Ballast water treatment system testing

Assessing novel treatments and validating compliance methods

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PhD Thesis

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May 2021
Cover photo: NIOZ, Simon Smit
Layout: Cees van Slooten
Printed by: GVO drukkers & vormgevers B.V.

The research reported in this thesis was carried out at the department of Biological Oceanography of the NIOZ, Royal Netherlands Institute for Sea Research, location Texel, The Netherlands.
This work has been co-funded by the North Sea Region Programme under the ERDF of the European Union.

rijksuniversiteit groningen

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Proefschrift

ter verkrijging van de graad van doctor aan de
Rijksuniversiteit Groningen
op gezag van de
rector magnificus prof. dr. C. Wijmenga
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op

vrijdag 18 juni 2021 om 14.30 uur

door

Cornelis van Slooten

geboren op 11 november 1982
te Urk

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Chapter 1

Introduction and thesis outline

1. International regulation on ballast water discharge

The spread of invasive species is recognized as one of the four main threats to global biodiversity. The other major threats are climate change, habitat destruction and overexploitation (Lawler, Aukema et al. 2006). Invasive species cause damage through outcompeting local species for available space, resources, and a lack of natural predators (Gittenberger and Moons 2011). Ballast water is essential for a ship's stability and ability to maneuver. However, by taking up water in one location and discharging this in the next port creates a major vector for the spread of aquatic invasive species (Gollasch, Minchin et al. 2015). To address the problem, the maritime branch of the United Nations, the International Maritime Organization (IMO) in 2004 adopted the Convention on ballast water and sediments (the Convention) (IMO 2004). The Convention aims to address the risk of ballast water mediated invasions by introducing limits on livings organisms in the discharged ballast water. The Convention would enter into force 12 months following the ratification of at least 30 countries representing at least 35% of world tonnage. Finland ratified the Convention in September 2016 and represented the country to reach 35% tonnage. Therefore, the Convention entered into force in September 2017. Through a phased implementation schedule ending in 2024, all ships on international voyages must comply with the Convention and to the incorporated Regulation D-2 Ballast water performance standard. The United States of America (USA) is not a signatory party to the Convention. Instead, independent from the ratification path of the IMO Convention, the USA adopted the Standards for Living Organisms in Ships' Ballast Water Discharged in U.S. Waters; Final Rule (Final Rule) in 2012 (USCG 2012). The Final Rule also addresses the risk of ballast water mediated invasions through imposing limits on the number of living organisms in discharged ballast water. In the USA, the United States Coast Guard (USCG) is tasked with the federal implementation and enforcement of the Final Rule. Both the Final Rule and the Convention introduced virtually identical ballast water discharge standards stating the following:

IMO Regulation D-2 Ballast water discharge standard

> *"Ships conducting Ballast Water Management in accordance with this regulation shall discharge less than 10 viable organisms per cubic meter greater than or equal to 50 micrometres in minimum dimension and less than 10 viable organisms per millilitre less than 50 micrometres in minimum dimension and greater than or equal to 10 micrometres in minimum*

dimension; and discharge of the indicator microbes shall not exceed the specified concentrations described in paragraph 2.
[paragraph] 2
Indicator microbes, as a human health standard, shall include:
.1 Toxicogenic Vibrio cholerae (O1 and O139) with less than 1 colony forming unit (cfu) per 100 millilitres or less than 1 cfu per 1 gram (wet weight) zooplankton samples;
.2 Escherichia coli less than 250 cfu per 100 millilitres;
.3 Intestinal Enterococci less than 100 cfu per 100 milliliters."

USCG 33 CFR 151.1511 – Ballast water discharge standard.

"(a) Vessels employing a Coast Guard approved ballast water management system (BWMS) must meet the following BWDS by the date in § 151.1512(b) of this subpart:
(1) For organisms greater than or equal to 50 micrometers in minimum dimension: discharge must include fewer than 10 living organisms per cubic meter of ballast water.
(2) For organisms less than 50 micrometers and greater than or equal to 10 micrometers: discharge must include fewer than 10 living organisms per milliliter (mL) of ballast water.
(3) Indicator microorganisms must not exceed:
(i) For Toxicogenic Vibrio cholera (serotypes O1 and O139): a concentration of less than 1 colony forming unit (cfu) per 100 mL.
(ii) For Escherichia coli: a concentration of fewer than 250 cfu per 100 mL"
(iii) For intestinal enterococci: a concentration of fewer than 100 cfu per 100 mL."

A key difference in the definition between the IMO and USCG Ballast Water Discharge Standard (BWDS) has been the subject of much debate. The IMO Convention refers to 'viable' organisms whereas the Final Rule refers to 'living' organisms. However, contrary to what has often been suggested, both BWDS used to be equivalent because the original IMO Convention defines the term 'viable' as follows*: "Viable Organisms are organisms and any life stages thereof that are living."* A discrepancy was introduced by the IMO via the Code

for approval of Ballast Water Management Systems (BWMS Code) (IMO 2018). The BWMS Code prescribes how a BWMS should be evaluated in order to obtain type approval from an IMO-member Administration. Type approval is a necessity for any BWMS to be allowed installation and use. However, the BWMS Code defines the term 'Viable' as: *"Viable organisms mean organisms that have the ability to successfully generate new individuals in order to reproduce the species."* In practice this means that the test requirements per the BWMS Code allow for the evaluation of discharged ballast water through a regrowth assessment of any surviving organisms. Therefore, the IMO accepted a Most Probable Number (MPN) serial dilution and incubation technique as one of the evaluation options (IMO 2017). In contrast, the USCG has thus far solely recognized methods that demonstrate whether an organism is 'living', rather than 'able to reproduce'. Despite fervent efforts from the industry, the MPN technique is not yet recognized by the USCG to determine the concentration of living organism in discharged ballast water (USCG 2019). In terms of curbing the introduction of invasive species, the term 'ability to successfully generate new individuals' as defined in the BWMS Code is more relevant than whether a specific organism is 'living' or 'dead'. In practice however, it is more straightforward to conduct a live/dead assessment then a viability assessment. Moreover, the definition of 'living' is arguably a more conservative approach, since solely living organisms may be able to reproduce, whereas 'non-viable' organisms may nevertheless be living.

For ships to comply with the BWDS, several management options are currently available in both IMO and USCG regulated waters:

Avoid discharge of ballast water in areas subject to the BWDS – The introduction of the compulsorily BWDS was an incentive for the shipowners to manage ballast water discharge in various ways. Ship's crew can decide to reduce or avoid the discharge of ballast water in certain ports, assuming the ship's safety is not compromised. More fundamentally, the BWDS has revived the interest in ballast-free ships or no-ballast ships. Several designs of ballast-free ships have been approved by leading class societies in recent years and maritime interest is growing due to projected costs savings by eliminating the need for ballast water infrastructure on board (Kakalis 2016)

Use potable water from recognized sources – The USCG recognized the use of potable water from a US source as an option to obtain compliance with the Final Rule. However, potable water from non-US sources is not automatically recognized. In 2016, the Dutch shipowner Van Oord B.V. was granted IMO G8 type approval for a system using potable water, up to 450 m^3 (Anonymous 2016).

Deliver the water to a port-reception facility – Port-reception facilities for ballast water have been presented as a contingency measure to allow ships to discharge their ballast water in the event of a BWMS failure (Anonymous 2017). A port-reception facility may store the non-compliant ballast water in shore-based tanks or dedicated barges for further treatment. In 2017, the Damen InvaSave BWMS received IMO G8 type approval from The Netherlands to disinfect the ballast water through a floating-barge reception facility, treating the ballast water from a ship on the barge and immediately discharging it overboard (Anonymous 2017). Similar initiatives are in development around the world, since it is expected that in the early phase of the implementation of BWDS, non-compliant ballast water will be a frequent occurrence (PACT 2021).

Use an onboard BWMS to disinfect the water prior to discharge – The majority of shipowners are considering the shipboard installation of a ballast water treatment system to disinfect the ballast water prior to discharge to comply with the BWDS. Having an onboard treatment system ensures the continued operation of the vessel independent of the third-party availability of freshwater or port-reception facilities. As a global enterprise, shipowners value self-reliance under the most challenging of circumstances. Dependence on shore-based solutions (tap water, reception facilities) is too often irreconcilable with ships' erratic sailing schedules and destinations.

2. BWMS type approval process

The BWDS has generated a market for shipboard solutions to the requirements of the IMO Convention and the USCG Final Rule. This has been an incentive for dozens of manufacturers to develop BWMS to disinfect ballast water. However, for ships to be allowed to carry a BWMS, the equipment requires an approval of the flag-state where the vessel is registered. Moreover, a ship-specific classification society must review and approve the equipment in accordance with its respective steel vessel rules, to ensure the safety of the ship and crew. Thus, to be allowed to discharge water treated with a BWMS, the equipment requires so-called type approval by the classification society and national administration signatory to the Convention or, in the case of U.S. waters, a USCG type approval, to enable discharge of treated water in area's subject to the Convention or the USCG, respectively. Generally speaking, the USCG and IMO type approval test requirements are comparable and the BWMS evaluation steps can be summarized as follows:

Readiness evaluation – The readiness evaluation aims to determine whether the BWMS is complete and in its final production configuration. Once testing has started the

BWMS is subjected to a design freeze and cannot be altered without the risk of voiding prior testing. The evaluation primarily consists of an engineering review to determine whether the proposed BWMS adheres to classification society's rules as well as the requirements of the Convention and Final Rule with respect to the design, construction, control and monitoring of the BWMS. This review is predominantly a desktop study of documentation describing the BWMS.

Component environmental testing – Once the BWMS has been determined ready for testing, its electrical and electronic components are subjected to environmental testing. The BWMS Code adopted the Unified Requirements E10 of the International Association of Classification Societies (IACS UR E10) (IACS 2014). In contrast, the USCG has incorporated distinct component environmental test requirements detailed in the Final Rule. The primary objective of the component environmental testing is to evaluate whether the electric and electronic components are safe and reliable to use in a shipboard environment with respect to electromagnetic interference, ship's vibration, temperature, humidity, sea-spray, voltage & frequency variation and inclination.

Land-based testing – Comprehensive testing protocols for land-based testing have been adopted by the IMO and the USCG. The Final Rule incorporated by reference the Environmental Technology Verification (ETV) protocol to specify land-based testing requirements (NSF-International 2010). Concurrently, since 2004 the IMO has implemented three iterations of its requirements for testing of ballast water treatment systems, the latest being the BWMS Code. Land-based testing consists of three distinct parts, Biological Efficacy (BE) testing, Operation and Maintenance (O&M) testing. Firstly, BE testing is aimed at determining whether the BWMS is able to disinfect the water to comply with the BWDS. Paramount among both land-based test programs is the requirement to conduct five consecutive successful test cycles at each of three pre-defined water salinity regimes, nominally called freshwater (0-1 PSU), brackish water (10-20 PSU) and seawater (28-36 PSU). Although technically incorrect, Practical Salinity Unit (PSU) is used as notation for salinity in all ballast water regulations. To avoid confusion, this thesis refers to salinity in PSU as well. See also (UNESCO-IOC 2010). Typically, the BWMS is containerized and tested at facilities that are purpose-built to conduct the land-based testing. The water used for BE testing must be sourced from natural origins and contain prescribed quantities of biota, organic carbon and sediments to challenge the BWMS. Augmentation of source water is permitted provided that it can be demonstrated that the augmented water presents an equivalent challenge for the BWMS to water that would have naturally contained sufficient

challenge densities of biota, organic carbon and sediments. A test cycle comprises of pumping water into designated hold tanks (>200 m^3) and stored for a minimum of 24 hours. Depending on treatment design, water is disinfected during the uptake, storage, discharge, or a combination of these three. The water used in the test cycles is characterized for physical, chemical and biological variables at least during the uptake to verify the challenge water characteristics, and during the discharge of the tank to verify compliance with the BWDS. Secondly, per USCG ETV Protocol (not required in the Convention), BWMS are required to operate for a total of 50 hours treating water in the process. During this evaluation the long-term mechanical robustness of the BWMS is evaluated by monitoring the technical performance of the BWMS and verifying the power requirements and maintenance intervals as indicated by the manufacturer. Discharged water during O&M testing is not required to be sampled and analyzed for physical, chemical and biological variables.

Environmental Acceptability evaluation – When the BWMS employs an active substance to disinfect the ballast water, additional evaluations are required to verify that the discharged ballast water poses no unacceptable environmental risk to the receiving waters. An active substance is defined by the Convention as follows: "*Active Substance means a substance or organism, including a virus or a fungus, that has a general or specific action on or against harmful aquatic organisms and pathogens*". The evaluations required for systems using active substances are stipulated in the IMO Guidelines G9 (IMO 2008) and further specified in the Methodology for information gathering and conduct of work of the GESAMP-Ballast Water Working Group (IMO 2017). In parallel, to use active substances that are designated as pesticides in the United States, a FIFRA registration is required for the sale, distribution and application of pesticides in the US as regulated by the Environmental Protection Agency (EPA) (EPA 2013). The EA evaluation of active substances focuses on characterizing and quantifying any Disinfectant Byproducts (DBPs) generated by the BWMS during land-based testing. Discharged treated water must also be subjected to multiple Whole-Effluent Toxicity (WET) assays. A WET test consists of subjecting model organisms to a dilution range of discharged water generated during land-based BE testing. Test data using model organisms of at least three trophic levels (invertebrate, vertebrate, fish) are required in the evaluation. Several endpoints can be evaluated during WET testing such as hatching, survival, reproduction and growth of the model organism. Commonly active substances used are generated onboard via electro-chlorination (EC) of seawater. When treating freshwater, an onboard brine-tank is used to provide the ions (chloride and bromide) needed in the EC process. EC is employed by ≈40% of IMO type approved systems

(ClassNK 2020). To protect the receiving environment, the IMO and USCG introduced a maximum allowable discharge concentration (MADC) of <0.1 mg/L of Total Residual Oxidants (TRO). TRO is the container term for all oxidants present in the water. Depending on the ion source, EC systems generate various active substances. TRO in seawater consists typically of various forms of chlorine and bromine as seawater contains abundant levels of chloride and bromide ions (Wong 1982). To comply with the MADC at discharge the TRO is neutralized prior to discharge by the injection of sodium thiosulfate or sodium sulfite in the ballast line. In-line TRO sensors typically control how much neutralization is required and subsequently monitor if the neutralization was successful. Chlorine injection and EC are well-known for its potential to produce DBPs in treated ballast water, such as haloacetic acids and trihalomethanes (Moreno-Andrés and Peperzak 2019) (David, Linders et al. 2018). As a result, the chlorinated effluent sometimes causes growth inhibition in various phytoplankton WET assays, demonstrating the importance of DBP and WET testing. In accordance with the IMO G9 procedure, the results of the WET test and the DBP's detected are subjected to a risk assessment to determine if the BWMS poses unacceptable risk to the ship's crew, general public or the environment. The risk assessment is evaluated by the IMO GESAMP-Ballast Water Working Group who recommends granting or denying approval for the BWMS (IMO 2019). Approval is formally granted or denied by IMO member states during the Marine Environmental Protection Committee (MEPC) meetings, organized roughly every 9 months at the IMO headquarters in London, UK.

Shipboard BE testing – The controlled environment of the land-based testing phase is well-suited to test the BWMS under standardized conditions. However, shipboard testing is primarily aimed at verifying the performance of the BWMS under actual real-world conditions that the BWMS have to operate in. The BWMS is often installed on a commercial (cargo) vessel in the engine room or another location that is consistent with its final intended use on vessels. The Convention requires continuous use of the BWMS over a period of at least 6 months. During that period, 3 BE cycles (IMO), or 5 BE cycles (USCG) are required to test the BWMS performance in at least two distinct geographical locations.

Scaling evaluation – To accommodate the wide variety of ships' ballast pump capacities, manufacturers usually offer a series of BWMS flow capacities. Due to practical constraints of the land-based test facilities, the land-based BE testing is performed at 200 – 500 m^3/h flow capacities, which represent the low-end of BWMS on offer. Depending on ship availability, shipboard BE testing is often performed using a BWMS model with a higher flow capacity than used for the land-based testing. However, manufacturers may apply

for type approval for more versions than solely tested in the land-based and shipboard environment. For an administration to consider such an application, the manufacturer needs to demonstrate via alternative means that the performance of the BWMS examined in land-based and shipboard tests can be extrapolated to the remaining models of the line-up. Depending on the specific technology used, Computational Fluid Dynamics (CFD) models are used to demonstrate that, for example, the mixing behavior of active substances is equivalent among all models of their line-up. In some cases land-based or shipboard testing of the (up)scaled units is required (IMO 2018).

3. Implementation of the Convention and Final Rule

Around the year 2024 the majority of ships will have to comply with the BWDS as detailed in regulation D-2 of the Convention. In most cases this requires installing an onboard BWMS. Concurrently, the USCG BWDS has already been implemented in 2012 by the Final Rule. However due to the lack of type approved systems, many ships have been able to obtain an extension to delay compliance to the US Final Rule. Voluntarily, shipowners have also had the opportunity to use an IMO type approved system as long as that system was recognized as an Alternate Management System (AMS) by the USCG. An AMS can be used in US-waters until a suitable USCG type approved system becomes available for a particular vessel (USCG 2012). Since December 2016, the number USCG type approved systems has been steadily increasing up to 39 systems as of December 30th, 2020, with another 8 systems under review (USCG 2021). With the increasing availability of USCG type approved systems it is expected that further granting of AMS or further extensions of existing AMS will be gradually phased out by the USCG in the coming years. When an AMS extension expires, the BWMS must either be upgraded or wholly replaced by a USCG type approved system to continue discharging in US waters.

Enforcement of the Convention and Final Rule – Any regulation that is not actively enforced is at risk of being ignored. In Article 9 of the Convention, it is outlined that three main tools are available to Port State Control (PSC) officers to determine whether a ship is in compliance with the Convention's D-2 discharge standard: (1) Verify the presence of the Type Approval certificate of the installed BWMS; (2) Inspection of the Ballast Water record book and; (3) Sampling of the ballast water provided this does not cause undue delay to the ship's schedule. To facilitate the enforcement of the Convention, and clarify ballast water sampling procedures, the IMO adopted the Guidelines for Ballast Water Sampling G2 (Guideline G2 (IMO 2008). Therein, it is recommended that ships provide a suitable ballast

water sampling point in the ballast water discharge line. Samples should be taken from the discharge line as close to the point of discharge as practicable. Guideline G2, article 6.3 mentions the following: "*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. […]*". Thus, the IMO clearly recognizes the value of indicative analyses to establish whether a ship is potentially non-compliant with the BWDS. Guideline G2 does not further discuss what form the indicative analysis should take. Instead, it recognizes in Article 6.6 that: "*given the complexity of ballast water sampling and analysis, new approaches may be developed to assess the composition, concentration and viability of organisms*". More recently, the IMO published Circular 42 in the requirements for compliance testing and a list of available compliance tools, which were yet to be developed at the start of this PhD research (IMarEST 2019, IMO 2020).

Paris-MOU – Guideline G2 laid out guidelines for the inspection of ships per Convention regulation D-1 (ballast water exchange) and regulation D-2 (BWDS) compliance. Generally, ships are inspected upon arriving in a port by a Port State Control (PSC) officer. PSC is employed or contracted by the national administration and is tasked with verifying and enforcing compliance of ships to national regulations and requirements. Because ships trade internationally, administrations in various global regions have harmonized in Memoranda of Understanding (MOU) their PSC inspections. The Paris-MOU describes how 27 European countries, including Russia and Canada, have harmonized their PSC inspections (ParisMOU 2018). Each PSC inspector is tasked with inspecting vessels against 17 conventions and protocols of which the Convention is the latest addition. Inspections are ranked in three orders of detail: Initial; More detailed or; Expanded Inspection. Depending on the type and age of the vessel PSC categorizes incoming ships for initial or more detailed inspection. Also, PSC across states may share information on particular ships, leading to a more detailed or expanded inspection for certain vessels in the next port of call. An Initial Inspection is limited to checking the certificates and documents that must be present onboard the ship. For example, ships are required to carry a valid ballast water management certificate and maintain a ballast water record book, as detailed in Article 9.1 (a) and (b) of the Convention respectively. A More detailed or Expanded inspection may involve the actual sampling of the ship's ballast discharge as detailed in Article 9.1 (c) of the Convention.

Commissioning testing – The need to sample ballast water is not limited to PSC inspections. The Survey Guidelines under the Harmonized System of Survey and

Certification outline how class societies should survey and certify newly installed BWMS upon commissioning of the system (IMO 2019). The 2019 HSSC guidelines call for actual sampling and analysis of the treated discharge water to verify compliance with the BWDS. At MEPC 73 (October 2018) a protocol was discussed how to sample and analyze the discharge during the commissioning of the BWMS. As a result, in November 2018 the IMO Guidance on Commissioning testing was issued with guidelines for a detailed sampling and indicative analysis of the discharged ballast water once all installation activities are completed, and was revised in 2020 at MEPC 75 (IMO 2020). From June 2022 onwards, the BWMS commissioning testing will become mandatory by all IMO member states via the incorporation of the guidance into the HSSC and the incorporation of the HSSC into the Convention.

US EPA Vessel General Permit – To regulate incidental discharges from ships in US waters, the EPA has implemented several iterations of the Vessel General Permit, which lays out the requirements for ships how to deal with the incidental discharge of graywater, bilgewater, exhaust gas scrubber wash water and treated ballast water effluent (EPA 2013). The effluent of all ballast water treatment systems needs to be monitored annually for total heterotrophic bacteria, *E. coli* and enterococci. When a BWTS employs a biocide to treat the ballast water, the VGP requires regular monitoring of the residual biocide and its derivatives in the discharge. In case of chlorination the relevant derivatives to be monitored are, chlorite, chlorate, total trihalomethanes and haloacetic acids.

Vessel Incidental Discharge Act (VIDA) – As listed above, the US has a myriad of regulations and two government agencies (EPA and USCG) governing and enforcing ballast water related matters. To streamline the rulemaking and enforcement tasks, the USA signed the Vessel Incidental Discharge Act (VIDA) into law in 2018 (US-CONGRESS 2018). In the coming years the VIDA requires the EPA and the USCG to update its regulations and enforcement requirements to comply with the VIDA. The EPA has published its national standards for incidental discharges (EPA 2020). By 2022 the USCG will be responsible for developing the corresponding compliance and enforcement requirements. This means that within several years the current framework of the VGP is scheduled to be revoked and replaced by the newly updated rules that are currently being developed.

4. Thesis aims and outline

At the start of this PhD project many new BWMS were in development and several active substances needed to be investigated for their applicability in ballast water treatment. Also,

the bacteria test requirements developed for type approval testing were criticized for prescribing labor-intensive plating methods whilst more efficient technologies were available. At the same time, it was generally unclear to PSC officers and other stakeholders how to conduct the compliance monitoring and enforcement in a cost effective, timely and easy manner. To address these challenges, the following more specific research topics were addressed which correspond to the various stages in the development, testing and compliance monitoring of ballast water management systems as described above:

1. Is a quaternary ammonium compound suitable as active substance to disinfect ballast water?
2. To enumerate heterotrophic bacteria, how do agar growth media compare to automated cell counting and molecular techniques? (Chapter 3)
3. Is the FlowCAM a suitable device to conduct indicative ballast water discharge analysis?
4. How do several proxy measurements perform in indicative ballast water compliance testing?

Each of the four questions were addressed in distinct research projects as described below:

In **chapter 2,** a quaternary ammonium compound was tested for use in ballast water treatment. In order to develop a BWMS, it is valuable that the disinfection potential of the treatment method is carefully assessed prior to any investment in the technological development into a commercial product. When investigating the potential disinfection properties in relation to ballast water treatment it is important to recognize two confounding factors that may cause problems in the application of commonly used water treatment technologies. In the first place, unlike stationary shore-based applications, the range of water quality conditions that a ship encounters varies considerably depending on its location and seasonal influences. Factors as salinity, temperature, sediment load, turbidity and pH of the water can notably impact the disinfection capacity of many treatment methods. Key is to recognize that a robust method is needed capable of responding predictably to achieve the BWDS in virtually any water quality it may encounter. Secondly, practical ship related matters should be considered such as the generation and storage of the disinfectant and its application method to treat the ballast water. Lastly, if the treatment method leaves a residual toxicity prior to discharge, an effective and practical neutralization method must be developed to ensure safe sampling practices and harmless discharge into the receiving

environment. The quaternary ammonium compound didecyldimethylammonium chloride (DDAC) was assessed for its potential to disinfect ballast water taking all the considerations mentioned above into account. An experiment was designed to assess the disinfection capacity of increasing doses of DDAC, using phytoplankton monocultures and natural plankton communities in seawater as challenge organisms. Subsequently, residual toxicity in the treated water was assessed using phytoplankton and regrowth potential as indicator. The residual concentration of DDAC was monitored using a colorimetric method. Lastly, addition of bentonite clay was assessed to test an inactivation method for the residual DDAC.

In **chapter 3,** a comparative analysis of heterotrophic plate counting (HPC), Flow Cytometry (FCM) and quantitative Polymerase Chain Reaction (qPCR) was conducted. For type approval purposes, the testing of treated ballast water has to be performed using prescribed standardized methods as found in the ETV protocol for USCG type approval and the BWMS Code for IMO type approval, respectively. These prescribed methods are often derived from industrial best practices and have, in some cases, been in use for decades. The major benefit of using standardized methods, where available, is to improve the comparativeness of BWMS tested for type approval by different laboratories. However, the downside of using standardized methods is, rather often, their archaic nature, as the industry moves forward in adopting more advanced technologies for improved accuracy, precision, cost and usability. We investigated one element of the water quality monitoring prescribed during the type approval process: total heterotrophic bacteria analysis. The various HPC methods as detailed in the ETV protocol were compared with each other and with two well-established techniques involving (qPCR) and FCM. Samples for HPC were spread onto solid agar medium plates with appropriate nutrients and incubated for several days at a controlled temperature. As bacterial growth on the agar plate is monitored, HPC produces results in the form of Colony Forming Units (CFU). In contrast to the other methods tested, HPC was the only viability test using cell replication as endpoint. Alternatively, qPCR was used to amplify the 16S ribosomal Ribonucleic Acid (16S rRNA) gene specific to heterotrophic bacteria. During the cumulative amplification cycles, the resulting increase in double stranded DNA fragments was measured via a fluorescent marker that was directly monitored by a fluorometer, yielding a real-time result as the amplification progresses. The basic premise is that the fewer cycles are needed before the fluorescence passes the detection limit, the higher the original concentration of heterotrophic bacteria must have been. Via comparison to a standard curve the original cell concentration was estimated. In FCM, samples were stained with a fluorescent DNA stain making intact cells fluorescent. Stained samples are analyzed

by a particle counter that is able to determine particle numbers and size by perturbations in the laser scatter when a particle crosses the light-beam. Each particle is simultaneously assessed for fluorescence intensity by a sensor detecting the DNA stain, thereby discriminating between debris and actual DNA containing cells. Fluorescent particles in the appropriate size were counted as bacteria. The comparative study was combined with a long-term sampling effort in the Wadden Sea to investigate the seasonal fluctuations in heterotrophic bacteria in seawater typically used for BWMS type approval testing. Additionally, freshwater was sampled from Lake NIOZ. Practically, it was assessed how much effort and training and analysis time is required to conduct each method. Results were assessed via regression analysis to compare various methods with each other. Finally, it was discussed with method yielded the most accurate and useful results with respect to type approval and compliance testing.

