface of cells resulting in cell lysis (Herwig et al. 2006). The advantages of using ozone are that no storage of toxic chemicals is required, and it is more environmentally friendly than other biocides (Gregg et al. 2009, Tsolaki & Diamadopoulos 2010). In seawater, the ozone generates oxidants from reaction with bromide and chloride ions which can cause environmental impacts even at low concentrations if not ozone is not properly neutralized at discharge (Oemcke & Van Leeuwen 2003). Corrosion of steel in vessel structures is an additional concern.

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Manuscript 5

Raman spectroscopy as a tool to measure viability of planktonic organisms in UV-treated ballast water

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Manuscript in preparation.

Raman spectroscopy as a tool for viability assessment of planktonic organisms in UV treated ballast water

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Abstract

To comply with regulations stated by the United Nation's International Maritime Organization, ballast water discharged by ships must be treated to avoid the spread of invasive organisms including algae. In this study, Raman spectroscopy and multivariate data analysis was used to obtain a model for discrimination between viable (potential invasive) and UV exposed non-viable organisms. To do this, a method was developed such that non-viable (and dying) algae consistently could be obtained. The method was based on UV exposure commonly used as ballast water treatment strategy. Raman spectra from both viable and UV treated algae of *Rhodomonas salina* and *Tetraselmis suecica* were measured A Partial Least Squares Discriminant Analysis model was obtained to form the normalized dataset, and the model was Cross-Validated using a Venetian blinds. It was possible to obtain 100% discrimination between the two algal species based on their individual Raman spectra. The model classified 92 and 91% of the viable algae correctly for *R. salina* and *T. suecica*, respectively, as opposed to 82 and 94% for non-viable algae. The classification model was acceptable but still with a total overall classification error of 13 and 7% for *R. salina* and *T. suecica*, respectively. In conclusion, Raman spectroscopy has a potential for algae species identification as well as discrimination between viable and non-viable algae.

Keywords

Raman spectroscopy, ballast water, invasive species, viability, UV irradiation

1. Introduction

Since the early 1900s it has been suspected that shipping could be a factor in the global introduction of non-native species, and it was later found that large numbers of different organisms were present in the ballast water tanks used to stabilize ships (Gollasch et al. 2007). Ballast water is taken in and discharged regularly in order to control the stability of modern steel hull ships. This is done in ports all over the globe and as a consequence of this, the International Maritime Organization (IMO), a specialized agency of the UN, passed the "*International Convention for the Control and Management of Ships' Ballast Water and Sediments*" in 2004 in order to limit the spreading of aquatic invasive species (IMO 2004). The focus of this present study is on microplanktonic organisms and originates specifically from regulation D-2 of the IMO convention which states that ships shall discharge "*... less than 10 viable organisms per milliliter less than 50 micrometers in minimum dimension and greater than or equal to 10 micrometers in minimum dimension; ...*". To comply with the convention, ships must in most cases process their ballast water before discharge. Ballast water treatment systems (BWTSs) are today commercially available typically using a prefiltration step followed by chemical- or UV disinfection of the ballast water (Tsolaki & Diamadopoulos 2010, Bircher 2016, Gerhard et al. *In prep.*).

Before installation on ships, BWTSs must obtain a Type Approval Certificate granted at IMO approved land-based test facilities after passing stated validation procedures (IMO 2016a). The performance of a BWTS is evaluated through a *comprehensive analysis* process where its ability to effectively remove or render organisms harmless is verified. Treatment strategies using active substances such as chemical that target and destroy cell membrane structure are typically evaluated using vitality assessment methods such as the Vital Stain (VS) method (Steinberg et al. 2011). The stain molecules are able to cross the cell membrane where unspecific enzyme activity of living cell convert them into fluorescent cell membrane impermeable molecules allowing quantification of cells using fluorescent microscopy or flow cytometry (Peperzak & Brussaard 2011, Steinberg et al. 2011, Adams et al. 2014).

Ultraviolet irradiation damage cellular DNA but leaves the cell membrane intact at low or intermediate fluences (doses) which result in living, but non-viable (not able to reproduce) organisms with delayed mortality (Olsen et al. 2016, Lundgreen et al. 2018). According to the adopted protocol, the VS method must be carried out within six hours after treatment (IMO 2016b) and within this time frame the UV treated organisms will appear alive with intact cell membranes. The stain cannot distinguish between viable and non-viable cells which still possess enzymatic activity and thus non-viable cells will activate the stain and will falsely be quantified although dying and posing no invasion risk (Cullen & MacIntyre 2016). For evaluation of treatment technologies based on UV irradiation the Most Probable Number (MPN) method (Throndsen 1978) is instead considered to be a robust viability assessment method (Madsen & Petersen 2015, Wright & Welschmeyer 2015, MacIntyre et al. 2017, Blatchley III et al. 2018, Cullen 2018, MacIntyre et al. 2018). The MPN method is based on the regrowth potential of organisms in the treated ballast water which is evaluated after an incubation period of two weeks (IMO 2016b). Thus, the MPN method takes into consideration the delayed mortality caused by the UV irradiation.

Although able to provide more accurate assessments of BWTS performance in relation to organism viability, the MPN method and VS method are both comprehensive methods that involve expensive and fragile equipment. Analyses are carried out by trained personnel who perform exhaustive analytical procedures that are both time and resource demanding. A number of *indicative analyses* of BWTS performance exist for assessment of viability of organisms in treated ballast water (IMO 2008, Gollasch et al. 2015). The idea behind indicative analyses is that the involved instrument should be quick and simple to operate without the need for special training. Such instruments can assist Port State Control officers that are obliged to monitor if ships are in compliance with ballast water discharge regulations and allow ship owners to carry out autonomous monitoring of BWTS performance. Applying robust indicative methods are essential for advising if ships are potentially compliant or non-compliant with regulations. Indicative analysis results showing gross exceedance can at an early stage prevent the discharge of non-compliant and biologically contaminated water (David & Gollasch 2015, Wright & Welschmeyer 2015). On the other hand, indicative analysis can also save compliant ships valuable time and costs. Some of the most common indicative analysis methods include pulse-amplitude modulated fluorometry (PAM) that measures the autofluorescent photochemical efficiency photosystem II in the chlorophyll protein complex, the adenosine triphosphate (ATP) assay based on measurements of bioluminescence in the presence of luciferase enzymes, and fluorescein diacetate (FDA) bulk analysis assay that exploits the leaking properties of fluorescein from the hydrolyzed cellular FDA to produce an extracellular fluid fluorescence signal which is directly proportional to living biomass (Welschmeyer & Maurer 2011, First & Drake 2014, van Slooten et al. 2015, Casas-Monroy et al. 2016, Outinen & Lehtiniemi 2017). Yet, no effective indicative method has been developed to specifically test for non-viable planktonic organisms after UV treatment. This means that even though the algae have sustained DNA damages that render them unable to reproduce, the currently available methodologies will identify them as living and possibly require additional treatment or storage time to fulfill discharge requirements (Olsen et al. 2016, Romero-Martinez et al. 2016, Lundgreen et al. 2018).

The aim of this study was to investigate if viable/non-viable classification of algae as well as species identification could be accomplished using Raman spectroscopy and multivariate data analysis. This was expected since classification of bacterial strains had been shown to be possible using Raman spectroscopy (Willemse-Erix et al. 2009). In this work a controlled system was constructed such that spectra from viable and non-viable monocultures of *Rhodomonas salina* and *Tetraselmis suecica* could be recorded. Background estimates for these spectra were performed and subtracted. The spectra were then normalized, and a classification model was build using Partial Least Squares Discriminant Analysis (PLS-DA). Raman spectroscopy was chosen as it is a non-invasive measurement technique that require no significant sample preparation and can be used in a wide array of measurement configurations. These include handheld and micro fluidic devices that will require minimum user interaction.

2. Materials and Methods

2.1. Algal monocultures and cultivation

Monocultures of *Rhodomonas salina* and *Tetraselmis suecica* (SCCAP, K-0297) were grown as semi-continuous cultures in Keller's Culture Medium (Keller et al. 1987) at 20 psu and 18°C in a temperature controlled room at 16:8 hour light/dark cycle.

2.2. Approach for UV exposure

To ensure that the algae were affected by UV exposure to such a degree that they were in a "dying phase" when measured, a criterium was made that no algae with intact cell membranes should be found in the sample 24 h after UV exposure. The criterium was made to ensure that it was the UV exposure that killed the algae and not poor storage. In control samples, no morphological changes were observed after 24 h (Figure 1a, b). A UV exposure of one minute resulted in a mix of obviously affected (ruptured cell membrane) and not affected individuals (Figure 1c, d) after 24 h. The optimal UV exposure time was three minutes since a combination of algae with intact and ruptured membranes were observed after 2 h and no algae with intact membrane were seen after 24 h (Figure 1e, f). Exposure times of three minutes and subsequent dark-hold of a minimum of 2 h were chosen to ensure that any changes introduced by the UV exposure would be visible.

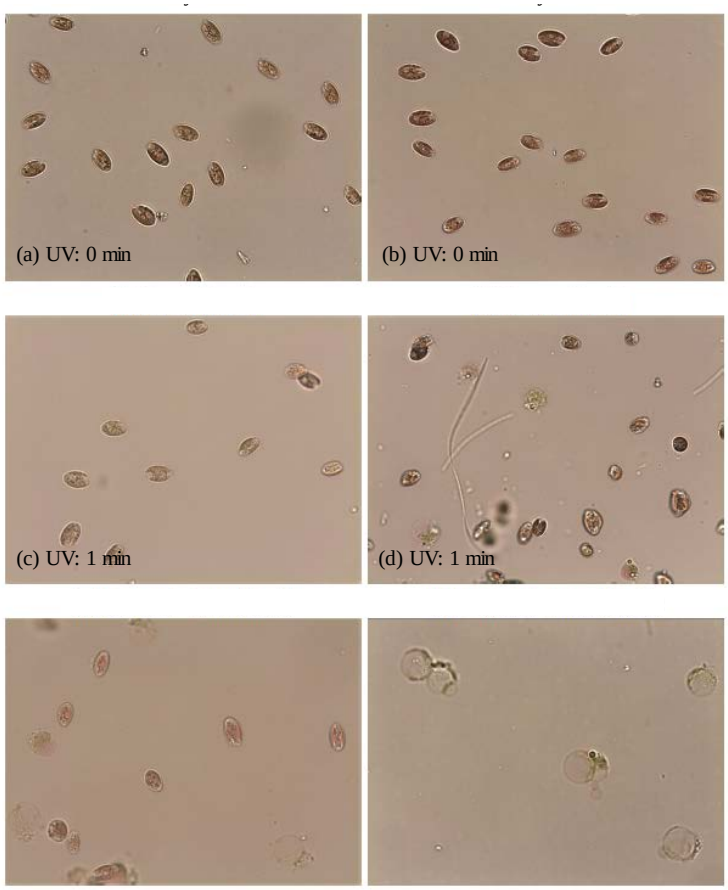


Figure 1. Effects of different UV exposure times on *R. salina* cell morphology over a 24 h period (Day 1 and Day 2). (a) and (b) no UV exposure; (c) and (d) 1 min UV exposure; and (e) and (f) 3 min UV exposure.

Algal monocultures were exposed using a Philips TUV PL-S 9W/2P UV lamp, emitting 2.3 W at a wavelength of 254 nm. The UV treatment of algae followed the procedure described below.

1. Two 50 ml beakers were first washed in demineralized water, rinsed with ethanol and left until the ethanol had evaporated.
2. Two samples of 10 ml (1:3 algae to seawater ratio) were prepared in the glass beakers.
3. The samples were mixed by inversion and placed right under the UV-lamp. The distance from the lamp to the table was 8.5 cm.
4. The lamp turned on and a timer was set to 3 min.
5. After the intended UV exposure, the lamp was turned off and the beakers were sealed with parafilm to prevent contamination.
6. The samples were then left at room temperature for a minimum of 2 h.
7. The samples were then inverted again and subsamples of approximately 1 ml were extracted and examined using both optical microscopy and Raman spectroscopy.
8. After examination, the leftover sample was placed at room temperature.
9. Approximately 24 h after exposure, step 7 was repeated but the samples were only examined using optical microscopy to verify that the algae were indeed non-viable.

2.3. Algal behavior after UV exposure

To make sure that the algae died as a consequence of the UV exposure and not potentially due to faulty storage overnight, an additional experiment was performed. The algae were exposed to UV irradiation and observed right thereafter instead of being left for 2 h. An image sequence was recorded using the same setup as that used for the Raman measurements however light microscopy was used instead (40× magnification). The aim of the image sequence was to demonstrate and document the observed mortal changes in morphology after UV exposure.

2.4. Data collection of Raman spectra

A drop of the sample (approximately 1 ml) was placed on a window of MgF_2 and transferred to a quartz petri dish to avoid any liquid being spilled on the instrumentation. The water immersion objective was inserted into the liquid and left for a few minutes. A Bruker Optics, Senterra Raman microscope spectrometer (model R200-L) was used for all measurements. The spectrometer is in this specific model build around an Olympus BX51 microscope with a motorized sample stage and an Infinity1 camera for video acquisition. The light source used was a 100 mW laser with a wavelength of 785 nm. An Olympus water immersion objective with 40× magnification and a numerical aperture of NA = 0.80 was used for all Raman measurements. The scattered light was filtered and then diffracted by a grating with 400 lines mm^{-1}. The diffracted light was then measured using an Andor DU420A - Open Electrode CCD cooled to –60°C using thermoelectric cooling with 1024×255 pixels. The spectral range for this setup was 80-3500 cm^{-1} or converted to wavelength 790-1082 nm. It must be noted that this wavelength region almost reaches the upper limit of the CCD detector, this since the quantum efficiency goes towards 0% at approximately 1100 nm.

Optical focus was then obtained on the algae which had settled at the liquid-MgF_2 interface using the microscope. Only algae which were settled on the interface was measured because it was necessary

that the algae were fixated during the spectroscopic measurement. For each algae a point in the middle of the algae was chosen and one spectrum was measured for each. When measuring the algae spectra, the integration time for a measurement was 5 sec. For every algal cell two spectra were measured, and the spectrum reported was the sum of the two spectra and corrected for cosmic spikes. In total, 1119 UV exposed and 1007 control of *R. salina* and 318 UV exposed and 468 control of *T. suecica* cells were measured, respectively.

2.5. Data Analysis

All classification models were performed using Partial Least Squares Discriminant Analysis (PLS-DA) models were obtained using an implemented in PLS Toolbox (PLS_toolbox, Eigenvector Research, Inc., Manson WA.). All data was interpolated to have equal spacing of 0.5 cm^{-1} and truncated to wave numbers in the range 455-1700 cm^{-1}. Low intensity spectra showing only background and very high intensity spectra were excluded using automated algorithms in MatLab (The Mathworks, Natick, MA). All spectra were then background corrected using an automated Whitaker filter. All spectra were normalized using Signal Normal Variate. For PLS-DA analysis all data were mean centered and where cross validated using venetian blinds.