In **chapter 4,** an imaging-in-flow system (FlowCAM) was assessed for systematic ballast water analysis. Traditionally, planktonic samples are analyzed for species composition and abundance via microscopy, which is an accurate albeit slow process. In recent decades the use of FCM gained popularity for its capability to automatically assess thousands of particles for dozens of variables in rapid fashion. Both microscopy and FCM have their advantages and limitations for the analysis of treated ballast water. As described above, PSC officers and other stakeholders have a significant interest in finding suitable analytical tools to rapidly assess ballast water for compliance to the BWDS. Microscopy is predominantly used by highly trained analysts during type approval testing of BWMS. Per the ETV protocol, microscopy is even the only method allowed for the enumeration of organisms. Its major drawback are the high training and qualification requirements needed to reliably use this tool to generate accurate and precise results. Also, the manual visual assessment of each particle makes this approach tedious, time-consuming and only allows for a limited volume to be analyzed. On the other hand, FCM solves a number of these limitations by offering an automated way of particle analysis. Additionally, FCM is able to capture dozens of particle characteristics for later analysis, such as (auto)fluorescence and size to identify the particles of interest. The major drawback of using FCM in PSC inspections is the lack of visual confirmation of the collected particle to substantiate it is an actual living organism. Also, most FCM systems are not able to process larger particles such as the ≥50 µm zooplankton fraction. In particular at the low concentrations close to the D-2 standard of <10 organisms per mL, the interference of debris in the sample becomes an ever more pressing problem, considering also the small volumes that FCM is able to process. For example, a single

particle detected in the 10-50 µm fraction in 100 µL sample volume, immediately converts to the exceedance of the discharge standard. In such cases it is important to be able to visually confirm if the detected particle is, in fact, an intact organism and not merely dead cell material or debris. Many FCM devices do not offer this possibility. The FlowCAM aims to solve this limitation by combining the best of both approaches. A sample is pumped through a glass flow cell via a computer-controlled syringe system. It then uses a magnification lens and camera to visualize and photograph individual microscopic particles as they pass through the flow cell. In this manner, individual particles are captured at up to 20 frames per second. Within minutes this method yields a suite of particle information from a single sample that would otherwise take hours to collect via microscopy. The device has two modes to trigger the capture of a particle: Auto Image Mode and Fluorescent Trigger Mode. In Auto Image Mode every particle that creates sufficient contrast to the background is captured. In Fluorescent Triggered Mode, particles are only photographed if they emit red fluorescence indicating the presence of chlorophyll. Additional to merely collecting the images, the proprietary Visual Spread Sheet software is able to analyze the pictures for over a dozen variables, such as length, width, area, and color, enabling the grouping and discrimination of phytoplankton cells, zooplankton and debris. Several experiments were conducted to determine the FlowCAM's performance in measuring the concentration of particles, using microbeads, UV-treated *Prorocentrum minimum* cells and natural water treated by a full-scale BWMS. The practical handling of the device in terms of ease-of-use, durability and endurance were assessed as well.

In **chapter 5,** two commercially available methods were compared with an inhouse developed method on UV-treated ballast water. The treated water was collected from samples obtained during land-based type approval testing of a full-scale BWMS. The BWMS treatment involved filtration and dosing medium pressure UV at uptake followed by a secondary medium pressure UV treatment at discharge after a 5-day holding time. The first commercially available method tested was an FDA kit (Hach), which filters 200 mL over a 10 µm nylon filter using a manual vacuum pump, collecting any remaining intact >10 µm sized organisms. The filter was subsequently placed into a solution with FDA and incubated for 15 minutes. FDA reacts with the functional esterase enzymes in organisms retained onto the filter. Following incubation, the FDA-induced fluorescence was measured using a handheld fluorometer as indication for compliance. The second method derived from Hach measured photosystem II (PSII) efficiency. PSII efficiency is considered a good indicator for the ability of chlorophyll to conduct photosynthesis. Without this ability phytoplankton cells

are either dying or dead. PSII was measured by chemically blocking the electron transfer in the PSII photosynthesis enzymes by adding the chemical 3-(3,4-dichlorophenyl)-1, 1-dimethylurea (DCMU). The DCMU based approach to measure PSII Efficiency was traditionally used before the development of the Pulse Amplitude Modulation (PAM) fluorometry using saturating light instead of DCMU. Hence, a PAM device (Walz) was used alongside the DCMU method for comparison. As third indicative method a commercially available ATP analysis tool was used (3M Clean-Trace™). To improve the performance of this generally applicable method for compliance testing, a concentration method was developed by using easy to use syringes and 10 µm nylon filter in single-use filter capsules that attached to the syringe. The resulting concentrate was analyzed using the 3M Clean-Trace™ total ATP swabs. The methods were evaluated specifically for ease of use, accuracy and sensitivity. Finally, an overall assessment was made as to which method was most promising in becoming a successful indicative tool for ballast water compliance testing.

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Chapter 2

Assessment of didecyldimethylammonium chloride as a ballast water treatment method

Assessment of didecyldimethylammonium chloride (DDAC) as ballast water treatment method

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Published in: Environmental Technology DOI 10.1080/09593330.2014.951401

Funding
This work has been co-funded by the North Sea Region Program under the ERDF of the European Union.

Abstract

Ballast water mediated transfer of aquatic invasive species is considered a major threat to marine biodiversity, marine industry and human health. Ballast water treatment is needed to comply with IMO ballast water discharge regulations. Didecyldimethylammonium chloride (DDAC) was tested for its applicability as ballast water treatment method. Treatment of the marine phytoplankton species *Tetraselmis suecica*, *Isochrysis galbana* and *Chaetoceros calcitrans* showed that at 2.5 µL L^{-1} DDAC was able to inactivate photosystem II (PSII) efficiency and disintegrate the cells after five days dark incubation. Treatment of natural marine plankton communities with 2.5 µL L^{-1} DDAC did not sufficiently decrease zooplankton abundance to comply with the IMO D-2 standard. Bivalve larvae showed the highest resistance to DDAC. PSII efficiency was inactivated within five days but phytoplankton cells remained intact. Regrowth occurred within two days of incubation in the light. However, untreated phytoplankton exposed to residual DDAC showed delayed cell growth and reduced PSII efficiency, indicating residual DDAC toxicity. Natural marine plankton communities treated with 5 µL L^{-1} DDAC showed sufficient disinfection of zooplankton and inactivation of PSII efficiency. Phytoplankton regrowth was not detected after nine days light incubation. Bacteria were initially reduced due to DDAC treatment, but regrowth was observed within five days dark incubation. Residual DDAC remained too high after five days to be safely discharged. Two neutralization cycles of 50 mg L^{-1} bentonite were needed to inactivate residual DDAC upon discharge. The inactivation of residual DDAC may seriously hamper the practical use of DDAC as a ballast water disinfectant.

Keywords

DDAC; ballast water treatment; IMO D-2 standard; zooplankton; phytoplankton

1. Introduction

The ongoing spread of aquatic invasive species through ballast water poses major risks to global biodiversity and may negatively impact marine industries and human health. (Bax, Williamson et al. 2003) Ballast water transport through shipping is considered a major vector in the spread of aquatic invasive species. (Gollasch 2006, Drake and Lodge 2007) To halt this spread the International Maritime Organisation (IMO) adopted the international convention for the control and management of ship's ballast water and sediments. (Anonymous 2004) The convention limits the maximum number of viable organisms allowed to be discharged through ballast water. These requirements are known as the D-2 Ballast Water Performance Standard (D-2 standard). (Anonymous 2004) In order to comply with the D-2 standard ship

owners will have to install Ballast Water Treatment Systems (BWTS) aboard their ships to disinfect the ballast water prior to discharge. In recent years many companies have developed on board treatment systems capable of disinfecting ballast water. (Gregg, Rigby et al. 2009) In order for a BWTS to be appreciated as a viable and effective system, the IMO has developed elaborate testing procedures. (Anonymous 2005, Anonymous 2008, Anonymous 2008) One of the major phases in the approval process is the execution of a full-scale land-based verification test. Prior to full-scale verification testing, pilot experiments are usually performed. Here the assessment of didecyldimethylammonium chloride (DDAC) as a potential ballast water treatment option is presented.

DDAC is a quaternary ammonium compound which is commonly used as a general disinfectant for a wide range of applications. It was registered by the United States environmental protection agency in 1962. DDAC is used to disinfect surface areas such as household, agricultural and medical equipment. DDAC is also used as disinfectant in swimming pools and as anti-sapstain agent in the wood industry. It is estimated that a total of 396 commercial products contain DDAC as active ingredient. (Anonymous 2006)

DDAC is a molecule with a positively charged cationic head side (ammonium) and a hydrophobic carbon tail side. Disinfection is achieved by binding of the hydrophobic tail into the lipid bilayer of cell membranes while the cationic head sticks out into the water phase. The binding causes rearrangement of the lipid bilayer which disrupts the cell membrane and leads to leakage of cell content and eventually cell death. (Ioannou, Hanlon et al. 2007)

To test the efficacy of DDAC in ballast water treatment, results of experiments were checked for compliance with the D-2 standard for organisms >50 µm (limit: <10 viable organisms m^{-3}), commonly referred to as zooplankton and 10-50 µm phytoplankton (limit: <10 viable organisms mL^{-1}). In addition to zooplankton and phytoplankton abundance, also photosystem II (PSII) efficiency, bacterial abundance and DDAC concentrations were monitored.

Experiments included lab scale trials using three phytoplankton monocultures to determine the appropriate DDAC dose needed for disinfection of marine phytoplankton. During these experiments phytoplankton concentration and fitness was followed during exposure to a range of DDAC concentrations. Three cubic metre vessel trials (cube trials) were performed, using natural seawater derived from a harbour adjacent to the institute. The cube trials were intended first of all to determine the DDAC dose needed for sufficient zooplankton and phytoplankton disinfection and secondly to test for any detrimental effects of high sediment loads on the efficacy of DDAC. The first and third cube trials were followed

by a lab scale regrowth experiment to test the potential for phytoplankton regrowth after treatment. Various BWTS do not physically disrupt cells immediately, so the potential for regrowth can be a decisive factor in the efficacy assessment of a potential BWTS. (Liebich, Stehouwer et al. 2012) Finally, a full-scale 100 m^3 tank trial was conducted using natural seawater to test the neutralization system that was needed to render the residual DDAC harmless upon discharge.

2. Materials and Methods

2.1. Experimental design

2.1.1. Lab trial

Three phytoplankton species, obtained from the National Centre for Marine Algae and Microbiota, were selected for the lab trial. The prasinophyte *Tetraselmis suecica* (CCMP 904), the prymnesiophyte *Isochrysis galbana* (CCMP 1323) and the diatom *Chaetoceros calcitrans* (CCMP 1315) were cultured in 500 mL polyethylene bottles (Nalgene) in a mix of 1:1 F/2 medium (Guillard and Ryther 1962) and enriched artificial seawater medium (Berges, Franklin et al. 2001) at 15ºC and a 16:8 light:dark cycle of 50 µmol photons m^{-2} s^{-1} light intensity. After reaching exponential growth phase, the cultures were treated with 0, 2.5, 5, 7.5 and 10 µL L^{-1} DDAC using an 8% DDAC working stock solution made by dissolving 1 mL Bardac® 2280, containing 80% DDAC (Lonza Inc.), in 9 mL milli-Q. Treated and control cultures were incubated at 15ºC in the dark. Samples of 50 mL were taken 24 hours before and one hour after the DDAC addition and subsequently for five days to monitor the following variables: phytoplankton abundance, PSII efficiency and DDAC concentration.

2.1.2.1. Cube trial 1

On 21 July 2010 natural seawater was pumped from the saltwater harbour adjacent to the institute at high tide. A 300 m^3 h^{-1} centrifugal pump was used to take up water through a pipeline normally used for filling 300 m^3 subterranean tanks. A bleeding valve was used to divert a side-stream to three opaque black polyethylene cubic meter containers (cube vessels). A one-litre working stock of 2.5 mL L^{-1} DDAC was made by mixing 6.25 mL Bardac® 2240, containing 40% DDAC (Lonza Inc.), in 993.75 mL milli-Q. The working stock was added to the second cube vessel when the vessel was 75% filled with seawater. After completely filling the cube vessel with 1000 L of seawater a DDAC concentration of 2.5 µL L^{-1} was reached. The firstly and thirdly seawater filled cube vessels were used as control. The cube vessels were stored inside to shield them from direct sunlight to prevent excess heating of the water in the vessels.

Before samples were taken from the cube vessels the content was stirred with a clean wooden paddle which was inserted through an opening on top of the vessel. Samples for zooplankton abundance were taken on day zero and day five from a tap at the base of the cube vessels. Zooplankton abundance before treatment from the DDAC-treated vessel was interpolated from the two adjacent control vessels. All cube vessels were sampled for phytoplankton abundance, PSII efficiency and DDAC concentration from a tap at the base of the vessel for five days.

2.1.2.2. Regrowth experiment

One-litre dilutions were made using treated water from Cube trial 1 at day five. These dilutions were made to detect a dose-response effect in case toxicity was observed in the undiluted treated water. As dilution water, 1.2 µm filtered and autoclaved seawater from the first cube vessel was used. Untreated water from Cube trial 1 was used for the control incubation. Undiluted, 10 times and 100 times diluted treated and control water was incubated at 15ºC with a 16:8 hours light:dark cycle at 50 µmol m^{-2} s^{-1} light intensity. A similar second dilution series was made to which living organisms were added by filtering one litre of freshly collected seawater over a GF/F filter (Whatman) and adding one filter to each incubation bottle. Alongside the dilutions a negative control (sterile seawater) and a positive control (sterile seawater with a freshly added filter containing living organisms) were incubated. For seven to ten days the phytoplankton abundance and PSII efficiency were monitored.

2.1.2.3. Cube trial 2

Two days after the termination of Cube trial 1, the second control vessel from trial 1was treated with 5 µL L^{-1} DDAC. This test was performed to determine the specific mortality of bivalve larvae which survived the initial treatment of 2.5 µL L^{-1} DDAC of Cube trial 1. A one-litre working stock of 5 mL L^{-1} DDAC was made by mixing 12.5 mL Bardac® 2240, in 987.5 mL milli-Q. The working stock was added to the second control vessel and thoroughly mixed with a wooden paddle to reach a concentration of 5 µL L^{-1} DDAC. Samples for zooplankton were taken at day zero and day five. Samples for DDAC concentration were taken at day zero, one, two, five and day six. As control for the zooplankton concentration, a 20 L polycarbonate bottle (Nalgene) was filled with water from the second control vessel before the DDAC addition and incubated in the dark alongside the cube vessel. At day five the 20 L bottle was completely analysed for zooplankton abundance.

2.1.3.1. Cube trial 3

Natural sediment was obtained from a saltwater bay adjacent to the institute. The sediment

was dried at 60ºC for three days to remove the water fraction. Three opaque black polyethylene cube vessels were used to perform an incubation in the dark. The first vessel was used as control. To the second and third cube vessel respectively 45 and 95 mg L^{-1} dried sediment was added. Two one-litre working stocks of 5 mL L^{-1} DDAC were made by mixing 12.5 mL Bardac® 2240, in 987.5 mL milli-Q. On 27 May 2011 the three cube vessels were filled with 1,000 L seawater from the saltwater harbour adjacent to the institute at low tide. The tanks were filled as in Cube trial 1. When the second and third cube vessel were 75% filled, DDAC was added from the working stocks to reach a final concentration of 5 µL L^{-1} DDAC.

In contrast to Cube trial 1, samples for zooplankton were taken directly from the bleeding valve at the beginning, middle and end of the hour it took to fill all three cube vessels. The average zooplankton count of the three samples was used as the zooplankton abundance on day zero before treatment for all cube vessels. Samples for DDAC treated zooplankton on day zero were taken from a tap at the base of the cube vessels. At day five all three cube vessels were sampled for zooplankton abundance. Before samples were taken from the cube vessels the content was stirred with a clean wooden paddle which was inserted through an opening on top of the vessel. Samples for phytoplankton and bacterial abundance, PSII efficiency and DDAC concentration were obtained from a tap at the base of the vessel during five days.

2.1.3.2. Regrowth experiment

At day five, 500 mL water from each cube vessel was incubated in polycarbonate bottles (Nalgene) at 15ºC with a 16:8 light dark cycle at a 50 µmol m^{-2} s^{-1} light intensity. After nine days the bottles were analysed for phytoplankton abundance and PSII efficiency.

2.1.4. Tank trial

On 16 September 2010, 100 m^3 of natural seawater was pumped into a 300 m^3 subterranean concrete tank situated onshore. The water was injected with DDAC (Bardac® 2240) using a dosing pump into the main pipeline after the pump to reach a final concentration of 5 µL L^{-1} DDAC. A second concrete subterranean tank was filled as a control. After five days the DDAC treated water was transferred to another tank. During the transfer 50 mg L^{-1} bentonite (natural clay mineral) was injected into the water to neutralize the residual DDAC. On day six a second neutralization step was carried out. At various moments during the incubation samples were taken for phytoplankton abundance, PSII efficiency, DDAC concentration and bacterial abundance using a tap at the base of the tank.

2.2. Analytical methods

2.2.1. Zooplankton enumeration.

Zooplankton samples containing natural untreated seawater were obtained by filtering 20 L of seawater over a 50 µm sieve either directly from the hose used for filling the cube vessels or from a tap at the base of the cube vessels. Cube vessels containing DDAC treated water were filtered entirely over a 50 µm plankton net.

Organisms retained in a 50 µm net or sieve after sampling were suspended in 100 mL 0.2 µm filtered seawater and immediately stained with neutral red vitality stain. (Crippen and Perrier 1974) Stained samples were distributed in a Bogorov dish and counted using a Zeiss microscope with a 20 times magnification. Organisms were determined alive on the basis of movement or whether they were stained by neutral red.

2.2.2. Enumeration of phytoplankton

Samples for phytoplankton abundance were analysed in duplicate. Analyses were performed within four hours after sampling using a Coulter Epics XL-MCL flow cytometer (Beckman Coulter). Phytoplankton cells were discriminated from other particles by detecting the red auto-fluorescence produced by chlorophyll when excited at 488 nm using the red fluorescence detector (620±15 nm band pass filter). (Anonymous 1998) Phytoplankton cells were enumerated by plotting red fluorescence against forward scatter. Subsequent data analysis was carried out using FCS Express 4 (De Novo Software). A selection gate was made based on the cluster of untreated cells. DDAC treated samples were analysed using the same gate used for untreated samples. Particles recorded outside of the gate were considered to be background noise or cell debris.

2.2.3. Enumeration of bacteria

Two methods for the enumeration of bacteria were used. In the first method samples for total bacterial counts were fixed with 1.8%/1% formalin/hexamine for 15 minutes at 4ºC, snap frozen in liquid nitrogen and stored at -80ºC until analysis. Prior to analysis bacterial samples were thawed at room temperature and stained with PicoGreen® (250 times commercial stock dilution, Invitrogen) which makes the genomic DNA green fluorescent.

The second method involved a live/dead determination. Unfixed samples were double stained with SYBR® Green (10,000 times commercial stock dilution, Invitrogen) and propidium iodide (500 times commercial stock dilution, Invitrogen). Cells with intact membranes were considered alive and cells with permeable membranes were considered dead. SYBR® Green is a membrane-permeant DNA stain making all bacteria (total bacteria) green fluorescent. Propidium iodide is a membrane-impermeant DNA stain making cells with

permeable membranes (dead bacteria) red fluorescent (Falcioni, Papa et al. 2008). Bacteria were analysed in duplicate using a Coulter Epics XL-MCL flow cytometer (Beckman Coulter) with a 488 nm excitation laser. Bacterial counts were discriminated from other particles on the basis of green or red fluorescence intensity and internal complexity using the green fluorescence detector (trigger, 525 ± 20 nm band pass filter), red fluorescence detector (620 ± 15 nm band pass filter) and side scatter detector, respectively.

2.2.4. PSII efficiency

Samples for PSII efficiency analysis were stored at 4ºC in the dark for thirty minutes to four hours prior to analysis. The PSII efficiency was measured in duplicate as Fv/Fm using a single-press saturation Pulse Amplitude Modulation (PAM) fluorometer (Walz, Germany). (Walz 2000)

2.2.5. DDAC analysis

DDAC samples were analysed colorimetrically within four hours after sampling according to HACH method 8337. (Anonymous 2012) Samples of the lab trial were analysed once. Samples of the cube trials, regrowth experiments and tank trial were analysed in triplicate. Per the manufacturer's instructions, concentrations of DDAC were expressed as parts per million (ppm, equivalent to $\mu L\ L^{-1}$). The density of DDAC at 20 ºC is 0.87 g cm^{-3} and the molar weight is 362.08 g mol^{-1} (ECHA 2007). As DDAC calibrations and analyses were conducted at room temperature, the DDAC conversion results in: 1.0 $\mu L\ L^{-1}$ = 0.87 mg L^{-1} = 2.4 $\mu mol\ L^{-1}$.

2.3. Statistical analysis

In all statistical analyses the null hypothesis was that there is no significant difference between treatment and control. When samples were analysed in triplicate, the 95% confidence intervals of the means (c.i.) were calculated using the MS Excel 2010 function CONFIDENCE.T. A Student's t distribution was used instead of a normal distribution because the former is more appropriate when dealing with small sample sizes. When the 95% c.i. did not overlap the difference between means was considered significant ($p<0.05$).

T-tests were carried out using the MS Excel function TTEST. A two-tailed distribution was assumed in all tests and $\alpha = 0.05$. Two types of t-tests were used depending on the equality of variance of the two samples. An F-test was performed to test for equality of variance using the MS Excel function FTEST using $\alpha = 0.05$ to decide which type of t-test should be used.

Least squares linear regression models were calculated using SYSTAT 13. When data were non-linearly distributed, they were transformed to the natural logarithm. To test whether

model coefficients were not significantly different from one another the 95% c.i. was calculated as: 95% c.i. = SE * t. Whereby SE is the coefficient's standard error calculated by SYSTAT 13 and t is the two-tailed t-value corresponding with $\alpha = 0.05$ and degrees of freedom (df) = n-1.

3. Results

3.1. Lab trial

3.1.1. Phytoplankton abundance

In the control the cell abundance of *C. calcitrans* remained between ~626,000 and ~713,000 cells mL^{-1} throughout the five-day dark incubation. The cell abundance of *T. suecica* ($t_0 \approx$ 36,000 cells mL^{-1}) and *I. galbana* ($t_0 \approx$ 464,000 cells mL^{-1}) decreased 58% and 52% respectively after 24 hours and fully or partly recovered respectively between one to five days dark incubation (Figure 1a). When cell abundance on day zero was compared with day six only *T. suecica* had not significantly changed (t-test: $p = 0.24$). Both the *I. galbana* and *C. calcitrans* cultured changed significantly between day zero and day six (t-test: $p = 0.03$; both cultures).

Several factors could have contributed to the apparent recovery in *T. suecica* and *I. galbana* cell abundance in the control incubations. Clumping of *T. suecica* cells has been observed in other studies, (Moheimani 2012) so it could be hypothesized that *T. suecica* cells were clumping at the start of the incubation and that the successive shaking of the bottles prior to sampling led to an apparent increase of cells over time. Cell clumps were not detected during flow cytometer data analysis, however, sometimes clumps or too large to enter the flow cytometer uptake needle. Also, cell clumps can be too rare to be picked up in the 92 µL sample volume analysed by the flow cytometer. It was deemed unlikely that actual growth occurred during the incubations, since the incubation was in the dark.

The patterns observed in the treated incubations were markedly different. Both *I. galbana* and *C. calcitrans* showed a significant decline (t-test: $p < 0.05$, all DDAC concentrations) directly after DDAC treatment at all concentrations tested (Figure 1b-1e). On day six the cell abundances of all DDAC treated cultures combined were on average (SD): 1,091 (557); 6,386 (1,845) and 35 (64) cells mL^{-1} for *I. galbana*, *C. calcitrans* and *T. suecica* cultures respectively. This is equivalent to a 99.8%, 99.0% and 99.9% decrease in cell abundance for treated *I. galbana*, *C. calcitrans* and *T. suecica* cultures respectively. Based on the shape of the clusters observed during flow cytometer data analysis, what appeared as

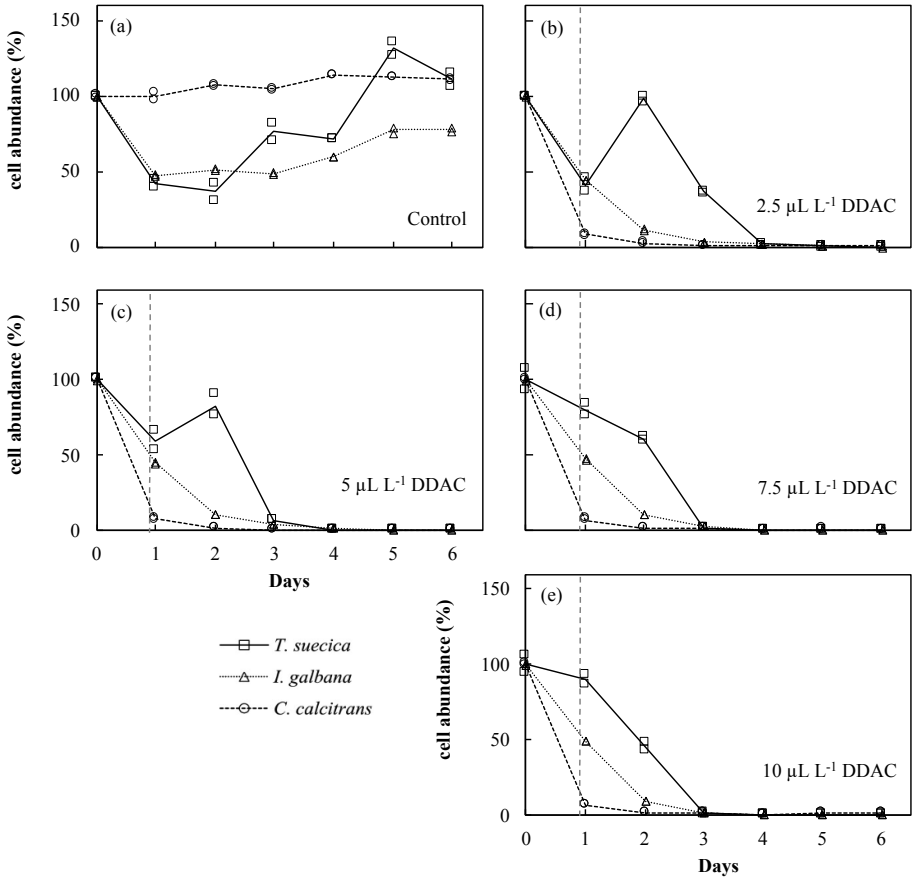

Figure 1. *Lab trial. Normalized cell abundance of T. suecica, I. galbana and C. calcitrans after the addition of control (a), 2.5 (b), 5 (c), 7.5 (d) and 10 µL L^{-1} DDAC (e). DDAC was added one hour prior to the sampling of t_1, indicated by the vertical dotted line. Results are duplicates with the average represented by a continuous line.*

intact cells in DDAC treated cultures on day six could also consist out of cell debris. However, no microscopic analysis was carried out to confirm this hypothesis.