3. Results

3.1. Algal behavior after UV exposure

After UV exposure, a single *R. salina* cell was focused upon and images at different points in time were recorded (Figure 2). The image sequence showed that the algal cell initially transforms slowly retaining its shape (Figure 2a-d), followed by a change in morphology (Figure 2e-h) and finally after approximately 18 min the cell was perceived dead (Figure 2i).

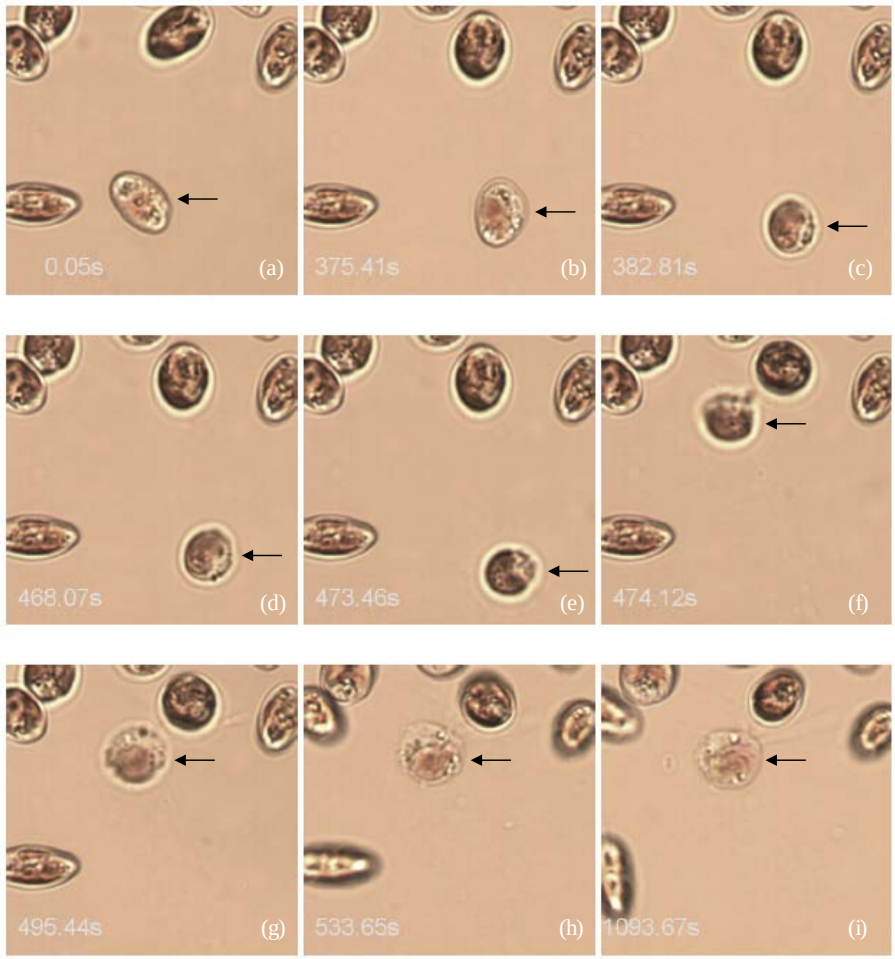


Figure 2. Image sequence a single *R. salina* cell (black arrows) following the change in cell morphology immediately after UV exposure 40× magnification.

3.2. Specific Raman spectra of T. suecica *and* R. salina *for species identification*

Comparison of the Raman spectra of *T. suecica* and *R. salina* showed that the discrimination between the two species was 100% using a two component PLS-DA model (Figure 3). The spectra are the mean of 786 and 2126 spectra of *T. suecica* and *R. salina respectively*.

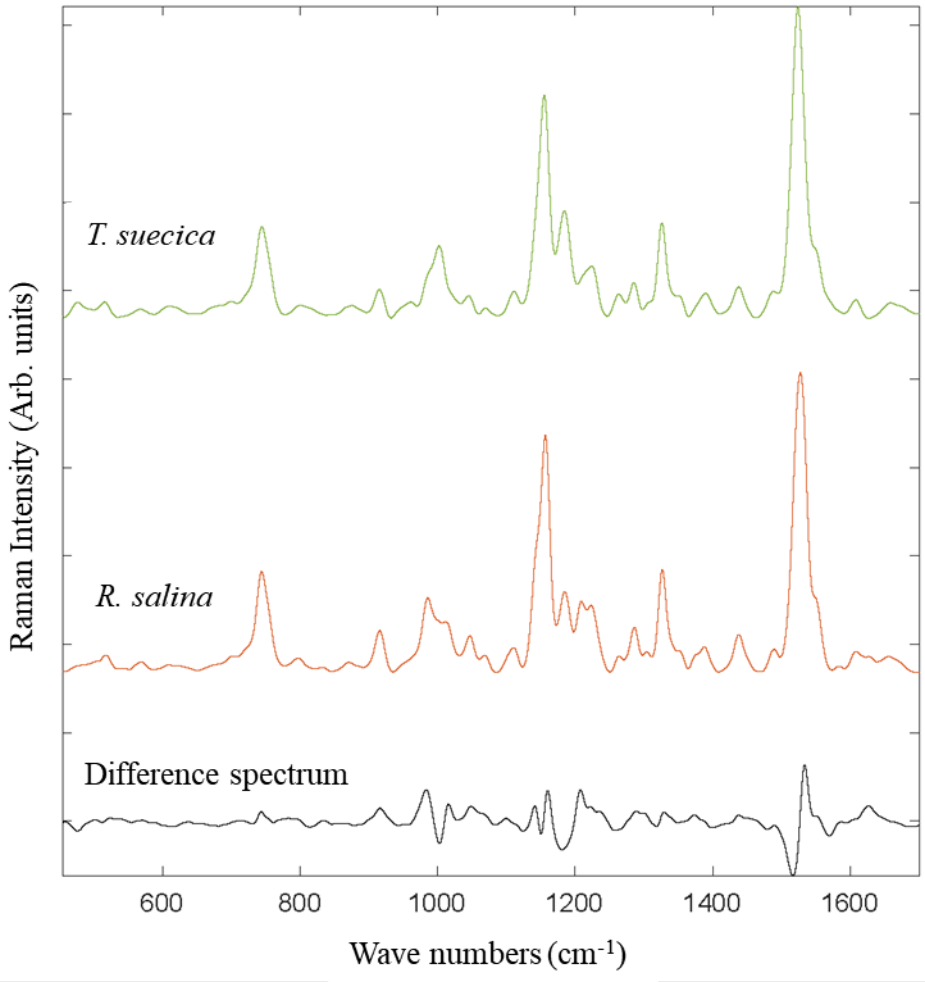
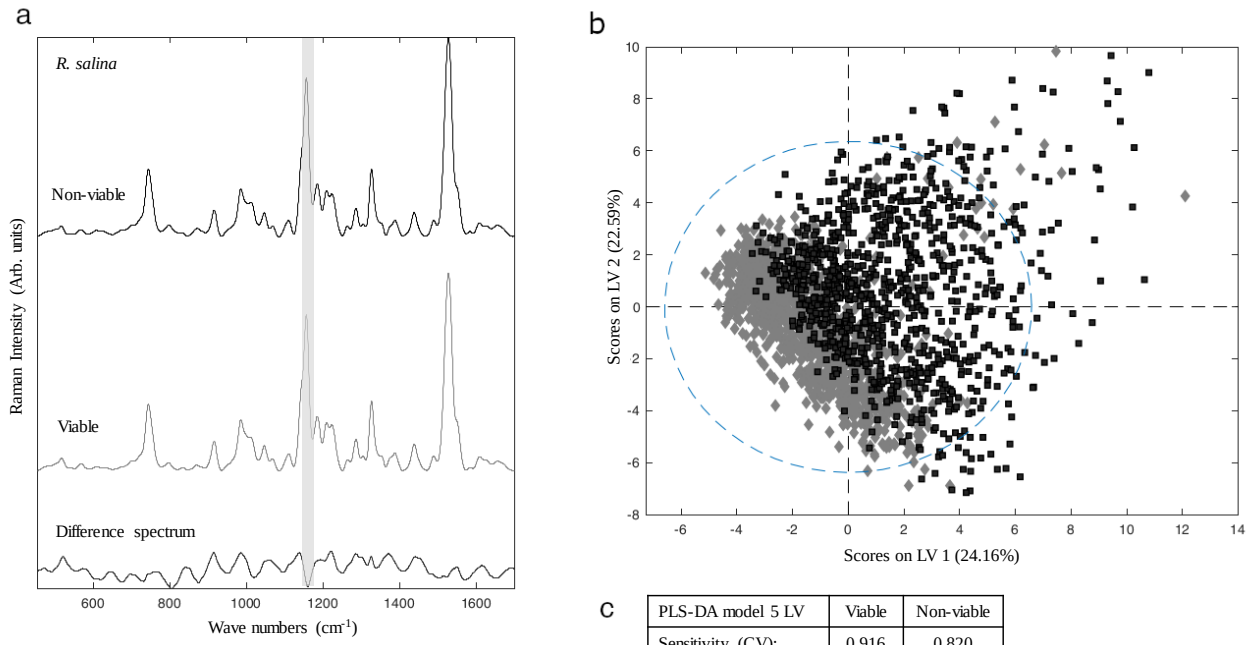