3.1.2. PSII efficiency

In the control incubations the PSII efficiency remained between 0.6 and 0.7 over the entire incubation time (Figure 2a). In the DDAC-treated incubations a complete inactivation of the PSII efficiency was observed in all three species at all DDAC concentrations tested up until the last incubation day. The PSII efficiency of *T. suecica* was still detectable within hours after the DDAC addition but was below detection limit after 24 hours at all DDAC

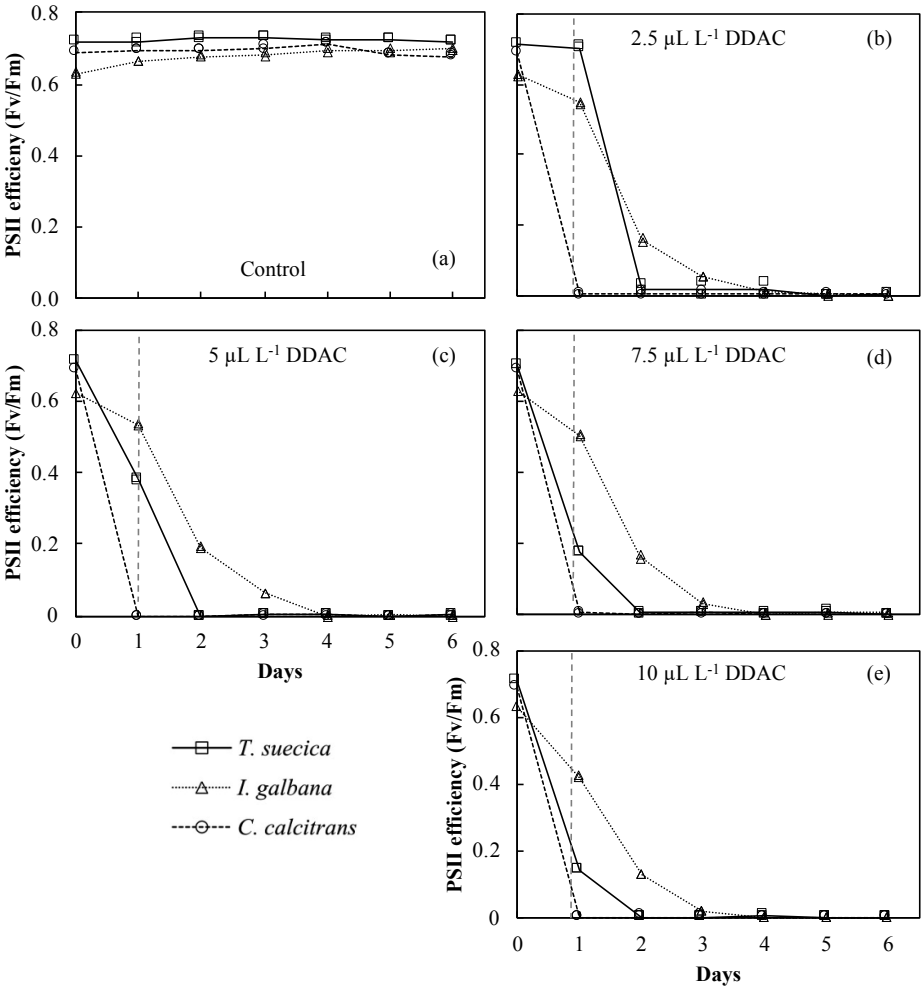

Figure 2. *Lab trial. PSII efficiency of T. suecica, I. galbana and C. calcitrans after the addition of control (a), 2.5 (b), 5 (c), 7.5 (d) and 10 µL L-1 DDAC (e). DDAC was added one hour prior to the sampling of t1, indicated by the vertical dotted line. Results are duplicates with the average represented by a continuous line.*

concentrations tested. The PSII efficiency of *I. galbana* remained detectable for two to three days after the DDAC addition. The PSII efficiency of *C. calcitrans* decreased to below detection limit directly after the addition of DDAC at all concentrations tested.

3.1.3. DDAC degradation

In most of the incubations the observed concentration of DDAC was lower than was actually added (Table 1). Especially at higher DDAC concentrations, much less was actually observed in the cultures. In all, except the 2.5 µL L^{-1} DDAC incubation, the highest concentrations

were observed in the *I. galbana* samples. The concentration of DDAC remained fairly constant during the incubation as indicated by the small standard deviation of the average DDAC concentration over the five -day incubation (Table 1).

Table 1. *Lab trial. Average DDAC concentration during the five-day incubation.*

	DDAC (µL L^{-1}); Average (SD)					
Treatment	*T. suecica*		*I. galbana*		*C. calcitrans*	
Control	0.0	(0.1)	0.0	(0.1)	0.0	(0.1)
2.5	1.9	(0.2)	2.3	(0.2)	2.7	(0.1)
5	2.9	(0.3)	4.9	(0.1)	2.7	(0.3)
7.5	4.4	(0.3)	5.9	(0.3)	4.1	(0.3)
10	5.4	(0.3)	6.6	(0.2)	5.4	(0.3)

3.2. Cube trial 1

3.2.1. Zooplankton abundance

Within hours after the addition of 2.5 µL L^{-1} DDAC, the abundance of living zooplankton decreased 51% from 82,050 to 40,250 organisms m^{-3}. After five days the number of living zooplankton was reduced 98% to 1,500 organisms m^{-3}. All remaining zooplankton in the DDAC treated cube vessel were bivalve larvae. In the control cube vessel living zooplankton decreased 42% from 82,050 to 47,550 organisms m^{-3} during the five-day incubation.

3.2.2. Phytoplankton abundance

Both the DDAC-treated and -control cube vessels showed a similar decrease in phytoplankton abundance during the five-day incubation (Figure 3a). The decrease in phytoplankton abundance was logarithmic in both the control and treated vessels. After five days the phytoplankton abundance decreased to approximately 4,000 cells mL^{-1} in the DDAC treated vessel which still exceeded the <10 viable cells mL^{-1} required by the IMO.

3.2.3. PSII efficiency

In contrast to phytoplankton abundance, a clear difference in PSII efficiency was observed between the control and DDAC-treated vessels. The PSII efficiency measured in samples from the control vessel resulted in yields associated with healthy phytoplankton (Figure 3b). Within two hours the PSII efficiency was reduced to 0.1 in the DDAC treated vessel and remained below 0.1 during the entire incubation.

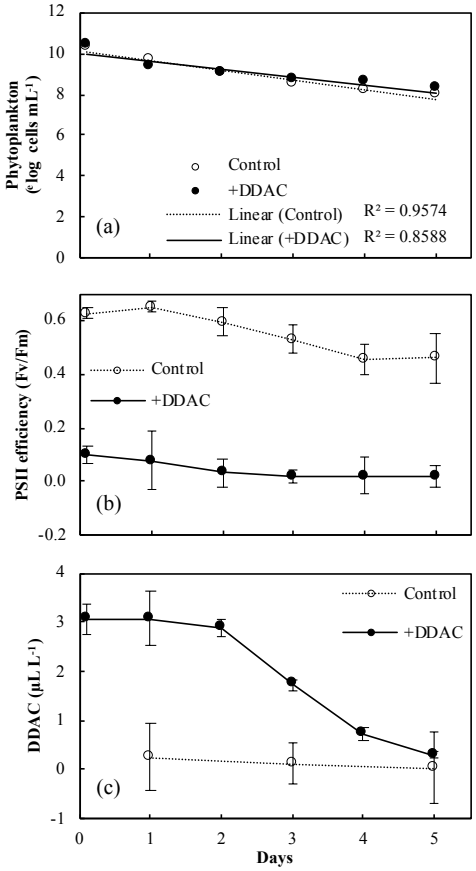

Figure 3. *Cube trial 1. Natural seawater treated with 2.5 µL L^{-1} DDAC. The phytoplankton abundance (a), the PSII efficiency (b) and the DDAC concentration (c) in the cube vessels. Error bars represent the 95% c.i. of triplicate measurements.*

3.2.4. DDAC degradation

The DDAC concentration remained constant until day two (Figure 3c). On day five the DDAC concentration in the treated vessel was not significantly different from the control vessel due to relatively high variability in control measurements. However, the average concentration in treated samples was still significantly different from zero on day five at 0.3 ± 0.1 µL L^{-1} DDAC (average ± 95% c.i.).

3.3. Regrowth experiment

3.3.1. Phytoplankton abundance

In all incubations except the negative control regrowth was observed (Figure 4). The incubations of DDAC treated water showed a one- to two- days lag time before regrowth. The control dilutions did not show this lag phase, except for the 100 times dilution which showed a one-day lag phase. When untreated phytoplankton was added to the DDAC treated water, the undiluted incubation showed a one-day lag phase before regrowth started (Figure 4c). The diluted incubations showed no lag phase prior to regrowth. Control water with untreated phytoplankton added showed no lag phase before regrowth.

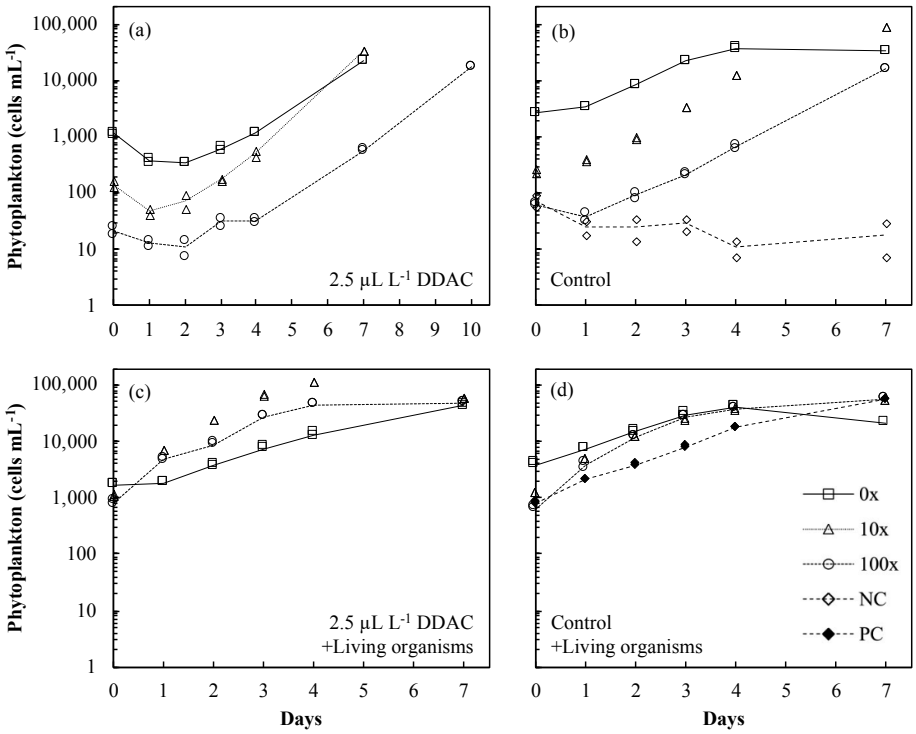

Figure 4. *Regrowth experiment. Phytoplankton abundance. DDAC treated water dilution series (a); control water dilution series (b); DDAC treated dilution series with addition of living organisms (c); control water dilution series with addition of living organisms (d). NC = negative control (sterile seawater). PC = positive control (sterile seawater with addition of living organisms). Results are duplicates with the average represented by a continuous line.*

3.3.2. PSII efficiency

In all incubations except the negative control a strong recovery of PSII efficiency was observed (Figure 5). Notably, the PSII efficiency of undiluted DDAC treated water with fresh phytoplankton was much lower on day zero than the other dilutions (Figure 5c).

3.4. Cube trial 2

3.4.1. Zooplankton abundance

Zooplankton abundance declined 62% from 23,850 to 9000 organisms m^{-3} within hours after the addition of 5 µL L^{-1} DDAC. At day five no zooplankton organisms were observed in the treated cube vessel. So, the zooplankton disinfection was 100% after treatment at day five.

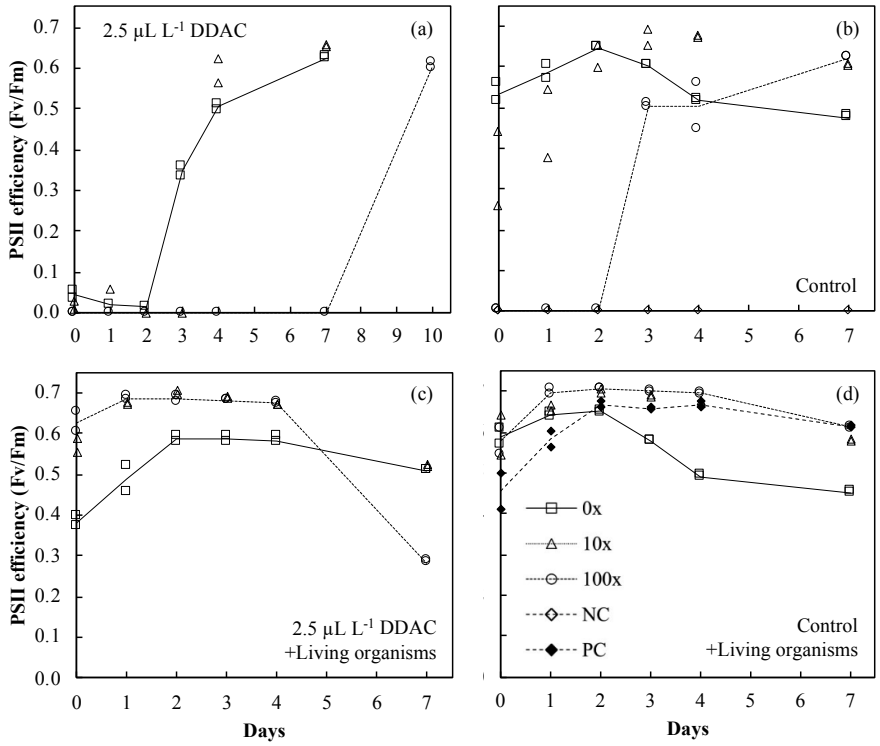

Figure 5. *Regrowth experiment. PSII efficiency. DDAC treated water dilution series (a); control water dilution series (b); DDAC treated dilution series with addition of living organisms (c); control water dilution series with addition of living organisms (d). NC = negative control (sterile seawater). PC = positive control (sterile seawater with addition of living organisms). Results are duplicates with the average represented by a continuous line.*

The zooplankton abundance in the control decreased by 82% from 23,850 at day zero to 4300 organisms m^{-3} at day five.

3.4.2. DDAC degradation

The observed concentration on day zero was 5.8 ± 0.3 µL L^{-1} DDAC (average ± 95% c.i.). On day five the DDAC concentration observed in the treated vessel remained significantly different from the control samples at 1.6 ± 0.4 µL L^{-1} DDAC (average ± 95% c.i.). On day six the DDAC concentration was not significantly different from day 5 (t-test: $p = 0.30$), indicating that a plateau was reached in the degradation process.

3.5. Cube trial 3

3.5.1. Zooplankton abundance

After five days 85% of the zooplankton present in the control vessel at day one was still alive. In the DDAC treated vessels with extra sediment virtually no organisms were left on

day five except for a phyllodocidae larvae and a nereididae larvae in the 45 mg L^{-1} TSS vessel (Table 2).

Table 2. *Cube trial 3. Natural seawater treated with 5 µL L^{-1} DDAC and extra TSS.*

	Zooplankton m^{-3}		
		Extra TSS mg L^{-1}	
Day	Control	45	95
0	14,733[a]	14,733	14,733
5	12,550	2	0

[a]*The average zooplankton concentration at intake for all three cube vessels.*

The dominant category of zooplankton organisms in the vessels on day one were bivalve larvae. The second most abundant category were balanidae nauplia. Both these types of zooplankton were considered as hard to kill by conventional ballast water treatment systems. The most difficult to kill zooplankton category found in the Wadden sea are balanidae cyprid larvae (personal communication with Frank Fuhr and Isabel van der Star). These were however not present at the time of testing.

3.5.2. Phytoplankton abundance

Just as observed in Cube trial 1, the phytoplankton abundance trends in control and treated vessel was remarkably similar (Figure 6a). Different from Cube trial 1 was that the decrease in phytoplankton abundance could be described by a linear regression model. The phytoplankton trends could be described by the following models: $y = -773x + 4{,}894$ (Control); $y = -667x + 4{,}421$ (45 mg L^{-1} TSS); $y = -640x + 4{,}591$ (95 mg L^{-1} TSS).

It was tested whether the decrease rate of phytoplankton was significantly different for the different treatments. The 95% c.i. for the coefficients was calculated using: SE * $t_{(df; 11)}$. The 95% c.i. for the three decrease rates were: 47.2 * 2.201 = 104 (Control); 62.2 * 2.201 = 137 (45 mg L^{-1} TSS); 78.3 * 2.201 = 173 (95 mg L^{-1} TSS). The coefficient ± 95% c.i. for the control and two treatments were: -773 ± 104 (Control); -667 ± 137 (45 mg L^{-1} TSS); -640 ± 173 (95 mg L^{-1} TSS)

All the 95% c.i. overlapped with each other so the decrease rates in phytoplankton were not significantly different among the control and two treatments.

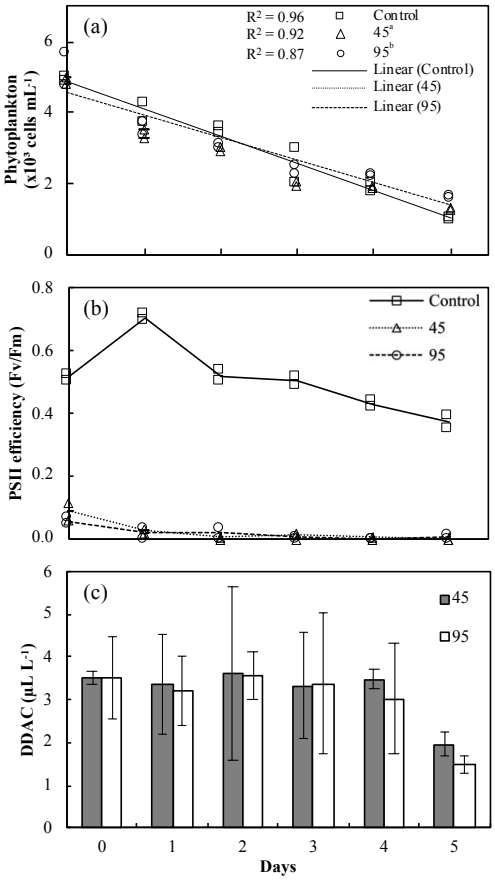

Figure 6. *Cube trial 3. Natural seawater treated with 5 µL L^{-1} DDAC. Phytoplankton abundance (a); PSII efficiency (b) and DDAC concentration (c) in the cube vessels. [a,b]45 and 95 refer to the amount of extra TSS in mg L^{-1} added to the DDAC treated vessels. Results of PSII efficiency are duplicates with the average represented by a continuous line. Error bars represent the 95% c.i. of triplicate DDAC measurements.*

3.5.3. PSII efficiency

Similar to Cube trial 1 the PSII efficiency of the treated vessels was reduced to below 0.1 within hours and remained close to the detection limit for the duration of the experiment (Figure 6b). The PSII efficiency observed in the control samples were associated with healthy phytoplankton cells.

3.5.4. DDAC degradation

On day one 70% of the added DDAC was measured in the vessels (Figure 6c). Day five marked the first day that a significant reduction in DDAC was observed in both vessels. In none of the vessels the DDAC concentration decreased below the detection limit.

3.5.5. Bacterial abundance

In the control vessel the majority of bacteria were alive at day one. After day three the bacterial abundance decreased strongly in the control (Figure 7a). The bacterial abundance in the DDAC-treated vessels was 30% - 40% lower than in the control vessels on day one (Figure 7b & 7c). Notably, the bacterial assembly in the treated vessels consisted almost entirely of dead bacteria. During the course of the incubation bacteria started to regrow in the DDAC treated vessels. On day five the bacterial abundance in both DDAC treated vessels exceeded the bacterial abundance in the control vessel.

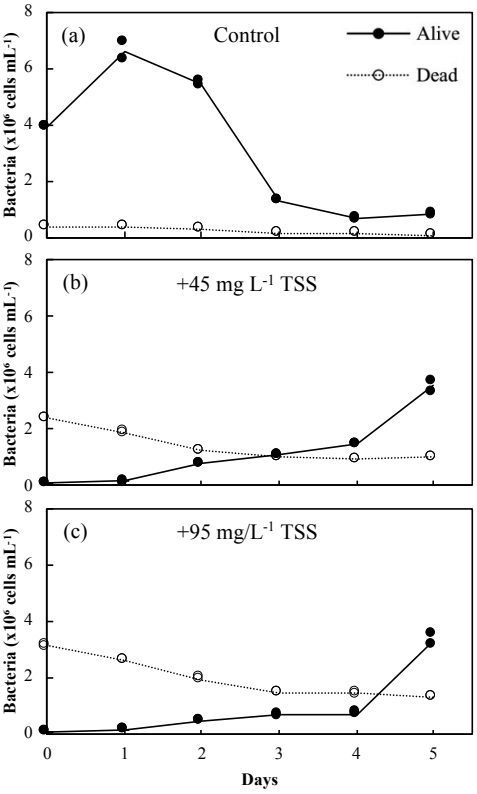

Figure 7. *Cube trial 3. Natural seawater treated with 5 µL L^{-1} DDAC. Bacterial abundance. Results are duplicates with the average represented by a continuous line.*

3.5.6. Regrowth experiment

After nine days of light incubation the control had increased from ~1,000 to ~8,400 phytoplankton cells mL^{-1}. Thus, clear regrowth was observed in the control. The PSII efficiency in the control had increased from 0.38 to 0.58, so a recovery was also observed there. No regrowth or PSII efficiency recovery was observed in DDAC treated samples. On the contrary, the intact phytoplankton cells at day five of the dark incubation had degraded after nine days in the light. Phytoplankton cell counts ranged from 0 to 7 cells mL^{-1} for the 45 and 95 mg L^{-1} TSS respectively.

3.6. Tank trial

3.6.1. Phytoplankton abundance

In contrast to cube trials, phytoplankton abundance did not decrease in the control tank. The control tank showed no significant decline in phytoplankton abundance on day four (t-test: $p = 0.06$) (Figure 8a). The 5 µL L^{-1} DDAC treatment immediately led to a decrease of 75% in phytoplankton abundance. At day 5 more than 500 cells mL^{-1} remained in the treated tank, which is 50 times higher than the <10 viable cells mL^{-1} allowed by the D-2 standard. (Anonymous 2004)

3.6.2. PSII efficiency

Within hours after the DDAC treatment the PSII efficiency was reduced to 0.04 and remained below 0.1 for the duration of the experiment (Figure 8b).

The strong decrease in phytoplankton numbers and the inactivation of PSII indicate that 5 µL L^{-1} DDAC was effective with respect to phytoplankton. However, a regrowth experiment was not performed, therefore the viability of the remaining 500 cells mL^{-1} at day five could not be determined.

3.6.3. DDAC neutralization

DDAC levels did not decrease significantly during the five day tank trial (t-test: $p = 0.40$) (Figure 8c). After an initial neutralization with 50 mg L^{-1} bentonite the DDAC concentration decreased to 0.75 ± 0.04 µL L^{-1} (average ± 95% c.i.) which was still significantly different from zero. At day six the neutralization procedure was repeated which reduced the DDAC concentration to non-detectable levels.

3.6.4. Bacterial abundance

Bacterial abundance was initially reduced due to the DDAC treatment. However, on day four and five clear regrowth of bacteria was observed (Figure 8d). So, just like in Cube trial 3 (Figure 7c) 5 µL L^{-1} DDAC was unable to prevent bacterial regrowth for a five-day period.

3.7. Overview

Results were classified as insufficient, inconclusive and sufficient with respect to disinfection efficacy (Table 3). Bacterial disinfection efficacy was also included in the summary despite the lack of IMO regulation of most bacteria. The classification of sufficient or insufficient disinfection was based on the assessment whether bacteria were initially affected by the treatment and whether regrowth occurred within the five-day dark incubation.

Table 3. *Overview of results classified as: insufficient (-), inconclusive (±) or sufficient (+).*

	T. suecica; *I. galbana*; *C. calcitrans*				Cube trials		Tank trial
	DDAC concentration (µL L^{-1})						
Variable	2.5	5	7.5	10	2,5	5	5
Zooplankton disinfection					-	+	
Phytoplankton disintegration	± ± ±[a]	± ± ±	± ± ±	± ± ±	±	±	+
PSII inactivation	+ + +[a]	+ + +	+ + +	+ + +	+	+	+
Phyto. regrowth prevention					-	+	
Bacterial disinfection						+	+
Bact. regrowth prevention						-	-
DDAC degradation	- - -	- - -	- - -	- - -	±	-	-

[a]*The three consecutive '+', '±' or '-' signs in the first four columns correspond with the results of T. suecica, I. galbana and C. calcitrans respectively.*

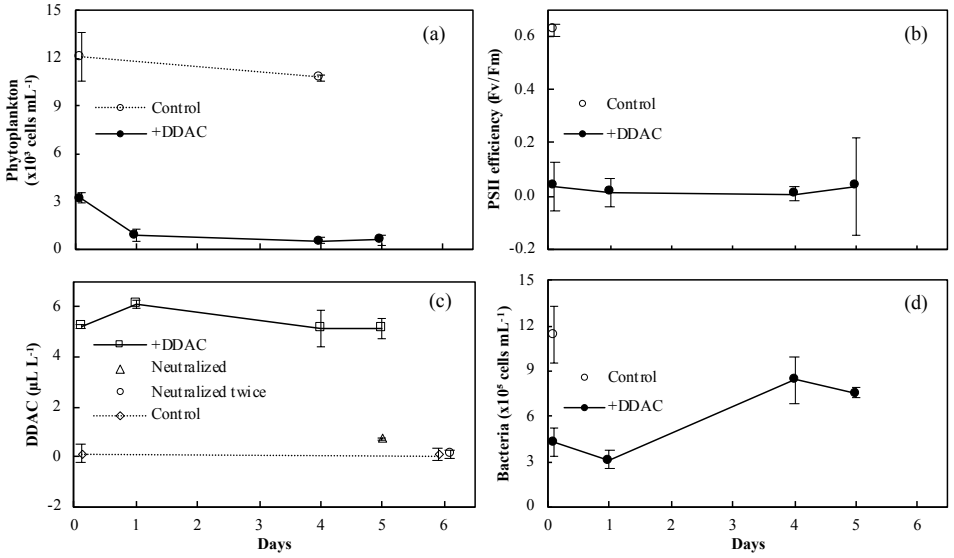

Figure 8. *Tank trial. Natural seawater treated with 5 µL L^{-1} DDAC. Phytoplankton abundance (a), PSII efficiency (b), DDAC degradation (c) and bacterial abundance (d). Error bars represent the 95% c.i. of triplicate DDAC measurements.*

4. Discussion

4.1. Establishing compliance with D-2

IMO type approved ballast water treatment systems should reduce the concentrations of certain groups of viable organism to below standard (D-2) values as well as being environmentally safe. (Anonymous 2008) For systems using toxic compounds environmentally safe means that the residual toxicity of discharged water should be below a certain threshold. The residual toxicity level can be reached by degradation of the toxic compounds during the five-day holding time, or if that is not sufficient, by adding a neutralizing agent. The merits and caveats of DDAC in ballast water treatment will be discussed for three organism groups, zooplankton, phytoplankton and heterotrophic bacteria, their viability as well as the environmental acceptability of DDAC.

4.1.1. Organism concentrations

DDAC treatment affected the physical integrity of phytoplankton cells in monocultures and the tank trial. In the cube vessels phytoplankton abundance also decreased in the control so no significant differences were observed. Since no clear differences in abundance and PSII efficiency were observed between 2.5 and 10 µL L^{-1} DDAC it appeared that 2.5 µL L^{-1} DDAC was sufficient to disinfect all three monocultures. However, in natural seawater, zooplankton and phytoplankton showed resistance to 2.5 µL L^{-1} DDAC. Eventually, 5 µL L^{-1}

DDAC proved sufficient to disinfect natural seawater in compliance with D-2. The rapid disappearance of cells after treatment is in congruence with other active substances like Peraclean® Ocean, (Veldhuis, Fuhr et al. 2006, de Lafontaine, Despatie et al. 2008) chlorine (Peperzak, Stehouwer et al. 2012) and chlorine dioxide (Veldhuis, Fuhr et al. 2009), which tend to disrupt cells directly after treatment.

In Cube trials 1 and 3, the cause of the similar decrease in phytoplankton abundance between treated and untreated vessels remained unclear. It could be hypothesized that the polyethylene cube vessels had adverse effects on phytoplankton survival. However, flocculation could also cause an apparent decline in phytoplankton cells because of sedimentation of phytoplankton clumps and the inability to count cell clumps as loose cells using a flow cytometer. (Ozbay and Jackson 2010) Grazing was ruled out as a cause for phytoplankton decline in the treated vessels, since zooplankton was almost completely eradicated by the DDAC treatment. On the other hand, the strong decline in phytoplankton abundance in the treated tank in the full-scale tank experiment was a good indicator that the DDAC treatment had been effective.

Although the 2.5 µL L^{-1} DDAC treatment in Cube trial 1 caused a reduction of 98% in living zooplankton, the abundance after five days was still well above the limit of <10 organisms m^{-3} allowed by the IMO. (Anonymous 2004) The apparent resistance of bivalve larvae to DDAC could be caused by the ability of bivalves to close their shells and thus temporarily avoid adverse environmental conditions. (Valenti, Cherry et al. 2006)

In Cube trial 2, the complete eradication of zooplankton organisms, including the bivalve larvae, indicated that 5 µL L^{-1} DDAC has sufficient zooplankton disinfection capacity to comply with the IMO D-2 standard. The large decrease in the control bottle indicated however, that the 12-day presence of the seawater in the cube vessel and 20 L polycarbonate bottle had negatively impacted the survival rate of the zooplankton.

The successful eradication of all but two worm larvae in the treated vessels of Cube trial 3 implied that the extra TSS added did not impair the disinfection capacity of 5 µL L^{-1} DDAC with respect to zooplankton. In the absence of the most challenging Balanidae cyprid larvae, 5 µL L^{-1} DDAC was capable of eradicating virtually all zooplankton organisms. So, these results were considered promising with respects to zooplankton disinfection efficacy of DDAC at current concentrations to comply with the IMO D-2 standard.

The reduction of bacterial abundance in the control of Cube trial 3 was probably the result of depletion of carbohydrates and continued grazing by microzooplankton. Carbohydrates are produced by phytoplankton under light conditions but in the dark no

photosynthesis can take place. Carbohydrates produced by phytoplankton are considered an important organic carbon source for bacteria. (Alderkamp, van Rijssel et al. 2007, Hahnke, Sperling et al. 2013) Apart from three indicator microbes, bacterial abundance is not regulated by the IMO, so the observed regrowth has no implications for assessing DDAC as a suitable ballast water treatment method.

4.1.2. Viability

The PSII efficiency results indicated that PSII of the phytoplankton in all experiments was severely damaged. When PSII efficiency levels drop below the detection limit, no photosynthesis can take place and hence no energy production. The inactivation of PSII could indicate that the phytoplankton cells were effectively killed by the DDAC treatment. However, recovery of PSII efficiency after removal of toxic compounds has been reported before. (Buma, Sjollema et al. 2009)

In the regrowth experiment of Cube trial 1 the decrease in PSII efficiency observed in undiluted treated water with untreated phytoplankton added could indicate that water containing 0.3 µL L^{-1} DDAC still contained residual toxicity to phytoplankton. Nevertheless, despite the inactivation of PSII during the five-day DDAC treatment, regrowth and PSII recovery occurred within three to four days. The short lag phase indicated that 2.5 µL L^{-1} DDAC had insufficient phytoplankton disinfection capacity to comply with the IMO D-2 standard. Also, it was shown that loss of PSII efficiency during treatment was no definitive indicator for loss of phytoplankton viability after treatment. In Cube trial 3 the loss of PSII efficiency at 5 µL L^{-1} DDAC was comparable to the effect observed at 2.5 µL L^{-1} DDAC. This indicated that the maximum effect of PSII disruption was indeed already reached at the lower DDAC concentration.

4.1.3. *Regrowth potential*

During the cube trials it became clear that phytoplankton cell integrity and PSII disruption were not reliable predictors of cell viability. In order to establish the effectiveness of a treatment, it was deemed essential to carry out regrowth experiments with treated water. The presence of regrowth in all 2.5 µL L^{-1} DDAC treated dilutions of Cube trial 1 showed that residual toxicity to phytoplankton probably was negligible after one- or two-days incubation in the light and that sufficient viable phytoplankton cells remained after treatment to enable regrowth. In contrast, the absence of regrowth and the continued degradation of any remaining cells in Cube trial 3 was a clear indication that intact phytoplankton cells at day five of the dark incubation were not viable. Although it cannot be ruled out that, in Cube trial 3, residual DDAC hampered regrowth. Nevertheless, the degradation to <10 cells mL^{-1} after

nine days in the tank experiment is a strong indication that 5 µL L^{-1} DDAC had sufficient phytoplankton disinfection capability to comply with the IMO D-2 standard. It can be challenging to establish how long it should take for regrowth to occur before it can be reliably concluded that the treatment was effective. In official G8 land-based tests it is custom to incubate up to 7 days after treatment. (Peperzak 2013) Since the regrowth incubation in Cube trial 3 lasted 9 days, without regrowth, it can be concluded with reasonable confidence that the treatment had effectively rendered all phytoplankton unviable.

Bacterial regrowth in DDAC treated vessels indicated that certain bacterial species were resistant to DDAC. Possibly a community shift in bacterial composition took place, since the bacteria at day one in Cube trial 3 were almost completely killed by the DDAC treatment. Bacterial resistance to quaternary ammonium compounds has been reported before (Mitchell, Brown et al. 1998) and it is therefore not improbable that DDAC resistant bacteria became dominant. It would be interesting to investigate the causal relationship between the increase in bacterial abundance and the significant decrease in DDAC observed on day five (Figure 6c). The decrease in DDAC concentration might be due to either chemical decomposition which could have enabled bacteria to regrow or, bacteria may have been responsible for the DDAC degradation (Henderson 1992).

4.1.4. Environmental acceptability

DDAC is immobile in sediment/soil, suggesting it binds strongly to organic substances (Anonymous 2006). Thus, the reason that the observed DDAC concentrations were lower than the added DDAC potentially was an immediate reaction of DDAC with organic matter in the phytoplankton culture after addition and adsorption to phytoplankton cells. In ballast water with a relatively high organic carbon content, the amount of DDAC added during ballasting may be higher than in water with a low carbon content. Ideally, the DDAC concentration is monitored directly after the addition, as is the case in electro chlorination systems where chlorine is measured, so that the disinfectant dose can be adjusted during the ships' ballasting operations, thereby reducing the chance of overdosing.

The persistence of DDAC during most incubations ensured that the disinfection process was continuous during the whole five-day period. In contrast to the monoculture tests, DDAC did degrade in natural seawater with a half-life of approximately three days. DDAC half-lives ranging from days to years in soil and sewage cultures have been reported before. Henderson (Henderson 1992) hypothesized that microbial population compositions could play a major role in the degradation rate of DDAC. Indeed, bacteria using DDAC as sole carbon source have been reported before. (Van Ginkel, Hoenderboom et al. 2003)

After adding 5 µL L^{-1} DDAC, degradation was either insufficient or even absent. So, to be responsibly used as ballast water treatment option a neutralization step is required, since it is not allowed by the IMO to discharge toxic ballast water. (Anonymous 2008) Therefore, monitoring the DDAC concentration upon discharge, in addition to monitoring at ballasting, appears critical to assess the need for a potential neutralization step in order to be environmentally acceptable.

Ideally, BWTS exclude or limit the use of active substances. Chemical-free treatment techniques are e.g. filtration, cavitation and UV-radiation. Whenever active substances are applied, preferably these are generated on-site e.g. in electro chlorination and chlorine dioxide treatment to eliminate the transport and storage of dangerous chemicals. Usually, residual chlorine is neutralized using a sulphur compound like sodium bisulphite, which is injected as a liquid in the discharge line during deballasting of a ballast tank. (Tsolaki and Diamadopoulos 2010). Compared to existing technologies, DDAC combined with bentonite neutralization has several disadvantages. DDAC and bentonite have to be transported and stored on-site creating potential logistical and health-related risks. In addition, bentonite is a clay mineral which is injected as a powder into the ballast water. Although precise turbidity measurements were not performed, the suspended bentonite was clearly visible with the naked eye after neutralization. Discharging turbid water could be an environmental risk in some areas and it is unclear whether port authorities would allow the discharge of bentonite-containing ballast water in their harbours.

5. Conclusions

Most BWTS apply physical separation to remove the larger particles from the ballast water prior to chemical treatment or UV-radiation. (Tsolaki and Diamadopoulos 2010) This is because chemical treatment alone is often not sufficient to reliably disinfect zooplankton organisms under all circumstances. Exceptions exist like the SeaKleen® trials which resulted in reliable disinfection of both zooplankton and phytoplankton using menadione as sole active substance without the use of filtration. (Wright, Dawson et al. 2009) At 2.5 µL L^{-1} DDAC zooplankton abundance was not reduced to below 10 organisms m^{-3}. In particular, bivalve larvae appeared resistant to the treatment. However, at 5 µL L^{-1} DDAC zooplankton abundance was twice reduced to below 10 organisms m^{-3}. It can be concluded that 5 µL L^{-1} DDAC is probably sufficient to disinfect zooplankton organisms from seawater in accordance with the IMO D-2 standard. (Anonymous 2004) Nevertheless, although DDAC results are promising, given the experience of other BWTS manufacturers that filtration seems

necessary, it is recommended that extensive zooplankton trials are carried out in full-scale tests in order to validate DDAC disinfection efficacy on a wide variety of zooplankton organisms.

In phytoplankton monocultures DDAC clearly disrupted cells at 2.5-10 µL L^{-1} DDAC. In the cube trials phytoplankton abundance decreased in the treated samples, but also in the control samples. So, it remained unclear whether DDAC was solely responsible for the decline. On the other hand, in the tank trial a clear difference in phytoplankton abundance between control and treated samples was observed. No regrowth was observed after nine days in the light after 5 µL L^{-1} DDAC treatment. It is therefore concluded that 5 µL L^{-1} DDAC was sufficient to achieve reliable disinfection in seawater with respect to phytoplankton in accordance with IMO. (Anonymous 2004)

Although initially reduced, bacterial regrowth was observed at 5 µL L^{-1} DDAC both in the cube vessels and tank trials. Therefore, the resistance of the three indicator microbes to DDAC or their capability of metabolizing the disinfectant still needs to be tested.

DDAC did not degrade to undetectable levels in any of the 5-day observations made in this study. Regrowth experiments hinted at adverse effects of residual DDAC on phytoplankton regrowth and PSII efficiency. Sufficient neutralization to comply with environmental acceptability of 5 µL L^{-1} DDAC was only achieved after two bentonite neutralization cycles. The addition of bentonite clay to ballast water considerably increased the particle load of the discharge water that may be subject to regulations in various countries and ports. In addition, the installation of a bentonite injection apparatus in addition to the transportation to the ship and storage of bentonite clay and DDAC on board will complicate the practical application of DDAC as a ballast water treatment method.

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Chapter 3

Quantifying heterotrophic bacteria in ballast water treatment systems: a comparative analysis of plate counting, flow cytometry and qPCR

Quantifying heterotrophic bacteria in ballast water treatment systems: a comparative analysis of plate counting, flow cytometry and qPCR

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To be submitted

Abstract

To enumerate culturable heterotrophic bacteria, during ballast water treatment system verification testing, it is mandated under the ETV protocol that cells are quantified using heterotrophic plate counting (HPC) techniques using media for fresh water and seawater respectively. Yet, it is well established that HPC techniques may underestimate the number of living bacteria present in natural water samples. In the present study HPC was compared with flow cytometry (FCM) and qPCR. All three approaches were applied on a stationary natural fresh water and seawater sample-point over a consecutive 30-week period. Bacterial abundances using HPC, FCM and qPCR generally yielded concentrations in the range of 10^4, 10^6 and 10^7 cells mL^{-1}, respectively. Substantial differences in abundance patterns were observed among the three techniques over time. With respect to FCM, glutaraldehyde-fixed and formalin/hexamine-fixed samples yielded similar results. The absence of a correlation between FCM and qPCR in freshwater samples was potentially caused by variation in gene copy number among various bacterial species. In contrast, no significant differences were observed when a monoculture of *E. coli* was quantified using FCM and qPCR over a 5-day storage period. In conclusion, FCM appears the more reliable technique to detect heterotrophic bacteria in natural water compared to qPCR.

1. Introduction

In 1883, Robert Koch described the first method to count bacteria in water by introducing the Heterotrophic Plate Count (HPC) method (Koch 1912). Since then, HPC has become an important method for water quality monitoring (Payment, Sartory et al. 2003). Applications vary from monitoring hygiene to mapping bacterial communities in natural environments (Oliver 1987, Sartory 2004, Nagvenkar and Ramaiah 2009). Water samples are inoculated on agar plates enriched with organic nutrients. The plates are incubated at a specific temperature and the amount of colony forming units (CFU) is counted after a set number of days. Each CFU may have originated from a single bacterium or perhaps a cluster of thousands of bacteria (Sutton 2011). Therefore, the CFU result is a conservative estimate of the number of culturable cells in a sample.

In recent years, HPC has been used to evaluate the disinfection performance of ballast water treatment systems (BWTS) (Cangelosi, Aliff et al. 2015). The use of HPC in testing BWTS is a direct result of international legislation prescribing limitations on the number of living organisms allowed in discharged ballast water. On March 23, 2012, the United States Coast Guard (USCG) issued the Standards for Living Organisms in Ships' Ballast Water Discharged in U.S. Waters (Final Rule) (USCG 2012). Furthermore, in September 2017, the International Convention for the Control and Management of Ships' Ballast Water and Sediments (BWM Convention) of the International Maritime Organization (IMO) entered into force (IMO 2004). Both the IMO and USCG set limitations to the number of living organisms discharged in ship's ballast water in their comparable Ballast Water Discharge Standards (BWDS). For ship-owners, to comply with the BWDS, the installation of a BWTS onboard their vessel is, in most cases, the optimal way to achieve compliance. Although, the current BWDS sets no limit to the number of heterotrophic bacteria, the Final Rule describes the commitment of the USCG to periodically assess the feasibility of implementing the Phase-Two BWDS. This Phase-Two is likely to introduce limits on the number of living heterotrophic bacteria in discharged ballast water.

In order for BWTS-manufacturers to install their equipment on seagoing vessels, they have to obtain IMO type approval from an IMO-member flag state. Similarly, to discharge BWTS-treated ballast water in U.S. waters, the BWTS has to obtain USCG type approval. Both the IMO (BWMS Code) (IMO 2018) and the USCG Final Rule (USCG 2012) have adopted a mandatory testing protocol to evaluate the BWTS for Type Approval certification. In the USCG Final Rule, the Generic Protocol for the Verification of Ballast Water Treatment Technology is incorporated by reference (ETV protocol, (NSF-International 2010). The ETV

protocol prescribes that the challenge water used to test BWTS for Type Approval must be evaluated for culturable heterotrophic bacteria. Specifically, HPC techniques must be used with 2216 Marine Agar (MA) and salt-modified R2A agar (R2A) for seawater and brackish water and Plate Count Agar (PCA) and Nutrient Broth (NB) agar for freshwater, using a 5-day incubation period.

It has been recognized that the number of living water-borne bacteria derived from immunofluorescent microscopy exceeds the number of CFU's as recorded by HPC (Xu, Roberts et al. 1982, Colwell 2002). For example, Fluorescent Microscopy (FM) using nucleic acid dyes such as Acridine Orange (AO) and 4',6-diamidino-2-fenylindool (DAPI) indicate that the number of DNA-containing bacteria in natural oligotrophic samples is two orders of magnitude higher than what is recorded by HPC and that this discrepancy disappears when eutrophic bacterial cultures are assessed (Zweifel and Hagström 1995). The discrepancy is also known as the "great plate count anomaly" (Staley and Konopka 1985). Bacteria that are not culturable but are still alive are considered to be in a Viable But Non-Culturable (VBNC) state (Oliver 2005). Several hypotheses for the great plate count anomaly have been proposed. For instance, a vast number of species is present in the sample and each one requires specific environmental conditions to reproduce effectively. In addition, the eutrophic plate media contain nutrients that are orders of magnitude higher than in natural water, thereby favoring only a limited number of species most adept at utilizing these resources (Connon and Giovannoni 2002). The FM observation suggesting that the concentrations of bacteria are underestimated by HPC has been corroborated by using Flow Cytometry (FCM) (Hoefel, Grooby et al. 2003). Similar to FM, in FCM analysis the cells are stained with a fluorescent dye and detected individually by a laser. Data acquisition using FCM is achieved objectively using particle scatter and fluorescence detectors. Although FCM uses well-defined parameters in counting bacterial cells, debris contained in the sample can obscure fluorescent cell signals. Also, the fraction of the bacterial community effectively stained by the fluorescent dyes is challenging to quantify in natural water samples (Gasol and Del Giorgio 2000).

A fourth enumeration technique that yields promising results is the quantitative Polymerase Chain Reaction (qPCR). This technique combines the traditional PCR reaction with real-time fluorescence analysis. Primers are used to target a specific DNA sequence (usually a gene coding region) and PCR amplifies that particular sequence. A double-stranded DNA (dsDNA) fluorescent stain is added to the reaction mix. As a result, fluorescence increases proportionally to the amount of DNA copies formed. Combined with

markers with a known DNA content, this technique can reliably estimate, in real-time, the number of gene copies during the PCR reaction (Skovhus, Ramsing et al. 2004). Two limitations are identified when this technique is used to enumerate organisms. First, genes often have multiple copies per organism, so the number of gene copies tends to overestimates the number of organisms (Nadkarni, Martin et al. 2002). Secondly, if the aim is to exclusively quantify heterotrophic bacteria, interference of genes amplified from autotrophic bacteria (cyanobacteria) and eukaryotic cell organelles (chloroplasts) may result in substantial overestimations when investigating natural water samples (Hodkinson and Lutzoni 2009). While it is recognized that HPC solely detects viable cells, which are the cells or interest, alternative methods like FCM and qPCR are providing important insight in the (otherwise overlooked) VBNC community related to ballast water treatment. Notably, it has been shown that bacteria in the VBNC state can be resuscitated (Chaiyanan, Huq et al. 2001, Fernandez-Delgado, Garcia-Amado et al. 2015). The dispersion of VBNC bacteria by ship's discharges could therefore interfere with the objective of the BWDS to reduce the spread of aquatic invasive species through ballast water. In order to obtain quantitative information on the accuracy of plate counting in BWTS test water, a thorough comparison between HPC, FCM and qPCR was made.

First, procedural tests were performed to compare the ETV-prescribed 5-day plate-observation period with shorter as well as with longer incubation times. In addition, two types of bacterial fixatives were compared to identify the most effective one for bacterial FCM enumeration. Furthermore, an *Escherichia coli* monoculture was analyzed with FCM and qPCR over a 5-day storage period. Finally, bacteria were enumerated using marine and freshwater that was sampled from late winter to autumn with HPC plates, FCM and qPCR.

2. Methods

2.1. Sampling procedure

Surface samples were taken every other week from week 8 until week 38, 2013 from the Marsdiep tidal inlet (marine) and Lake NIOZ (fresh water) using a clean bucket. Salinity and temperature were measured directly in the bucket using a calibrated Conductivity Meter with a Pt sensor (GMH 3430, Greisinger). The sensor was calibrated with the following Laboratory Salinity References: 3, 22, and 32 g KCl kg^{-1}. One liter of the bucket sample was transferred into a polycarbonate bottle and transported to the laboratory for further processing.

2.2. Heterotrophic plate counting

Media for HPC were chosen, based on the recommendations in the ETV protocol (NSF-International 2010). For seawater samples, 27.6-g Difco™ 2216 Marine Agar (MA) was dissolved in 500-mL milli-Q™. The second seawater medium was 9.1-g Difco™ R2A agar (R2A) dissolved in 500-mL Enriched Seawater, Artificial Water (ESAW) medium excluding the ESAW-prescribed nutrients (Harrison, Waters et al. 1980), because R2A agar is an oligotrophic medium intended to cultivate stressed and poor-growing bacteria. For freshwater samples 11.8-g Difco™ Plate Count Agar (PCA) was dissolved in 500-mL milli-Q™. The second freshwater medium was prepared using 4.0-g Difco™ Nutrient Broth and 7.5-g Difco™ Nutrient Agar (NB) dissolved in 500-mL milli-Q™. All media were dissolved in 1-L autoclavable bottles using a microwave oven and subsequently autoclaved 20 minutes (120°C, 0.2 MPa) in a Laboklav (SHP Steriltechnik AG). The media were poured in petri dishes (Ø 100mm x 15mm, VWR) inside a laminar flow bench (Interflow) and stored in a SI-900R incubator (Jeiotech) at 25°C for three days to check for contamination. Positive controls for each plate were made by inoculating them with a swab from a keyboard surface. After collection, samples were diluted 10 times using 0.2-µm filtered sample water. From this dilution, a 100-µL volume was spread onto the plates within 30 minutes after sampling. Triplicate plates were incubated in a SI-900R incubator (Jeiotech) at 25°C.

In week 8, at day 4, day 5 and day 7, Colony Forming Units (CFU) were counted semi-automatically with the aid of a HG/ColonyCounter application (HyperGEAR) installed on an iPad mini (Apple, Model A1432). From week 10 onwards, colonies were solely counted after 7 days of incubation. A digital picture was made of each agar plate, placed on a black background for contrast. A standard setup was used to make sure all plates were photographed under similar circumstances, resulting in a digital database of all plates. The Colony counter software automatically detected colonies on the plate's pictures and the results were immediately subjected to a visual correction by using the add/delete option in the software. Furthermore, the plate itself was examined to verify if a putative colony on the photograph actually was a colony. A detailed comparison between automatic and corrected counting results was not performed, because substantial corrections were required in all cases.

2.3. Flow cytometry

Triplicate samples of 1.5-mL were transferred into 2-mL cryovials (Greiner Bio-One) containing 150-µL 25% (w/v) glutaraldehyde (GA; 2.3% final concentration) or 150-µL 18% (w/v) Formalin/Hexamine (FH 1.6% final concentration), incubated for 30 minutes at 4°C,

frozen in liquid nitrogen and stored at -80°C until further analysis. After thawing at room temperature, samples were diluted 10 times by adding 100-µL sample to 900-µL 0.2-µm filtered Tris-Ethylenediaminetetraacetic acid buffer (Tris-EDTA buffer, pH 8.0). Subsequently, PicoGreen® (ThermoFisher; 500 times commercial stock dilution) was added. The 'total bacteria' stain PicoGreen® was used to target all intact bacterial cells. The results of the 'dead bacteria' stain SYTOX™ Green (ThermoFisher) are not reported because in the natural samples the fluorescent signal of the dead cells stained with SYTOX™ was obscured by fluorescent debris, leading to inconclusive results. Samples were incubated for 15-30 minutes in the dark at room temperature and analyzed using a FACSCanto™ flow cytometer (Becton Dickinson) with a 488 nm laser. As particle-detection trigger, green fluorescence was used (FBG channel, 530 nm). The flow rate was monitored twice per day using Trucount™ beads (Becton Dickinson) diluted in Tris-EDTA buffer. Performance of fluorescence detectors was checked using Cytometer Setup & Tracking beads (Becton Dickinson). Coefficients of variations were maintained below 6%. Results were analyzed using FCS Express version 4 software (De Novo Software).

2.4. qPCR

Within 15 minutes after sampling, 50 mL sample was filtered over a 45-mm 0.2 µm polycarbonate filter (Millipore, Sigma-Aldrich). Filters were stored in cryovials at -80 °C. DNA extraction from the filters was performed using the PowerSoil® DNA isolation kit (MO BIO). After extraction, the resulting 100 µL DNA solution was divided over 3 aliquots: 15 µL for quality control, 20 µL as working solution and 65 µL as backup. All aliquots were stored at -20 °C.