Figure 3. Raman spectra of untreated *T. suecica* and *R. salina*, and their difference spectrum showing the inter species differences.

3.3. *Classification of viable and non-viable* R. salina *and* T. suecica *from Raman spectra*

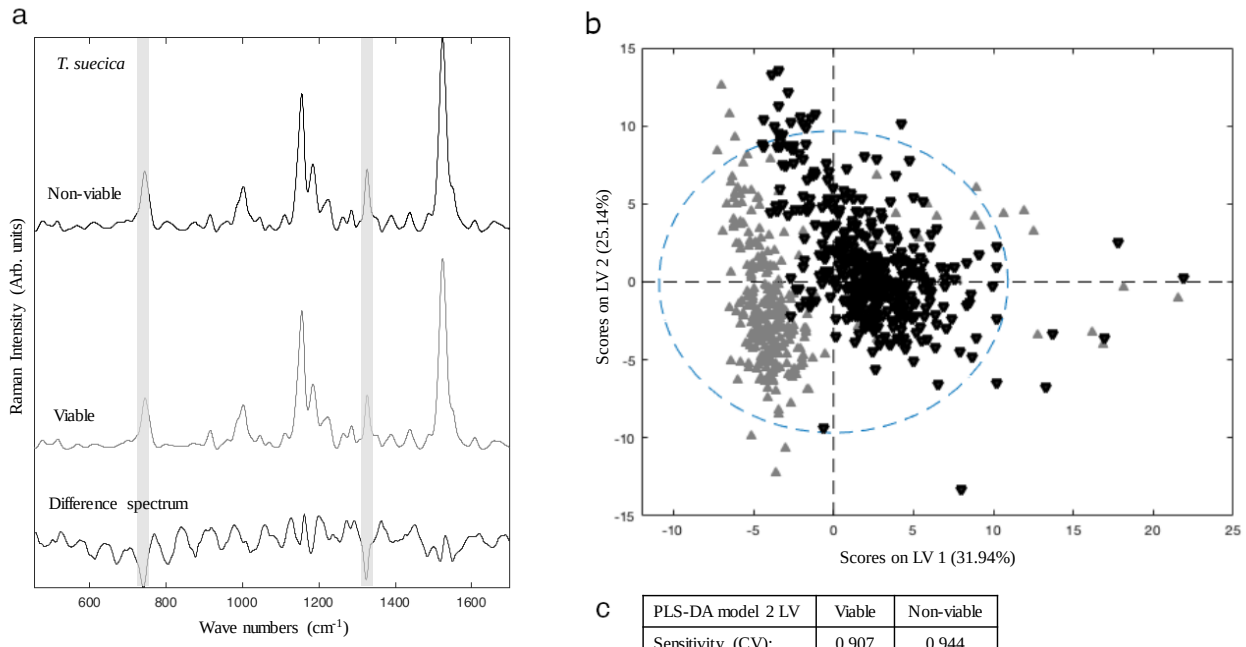
For *R. salina*, small differences between viable and non-viable cells where recorded as shown in the difference spectrum in Figure 4a. The largest deviation between the two spectra was observed to be at 1160 cm^{-1} where an upregulation was recorded in the non-viable spectrum compared to the viable spectrum (Figure 4a, grey band). Discrimination using a 5-component cross validated PLS-DA showed reasonable discrimination with a total overall classification error of 13% (Figure 4b, c). The spectra and score plots are the result of 2126 spectra of individual cells and it is visible that the viable group together and thereby generate a group with very high sensitivity, but lower specificity as non-viable mixes with this group. It is also visible that we have a similar distribution of samples as seen in traditional live/dead cell counting using fluorescence.



PLS-DA model 5 LV	Viable	Non-viable
Sensitivity (CV):	0.916	0.820
Specificity (CV):	0.820	0.916

Figure 4. (a) Raman spectra of viable and UV exposed non-viable *R. salina* cells from which the difference spectrum could be obtained from subtraction of the two spectra. The grey band signifies the wave number (1160 cm^{-1}) at which the largest difference between the two spectra was recorded. (b) score plots for LV 1 and LV 2 showing the cell sorting pattern of viable (gray) and non-viable cell (black) using the PLS_ToolBox model. (c) Cross validated sensitivity and specificity of PLS-DA model using 5 Latent Variables showing high specificity and sensitivity.

For *T. suecica*, larger differences were visible in the difference spectrum (Figure 5a). Upregulations were observed in the non-viable spectrum at 740 and 1324 cm^{-1} compared to the viable spectrum (Figure 5a, grey bands). Using a 2-component cross validated PLS-DA a total overall classification error of 93% was achieved (Figure 5b, c). The spectra and score plots are the result of 786 spectra of individual cells and show similar results to the *R. salina* data, but with a better separation of sample groups and thereby higher sensitivity and selectivity for both sample groups.



c

PLS-DA model 2 LV	Viable	Non-viable
Sensitivity (CV):	0.907	0.944
Specificity (CV):	0.944	0.907

Figure 5. (a) Raman spectra of viable and UV exposed non-viable *T. suecica* cells from which the difference spectrum could be obtained from subtraction of the two spectra. The grey bands signify the wave numbers (740 and 1324 cm^{-1}) at which the largest differences between the two spectra were recorded. (b) score plots for LV 1 and LV 2 showing the cell sorting pattern of viable (gray) and non-viable cell (black) using the PLS ToolBox model. (c) Cross validated sensitivity and specificity of PLS-DA model using 2 Latent Variables showing high specificity and sensitivity.