DNA extraction performance was estimated using a NanoDrop (Thermo Fisher Scientific) assessment. Subsequently, DNA concentrations were determined in duplicate using PicoGreen® (250-times commercial stock dilution) and a fluorescence analyzer (488 -> 520 nm) (Spectramax FS2500). The resulting DNA concentration was used to add equal amounts of DNA to each PCR reaction. In addition, a gel electrophoresis including SmartLadder (Eurogentec) was performed to estimate the size and weight of the extracted DNA fragments. As loading dye, Bromophenol Blue was used. All electrophoresis gels contained 1.5% agarose and were run on 80-V for 45 minutes.

The master mix for the standard curve was produced using the following primers for the 16S rRNA gene. F: 341 (5'-CCTACGGAGGCAGCAG-3'), R: 907(A) (5'-CCGTCAATTCATTTGAGTTT-3') and R: 907(C) (5'-CCGTCAATTCCTTTGAGTTT-3'). For one reaction: H_2O (36 µL), 10X PicoMaxx PCR buffer (5 µL, Agilent Technologies),

10X dNTP (5 µL), 50X Bovine Serum Albumin to relief PCR amplification inhibitors (Kreader 1996) (BSA, 1 µL), F:341 primer (0.2 µL), R:907A primer (0.2 µL), R:907C primer (0.2 µL), 125X Taq polymerase (PicoMaxx, 0.4-µL) and template DNA (2-µL) was used. The DNA template originated from extracted DNA of sample water obtained in week 10, for both seawater and fresh water. The PCR conditions were: 95°C (4 min.); 36 cycles: 95°C (30-sec.), 55°C (30 sec.), 72°C (1 min.). Followed by 72°C (7 min.), 4°C (10 min.) and 15°C (∞). Resulting PCR products were used to make a standard curve for qPCR. The length of the standard curve bands was 566 bp which has an average weight of 373,560 Dalton, which results in $6.2 * 10^{-10}$ ng copy^{-1}. Fluorescence analysis indicated that the DNA concentration of the sea water template sample was 1.6 ng µL^{-1}. In order to obtain $1.0 * 10^9$ copies µL^{-1} in 100-µL volume, 38.76 µL sample was diluted in 64.24 µL SPUDA 10^6 + Tris. The standard curve was made by diluting 4 µL of the initial stock to 36 µL of SPUDA 10^6 + Tris and repeating this step nine times. An additional standard curve of fresh water was made in a similar fashion.

The master mix for the samples was made using the following primers targeting the 16S rRNA gene. F: arch519aS15 (5'-CAGCMGCCGCGGTAA-3') and R: bact785bA18 (5'-TACNVGGGTATCTAATCC-3'). Per sample: H_2O (14.2 µL), 10X PCR buffer (PicoMaxx, 2 µL), 10X dNTP (2 µL), 50X BSA (0.4 µL), F:arch519aS15 primer (0.2 µL), R:bact785bA18 primer (0.2 µL), 125X Taq polymerase (PicoMaxx, 0.2 µL), 50X SYBR® Green (0.4 µL) and template DNA (2 µL). As template for freshwater samples, pure sample water was used. For templates of seawater samples, 10 times diluted (milli-Q™) sample water was used to mitigate the inhibitory effect of the seawater minerals on the PCR reaction. The PCR conditions were: 95°C (2 min.), 42 cycles: 95°C (30 sec.), 48°C (40 sec.), 72°C (40 sec.), followed by a fluorescence scan. After 42 cycles: 95°C (10 sec.) followed by a melt-curve from 65°C to 95°C using increments of 0.5°C for 5 sec., including a fluorescence scan, concluded by 15°C (∞).

2.5. FCM and qPCR comparison using E.coli

To compare qPCR with FCM and to study the impact of storage time on bacterial enumeration in a controlled manner, a standardized laboratory test setup was devised. *E.coli* (Vitroids™, Sigma-Aldrich) was incubated in a culture vessel with MacConkey medium at 44°C for 24 h in a Speedy Breedy incubator (BacTest®). To enable linear regression analysis between qPCR and FCM, a serial-dilution was made in four-fold by diluting the source culture 10^1-, 10^2-, 10^3-, 10^4-, 10^5-, 10^6- and 10^7-times with MacConkey medium. Samples for qPCR and FCM were taken from the first two dilution series on day 0. The remaining two

dilution series were stored at 4°C for five days. On day 5 the stored dilution series were sampled for qPCR and FCM analysis. FCM samples were fixed with GA, samples for qPCR were filtered, stored and extracted as described above.

A qPCR analysis was performed using a NovaQUANT™ Coli qPCR Kit (Novagen®), containing the proprietary primers and *E. coli* DNA standard. The DNA standard was used to make a calibration dilution ranging from 1 ng μL^{-1} through 0.1 fg μL^{-1}. To calculate *E. coli* cell concentrations, it was assumed that 10-ng genomic *E. coli* DNA standard was equal to 2,000,000 *E. coli* cells. The mastermix consisted of 10 µL SsoFast™ EvaGreen® Supermix (Bio-Rad), 2 µL NovaQUANT™, 6 µL PCR grade water and 2 µL *E. coli* standard or sample. Samples were run on a CFX96-Real Time System (Bio-Rad) using the following protocol: 98°C (2 min.), 98°C (10 sec.), 60°C (25 sec.), measure fluorescence, for 41 cycles. The DNA melt-curve was determined using 0.2°C increments for 10 sec. from 75°C to 95°C. *E. coli* samples for FCM were processed and analyzed as described in Chapter 2.3, with the change in DNA stain to SYBR® Green in lieu of PicoGreen®.

2.6. Statistical analysis

A least squares linear regression model was used for testing the null hypothesis (H_0) that there is no significant correlation between two groups. Because there was little temporal variation in bacteria concentrations, additional single-factor ANOVAs on log(x+1) transformed data were performed to test H_0 that there is no difference between the means of two analysis methods. H_0 was rejected at $P<0.05$. All statistical analyses were performed in Microsoft Excel for Mac v16.45.

3. Results

3.1. Procedural

3.1.1. Fungal overgrowth and smear

Of the 204 petri dishes included in the HPC experiment, five plates (2%) were excluded from analysis due to fungal overgrowth. Furthermore, twelve plates (6%) were excluded from analysis due to bacterial growth smear that obscured distinct colonies. Fungal growth was considered interfering with the analysis solely when the whole plate was overgrown. Fungal interference was observed four times in PCA and once in NB medium. No fungal interference was observed in both saltwater media. The presence of smear was more or less evenly observed over the four media types.

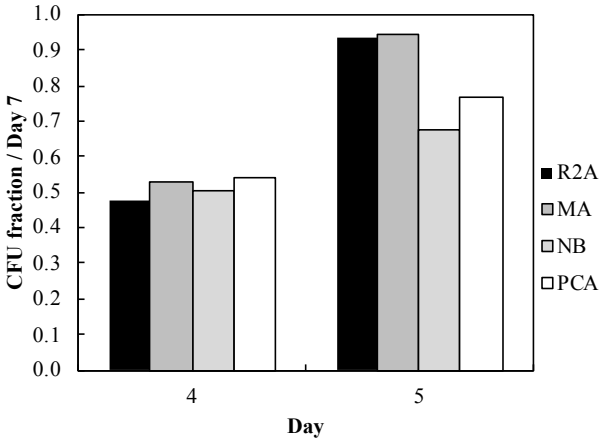

Figure 1. *Number of colony forming units (CFU) detected on four HPC media after 4 and 5 days incubation relative to a 7-day incubation period.*

3.1.2. Incubation time effect on CFU counts

In week 8, at the ETV-prescribed 5-day incubation time CFU numbers were in 84% of cases lower then after 7 days of incubation. Furthermore, CFU counts were at 50% after 4 days relative to 7 days for both freshwater and saltwater media. At day 5, the saltwater and freshwater media yielded 94% and 71% of CFU's compared to day 7, respectively (Figure 1). Subsequent HPC incubations were solely counted on day 7 to obtain the maximum number of CFU's.

Table 1. *Average bacteria concentrations during week 8 to 38, 2013.*

Method	Salinity	Sub-method	Average (mL^{-1})	CV (%)	Min – Max	
HPC	Freshwater	NB	1.5×10^4	72	0.7 – 42.0	$\times 10^3$
		PCA	8.6×10^3	45	0.0 – 1.7	$\times 10^4$
	Seawater	R2A	3.3×10^3	92	0.0 – 1.3	$\times 10^4$
		MA	3.9×10^4	91	0.1 – 14.1	$\times 10^4$
FCM	Freshwater	FH	3.8×10^6	84	0.7 – 8.9	$\times 10^6$
		GA	4.5×10^6	84	0.3 – 11.4	$\times 10^6$
	Seawater	FH	4.4×10^6	52	2.1 – 9.7	$\times 10^6$
		GA	4.5×10^6	63	1.2 – 11.9	$\times 10^6$
qPCR	Freshwater		5.2×10^7	52	0.4 – 11.7	$\times 10^7$
	Seawater		2.2×10^7	65	0.5 – 6.8	$\times 10^7$

CV = Coefficient of Variance; HPC = Heterotrophic Plate Count Agar; FCM = Flow Cytometry; qPCR = quantitative PCR; NB = Nutrient Broth; PCA = Plate Count Agar; FH = Formalin/Hexamine; GA = Glutaraldehyde; MA = Marine Agar; R2A = salt-modified R2A agar;

3.1.3. Comparing fixatives for FCM

Samples fixed with GA showed a slightly higher bacterial abundance than samples fixed with FH, both in fresh water and seawater (Table 1). However, when comparing the log(x+1) transformed data with a single-factor ANOVA, the means were not significantly different from each other (Table 2a and 2b). Regression analysis showed an obvious correlation between the two fixative results (Figure 4c and 4d) with high R^2 values of 0.99 and 0.98 in freshwater and seawater, respectively (Table 3).

3.1.4. E.coli experiment

Using an *E. coli* monoculture, cell concentrations obtained from qPCR and FCM were highly correlated. Results were log-transformed and a least squares linear regression model on Day 0 and Day 5 concluded significant correlations within and between days and methods ($p < 0.001$ in all cases) (Figure 2). An ANOVA test found no significant differences between the means of qPCR and FCM irrespective of the day of sampling ($p = 0.88$). In other words, neither the use of different detection techniques nor the 5-day storage at 4°C yielded significant differences in the abundance of *E. coli* cells.

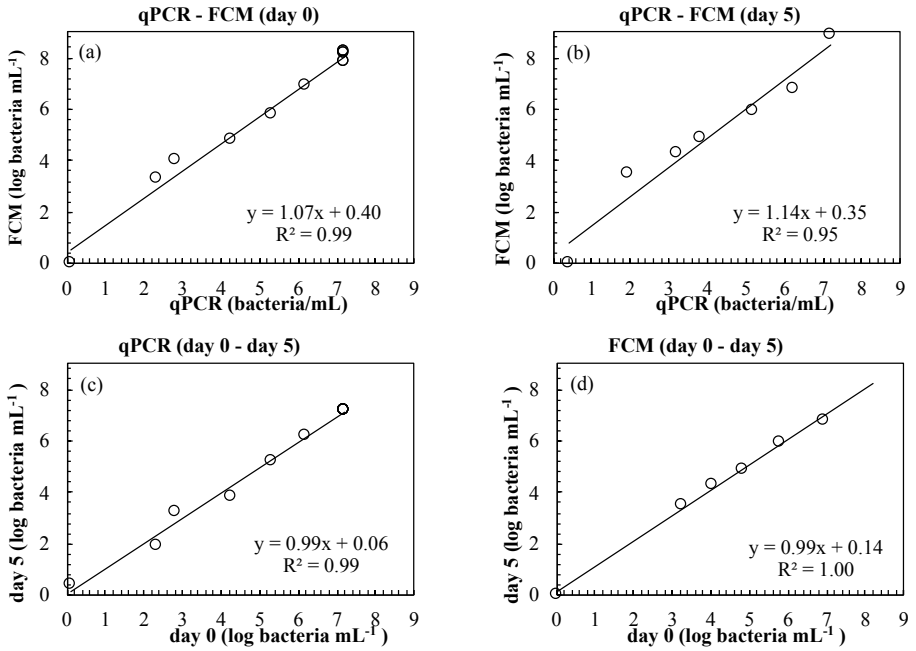

Figure 2. *E. coli experiment, comparing FCM (flow cytometry) and qPCR within (**a**, **b**) and between days (**c**, **d**).*

3.2. Experimental

3.2.1. Temporal development and technical differences

In Figure 3, Table 1 and Table 2, it is shown that bacterial concentrations obtained by the HPC, FCM and qPCR were significantly different from each other. These differences were similar between freshwater and seawater. The HPC methods yielded the lowest bacterial concentrations, ranging from 10^3 and 10^4 cells mL^{-1}. Samples analyzed using FCM yielded bacterial concentrations two to three orders of magnitude higher at 10^6 cells mL^{-1}. The highest estimates of bacterial abundances were observed using qPCR at 10^7 cells mL^{-1}. The coefficient of variation (CV) of the various techniques ranged from 45-91% (Table 1).

3.2.2. Regression models of HPC, FCM and qPCR

In general, the correlations between the three techniques were poor (Figure 4, Table 3). The techniques yielded significant differences (Table 2). The best correlations were found between FCM and qPCR, notably in seawater. (Figure 4e and h). Large positive intercepts as in the ETV-required plate count techniques indicate systematically higher bacteria concentrations in NB compared to PCA and R2A compared to MA (Figure 4a and b).

Table 2. *Results of a single-factor ANOVA on log(x+1) converted seawater and freshwater data to test the null hypothesis (H_0) that there is no difference between the means of two analysis methods in **a** freshwater and **b** seawater. $P<0.05$ (in italics) indicates a significant difference between the means.*

Table 2a

P Freshwater	NB	PCA	FH	GA
NB				
PCA	*0.03*			
FH	*0.00*	*0.00*		
GA	*0.00*	*0.00*	0.40	
qPCR	*0.00*	*0.00*	*0.00*	*0.00*

Table 2b

P Seawater	MA	R2A	FH	GA
MA				
R2A	*0.00*			
FH	*0.00*	*0.00*		
GA	*0.00*	*0.00*	0.71	
qPCR	*0.00*	*0.00*	*0.00*	*0.00*

NB = Nutrient Broth; PCA = Plate Count Agar; FH = Formalin/Hexamine; GA = Glutaraldehyde; qPCR = quantitative PCR; MA = Marine Agar; R2A = salt-modified R2A agar;

Table 3. *R^2 results of correlation plots between two datasets in **a** freshwater and **b** seawater.*

Table 3a

R^2 Freshwater	NB	PCA	FH	GA
NB				
PCA	0.11			
FH	0.01	0.01		
GA	0.00	0.02	0.99	
qPCR	0.01	0.00	0.02	0.11

Table 3b

R^2 Seawater	MA	R2A	FH	GA
MA				
R2A	0.10			
FH	0.11	0.34		
GA	0.00	0.32	0.98	
qPCR	0.01	0.02	0.40	0.48

NB = Nutrient Broth; PCA = Plate Count Agar; FH = Formalin/Hexamine; GA = Glutaraldehyde; qPCR = quantitative PCR; MA = Marine Agar; R2A = salt-modified R2A agar;

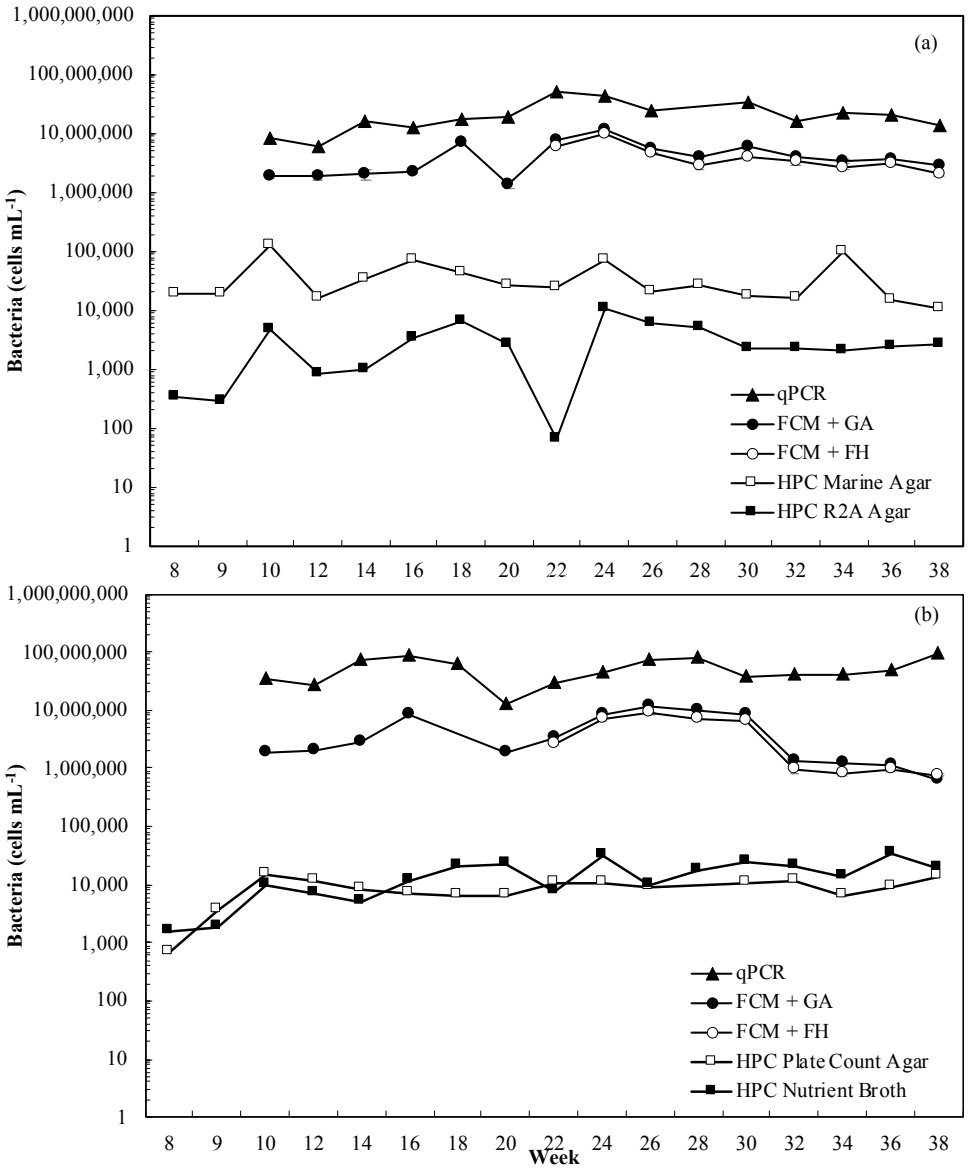

Figure 3. *Average concentrations of bacteria in* ***a*** *Seawater and* ***b*** *Freshwater from week 8 to week 38 (2013) using five different quantification techniques. qPCR in gene copy numbers* mL^{-1}*; FCM in cells* mL^{-1}*; HPC in colony forming units* mL^{-1}*.*

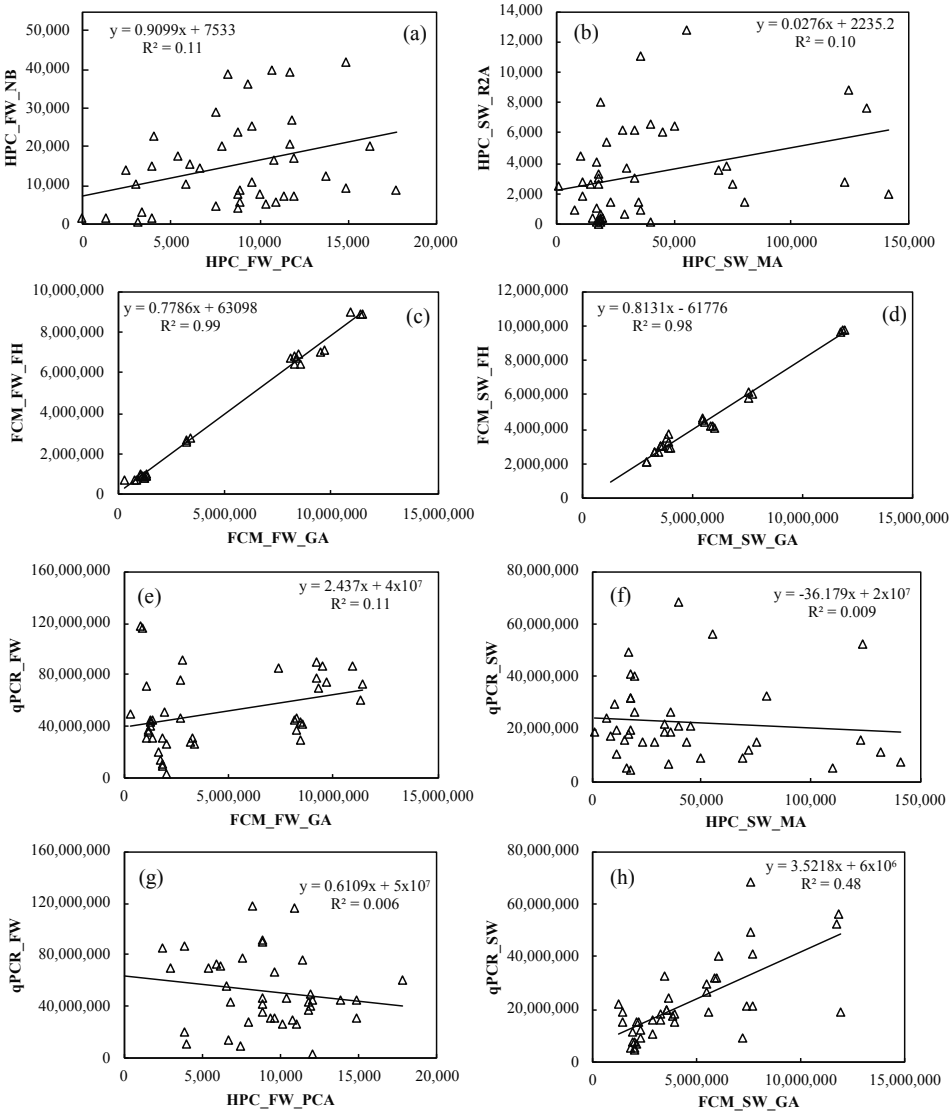

Figure 4. *Regression analysis between* ***a*** *PCA (plate count agar) and NB (nutrient broth) in freshwater.* ***b*** *MA (marine agar) and R2A (salt-modified) in seawater. FCM (flow cytometry) in* ***c*** *freshwater and* ***d*** *seawater comparing GA (glutaraldehyde) and FH (formalin/hexamine) fixative. qPCR and FCM in seawater using* ***e*** *GA and* ***f*** *FH fixative and similarly for freshwater in* ***g*** *and* ***h.***

4. Discussion

4.1. Procedural

The observed fungal interference in freshwater media was also reported in a HPC study of groundwater using the low-nutrient NWRI agar and R2A agar, but similar to the salt-modified R2A agar results reported above, the referenced study did not report fungal interference from groundwater samples on R2A agar (Lillis and Bissonnette 2001). Fungal interference could not be related to low-nutrient agar, since both R2A and NWRI are low-nutrient media. The frequent observation of fungal growth on HPC plates raises the question whether fungi should also be included in future ballast water treatment studies. The significance of fungi in water systems is still poorly understood, however it has been reported that certain fungi are known to infect phytoplankton species (Wetsteijn and Peperzak 1991).

Generally, colony count software provides a time-saving benefit in case of >100 colonies per plate. Also, the manual addition or deletion of CFU's in such software is helpful to keep track of the number of colonies. The resulting photos provide a convenient database for later reference.

In FCM analysis both GA and FH samples fixed with GA yielded results that were not significantly different from each other in both seawater and freshwater. Both GA and FH fixatives perform relatively similar to each other. Due to an apparent higher result when using GA compared to FH it is not recommended to use the fixatives interchangeably when comparing relative differences among samples.

The 3- to 5-day plate-incubation time, as prescribed in the ETV protocol for HPC, underestimates the concentration of culturable heterotrophic bacteria by 6% (saltwater) to 29% (freshwater) compared to a 7-day incubation time. Considering that culturable aerobic heterotrophic bacteria are subject to a minimum test water uptake requirement of 10^3 CFU mL^{-1}, this may lead to the erroneous conclusion that this requirement was not met. At present there is no risk of a false-negative result for discharged ballast water since the BWDS does not currently prescribe discharge limitations on heterotrophic bacteria. However, this may change in future revisions of ballast water regulations such as the USCG Phase-Two Standard (USCG 2012). In general, it appears that the minimum challenge conditions for culturable heterotrophic bacteria in the ETV protocol and the BWMS Code are too high, considering that the amount of VBNC's as measured by FCM or qPCR consistently exceed the minimum challenge conditions by orders of magnitude.

4.2. Experimental

It was noteworthy to observe low levels of correlation among HPC, FCM and qPCR

methods. Although it was anticipated that absolute quantities would be different, it was nevertheless expected that growth patterns among various methods would indicate similar trends. Instead, growth patterns were reasonably stable during the 30-week sampling period as the CV was <100% in all methods. Among techniques opposing growth trends were occasionally observed. For example, qPCR results increased from week 30 through week 38 while conversely, FCM methods showed a decline in bacterial abundance (Figure 3b). In short, growth patterns showed a completely different pattern among HPC and FCM and qPCR. The absence of meaningful correlations between different enumeration methods is in addition to the significant differences in the means. HPC, FCM and qPCR result in significantly different mean result and are not significantly correlated.

An important consideration is the presence of VBNC's. In recent years the widespread presence of these bacteria has been elucidated. Some VBNC's are normally culturable but in response to stress-factors become non-culturable. They are not dead however, and under the right circumstances they can regain their ability to grow and divide (Oliver 2010). Among the identified VBNC are all human pathogens included in the IMO and USCG ballast water discharge regulations. Most notably, VBNC *Vibrio cholerae* O1 was identified in Bangladesh in 1994 (Islam, Miah et al. 1994). And in 1996 it was observed that VBNC *V. cholera* O1 could revert to a culturable state in the human intestine (Colwell, Brayton et al. 1996). Ballast water treatment can be regarded as a stress factor for many bacterial species, potentially inducing VBNC-state. So, the presence of VBNC bacteria, which are thus undetectable by HPC methods, but remain a potential threat, is recommended to be considered in the choice of detection method. Furthermore, as long as the heterotrophic bacteria are solely monitored for the challenge they pose to a BWMS, it could be argued that the total number of bacteria is more relevant than the culturable number of bacteria, as VBNC bacteria presumably pose a similar BWMS challenge as culturable bacteria (e.g., active substance degradation or light-attenuation)

4.3. qPCR and quantifying bacteria

The high degree of correlation between *E. coli* concentrations obtained with qPCR and FCM was remarkable because in natural samples these correlations were lower or absent. This discrepancy may partly be attributed to varying 16S rRNA gene copy numbers among bacterial species. When assessing a monoculture using the NovaQUANT™ assay, calibration material based on known gene copy numbers is readily available for *E. coli*. In natural samples, the bacterial species assembly is unknown, so the average gene copy number needed to convert the gene-copy results to actual bacterial cells is not readily available. Studies have

been dedicated to estimate 16S rRNA gene copy numbers more reliably (Vetrovsky and Baldrian 2013), and some estimates, marginally improved the comparison between FCM and qPCR (data not shown). Due to the legal implications of the BWDS, to introduce qPCR as quantitative tool in ballast water compliance testing, the average gene copy number of 16S rRNA is required per sample. In order to obtain this, the bacterial species assembly of each ballast water sample should be determined using complicated and time-consuming molecular techniques. If, finally, a reliable gene copy estimate is obtained, it is expected (based on the *E. coli* experiment) that the resulting cell abundance will largely resemble cells concentrations obtained using FCM. Therefore, FCM is considered more user-friendly, cheaper and quicker than qPCR to reach a similar endpoint. Thanks to its specificity however, for the indicator microbes ballast water compliance methods based on qPCR have been developed for *E.coli, Enterococci* and *V. cholerae* (Darling and Frederick 2018).

4.4. Concluding remarks

In conclusion, FCM appears more reliable than qPCR to detect total bacterial abundance in natural water samples. Most importantly, there was no correlation between HPC and FCM results in bacterial trends over time. Therefore, these results support the notion that the prescribed HPC techniques are not predictive of the actual challenge posed by bacteria in the challenge water. More fundamentally, it is unclear whether the challenge posed by bacteria can be distinguished from other organic matter sources contained in the Dissolved and Particulate Organic Carbon (DOC, POC) pools. Organic matter poses a challenge to oxidant-based systems by reacting with hypochlorite, thus (potentially) forming disinfection byproducts and lowering the Total Residual Oxidants (TRO) available to kill the organisms in the treated water. This chemical process supposedly does not discriminate between organic matter originating from dead or living material. Compared to the challenge water POC requirements for type approval testing the amount of Carbon of 10^6 bacteria/mL is negligeable (5 mg C/L vs 26 µg C/L, respectively) (Trousselier, Bouvy et al. 1997). In UV-systems, the main challenge is low ultraviolet-transmission (UV-transmission) in the water caused by attenuating substances. The main contributors to UV attenuation are humic and fulvic acids as part of the DOC fraction. It is unclear how living bacteria contribute to the challenge posed to UV-based BWMS other than being part of the POC fraction, which has its own minimum required concentration in the challenge water. Therefore, in the absence of discharge limits, the relevance of the heterotrophic bacterial challenge requirements to the type approval process must be further investigated. At the same time, it must be noted that in the absence of regulating heterotrophic bacteria, high bacterial growth in treated ballast water

can occur as a side-effect of treatment, which impact should also be further assessed (Stehouwer, van Slooten et al. 2013).

5. Acknowledgements

This work would have been impossible without the dedication and enthusiasm of Merlina Kranenburg, who for 30 weeks conducted the sampling and analysis impervious to the harsh weather conditions in winter and fall on Texel. Judith van Bleijswijk and Harry Witte of the NIOZ molecular lab were essential in conducting the qPCR analysis and help identifying 16S rRNA primers that were selective for bacteria but excluded the cell-organelles and cyanobacteria. Furthermore, the colleagues of the NIOZ plankton, Anna Noordeloos, Kirsten Kooijman and Alma Lamers for supporting the plate counting and flow cytometry work.

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Chapter 4

Assessment of imaging-in-flow system (FlowCAM) for systematic ballast water management

Assessment of imaging-in-flow system (FlowCAM) for systematic ballast water management

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Published in: Science of the Total Environment. DOI 10.1016/j.scitotenv.2017.06.070

Abstract

Assessing the disinfection of ballast water and its compliance with international standards requires determining the size, viability, and concentration of planktonic organisms. The FlowCAM (Flow Cytometer and Microscope) is an Imaging Flow Cytometry designed to obtain the particle concentration, images, and quantitative morphologic information. The objective in this paper is to establish the basis for transforming the FlowCAM from being a laboratory analyzer into a tool for systematic monitoring of ballast water. The capacity of the FlowCAM was evaluated by analyzing artificial microbeads, phytoplankton monocultures, and real seawater samples. Microbead analyses reported high accuracy and precision in size and concentration measurements. Monoculture analyses showed the effect of disinfection treatments in cell appearance and growth. Low concentration and heterogeneity of particles in real seawater analyses require the comprehensive observation of images by experts. Additionally, some physical characteristics of the device must be improved. The optimization of device configuration enables the quick transferring of files and information between parties involved in ballast water management. FlowCAM may become a feasible technology for this after the device and protocols are adapted.

Keywords

Phytoplankton; exotic species; FlowCAM; ballast water; *Prorocentrum minimum*; flow cytometry

1. Introduction

Ballast water is essential for the correct seaworthiness of vessels and represents a major vector for the transport of non-indigenous species beyond their natural borders (David, 2015; Molnar et al., 2008). Once discharged, these species may become invasive if they overcome the environmental change (Leppäkoski and Gollasch, 2006) and, consequently, they may cause ecological, economical, and even human health damage at the ballast water receptor area (Briski et al., 2014; Liu et al., 2014; Monterroso et al., 2011; Ranjan et al., 2008; Shipway et al., 2014; Williams et al., 1988). National and international regulations have been adopted to mitigate and prevent further ecosystem invasions by organisms released with ballast water.

The International Maritime Organization (IMO) adopted the International Convention for the Control and Management of Ships' Ballast Water and Sediments (BWMC) in 2004, which entered into force in September 2017. As a complement for supporting the implementation of it, a series of technical Guidelines have been developed. The BWMC D-2 standards establish the limitations of organisms released with ballast water discharge: the concentration of viable organisms whose minimum dimension is greater than or equal to 10 μm and less than 50 μm should be less than ten organisms per mL, as well as the concentration of organisms greater than or equal to 50 μm in minimum dimension should be less than ten viable individuals per m^3; additionally, three bacteria indicators are controlled. The achievement of the BWMC D-2 standards may require the application of approved ballast water treatment systems (BWTSs). The filtration followed by either chlorination or UV irradiation are the most frequent options (Lloyd's Register, 2015). The procedures for BWTSs Type Approval include land-based tests with cultured organisms (D'Agostino et al., 2015) or natural seawater, and full-scale shipboard tests (Guideline 8). Land-based testing for BWTSs' approval using cultured organisms requires a combination of at least five species belonging to a minimum of three phyla or divisions with a recommended concentration of 10^4 individuals mL^{-1} in case of 10-50 μm organisms (Guideline 8). The dinoflagellate *Prorocentrum minimum* is a bloom-forming dinoflagellate distributed in temperate and subtropical waters and is potentially harmful to humans via shellfish poisoning and is also related with coastal eutrophication (Hajdu et al., 2005; Heil et al., 2005; Leppäkoski and Gollasch, 2006); therefore, it has been commonly used in studies on ballast water management (David et al., 2013; Gregg et al., 2009; Sutherland et al., 2001). Ships may be subjected to inspection by an authorized party in order to determine its compliance with the BWMC (Article 9) which includes ballast water sampling (Guideline 2). To enhance the

applicability of the BWMC, the operation of BWTSs and the inspections should minimize interferences with the normal schedule of the ships which requires precise yet fast methods for monitoring the size, concentration, and viability of organisms in ballast water.

A framework has been proposed for the development and validation of official monitoring tools based on proof-of-concept pilot studies with increasing complexity to evaluate a series of items such as the data quality, physical characteristics, maintenance, costs, ease of use, and the technical expertise that is required (Drake et al., 2014). Instrumental analyzers are based on different approaches for obtaining information about size, concentration, and viability of organisms such as direct microscopy observation and enumeration, flow cytometry, and indirect biochemical measurements (Bakalar, 2014; Olsen et al., 2016; Peperzak and Gollasch, 2013; Poulton and Martin, 2010; Stehouwer et al., 2013; van Slooten et al., 2015). This study focuses on the assessment of the FlowCAM™ (Flow Cytometer and Microscope; Fluid Imaging Technologies, ME, USA) as an instrument to be used for systematic ballast water monitoring in the context of the BWMC.

The FlowCAM™ combines optics, fluidics, and electronic parts to automatically photograph, measure, and enumerate the particles passing continuously through a glass flow cell (Sieracki et al., 1998). The device can automatically capture images at established rates up to 20 images per second (Auto Image Mode) or be triggered by the detection of a fluorescent signal from chlorophyll a using a laser beam (Fluorescence Triggered Mode). Working on Fluorescence Triggered Mode, the device cannot determine which particle triggered the capture if more than one particle appears concurrently within the field of view and dismisses the capture of heterotrophic organisms; additionally, the Fluorescence Triggered Mode also requires calibration of parameters such as gain and thresholds (Garmendia et al., 2013; Poulton and Martin, 2010). Therefore, the Auto Imaging Mode was considered more appropriate for this pilot study and for subsequently setting the basis for studying the Fluorescence Triggered Mode. Concentration measurement in Auto Image Mode is based on the count of particles and the volume imaged. This is calculated through the length, width, and depth of the field of view and the total number of captures in the analysis. The shape of each particle detected in the photographs is recognized and then a series of morphologic quantitative measurements are determined (Table 1) based on the characteristics of the pixels enclosed by the particle edge. Size variables are Feret measurements or the conversion of the shape into a rectangle. Color variables are based on the average pixel value for each color (Fluid Imaging Technologies, 2012). Therefore, three batches of information are retrieved by the FlowCAM for assessing the size, concentration, and viability of

organisms as required by the BWMC: images, quantitative morphological information, and concentration measurements.

Table 1. *FlowCAM morphologic size and color quantitative variables studied*

Feret based size	ESD	Equivalent spherical diameter
	L	Length
	W	Width
Area based size	ABD	Area based diameter
	GL	Geodesic length
	GT	Geodesic thickness
Color	AB	Average blue
	AG	Average green
	AR	Average red
	Int	Mean grayscale intensity
Color ratios	B/G	Average blue/ Average green
	R/B	Average red/ Average blue
	R/G	Average red/ Average green

Applications of the FlowCAM are embraced in a wide variety of studies (Bowers et al., 2008; Brown, 2011; Brzezinski et al., 2011; Reynolds et al., 2010; Sutherland et al., 2001; Zetsche and Meysman, 2012) that used microalgae cultures (Steinberg et al., 2012) and a natural water analysis to classify and determine the abundance of particles (Morillo-García et al., 2014; See et al., 2005), biomass, and biovolume (Álvarez et al., 2014) as well as to generate size spectra (Álvarez et al., 2011; Schartau et al., 2010). However, the features and configurations that enable its systematic use as a ballast water monitoring tool have not yet been explored. The information embedded in the output files can be exported systematically as image and Excel files thus allowing their transfer into standard computers for remote storage, processing, and interpretation. The correct configuration and the establishment of protocols for analysis and data management enable transferring and recording original and traceable data that is unbiased by the subjective criteria of the device operator. These original data represent an objective proof for evaluating the efficacy of the BWTSs throughout their development, approval, and operation; facilitate the inspections that are required by Port

State Authorities; and help with the early detection of threat species and environmental monitoring for risk assessment (Gómez et al., 2015; Larson et al., 2011; Zhang et al., 2014).

The aim of this study is the execution of a proof-of-concept pilot study for the FlowCAM as a systematic ballast water analyzer in the context of the BWMC and is focused on determining the size, concentration, and viability of the 10-50 µm organisms. The study consists of an analysis of spherical microbeads, monoculture of *Prorocentrum minimum* subjected to laboratory UV treatment, and natural seawater samples subjected to full-scale UV-based BWTS. Items related with data quality, physical characteristics and performance, ease of use, and technical expertise required were explored to assess and improve the feasibility of the FlowCAM for systematic ballast water monitoring.

2. Methods

2.1 Description of samples

2.1.1. Calibrated microbead suspensions

The ability of the FlowCAM for measuring the size and concentration of particles was checked with spherical microbead tests. 1) Suspensions with mixtures of 10 and 50 µm microbeads (Duke Scientific Corporation; Palo Alto, CA, USA) with a calibrated mean and standard deviation (10.03 ± 0.05 and 49.7 ± 0.7 respectively). Arbitrary amounts (four to ten drops) of 10 and 50 µm microbeads stocks were mixed in vials containing 5 mL of Milli-Q™ water for analysis with the FlowCAM. 2) Microbead suspension with a factory-calibrated concentration of 3000 (± 10%) microbeads mL^{-1} in stock (Thermo Scientific, Waltham, MA, USA) subjected to serial dilutions obtaining reference concentrations ranged from five to 1000 microbeads mL^{-1}.

2.1.2. Phytoplankton monoculture disinfection treatments

The effects of disinfection treatment on cell morphology and growth were measured in cultures of *P. minimum* (Heil et al., 2005; Pavillard, 1916) from a NIOZ culture collection. Stock culture was incubated at 15°C in an 18:6 light:dark cycle at ca. 100 µmol photons m^{-2} s^{-1}. Once the culture reached a density of approximately $3 \cdot 10^4$ cells mL^{-1}, it was split into aliquots of 1.5 L and subjected to UV treatment. The UV treatment was applied by a 3-times recirculation of 1.5 L *P. minimum* culture (254 nm absorbance of 0.6) in a flow-through reactor (volume of 287 mL, UV pathlength between quartz sleeve and reactor wall of 0.825 cm) equipped with a low-pressure monochromatic lamp (monochromatic UV-C_{254}, germicidal UV power of 3.6 W, length of 14.6 cm). The total UV_{254} dose of 540 mJ cm^{-2} was calculated using the calculated dose approach as described in (USEPA, 2006). Control was subjected to

the same recirculation procedure but with the UV lamp turned off. Control and treated samples were re-incubated in the same previous culture conditions. Aliquots of each sample were analyzed daily by triplicate with the FlowCAM until changes in the morphology of cells and concentration were not detected between consecutive daily analyses.

The accuracy of the FlowCAM phytoplankton counts was checked with several approaches. First, the previous experimental setup counted a *Tetraselmis chuii* (Butcher, 1959) dilution series with the FlowCAM and manually by microscope. Fresh samples were placed on a Neubauer counting chamber and then a photo was taken using an LAS EZ camera and capturing software (Leica Microsystems, Switzerland) and 100X magnification objective and then the cells were enumerated in a computer display. A *T. chuii* culture was provided by Marine Reserves' Service of the University of Cadiz. Both cultured species have comparable size features: 12 - 16 µm long and 7 - 10 µm broad for *T. chuii* (Hori et al., 1982) and 15 - 23 µm long and 10 - 15 µm broad for *P. minimum* (Hajdu et al., 2005; Lu et al., 2005). Samples of *P. minimum* were counted by both the FlowCAM and concurrently with a BD Accuri™ C6 flow cytometer (Becton Dickinson, New Jersey) throughout the incubation after the UV irradiation. The flow cytometer was configured to analyze 50 µL of sample to detect particles using a 488-nm laser and discriminating phytoplankton cells by autofluorescence of the chlorophyll detected by 670-nm long pass filter.

2.1.3. BWTS application to real seawater

The disinfection of NIOZ harbor water with a full-scale UV ballast water treatment system was tested in five uptake-discharge cycles. On Day 0 of each test cycle, water was stored in three 300 m^3 tanks, two containing filtered and UV-treated water, and one with untreated water as a control. On Day 5, the treated water was again UV-treated (Stehouwer et al., 2010) and then discharged. Three samples were taken during the early, middle, and late stages of uptake and discharge into 1 L PET bottles and kept in the refrigerator until the FlowCAM analysis. Analyses were performed as soon as possible between one and six hours after the sample taking with occasional exceptions taking a maximum of ten hours in the most unfavorable case. Triplicate analyses were performed on 25 mL aliquots from each bottle, leading to nine analyses per sample at the uptake or discharge procedure. The first two cycles were used to properly configure the FlowCAM.

2.2. FlowCAM setting and analysis procedure

A device FlowCAM Benchtop B3 Series (Fluid Imaging Technologies, **Yarmouth, ME, USA**) was used that was equipped with a syringe pump and managed with Visual Spread Sheet™ software (VSS), Version 3.2 (Fluid Imaging Technologies). Objective of 10X (the

10X eyepiece led to overall magnification of 100X), and a 100 µm depth flow cell were mounted in the FlowCAM device. The Auto Image mode was used at a capturing rate of 20 images per second. The flow rate was 140 µL min^{-1}. The acquisition data filter was set at 4 µm ESD; particles below this value were ignored. The analysis stop condition for individual analysis was set by different parameters depending on the purpose of each experimental series. Since the purpose of 10 and 50 µm microbeads analyses was studying the accuracy of size measurements, the analyses were stopped manually after detecting more than 15,000 images in a single analysis. On the other hand, 20 µm microbead analyses, with the purpose of calibrating the concentration measurements, were automatically stopped after the detection of 100 particles. Analyses of culture and seawater were configured to stop after the processing of 1 mL of sample, corresponding to approximately 0.3 mL of sample photographed according to the established imaging and flow rates which determine an efficiency of approximately 30% (ratio of imaged volume respect with total processed volume). The actual values of volume imaged and efficiency for each analysis were recorded in the "Run Summary" text files that were embedded in the output folder.

Triplicate aliquots from microbeads, culture, or seawater sample were poured into 25 mL glass flasks previously rinsed with the proper sample and gently stirred throughout the analysis. Between samples, the system was rinsed by demineralized water filtered additionally by 0.2 µm and then primed with the next sample. Two FlowCAM real-time software tools were continuously observed throughout the processing of the samples in order to detect possible failures during the analysis: the X/Y diagram of particle position within the field of view and the particles detected per time interval; heterogeneities in both plots indicate issues in the sample flow. Analyses with problems were cancelled and reinitiated after dealing with the issue causing the failure, principally the piping system cleaning by backwash. Fixatives were not used in any case. Ethanol and isopropanol were used to clean the hydraulics system before storage. The data collected from analyses consisted of total particle concentration, the image of each particle detected, and the series of quantitative morphological and color variables data for microbeads, culture, and seawater samples.

2.3. Output files management

After each series of analyses, output files were exported from the FlowCAM into a standard computer without access to VSS® software. Collages displaying the images of particles were systematically saved by the FlowCAM as a tagged image (TIF) format file; therefore, the VSS® features for automatic selection and classification of images were disabled which

improved the accessibility of the data. The individual quantitative measurements were exported as Data Export files and particles count and concentration data as Summary Export files both with comma separated values (CSV) format.

2.3.1. Data filtering and classification

Image files in a tagged image format (TIF) and object measurements in a comma-separated value (CSV) format were used for the classification and enumeration of particles according to their visible appearance or quantitative size and color variables (Table 2). Manual counts by human were performed by displaying direct enumeration of specific particles in image files. For managing the information in Data Export and Summary Export files, their data contents were merged into one consolidated sheet. The MS Excel conditional function was applied to all individual particles recorded in Data Export files for classifying particles matching the selected filtering parameters. The concentration of particles belonging to one specific category (C_n) was calculated as the quotient between the count of particles in the corresponding category (N_n) and the processed volume calculated systematically as the ratio between the total count (N_t) and total concentration (C_t) in consolidated Summary Export files ($C_n = N_n \cdot C_t / N_t$).

Table 2. *Variables and values used for automatic filtering of images in artificial microspheres, Prorocentrum minimum monoculture and seawater analyses.*

Sample	Variables	Values (min-max)
20 µm microbeads	ESD	15 – 25 µm
P. minimum	W	7 – 18 µm
	L	13 – 21 µm
Seawater	W	> 9

2.3.2. Abundance spectra of size and color variables

Abundance distributions of size and color variables were determined using the quantitative information in Data Export files obtained in analyses. Size spectra of 10 and 50 µm microbeads were used to assess the accuracy and precision of size measurements by observing the position and broadness of abundance peaks with respect to their reference values. Similarly, abundance distributions and Box-Whisker plotting enabled the quantitative study of morphology in natural cultured organisms as well as detecting and quantifying the cellular deformations and damages caused by the application of the treatments.

2.3.3. Accuracy and precision of concentration measurements

The quality of the concentration measurements was assessed by linear regression of data obtained by the FlowCAM with reference values: theoretical standard concentrations in microbeads experiments, Neubauer counts done with *T. chuii* culture, and the comparison of the FlowCAM with flow cytometer results. The precision of concentration measurements was studied by the values and statistical significance of linear regression parameters (non-significant intercept and significant slope of 1 indicate appropriate precision) and the study of the coefficient of variation between triplicate analyses.

2.3.4. UV treatment efficacy by growth modeling

The growth curves of the *P. minimum* after the UV treatment were modeled to obtain the concentration of viable organisms after the irradiation (Romero-Martínez et al., 2016). Daily concentration measurements of the control sample were fitted to a logistic model to obtain the growth rate (r), the carrying capacity (N_{max}), and the initial concentration (N_0) by minimization of the mean square error. The organisms remaining viable after the treatment were considered to follow the same parameters "r" and "N_{max}" as the control samples, but with an initial concentration (N_{0v}) reduced by the UV irradiation. The treatment inactivation efficacy was determined as 1- N_{0v}/N_0.

3. Results and discussion

3.1. Standard microbeads

3.1.1. Individual recognition and size calibration

Images recorded in the analyses of 10 and 50 µm microbeads mixtures reported the appropriate recognition of individual particles (Fig. 1a) and their edges (Fig. 1b) with an occasional presence of contaminations (unidentified particles) and images containing more than one bead. In the case of concentrated solutions of 10 and 50 µm microbeads experiments

with concentrations higher than 10^5 microbeads mL^{-1}, the ratio of contaminations and misrecognized images represented less than 1% of the total images. Although the sharpness of 10 µm microbeads images is heterogeneous due to the wide range of focusing within the 100 µm depth of the flow cell, their shape is visually recognizable. The focusing of 50 µm microbeads was homogeneous.

The six size variables accurately measured the 10 µm microbeads (Fig. 1c). On the other hand, Feret-based measurements ESD, L, and W accurately determined the size of 50 µm microbeads, whereas the area-based variables ABD, GL, and GT underestimated their diameter (Fig. 1d). Among the six variables that were analyzed, W provided an approximation of the minimum dimension of organisms according to the BWMC precepts. According to the size spectra, the W of 10 µm microbeads was determined between 9 and 12 µm and between 47 and 53 µm for 50 µm microbeads; therefore, individual particles in which W was between 9 and 53 µm could be considered within the 10-50 µm range restricted by the D-2 standards.

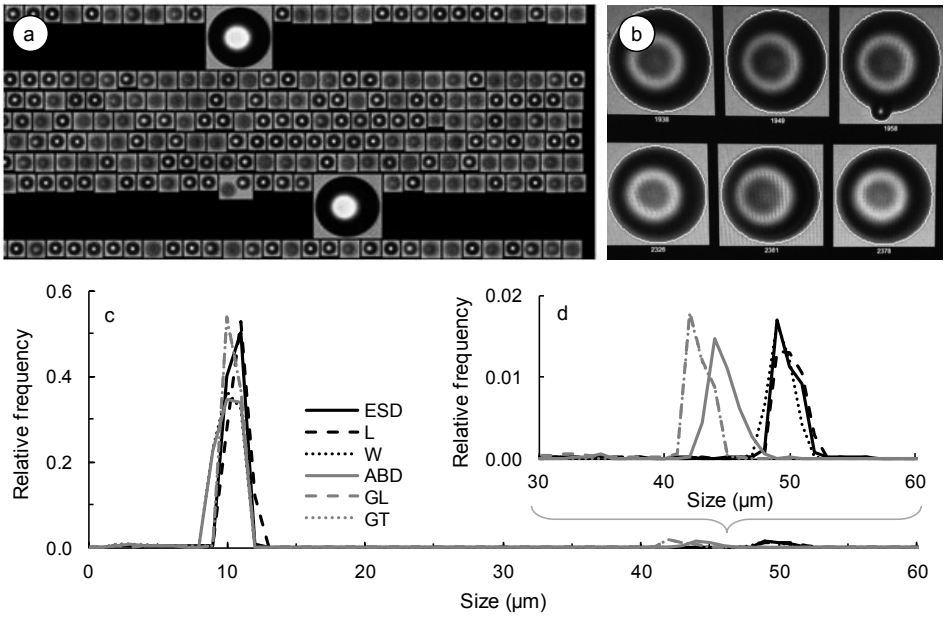


Figure 1. *Representative section of images collages registered in 10 - 50 µm microbeads mixtures analyses (a), selection of 50 µm microbeads images surrounded with thin a thin line representing their recognized edge (b) and abundance peaks (c and d) detected at size spectra.*

3.1.2. Calibration of concentration measurements

The calibration of concentration measurements was based on the analyses of a 20 µm microbeads stock subjected to different dilution factors and then comparing the concentration provided by the FlowCAM with their theoretical values. Images captured included entire microbeads, partial microbeads coinciding with the edges of the field of view, and contaminations (Fig. 1a). These contaminations are the result of undetermined particles existing in materials, hydraulic systems, dilution, or rising water, and their concentration varies throughout the course of the experimentation. Analyses using Milli-Q water as a blank indicated the existence of 98 contaminations per mL in average, thus compromising the accuracy of the direct concentration measurements, especially for low concentrated samples. Due to the unspecific origin of the contaminations and their variable amount, a systematic blank subtraction is not appropriate. Similarly, extremely clean materials and products reduce the presence of contaminations, although this extreme cleanness becomes unfeasible, especially for using the FlowCAM as field instrument.

The total concentration data were corrected into the actual concentration of microbeads by two different mechanisms: manual enumeration based on images and automatic selection based on a software filter using the quantitative size measurements. Concentrations of microbeads subjected to manual enumeration were compared with their theoretical values, indicating strong correlation ($R^2 = 0.99$) respect, with non-significant ($p = 0.075$) intercept of -0.04 and significant ($p < 0.001$) slope of 1.01. Similarly, the automatic data filtering presented strong correlation ($R^2 = 0.93$) with a significant ($p = 0.003$) intercept of 0.23 and significant ($p < 0.001$) slope of 0.92. Both methods showed different advantages and drawbacks. The manual enumeration of particles was precise and quick under the stop condition established in 100 images per analysis and ensured full certainty in the identification of the particles. On the other hand, the automatic selection was slightly less precise than the manual enumeration which may exclude partial captures or include contaminations with a similar appearance to the target particles. In this sense, the manual enumeration is recommended in low concentrated samples with fewer of images to be observed whereas the automatic data selection is a better approach for analysis for highly concentrated samples.