4. Discussion

4.1. Algal behavior after UV exposure

The images shown in Figure 2 illustrate one way for the algae to die. Natural death causes cannot be ruled out. However, the final state shown in Figure 2i was observed in numerous places in the sample shortly after UV exposure which indicate that the treatment caused cellular damages that eventually caused cell death at different rates. Some died relatively fast after UV exposure, whereas some died hours later. After 24 h, all cells in the samples were recorded as dead. In the image sequence the algae (and many more) died within the first 40 minutes after the UV exposure. In combination with the control samples (no treatment) where no change in algal concentration and morphology was observed this indicates that the storage at room temperature overnight did not cause an increase in cell death.

4.2. Specific Raman spectra features of T. suecica *and* R. salina *for species identification*

The majority of the existing comprehensive and indicative analysis approaches for viability assessment are not able to simultaneously identify algal species in the quantification or bulk analysis process. Conventional microscopy can identify down to species level but is laborious and time-consuming (First & Drake 2012, Lundgreen et al. 2018, Lundgreen et al. *In prep.-a*). Only a few automated approaches exist with the potential for taxonomic classification of classes or even species of phytoplankton. FlowCAM and PlanktoVision are examples of such approaches which both use

image analysis systems to automatically estimate the classification of the recorded organisms (Buskey & Hyatt 2006, Schulze et al. 2013, Álvarez et al. 2014, Camoying & Yñiguez 2016).

FlowCAM combines traditional flow cytometry and digital imaging microscopy but phytoplankton taxonomic classification resolution is limited up to genus level or higher (Álvarez et al. 2014). Species identification of formalin fixed cells was achieved by Camoying & Yñiguez (2016) but was limited to only include phytoplankton groups with simple shapes such as spherical *Coscinodiscus sp.* and thin-elongated *Pseudo-nitzschia* because changing orientation of organisms in the flow chamber hampered the identification process. In addition, the method is challenged by occurrences of poor image quality (Álvarez et al. 2014).

Another study examined the taxonomic classification performance of the open source image analysis system, PlanktoVision (open source image analysis system, PlanktoVision (2013)), on a set of images obtained from automated scanning microscopy that simultaneously obtained brightfield and fluorescent images from a sample in an Utermöhl chamber (Schulze et al. 2013). The sample consisted of 10 different taxa fixed with paraformaldehyde. PlanktoVision showed an average recognition rate of 94.7% and average error rate of 5.5% which were within the same range as routinely engaged personnel (84-95%) but higher than trained but not routinely engaged personnel (67-83%) (Schulze et al. 2013).

Raman spectroscopy is unaffected by orientation of the organism and the method was able to achieve 100% discrimination of fresh (living) samples between the two studied species based on very distinct features in each of their Raman spectra (Figure 3). In combination with a more automated approach this could be a promising tool for fast and accurate identification of certain key species or classes of algae present in treated or untreated ballast water samples or in relation to harmful algal blooms (HABs) events.

4.3. *Classification of viable and non-viable* R. salina *and* T. suecica *from Raman spectra features*

When looking at the results obtained from the PLS-DA models, it was found that these generally trend to classify algae as viable for *R. salina* based on the PLS Toolbox model. Here, sensitivity was found to be 92% for correctly classifying viable *R. salina* compared to only 82% of the UV exposed non-viable *R. salina*. The model obtained exhibited a bias towards identifying *R. salina* cells as viable, however this increased the specificity for the non-viable cells. Even though the non-viable cells to some degree were not classified optimally, the misclassification of non-viable cells was relatively high. This is important if this type of classification is to be used for the validation of BWTSs. The most important criteria for such a test would be that it is not overly optimistic towards the effectiveness of the treatment. This would be the case for the model build in this work. The bias of this model most likely originate in the spread of the data e.g. illustrated by the score plot in Figure 4b, but also in the uncertainty of the dataset itself. In the system used, a build in uncertainty exists which will be transferred to the PLS-DA model. This comes from the spectra classified as viable but originates from algae dying due to natural causes in the sample. This cannot be controlled and will always make the line blurry between viable and non-viable cells in such models. The overall performance of the model was acceptable with expected misclassifications mainly due to algal cells dying of natural causes. However, classification errors of 13% and 7% for the two studied species are

still a considerable proportion if this was to be used for a viability assessment approach. It is also seen that the differences in viability is larger in *T. suecica* compared to *R. salina* and thereby easier to separate using PLS-DA models.

For *R. salina* the largest change in the viable to non-viable spectra was at 1160 cm^{-1} which has previously been shown to be the main discriminator band of carotenoids (Koljenović et al. 2005, Jehlicka et al. 2014). Several studies have reported a similar upregulation of carotenoids in algal cells which had been exposed to environmental stressors such as UV exposure (Sharma et al. 2015, Al-Rashed et al. 2016, Ganapathy et al. 2017). The upregulation of carotenoids in microalgae (and other plants) is an antioxidant defense mechanism that includes a non-enzymatic metabolite to alleviate the photooxidation damage of the UV induced production of reactive oxygen species (ROS) (Sharma et al. 2015, Al-Rashed et al. 2016, Ganapathy et al. 2017). The emission spectrum of carotenoids is around 560-640 nm (Gillbro & Cogdell 1989) and the recorded upregulation of carotenoids might explain the redshifted fluorescence which was observed in UV exposed *R. salina* cells and could be a promising non-viability indicator

The largest differences between the viable and non-viable *T. suecica* appeared at the wave numbers 740 and 1324 cm^{-1} which indicated upregulation of certain carbohydrates (Arboleda & Loppnow 2000). The UV induced formation of ROS interact and cause oxidative damage to cellular macromolecules such as DNA, proteins, fatty acids and saccharides (Duan & Kasper 2011). Certain polysaccharides have been suggested to play important roles in scavenging free radicals and acting as antioxidants to minimize oxidative damage (Ruperez et al. 2002, Sokolova et al. 2011, Wang et al. 2013). In the present study, the observed upregulation of saccharides in UV exposed cells could therefore be a defense strategy adapted by *T. suecica* where the saccharides act as free radical scavengers to protect more sensitive macromolecules such as DNA.

The different responses to UV exposure observed in the two species could be explained by differences in cell wall structure and cellular components. The green algae *T. suecica* is rich in intracellular polysaccharides and the cell wall consist of complex carbohydrates (Becker et al. 1998, Kermanshahi-Pour et al. 2014). Instead of a cell wall, the cryptophyte *R. salina* has a unique cell covering termed the periplast that is made up of thin and fragile rectangular proteinaceous plates underlying the plasma membrane which make the cell quite elastic but also fragile (Goldman & Dennett 1985, Brett et al. 1994).

No other major bands were significant in the PLS-DA model loadings including that we saw no significant changes in chlorophyll for viable and non-viable cells which suggests that no regulatory change was induced by the UV exposure. Similar observations were made by Guihéneuf et al. (2010) in two microalgal species (*Pavlova lutheri* and *Odontella aurita*) exposed to a combination of UVA and UVB irradiation.

A study by Schulze et al. (2011) used a red-green fluorescence assay to distinguish between viable and non-viable *Synechocystis* sp. cells. The method was based on red autofluorescence of “healthy” chlorophyll and unspecific green autofluorescence which is known to signify a degradation of chlorophyll because this fluorescence type is normally superimposed by healthy chlorophyll (Hortensteiner 2006). The study showed the method to be simple, fast and accurate in determination

of viable and non-viable *Synechocystis* sp. cells (Schulze et al. 2011). However, red autofluorescence has been reported to last for weeks in dead cells (Olsen et al. 2015) and has been considered not to be a good marker for viable/non-viable classification of autotrophic cells (Garvey et al. 2007, Olsen et al. 2015). Although the method showed promising results it is limited to be able to asses only autotrophic phytoplankton and not heterotrophic species.

Raman spectroscopy was shown to hold the potential to simultaneously determine the taxonomic classification and the viability state of algal cells and the method is potentially also able to include heterotrophic cells. Algal species known to be robust towards UV treatments, such as *T. suecica* (Lundgreen et al. *In prep.-b*), could be used as indicator species to determine the efficacy of a BWTS by combining the Raman spectra features for species identification and corresponding PLS-DA model with score plots of non-viable and viable.

4.4. Concluding remarks

The present results indicate that is possible to use Raman spectroscopy to classify viable/non-viable *R. salina* and *T. suecica* cells. It would most likely be possible to improve the classification model obtained and decrease the relatively high classification error. To do this, one might try and identify the regions in the spectra which impact the classification most, this is called interval PLS. Here a new model would then be based solely on spectral points selected for maximizing the discrimination, instead of hole spectra.

If one could look beyond these improvements and take this type of classification further, the next step could be to try and replicate the measurements with several types of algal species from monocultures or natural samples. This type of classification could possibly in the future be used to assess the viability of microplanktonic organisms in UV treated ballast water, but more research would lie ahead. If a viability assessment method based on Raman spectroscopy was to be developed to identify viable and non-viable organisms, a setup where a sample is completely screened would first need to be constructed. A possibility for the basis of such a setup could be a lab-on-a-chip, possibly coupled with optical tweezing to fixate the algae doing the measurements. With regards to the data analysis of such a classification setup, it would probably be unwise to make a model with a large number of dependent variables (classes). Instead a stepwise classification, first identifying the species of the organism and then assessing the viability, would most likely yield the best results. It should also be noted that all measurements performed in this work was done in a controlled environment. In the experimental setup the signal contribution from the medium could to some extend be known and controlled. However, this is not what could be expected for a sample of ballast water.

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8. Appendix A - Popular science article

Fighting the Invasion

Environment, Coastal & Offshore (ECO) Magazine (Published in the July/August 2018 issue)

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Fighting the Invasion

Today, invasive species are one of the most serious threats to aquatic ecosystems worldwide. ECO speaks with Kim Lundgreen, Ph.D. Student at the University of Southern Denmark, as he reveals the extent of this international problem as well as the steps being taken to reduce the spread and restore the balance in favor of native communities.

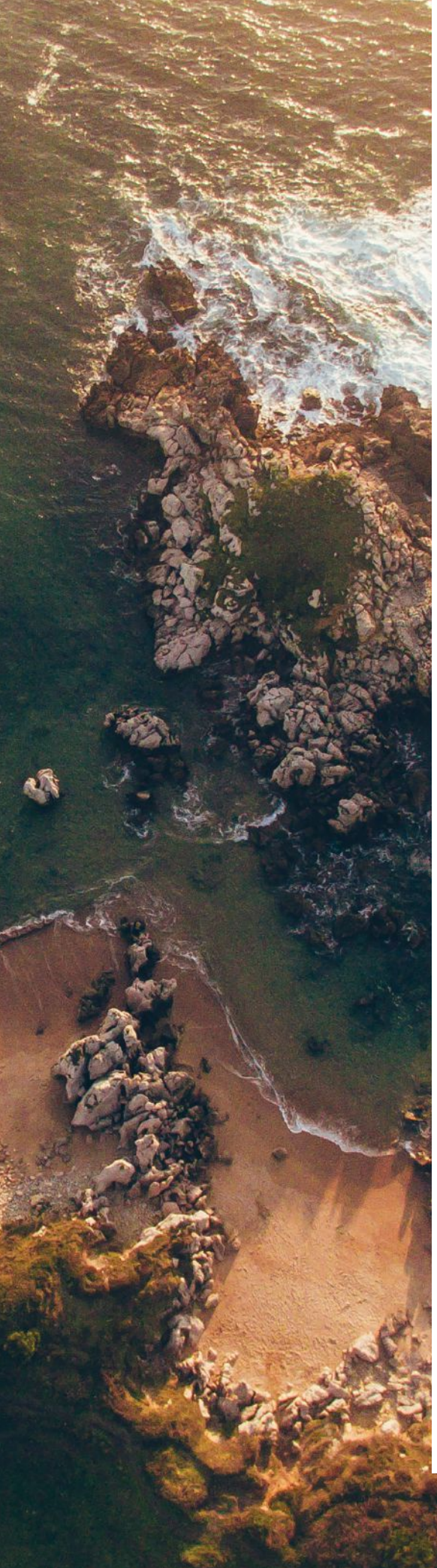
ECO: The spread of aquatic invasive species is a well-known issue impacting coastal ecosystems around the world. But how serious is the problem?

I would say it is very serious, and I don't think many people are truly aware of the extent of the problem, or the huge economic costs when combining both aquatic and land-based invasive species. It was estimated some years ago, that the negative impact of all invasive species put together was equivalent to five percent of the annual world economy – something in the order of $1,400,000,000,000. This is a number that is hard to grasp; even if the costs are overestimated and it is only perhaps half this value, it is still an astonishing number.

Todays' economic burden of land-based invasive species is estimated to be much higher than for aquatic but the continuous introduction of new invasive species in aquatic environments means this will likely increase. A 2012 report from the United Nations Environment Programme estimated annual global costs to be in the order of $100 billion for controlling and preventing the spread of aquatic invasive species. I find this number to be frighteningly high.

I don't think people had foreseen that "just" moving some organisms around a bit would have such severe consequences, not only economically but also regarding harmful effects on ecosystems and human health.





ECO: What are the main ways organisms are being transported?
Organisms can be transported via hull fouling, where they attach themselves on the outside of the ship's hull, but the major transport occurs in the ships' ballast water. Not many people know what ballast water actually is or what it's used for, and that it is the most important player in the spreading of aquatic invasive species.

Ballast water is a necessity for all large ships for safety and economic reasons. It is used for reinforcement of the hull and for adjusting and trimming the position of the ship during voyage in relation to cargo load for better stability and fuel consumption. Some of the biggest ships out there can carry up to 100,000 m^3 of ballast water. That's the same as 40 Olympic sized swimming pools.

In theory, there can be thousands of different species and billions of planktonic cells inside ships' ballast water tanks during voyages.

ECO: Are there species that are more likely to spread than others?
To be acknowledged as an 'invasive species,' an organism must first survive the treatment process during intake. The ballast water is cleaned by treatment systems which are very efficient in removing the larger aquatic animals, but also costly for the ship-owners: the price for some of the treatment systems that can handle large volumes of water are within the range $1-3 million.

Some of the smaller and tougher species can potentially survive if treatment is not optimal, and they are therefore more likely to spread. This especially includes organisms with egg or larval stages such as crustaceans and mollusks, but some algal species have also shown to be robust towards treatment procedures.

Next, they must survive the potentially long passage in the dark ballast water tanks, and eventually a second treatment that happens before the water is discharged into a new environment.