The coefficient of the variation between triplicate analyses of one sample ranged from 8.2% in high concentrated samples up to 63.5% in low concentrated samples. Volumes analyzed to reach the stop condition (100 particles detected) ranged from 0.036 to 0.360 mL, imaged between 1.15 and 11.05 min depending on the concentration of particles in the

solution. Precision in concentration measurements depends on the number of particles that are counted (Lund et al., 1958) and thus the volume analyzed can be adapted to increase the precision in cases of low concentrated samples.

3.2. Prorocentrum minimum subjected to UV treatment

3.2.1. Images displaying

Most images of untreated cultures correspond to the cultured organism captured in different orientations as well as occasional debris or contaminations (Fig. 2). Each individual analysis of 0.33 mL provided between 1,000 and 13,000 images. In this sense, the manual enumeration of cells in images becomes unfeasible at the used configuration. Although there are possibilities to enhance the manual enumeration by diluting the samples, analyzing shorter volumes, or displaying only a sub-set of the images, these procedures are time consuming and introduce uncertainty. Additionally, unlike the displaying of microbeads, the manual enumeration of living cells is subjected to the criteria of the analyst, especially after the UV treatment. Therefore, the concentration of organisms was approached by either the total concentration or the automatic classification of particles according to their quantitative graphic properties.

The UV irradiation caused modifications in the *P. minimum* cells' color and induced the formation of aggregates not observed in control samples (Fig. 2) becoming larger and more numerous during the course of the incubation. The cells belonging to aggregates appeared empty or clearly damaged and were surrounded by spilled intracellular content as a sticky matrix. Cellular spill out and aggregation has been reported for microalgae after disinfection treatments (Bai et al., 2010; Imase et al., 2013; Oukarroum et al., 2012). Therefore, the recorded images can address the application of the UV treatment with a visual approach.

3.2.2. Size and color measurements

For untreated organisms, the variable L determined an abundance peak ranging from 13 up to 21 μm (Fig. 3) whereas the variable W showed a broader peak between 7 and 18 μm; both L and W were consistent with data in literature (Hajdu et al., 2005; Lu et al., 2005). These values were used to establish a classification filter for selecting the *P. minimum* organisms among the total number of particles that were detected (Table 2).

Part of the heterogeneity of L and W is attributable to the orientation of organisms in images. The variable W, calculated as the minimum Feret measurement, is more sensitive to the orientation whereas the L, calculated as the maximum Feret measurement, is more stable along the different orientations. According to the BWTS D-2 standards specifications, the

size measurement refers to “the minimum dimension between main body surfaces of an individual when looked at from all perspectives”, affecting all individual organisms and not per species (Gollasch et al., 2007). This fact adds extra difficulty for determining the belonging of a certain organism to the 10-50 µm controlled by the BWTS (Folkunger, 2014) and questions the suitability of the D-2 standards criteria for classifying organisms by size (Drillet et al., 2013; Folkunger, 2014; van der Star et al., 2011). The images and size measurements taken by the FlowCAM can assist in the selection of species for land-based testing of BWTS with cultured organisms (Guideline 8), requiring species where individuals always show W greater than 10 µm.

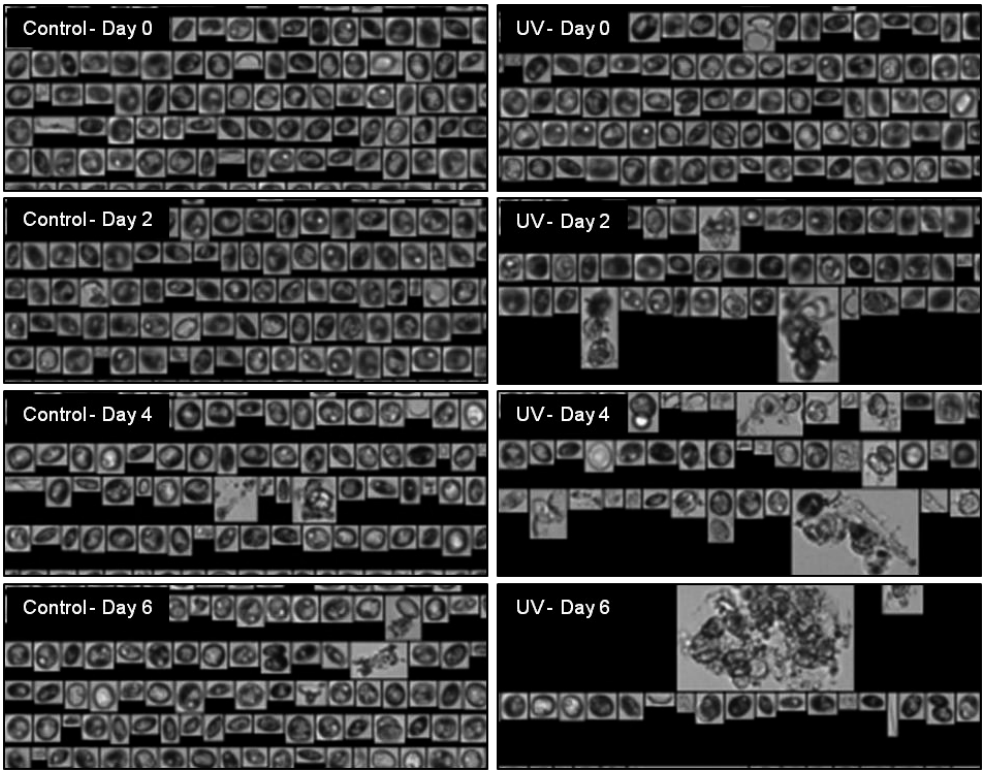


Figure 2. *A representative section of images collages throughout six days after the UV treatment of Prorocentrum minimum.*

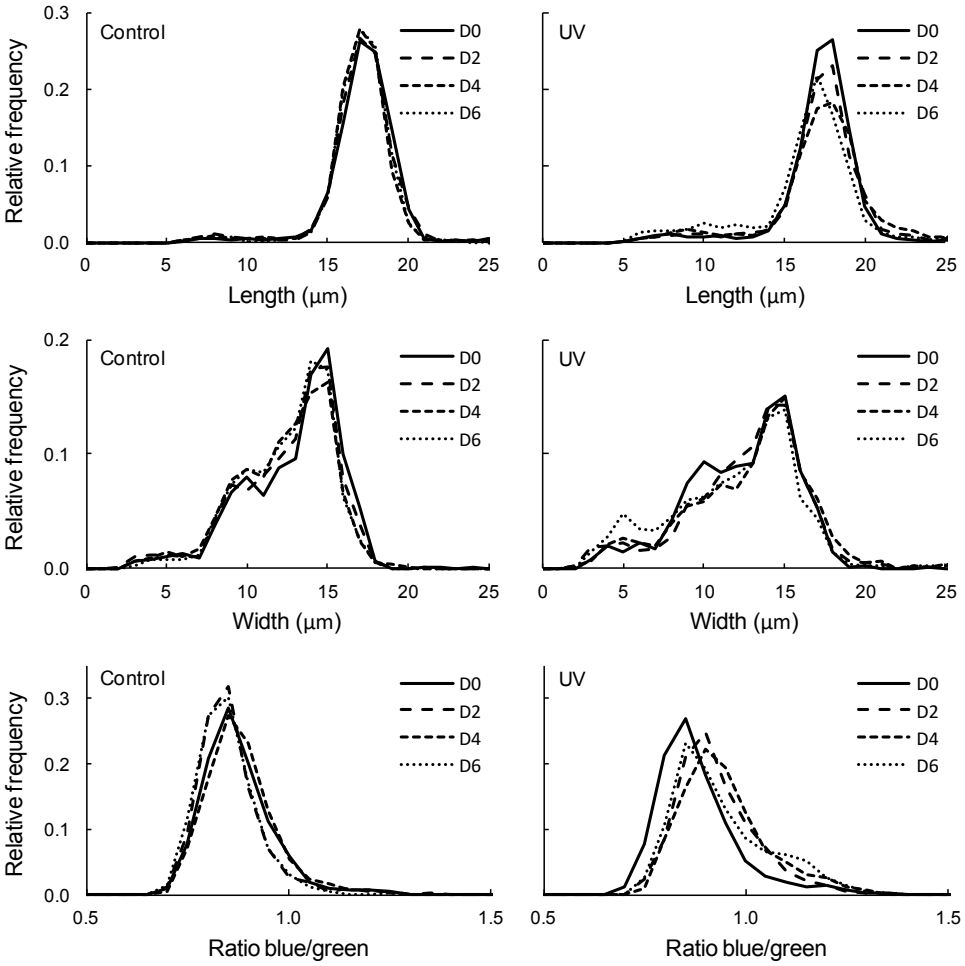


Figure 3. *Evolution of different graphic properties of Prorocentrum minimum after the UV irradiation. Representation of 1500 images per sample. Complete profiles are in Supplementary Material.*

The color and size variables partially supported the effects caused by the UV treatment on the cells morphology that was observed through the images. Abundance spectra of variables L, W, ABD, and GT showed an increase of smaller particles (Fig. 3). Similarly, the Ratio B/G and Ratio R/B varied their distribution but without an evident relationship with the treatment application and the elapsed incubation time. The most consistent effect of UV treatment in particle appearance was the increasing number and size of aggregates as observed in images (Fig. 2) and addressed by the increase of time of the upper outliers in Box-Whisker plots of size variables (Fig. 4).

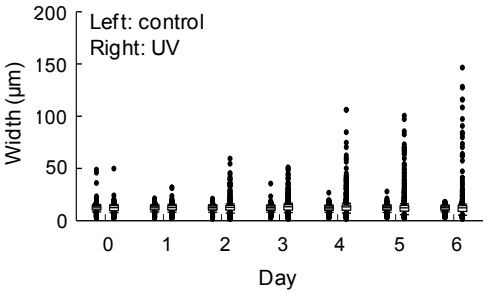


Figure 4. *Evolution of variable Width addressing the aggregates formation after the UV irradiation of Prorocentrum minimum. Representation of 1500 images per sample.*

Despite the UV treatment caused deformations in cell morphology, the variations in morphological quantitative data were weak and mostly inconsistent. The variations in controls were considerable which hampered the addressing of UV damage in the *P. minimum* individuals.

3.2.3. Concentration measurements and growth monitoring

The ability of the FlowCAM to monitor the concentration of cultured organisms was controlled by a previous calibration with the organism *T. chuii.* Concentrations used in culture analyses were high enough to minimize the bias caused by contaminations. Total concentrations calculated with the FlowCAM showed strong linear correlation with microscopy counts (Fig. 5), non-significant ($p = 0.166$) intercept, and significant ($p < 0.001$) slope. Variation coefficients between triplicate values ranged from 1 up to 62% in microscopy counts, whereas the FlowCAM measurements ranged from 0.2 up to 8.7% thus increasing the precision. Total concentration data obtained by the FlowCAM showed linear correlation with their respective measurements with a flow cytometer (Fig. 6) with non-significant ($p = 0.973$) intercept and significant ($p < 0.001$) slope. Concentration data were filtered to remove the particles when L and W did not match with intact organisms (Table 2) with short variations in correlation parameters if compared with total concentration data (Fig. 6). The coefficient of variation of triplicate measurements was, on average, 3.50% for the FlowCAM measurements and 7.59% for the flow cytometer. Therefore, the FlowCAM provided accurate concentration measurements with lower dispersion than microscopy and flow cytometer at the setting used in this study.

Figure 5. *Linear regression for mean values of triplicate concentration measurements of Tetraselmis chuii obtained by the FlowCAM and their respective measurements using microscopy manual counts. Error bars standard deviation between triplicate measurements. Number in italics represent non-significant parameters.*

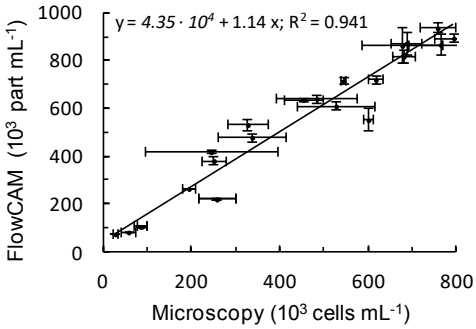
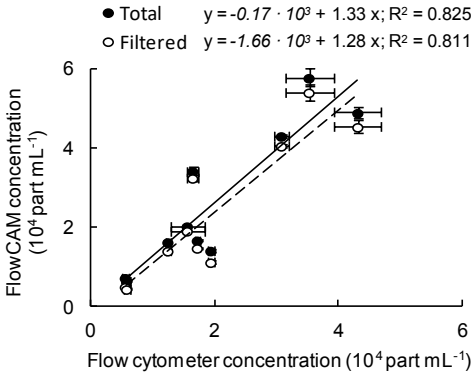


Figure 6. *Linear regression for mean values of triplicate concentration measurements obtained by the FlowCAM and their respective measurements using flow cytometer. Error bars standard deviation between triplicate measurements. Numbers in italics represent non-significant parameters.*



The inactivating efficacy of UV treatment was evaluated by modeling the growth curves according to a logistic model. Both total and filtered particle concentrations showed similar trends following logistic growth in control and shrinkage in the UV treated sample (Fig. 7). Experimental data of control were fitted to a logistic model to determine the growth rate (r) and carrying capacity (N_{max}) (Table 3). In UV treated samples, the organisms kept their viability growth, in general, following the same "r" and "N_{max}" as control organisms with a reduction in the initial concentration of organisms (Romero-Martínez et al., 2016). The concentration of viable organisms after the UV irradiation ($N_{0\,UV}$) can be estimated by modeling the growth curve of treated samples. According to the logistic model parameters, values of "$N_{0\,UV}$" greater than 52 cells mL^{-1} produces regrowth that is detectable on the sixth day of incubation; therefore, since regrowth was not observed, the actual value of "$N_{0\,UV}$" is

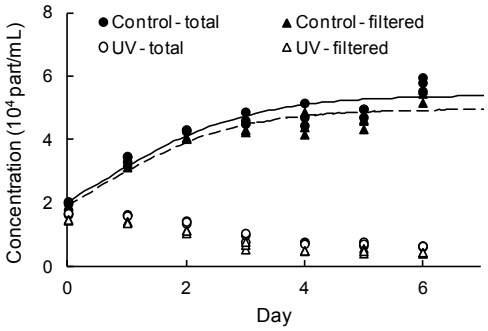


Figure 7. *Evolution of total and filtered concentration values after the UV irradiation of Prorocentrum minimum. Lines represent the logistic model fitted to total (solid) and filtered (dashed) experimental values of control samples.*

lower than 52 cells mL^{-1}. The inactivating efficacy of UV treatment ($N_{0\ UV}$ / $N_{0\ control}$) was greater than the 99.75%. These values exceed the disinfection achieved for *P. minimum* by full-scale UV units between the 84% and the 87% (Sassi et al., 2005; Sutherland et al., 2001; Tsolaki and Diamadopoulos, 2010). The same modeling procedure applied to filtered concentration data which determined an inactivation efficacy greater than the 99.87%. Since the automatic data filtering is a complex procedure that introduces uncertainty, the total concentration measured directly by the FlowCAM supposes a better approach for determining the inactivation efficacy by UV treatment in *P. minimum* monocultures by growth modeling.

Table 3. *Logistic model parameters for Prorocentrum minimum UV experiment: growth rate (r), carrying capacity (N_{max}), initial concentration ($N_{0\ control}$), maximum value of estimated concentration of viable organisms in UV treated sample ($N_{0\ UV}$) and minimum value of inactivating efficacy percentage.*

	r (d^{-1})	N_{max} (cells mL^{-1})	$N_{0\ control}$ (cells mL^{-1})	$N_{0\ UV}$ (cells mL^{-1})	Efficacy (%)
Total	0.827	53926	20332	52	99.75
Filtered	0.879	49509	19162	25	99.87

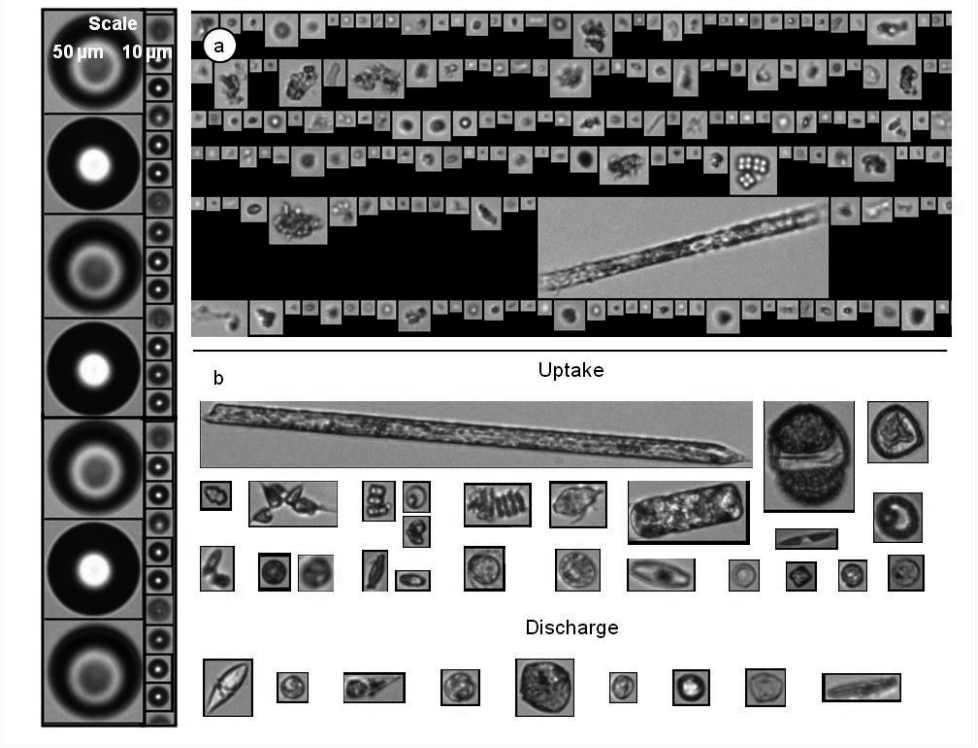


Figure 8. *Section of images collage obtained in seawater analyses (a) and images corresponding to individual, aggregated or chained organisms after a non-exhaustive observation (b). A visual size scale is available from 10 and 50 µm microbeads analyses.*

3.3. Analysis of natural seawater

3.3.1 Optimization of natural seawater analyses and data management

The images obtained from seawater analyses (Fig. 8a) include organisms (Fig. 8b), debris, contaminations, and other material. Since single organisms appear as part of a wide variety of more complex compositions such as chains, aggregates, or those associated to inert material, the automatic data filtering based on the quantitative graphic properties is not suitable (Álvarez et al., 2012), requiring the exhaustive observation of images by specialist staff to determine the actual concentration of organisms (First and Drake, 2012). The feasibility of the systematic enumeration of organisms is conditioned primarily by the availability of trained staff, the number of images to be observed, and the statistical significance of the retrieved data (Frazier et al., 2013; Miller et al., 2011). The output files that embed the images, quantitative morphologic properties, and concentration data can be transferred to offices with the staff specialized in identification and enumeration of organisms.

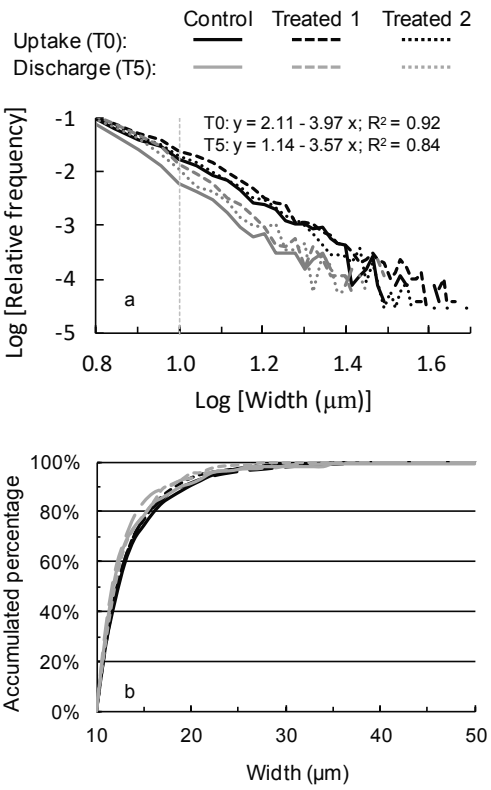


Figure 9. *(a) Double logarithmic representation of size spectra obtained in uptake (T0) and discharge (T5) of three testing cycles of a BWTS. Linear regression parameters are provided for control samples using particles sized between 10 and 50 µm. (b) Accumulated relative frequency for particles between 10 and 50 µm. Both figures correspond to one of the three tests cycles; results are similar for the two other cycles.*

To make the quick data transferring and enumeration of organisms to remote offices feasible, the output files must be optimized to eliminate interferences and reduce their size.

The predominance of small particles, as observed in images and size spectra (Fig. 9a), is a common feature in the analysis of natural water samples (Li and Logan, 1995; Quinones et al., 2003). According to the size measurements of 10 µm microbeads (Fig. 1), particles whose W is below 9 µm can be considered smaller than 10 µm thus not controlled by the BWMC. Therefore, a lower limit value for the "Acquisition Filter" of 9 µm W prevents the capture of particles whose minimum dimension is below 10 µm, leading to the optimization of the output files without loss of information about organisms controlled by the BWMC D-2 standards. Since natural seawater organisms may appear in the form of aggregates or chains, particles over 53 µm may contain one or several organisms actually between 10 and 50 µm thus the use of upper limit for "Acquisition Filter" is not recommended. The number of images below 9 µm supposes an overload of information of particles not controlled by the BWMC D-2 standards which interferes with the observation of images and unnecessarily increases the size of recorded files, complicating data management, storing, and transferring. According to the data obtained, the concentrations of particles over 9 µm that may correspond with organisms controlled by the BWMC and require the observation by an analyst are, on average, 1,365 particles mL^{-1} in the uptake and 177 particles mL^{-1} in the discharge (Table 4). In this sense, avoiding the detection of particles below 9 µm results in

the elimination of approximately 95% of the total number of images, reducing the number to be observed by the analyst as well as the weight of the output files thereby enhancing the manual enumeration and data management.

Table 4. *Concentration measurements of total (using 4 µm ESD as acquisition filter) and 10-50 µm particles (Width > 9 µm) throughout three BWTS testing cycles of uptake (T0) and discharge (T5).*

Cycle	Sample	Total concentration (particles mL^{-1})		> 9 µm concentration (particles mL^{-1})	
		Uptake	Discharge	Uptake	Discharge
1	Control	19800	5730	1128	142
	Treated 1	28229	12860	1595	324
	Treated 2	15693	9114	825	184
2	Control	9411	4786	461	64
	Treated 1	13849	6564	797	174
	Treated 2	12679	6365	672	119
3	Control	50435	5467	2855	79
	Treated 1	34924	13183	1586	243
	Treated 2	47442	15442	2363	269

The abundance of 10 µm particles is approximately three orders of magnitude greater than 50 µm particles (Fig. 9a). The percentile analysis of particles potentially within the 10-50 µm (filtered as W > 9 µm) reflects that approximately 50% of the particles are sized between 9.00 and 11.04 µm, on average, and the 90% of particles measure less than 17.35 µm (Fig. 9b). For values between 17.35 µm and 50 µm, corresponding to the remaining 10% of detected particles, strong discontinuities and gaps are observable in size spectra plots indicating a lack of representativeness of large particles in relation with small particles. This implies a misbalance in the statistical representativeness of individuals along the 10-50 µm range; obtaining representative samples of large organisms requires processing much larger volumes with respect to small organisms. Because of their higher abundance with respect to larger organisms, the nonconformities with the BWMC D-2 standards is, in general, primarily due to small organisms (with exemptions, for example, for things such as a bloom of a large specie) whose assessment requires lower sample volume for obtaining representative results (Carney et al., 2013; Lund et al., 1958). On the other hand, the processing of larger sample

volumes is required to increase the representation of larger particles, although it supposes larger analysis runtime and the excessive detection of more abundant, smaller particles. The splitting of the 10-50 µm range analysis into several sub-ranges may correct the misbalance in the particle representation.

3.3.2. Effects of disinfection treatment

The five days of storage in tanks removed over the 75% of 10-50 µm particles existing at the uptake procedure (Table 4). The size spectra (Fig. 9a) also depicted a relatively low presence of 10-50 µm particles after the five days of storage. The loss of particles larger than 9 µm was, on average, 90.21% in controls and 81.88% in treated samples after five days of storage. This greater loss in control with respect to filtered and UV irradiated samples was consistent throughout the three studied cycles and resulted in being paradoxical. Besides the treatment application, the evolution of natural biological communities over their storage in tanks is conditioned by factors such as sedimentation and vertical migration processes (First et al., 2013). In this sense, the exhaustive observation of images is required to determine the actual concentration of 10-50 µm organisms among the detected particles, as well as elucidating the processes that condition the evolution of organisms in tanks. The noise caused by small (W < 9 µm) particles hampered the exhaustive observation of images, requiring the adequate setting developed in this study: lower "Acquisition Filter" of 9 µm W and splitting the analysis of 10-50 µm range into several size sub-ranges. Further studies will be focused on monitoring the evolution of organisms in tanks by taking and analyzing samples periodically throughout the storage time.

In general, changes in the concentration of organisms are not addressed immediately after the UV treatment, however, the subsequent growth monitoring reveals loss in their capacity to reproduce according to the UV dose applied and their UV-resistance (Cullen and MacIntyre, 2016; Liebich et al., 2012; Romero-Martínez et al., 2016; Sakai et al., 2007; Stehouwer et al., 2015; Tao et al., 2010). Therefore, the evolution of the organisms once discharged from the ballast into the ocean is conditioned by treatment features such as the UV dose applied, the time under light limitation in tanks, and the species composition and their interactions (First et al., 2016; Viitasalo and Sassi, 2005). Although the growth monitoring is so lengthy that it does not allow the assessment of BWTS effects during its normal operation (First and Drake, 2014), it is considered a suitable approach to evaluate the UV treatment effects (First and Drake, 2013). The images and concentration measurements retrieved by the FlowCAM enable studying the evolution followed by the different species after the irradiation with UV light. Similarly, the FlowCAM enables continuous, precise, and

a relatively quick determination of the abundance and diversity of organisms in ballast water as well as the detection of species especially involved in ecosystem invasions, thus improving the management of the ballast water.

3.4. Validation and feasibility of FlowCAM for ballast water management

Beside the capacity for accurately determining the size, concentration, and viability of organisms, the feasibility of the FlowCAM as a ballast water monitor is subjected to a series of technical and logistic requirements (Drake et al., 2014; Erickson et al., 2012). This study assesses the use of a FlowCAM as a supporting tool in the development and approval of disinfection technologies and BWTSs with cultured or natural organisms and its applicability as an on board BWMC compliance tool or as an early detector of threats related with the discharge of ballast water.