Organisms must also be able to survive and overcome the new conditions where different ecological factors might dominate in relation to the area they originally came from. Finally, it must be able to adapt to a degree where it succeeds in reproducing and establish a population. Once an invasive species has established a reproductive population it can out-compete local species and multiply into pest proportions.

The damage invasive species exert on the environment and biodiversity is considered one of the most serious threats to aquatic ecosystems worldwide. These damages are often irreversible as it is almost impossible to eliminate an invasive species once a population has been established.

One of the most "successful" – and thereby least wanted – invasive species is the zebra mussel in the Great Lakes. It is native in Eastern Europe where the population is controlled by natural predators. Consequently, the absence of these predators in the Great Lakes and the perfect ecological conditions led to a population explosion after it was introduced via the discharge of ballast water. This caused the near extinction of some local species and, as the mussels consume large quantities of phytoplankton, there was a decrease in the food availability for commercial and game fish in the area. It is estimated that zebra mussels will outcompete 50 percent of the native

mussels, eventually causing extinction of more than 100 species. The major economic impacts include clogging the pipes of water treatment facilities and power plants, resulting in huge expenses and mobilization of resources in cleaning operations, as well as costly damages on boats and harbor areas.

Other successful invasive species that have caused major ecological and economic damages include the North American comb jellyfish, the European crab, and the North Pacific sea star which have all had devastating impacts on the fishing industry. The parasitic salmon flukes that infect salmon can cause catastrophic losses in aquaculture productions. And finally, toxic algal blooms can kill or harm other organisms – including humans - and blooms also cause huge economic losses for areas reliant on tourism due to the periodic damage and closing of beaches.

ECO: How has this issue evolved with the growth of the shipping industry?

Until the 19th century, ships' ballasts were solid, typically rocks, but with the introduction of steel ships the solid ballast was replaced by water which was more flexible and could be adjusted according to cargo load.

The use of water as ballast introduced the transportation of unwanted organisms to different corners of the world where some were able to establish stable populations and became the first harmful invasive species.

Shipping is the most cost-efficient way of moving goods and, for that reason, shipping accounts for about 80 percent of the global transportation of goods today. It has been estimated that between four and ten billion tons of ballast water is moved around the world annually. With the growing maritime trade caused by globalization, the demand for more and larger ships will, in my opinion, also increase the risk of transporting potential invasive species in ships' ballast water.

One of the most recent concerns today in terms of invasive species is focused on the opening of Arctic Ocean shipping routes. The Arctic has, until now, been isolated from potential invasions due to its harsh climate but with the opening of shipping routes, the pristine Arctic ecosystems now also become vulnerable.

ECO: What is being done to reduce the spread?

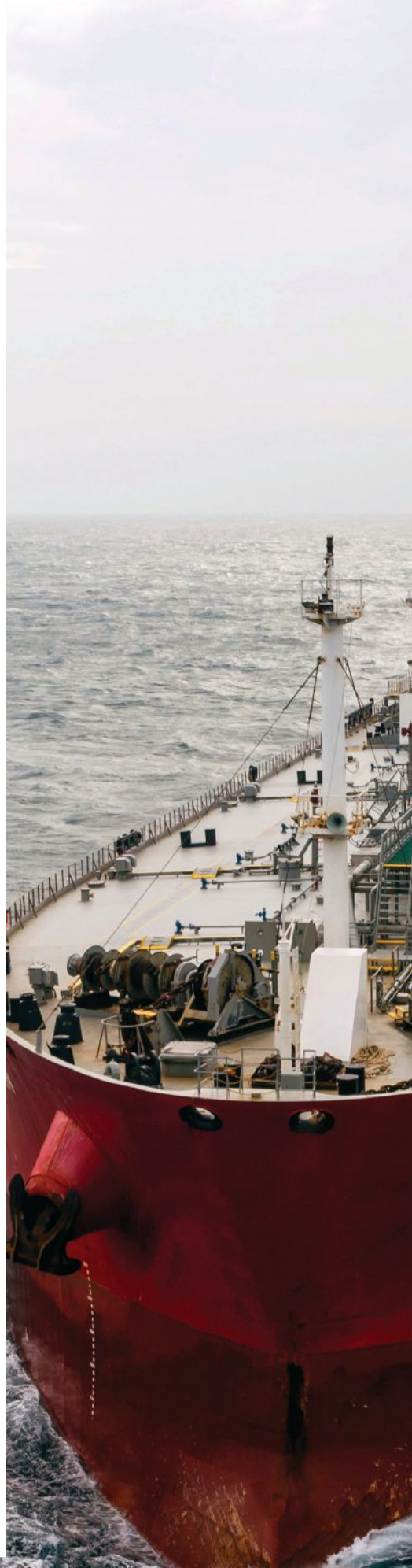
I think it is fair to say that the impact of aquatic invasive species was underestimated, and our efforts to implement controls to encircle and contain the problems were initiated too late and too slow.

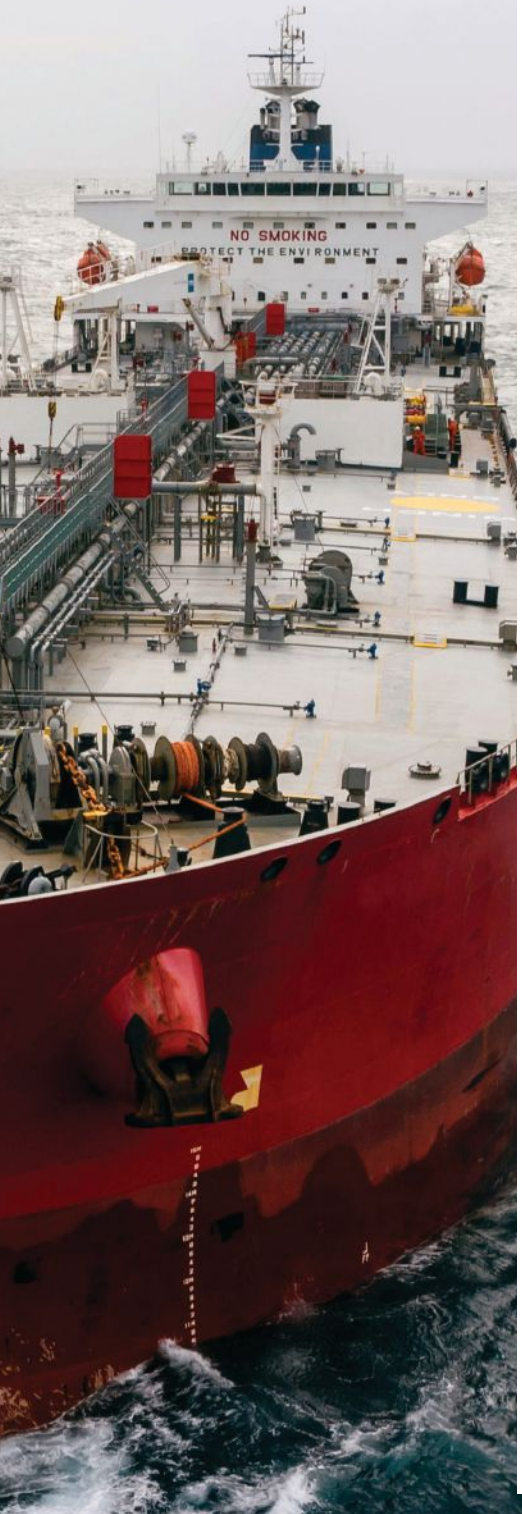
The "International Convention for the Control and Management of Ships' Ballast Water and Sediments" was adopted by the International Maritime Organization (IMO) in 2004 – many years after it was globally accepted that this was a huge environmental and economic issue. It wasn't until September 2017 that the convention was ratified. All ships are now required to have a ballast water treatment system on board that has been thoroughly verified by testing facilities approved by the IMO.

But many invasions have already occurred, and it seems like there is no saturation tendency in the accumulation of aquatic invasive species. However, it is important to keep complying with current regulations regarding ballast water treatment systems. The regulations are constantly being evaluated and work done by initiatives such as Global TestNet and GloBallast Partnership Programme are central in optimizing current regulations for better protection. Both initiatives aim to increase levels of standardization, support development of new technologies, as well as uphold transparency and openness in testing of ballast water management systems.

ECO: What challenges does the industry face in stemming this issue and meeting new regulation requirements?

Implementing new technologies for treatment systems onboard existing ships is always a challenge, both regarding space limitations and economy. The so-called retrofitting is necessary in thousands of ships, so dry dock capacity is also a challenge.





When a treatment system is installed and running onboard a ship, there isn't currently enough technology to help verify the efficiency of the system, and thereby whether ships comply with the regulations set by the IMO and U.S. Coast Guard.

It's my impression that research communities and the industry are working closely together to find solutions. And it's important we that collaborate closely to uncover the most relevant and pressing technical and biological issues that science needs to address to keep improving treatment systems and testing procedures, and help limit any further spreading of aquatic invasive species.

For example, my Ph.D. project at University of Southern Denmark is a collaboration with DHI Water & Environment, which is an IMO approved testing facility. Together, we are addressing the question: Does the use of laboratory cultured standard test organisms mixed with natural algal populations reduce the sensitivity of the testing procedures for organisms in the size class 10-50 µm?

The use of a standard test organisms is necessary for most testing facilities because natural algal population concentrations are not always high enough to fulfil the concentration requirements for testing ballast water treatment systems. Because of this, it is common practice to add standard test organisms to reach the requirements for testing. But it has also been asked if these organisms are "weaker" than natural algal species and whether the use of them lowers test water quality.

If that is the case, the result will be less conservative testing conditions and there is a risk of approving treatment systems that are too weak. On the other hand, if standard test organisms turn out to be as or even more robust than natural species, the practice of mixing can be considered safer. Our research has shown that standard test organisms are promising candidates to use together with natural algal populations for sounder validation of treatment systems. All treatment systems need to fulfil some discharge standards set by the IMO and U.S. Coast Guard. This means that after the treatment process water samples must be collected and quantified for number of living organisms in different size classes. The current method for quantification of the size group 10-50 µm, which mainly consists of phytoplankton, requires labor intensive microscope counting, is relatively slow, requires specialized personnel, and is challenged by subjectivity.

In my research, we are currently developing an automated, faster and more objective method by using a high content screening platform and image analysis software and have produced some promising results for an alternative quantification assessment method for validation of treatment systems.

ECO: Do you think with continued collaboration between science and industry, we can one day stop the spread of invasive species?

Overall, I think the scientific community is very interested and motivated in helping the industry as the shared goal is protection of the environment, human health, and the conservation of pristine ecosystems. And the industry can benefit a lot from collaborations with researchers: often in a highly competitive industries they lack the resources to explore the technical and biological challenges they face. In the scientific community, that resource can be mobilized if the outcome of the collaborations results in publications. But for these mutual collaborations to bloom it is essential that there is effective communication between the two communities.

Still, I do not think we will ever be able to stop the spread of aquatic invasive species. With the right attitude and the combined use of expert knowledge from the IMO, U.S. Coast Guard, initiatives such as the GloBallast Project and Global TestNet, the industry, politicians and the science community, I am optimistic about at least reducing the flow. Hopefully this will result in partial recovery of some affected habitats and improve conditions for sensitive native species worldwide.

9. Appendix B - PhD Programme requirements

Research project requirements

The research project of the PhD student is the main element of the training programme and is carried out under supervision by one or more supervisors. The project is concluded with the delivery of a thesis and a public defense of the thesis. A PhD thesis must as a main rule contain articles of the highest academic level, which can be published internationally. Respecting, of course, the publishing traditions of the research area.

Department of Biology manuscript requirements

A PhD thesis will typically contain three or more full-length articles. Of these, at least one should be submitted, to an internationally recognized journal and at least two articles should have the PhD student as first author. In cases where only manuscripts are included that have not been accepted for publication in an internationally recognized journal, it is up to the assessment committee to assess whether they have the sufficient quality to be publicized in an internationally recognized journal.

Knowledge dissemination / Teaching hours (840 working hours)

Activities that can be credited as knowledge dissemination: External presentations and posters, articles conveying scientific information to the general public, in certain cases scientific articles, production of books, commissioned research, and internal presentations. Posters and short presentation (less than 40 min.) held externally count for 30 knowledge dissemination hours.

Department requirement: 840 working hours.

Activity/course	**Teaching hours**
Scientific Diver Course (2016, 2017, 2018)	259+259+262.5 = 780.5
The Maritime House (high school students)	12+32 = 44
Studie-praktik (high school students)	10.5+5 = 15.5
Mentoring of new PhD students	10
Open House (Biology)	6
TOTAL	**856**

In addition, a popular science article (**10 hours**), an external poster presentation (**30 hours**) and an oral conference presentation (**30 hours**) were completed but these were not part of the official approval of teaching hours.

PhD courses (30 ECTS)

Department requirements: 30 ECTS (29 ECTS was approved by the Department)

Course	Institution	ECTS
An introductory meeting with the Head of the PhD School	SDU	0.5
Course in Project management	SDU	3
Responsible conduct of research	SDU	2
Flow cytometry and cell sorting - A	SDU	2.5
Presentation techniques for PhD students and young researchers	External	1
Doctoral Writing Boot Camp	External	3
Getting started on your teaching	SDU	2
Bioimaging (BMB825)	SDU	5
Advanced Methods in Microscopy Module E: Summer course	KU	5
A guided tour of essential statistical methods for environmental scientists	RUC	5
TOTAL		**29**

Environmental exchange

In the interests of the international level of education, this should normally be a lengthy change in study environment of 2 to 6 months at a foreign research institution that is academically relevant to the PhD programme. Residence should at any rate have a minimum length of 3 consecutive weeks.

- DHI – Singapore 25 Oct – 16 Nov 2016 (23 working days)
- DHI – Denmark visits 2016-2018 (total of 97 working days)

Other activities

The scientific ones

- **Oral presentation** (15 min) at the Aquanvad-ED special session of NEOBIOTA Conference 2018 at the Royal Marine Hotel, Dun Laoghaire, Dublin, Ireland (3-7 Sep 2018). *Title: Development of a novel analytical method for automated viability assessment of planktonic organisms used for validation of ballast water treatment systems*
- **Interview/popular science article** in Environment, Coastal & Offshore (ECO) Magazine (July/August 2018). *Title: Fighting the invasion*
- **Poster presentation** as part of Advanced Methods in Microscopy course at Copenhagen University (19-23 June 2017). *Title: Viability prediction of potential invasive species of microplankton in ship's treated ballast water*

The not so scientific ones

- Organizer of some writing retreats: *Shut up and write!* and *Shut up and write… some more!*
- PhD student representative for work environment well-being/stress issues
- Party Committee member
- Commercial diving committee member and diving activities advisor