3.4.1. Physical characteristics and maintenance

Some issues related with the physical characteristics of the device were encountered throughout the proof-of-concept which may compromise the continuity of systematic studies based on the FlowCAM analyses. Most of these inconveniences can be corrected with the establishment of procedures and maintenance protocols and the re-design of certain parts of the device.

Problems may occur during the analysis runtime caused by background decalibration, clogging, and bubbles formation. Although a re-processing of the output file is possible, the deletion and repetition of the analysis was considered a better approach in systematic studies. Background decalibration is occasionally caused by vibrations and taps on the device which induces the background shapes to be recognized as particles in the sample that are then continuously captured. Although it is represented as an infrequent failure, the FlowCAM location should be as isolated and stable as possible. The routine analyses of cultures or natural seawater soiled the pipes and the flow cell, causing bubbles formation and clogging whereby continuous detection resulted in a failed analysis. The most effective cleaning protocol for preventing the bubbles formation was a rinse with distilled water ("No Save" mode simulates an analysis without recording of data, which is useful for determining when the particles from the previous samples have been removed), followed by a rinse with ethanol, isopropanol and, finally, ethanol again. This protocol was applied systematically at the end of a daily series of analyses prior to the device storage. Clogging was detected in real-time by the heterogeneities in the particles per time interval plot and the diagram of X/Y location of particles in the field of view. In the case of clogging, a backwash is recommended followed by a cleaning protocol if necessary. If the cleaning protocol fails in soil removal, an

ultrasound bath is recommended which requires removing the flow cell (XX). Since these failures are fortuitous facts during analyses, it is recommended to split one analysis of a certain sample volume into a series of shorter analyses to minimize the loss of data in case of failure.

The flow cell replacement is required when the hydraulic circuit becomes hardly soiled, or a change of magnification is necessary. This procedure was certainly the most bothersome and often led to breaking the flow cell, increasing costs, and leading to potentially running out of replacement parts thus compromising the systematic study. The systematic use of the FlowCAM in the laboratory and especially in field studies requires the development of a new compact and user-friendly model of a flow cell, enhancing the continuity of the analyses and the possibility of working quickly with different magnifications.

3.4.2. Ease of use and expertise required

The technical and logistic features of the FlowCAM enable the separation in space and time of the analytical procedure, the interpretation of the output files, and the decision making by third parties such as the BWTSs developers, ship operators and Environmental or Maritime Authorities who are implied in the control of alien species (Monterroso et al., 2011). Similarly, this device may enhance the *in situ* data collection by vessels and ports thus enhancing their environmental control (Darbra et al., 2009). Each stage in this sequential process requires different expertise which optimizes the human resources involved:

1. The acquisition of data in a laboratory or the field was focused on processing fresh samples without the necessity of chemical reagents or fixatives or a procedure of dilution and concentration (Carney et al., 2013; Jakobsen and Carstensen, 2011; Zarauz and Irigoien, 2008). In this sense, the manipulation of samples, subjective interpretations, and responsibility by a device operator becomes greatly reduced.
2. Secondly, the original output files are received by experts trained in the identification and classification of particles with the aim of enumerating the organisms among the particles in image files as well as detecting possible threats such as the presence of potential invaders or toxic species (Briski et al., 2012). Images, morphologic, and concentration data are embedded in output files; therefore, this stage may be carried out with standard computers without special hardware or software requirements. The data transferring allows the centralization

of expertise in offices placed anywhere thus avoiding their displacement to the field.

3. The final stage corresponds with the decision-making by third parties based on the interpretation carried out by experts. Decisions may involve the acquisition of new data under similar or different configurations, the necessity of extra treatment applications, or the acceptance of ballast as a safe discharge according to the BWMC standards.

This networking requires quasi real-time communication and data transferring between the shipboard team and the interpretation offices in which feasibility is conditioned primarily by the size of files and the quality of the data transmission channels. Further studies will be focused on the optimization of hardware and software configurations for obtaining files that are more representative and manageable thereby enhancing the procedures for BWTSs development, ballast water monitoring for BWMC compliance, and prevention of alien species introductions.

4. Conclusions

Through the experimental plan carried out with several types of artificial and biologic particles, it has been showed that the FlowCAM possesses the capacity to detect and measure the size and concentration of particles within the 10 – 50 µm range, according to the BMWC D-2 standards. The effects of BWTSs on dinoflagellate *P. minimum* monoculture could be assessed by their appearance in images and the total concentration measurements whereas the changes in quantitative morphological information were not evident enough. Heterogeneous low concentrated samples such as seawater require comprehensive examination by experts for identifying and enumerating the organisms among the debris and contamination images. Under the correct device configuration, this procedure can be carried out remotely in centralized offices thus avoiding the displacement of expert staff. In contrast, some parts of the device require substantial re-design to ensure the systematic use in middle and long-term. The correction of physical failures and the establishment of protocols for data retrieving, transferring, and interpretation can enhance the quickness and effectiveness of ballast water management.

Acknowledgements

This work has been co-funded by the North Sea Region Programme (ERDF) and the Spanish Interministerial Science and Technology Committee (CTM2014-52116-R), and FPI Grant (BES-2010-029938) by the Spanish Government. The authors declare that they have no conflict of interest.

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Chapter 5

Development and testing of a rapid, sensitive ATP assay to detect living organisms in ballast water

Development and testing of a rapid, sensitive ATP assay to detect living organisms in ballast water

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Published in: Journal of Applied Phycology. DOI 10.1007/s10811-014-0518-9

Funding:
This work has been co-funded by the North Sea Region Program under the European Regional Development Fund of the European Union.

Abstract

To reduce the spread of aquatic invasive species, the discharge of ballast water by ships will soon be compulsorily regulated by the International Maritime Organization (IMO) and the United States Coast Guard (USCG). Compliance with their regulations will have to be achieved by onboard ballast water management systems. To monitor the treatment system performance, rapid and easy compliance techniques are required. This paper reports on the suitability of Adenosine Triphosphate (ATP) to quantify living 10 to 50 µm organisms at <10 cells mL^{-1}, which is the upper limit of the IMO D-2 and USCG regulations. Initial tests revealed that commercially available ATP assays lacked sufficient sensitivity to monitor ATP in treated ballast water. A rapid and easy concentration method was developed to increase sensitivity and remove interfering salts, non-target organisms (*Micromonas pusilla*) and dissolved ATP. Laboratory experiments revealed that, after concentration, salinity was reduced 97% and concentration efficiencies reached 85%. The ATP assay was tested in a UV-based full-scale ballast water management system, treating seawater and fresh water. ATP levels were compared with two alternative compliance tools: FDA and Photosystem II efficiency. Results showed a 10-fold decrease in ATP levels after treatment compared to a 5-fold decrease in alternative compliance techniques. Following refinements, the ATP assay's detection limit reached 2.5 ± 0.5 cells mL^{-1}, using a *Thalassiosira rotula* monoculture. Initial estimates of the pass and fail level were 50 and 6,000 relative luminescence units, respectively. Further validation is recommended. The ATP assay is a promising tool for ballast water compliance testing.

Keywords

CME; ATP; ballast water; IMO D-2; PSII efficiency; FDA

1. Introduction

Ballast water plays an essential function in a ship's stability, trim, draft and structural integrity. Thus, ballast water is critical to enable safe shipping. However, through ballast water transport, huge quantities of viable (able to reproduce) organisms are transported around the world and discharged into to foreign ecosystems (Drake and Lodge 2007). These newly introduced species may become invasive and outcompete local species for habitat and food availability. The ongoing spread of aquatic invasive species can lead to major damage to biodiversity and economic losses (Molnar, Gamboa et al. 2008). To prevent the dispersal of aquatic invasive species through ballast water, the International Maritime Organization (IMO) and United States Coast Guard (USCG) have enacted legislation which limits the number of viable organisms that are allowed to be discharged through ballast water (Anonymous 2004, Anonymous 2012). Both IMO's D-2 regulation and the USCG regulation limit, among others, the discharge of viable 10 to 50 µm organisms to <10 mL^{-1} and the discharge of viable >50 µm organisms to <10 m^{-3}.

To comply with the upcoming discharge regulations, most ships will have to be fitted with ballast water management systems (BWMSs), to disinfect ballast water before discharge. After acquisition and implementation of a BWMS, ship owners may want to monitor the biological efficacy of their BWMS over time and in various water types and qualities. In addition, Port State Control (PSC) officers are obliged to monitor the compliance of ships to the ballast water convention. In accordance with the recommendations outlined in the IMO ballast water sampling guidelines (G2), a quick screening method to identify ships that are potentially in violation of the D-2 standard is needed (Anonymous 2008). Sampling and monitoring obligations require that ballast water discharge should be analyzed for the presence of viable organisms. Due to their low abundance, accurate zooplankton (>50 µm) estimates require cubic meters of water to be sampled and analyzed microscopically. For the smaller phytoplankton and micro-zooplankton organisms (10 to 50 µm), analysis often requires expensive and complicated equipment such as flow cytometry. All of these analyses require trained personnel to produce reliable results. In practice therefore, detailed quantitative biological analysis of ballast water is time-consuming, tedious and expensive.

Commonly, ship owners and PSC will not have the capabilities to carry out specialistic quantitative biological analyses. Although they are authorized to sample ballast water, PSC inspectors will mainly focus on checking the presence of a treatment system, the availability of qualified personnel to run the system and whether the system has reported any errors in its mechanical or chemical operation specifications (personal communication K.

Hak, inspector of the Ministry of Infrastructure and the Environment, The Netherlands). To improve the capabilities of ship owners and PSC to monitor the biological efficacy of BWMS, tools are needed that can estimate the concentrations of viable organisms. In addition, these so-called Compliance, Monitoring and Enforcement (CME) techniques will have to be reliable, yet quick and simple enough to be used by minimally trained crew on board ships. In recent years several CME techniques have been developed to monitor viable organisms in discharged ballast water (Welschmeyer and Maurer 2011, Delacroix and Liltved 2013, Anonymous 2014). Usually, sexually reproducing large zooplankton are excluded from CME techniques, since sampling cubic meters of seawater would be too time-consuming and logistically challenging in a ship's engine room. The development of the ATP assay presented here, solely focused on the 10-50 µm size fraction of the IMO and USCG discharge standards.

Whenever a chemical reaction inside a living organism is carried out that requires energy, this energy is provided by ATP (Lipmann 1939, Lipmann 1939, Lipmann 1940, Lipmann 1941). For decades, the presence of ATP has been considered a good indicator for the presence of metabolically active organisms (Karl 1993). Although metabolic activity does not guarantee viability it is considered to be a good viability indicator for unicellular organisms since they usually reproduce asexually. ATP quantification is usually based on bioluminescence derived from firefly (*Photinus pyralis*) luciferin/luciferase complexes. Several ATP assays are globally available such as the ENLITEN® ATP assay (Promega, Wisconsin, USA), Molecular Probes® ATP Determination Kit (Invitrogen, California, USA) and the Clean-Trace™ system (3M, Minnesota, USA). These commercial ATP assays require less than $5,000 to acquire and cost no more than $10 per analysis. In seawater however, the large amount of metal ions interfere with the luciferin/luciferase reaction which inhibits the light production (Sudhaharan and Reddy 2000). To solve this, elaborate pre-treatment steps were developed involving ATP extraction using boiling Tromethamine (Tris), H_2SO_4 or activated carbon (Hodson, Holm-Hansen et al. 1976), which are still in use to date (Maurer 2013). Using these extractions techniques, much research has been devoted to correlate ATP to marine microbial biomass (Novitsky 1987), phytoplankton biomass (Hunter and Laws 1981) and zooplankton biomass (Maranda and Lacroix 1983). Though proven effective, these extraction techniques are too complicated and time consuming to be used by PSC officers and ship's personnel.

In the present study, Clean Trace™ ATP assay (3M, Minnesota, USA) was applied. To remove metal ions, concentrate and extract ATP from relevant organisms, a simple and

straightforward concentration method was developed. Ships sail in polar as well as tropical regions and both fresh water and seawater are used as ballast. Therefore, the ATP assay was tested at various ambient temperatures and salinities. Chlorine-disinfection is commonly used in BWMSs, therefore the effect of chlorine on the ATP assay was also examined.

Early on in the development of the ATP-based CME technique, the opportunity arose to test the assay on a full-scale UV-based BWMS. The performance of the ATP assay was compared with three additional CME techniques. Firstly, esterase activity using bulk fluorescein-diacetate (FDA) fluorescence was determined using a proprietary system provided by Hach (Colorado, USA). Secondly, photosystem II (PSII) efficiency was estimated using [3-(3,4-dichlorophenyl)-1, 1-dimethylurea] (DCMU), also provided by Hach. Thirdly, PSII efficiency was determined using Pulse Amplitude Modulation (PAM) fluorometry (Walz 2000).

Esterase enzymes are exclusively produced by living organisms and thus considered a proxy for the presence of living organisms (Rotman and Papermaster 1966). Before the development of PAM fluorometry, the PSII efficiency of active chlorophyll was estimated using the photosynthetic inhibitor DCMU (Cullen and Renger 1979). Results of the tests using a full-scale BWMS are presented early on, to reflect the chronology of the development process. Following these tests, modifications to the concentration method were made to increase the usability, precision and sensitivity of the ATP assay. The practical use of the concentration method in combination with ATP analysis in ballast water compliance testing will be discussed.

2. Methods

Firstly, all analytical methods applied in the research are explained. In order to comprehend the development process, a separate section was devoted to explaining all concentration methods applied during the research (see also Table 1). Finally, the experiments carried out are explained in detail (see also Table 2).

Table 1 *Overview of all experiments conducted. The number of independent trials is denoted as 'n'. The null-hypothesis describes the result if no significant effect was found*

Experiment	n	Null-hypothesis (H_0)
The influence of hypochlorite on ATP detection.	1	Hypochlorite up to 10 mg L^{-1} does not influence the light output of the 3M Clean Trace™ ATP assay[a] using the BDK[b].
The relationship between the ATP concentration and the resulting RLU signal.	1	There is no linear correlation between the ATP concentration and light produced during ATP analysis using the BDK.
The influence of salinity on ATP detection at 4°C, 15°C and 26°C.	1	1. Salts have no effect on the light production of the ATP assay using the BDK. 2. Temperatures of 4°C, 15°C and 26°C have no relative effect on the light production of the ATP assay using the BDK.
UV-C treatment of *T. rotula.*	1	1. A dose of 139 mJ cm^{-2} UV-C (254 nm) has no effect on the viability of *T. rotula* cells. 2. The effect of UV-C treatment on *T. rotula* cannot be effectively monitored using: a. Flow cytometry b. Variable fluorescence c. FDA analysis d. ATP analysis 3. Data resulting from flow cytometry, variable fluorescence, FDA analysis and ATP analysis are not correlated.
Test compliance kits during IMO G8 land-based testing.	6[c]/10[d]	Organism concentrations derived from flow cytometry and microscopy (the official land-based test data) cannot be correlated with the indicative compliance tools: a. DCMU b. FDA c. ATP
Detection limit of ATP analysis using CM3.	1	1. ATP analysis using the ATP assay with either the ATP swabs or the BDK following CM3 is not linearly correlated with the concentration of *T. rotula.* 2. ATP analysis using either the ATP swabs or the BDK following CM3 is not able to detect <10 *T. rotula* cells mL^{-1}.
Improving the concentration efficiency and salinity reduction of the CM.	1	1. Flushing 5 mL milli-Q™ back and forth five times instead of one flush does not improve the collection of particles from the concentration filter. 2. Replacing the salt-contaminated 50 mL syringe with a sterile 5 mL syringe when back flushing, does not improve the removal of salts in the concentrate.
Comparing the precision of CM3 and CM5.	1	Changes to the back flush procedure do not lead to less variation among replicate measurements of natural seawater.
Detection limit of ATP analysis using CM5.	1	1. ATP analysis using the ATP swabs following CM5 is not linearly correlated with the concentration of *T. rotula.* 2. The ATP assay using the ATP swabs following CM5 is not able to detect <10 *T. rotula* cells mL^{-1}.

[a]All ATP analyses were performed using the 3M Clean Trace™ ATP assay. [b]BDK: Biomass Detection Kit. [c]Control tanks. [d]Treated tanks

Table 2 *Overview of the development process of the concentration method, compared with the FDA- and DCMU-based methods.*

	Concentration Method (CM)					Hach	
Feature	CM1	CM2	CM3	CM4	CM5	FDA	DCMU
Sample volume (mL)	200	100	100	100	50	200	3
Extractant volume (mL)	2	5	5	5	5	2	
Concentration factor	100x	20x	20x	20x	10x	100x	
Salinity reduction factor	nd[b]	nd	17x	nd	33x	nd	
Concentration efficiency	nd	nd	63%	85%	85%[c]	nd	
Detection limit (cells mL^{-1}; average ± CI)	nd	nd	>50	nd	2.5 ± 0.5	nd	nd
Time required (minutes)	~5	~3	~3	~3	~3	~40	~5
Usability at dock	-	-	+	+	+	-	++
10 µm pore size / 25 mm Ø nylon screen filter	X	X	X	X	X	X	
Beaker-flask-cuvette filtration manifold	X					X	
Syringe filtration system		X	X	X	X		
Reusable stainless steel syringe filter capsule		X					
Disposable polypropylene filter capsule			X	X	X		
Pipettes and tweezers needed	X					X	
Five times back flush			X	X	X		

[a]not applicable. [b]not determined. [c]derived from CM4

2.1. Analytical methods

The 3M Clean-Trace™ NG luminometer was used in combination with either the 3M Clean-Trace™ Biomass Detection Kit (BDK), or the 3M Clean-Trace™ Water Total ATP swabs (ATP swabs). The BDK was considered more appropriate in a laboratory setting and resulted in more accurate results, however due to the need for pipetting small volumes it was not

deemed suitable for use by untrained crewmembers. The ATP swabs required immersing a dip-stick in the sample, which was considered more user-friendly. The methods were used as according to the manufacturer's prescription:

BDK: Firstly, 100 µL sample was pipetted into a cuvette. Secondly, 100 µL of proprietary cell lysing extractant was added and incubated for one minute. Finally, 100 µL of 3M luciferin/luciferase reagent was added to the cuvette and mixed. The resulting luminescence was immediately determined using a luminometer and recorded as Relative Luminescence Units (RLU).

ATP swabs: The swabs arrived pre-moistened with extractant on delivery. A swap was dipped into a water sample and inserted into a tube containing the luciferin/luciferase reagents. The sample volume was 157 ± 3 µL (average ± 95% CI). The sample was mixed with the reagents by pressing the dip-stick through two membranes and the RLU was immediately measured using the 3M luminometer .

FDA analysis: A 200 mL sample was filtered over a nylon screen filter (10 µm pore size, 25 mm diameter). The filter was transferred to a 4 ml polyethylene cuvette and immersed in 2 mL proprietary buffer. One drop of FDA was added to the cuvette and incubated for 30 minutes. During incubation, FDA was cleaved by intracellular esterase enzymes thereby producing green-fluorescent fluorescein. After a vigorous shake, the filter was removed from the cuvette. The fluorescence in the cuvette was measured (495/517 nm, excitation/emission) using a proprietary Hach fluorometer (Welschmeyer and Maurer 2011).

The terminology for PSII efficiency analyses was adopted from Kromkamp and Forster (Kromkamp and Forster 2003). The Hach DCMU-based method was applied as follows. Initially, the fluorescence (F_0) of a 2-minute dark-adapted sample was measured, with a proprietary Hach fluorometer using a single turnover (ST) light pulse. Subsequently, the chlorophyll was inactivated by adding DCMU and fluorescence was measured again after 2 minutes dark incubation (F_{DCMU}). From the difference in fluorescence the PSII efficiency was calculated: $(F_{DCMU}–F_0)/F_{DCMU} = F_v/F_{DCMU}$.

PAM fluorometry (Water-PAM, Walz, Bavaria, Germany), using a multiple turnover (MT) light pulse, was used to measure the PSII efficiency of active chlorophyll and expressed as: $(F_0\text{-}F_m)/F_m = F_v/F_m$. Samples were dark acclimatized for 30 minutes.

To enumerate phytoplankton cells in laboratory trials, a BD Accuri™ C6 flow cytometer (Becton Dickinson, New Jersey, USA) was used. Particles were detected using a 488 nm laser. Phytoplankton cells were discriminated from other particles based on red auto fluorescence of the chlorophyll detected by the FL3 channel (670 nm long pass filter).

For a live/dead determination of phytoplankton 0.5 µM SYTOX® Green nucleic acid stain (Invitrogen, California, USA) was used. This stain enters permeable cells where it causes green fluorescence when bound to DNA. The method is based on the assumption that permeable, stained cells are dead and non-stained cells are alive. Stained cells were discriminated from other cells using the FL1 channel (530 ± 30 nm band pass filter).

2.2. Developing the concentration method

Concentration method 1 (CM1), was based on a traditional flask-filter-beaker assembly. A sample of 200 mL was filtered (nylon screen; 10 µm pore size, 25 mm diameter) (Millipore, Massachusetts, USA) using a 1 L flask with filter beaker on top. After filtration the filter was placed in a 4 mL polyethylene cuvette with 2 mL of sterile milli-Q™ (Millipore), resulting in a 100 times concentration of >10 µm particles. After a vigorous shake the RLU was determined using ATP swaps.

To simplify the filtration procedure, concentration method 2 (CM2) was developed. A 100 mL sample was taken up using a 100 mL syringe (Plastipak™, Becton Dickinson). The sample was gently filtered over a nylon screen filter (10 µm pore size, 25 mm diameter, Millipore), contained in a stainless-steel reusable filter holder (Millipore). Particles retained in the filter were flushed out with a 5 mL syringe (Terumo, Tokyo, Japan) containing 5 mL milli-Q™ into a 15 mL polypropylene tube (Greiner Bio-One, North Carolina, USA). The concentrate was analyzed for the RLU either with ATP swabs or the BDK.

To further simplify the procedure for onboard use, concentration method 3 (CM3) was developed. The stainless-steel filter capsule of CM2 was replaced with a custom-made polypropylene disposable filter capsule, containing a non-replaceable nylon screen filter (10 µm pore size, 25 mm diameter (Sterlitech, Washington, USA).

It was suspected that the concentrate was not extracted sufficiently by the single rinse of 5 mL milli-Q™. To improve the extraction efficiency, concentration method 4 (CM4) was developed. Instead of directly removing the 100 mL syringe after filtration, the 5 mL milli-Q™ was flushed back and forth into the 100 mL syringe five times, to release particles from the filter more effectively.

It was noted that in turbid water, 100 mL sample could easily clog the filter. Also, residual salinity could be substantial in concentrated samples. To avoid clogging and increase the salinity removal, concentration method 5 (CM5) was developed. The sample volume was reduced to 50 mL using 50 mL syringe (Terumo). After filtration, a 5 mL syringe containing 5 mL milli-Q™ was connected to the outlet side of the filter. The 50 mL filter, contaminated with salts, was removed and on the inlet fitting of the filter a sterile 5 mL syringe (Terumo)

was attached. The concentrate was flushed back and forth five times so that the concentrate ended up in the syringe connected to the inlet side of the filter. After removal of the piston the concentrate was sampled directly from the syringe using the ATP swabs.

Because various concentration factors among experiments were used it was deemed inappropriate to convert RLU values to absolute ATP concentrations. In addition, due to inherent uncertainties in concentration efficiencies, presenting absolute ATP levels would give a false impression of comparability among different experiments. To evaluate ATP analysis, it was considered most important that <10 cells mL^{-1} were above the detection limit of the device, and that substantial differences were observed between disinfected water (D-2 compliant) and control water. For both objectives, reporting results in RLU was considered sufficient.

2.3. Experimental design

2.3.1. Linearity and abiotic influences on the ATP assay

Many BWMS use electro-chlorination to produce hypochlorite (ClO^-) as an active substance, to achieve disinfection of ballast water (Anonymous 2013). Therefore, the effect of hypochlorite on a standard solution of ATP was tested. Test solutions were made by diluting a 10-15% sodium hypochlorite solution (Sigma-Aldrich, Missouri, USA) in milli-Q™. Concentrations were determined using DPD Chlorine Total powder pillows for analysis in a Hach DR/890 Colorimeter (Anonymous 2009). As test concentrations 0, 0.25, 5 and 10 mg L^{-1} Cl_2 were used. The ATP concentration in all four test solutions was 0.6 ng mL^{-1} by adding an ATP standard (contained in bovine serum albumin, 3M). Test solutions were analyzed in triplicate using the BDK.

To verify the linearity between ATP concentration and RLU signal, a test solution was made using milli-Q™ water and an ATP standard. (contained in bovine serum albumin, 3M). A calibration series was prepared by dissolving the ATP standard with milli-Q™ water to reach a concentration of 0, 0.12, 0.6, 1.5, 3, 7.5, 15, 30, 45 and 60 ng mL^{-1} ATP. The RLU signals were determined in triplicate for each of the dilutions. To investigate the effect of temperature, all equipment and test solutions were acclimated for one hour in climate rooms at 4°C, 15°C and 26°C prior to analysis.

Salinity test solutions (30 mL) were prepared in 60 mL glass bottles with aluminum caps using mixtures of milli-Q™ and seawater (0.2 µm filtered and autoclaved) to reach the desired salinities of 0, 4.5, 9, 18, 27, 31.5 and 36 g kg^{-1}. Temperatures were set at 4°C, 15°C or 26°C by acclimating all test solutions and equipment into climate chambers at least one hour before starting the analyses. The test solutions were spiked with 6 ng mL^{-1} of ATP

analyzed in triplicate using the BDK.

To test the effect of 0-2 g kg^{-1} salinity on ATP analysis, sterile seawater (0.2 µm filtered and autoclaved) was added to milli-Q™, to reach salinities of 0, 0.5, 1 and 2 g kg^{-1}. Two salinity dilution series were prepared, containing 0.3 ng mL^{-1} and 3 ng mL^{-1} ATP respectively. The series were analyzed in triplicate using the BDK.

2.3.2. UV-C treatment of Thalassiosira rotula

The marine diatom *Thalassiosira rotula* (CCMP 1018) was obtained from the National Center for Marine Algae and Microbiota (NCMA). To investigate the effect of UV-C (254nm) radiation on the survival of *T. rotula* and on ATP levels, a laboratory experiment was carried out. *T. rotula* is a chain forming species of approximately 15 µm in minimum dimension. *T. rotula* was cultured in 0.2 µm filtered and autoclaved seawater (salinity: 28 g kg^{-1}) with excess nutrients at 15°C under a 16:8 light:dark regime (50 µmol photons m^{-2} s^{-1}). When the culture was in the exponential growth phase, it was diluted with 0.2 µm filtered and autoclaved seawater to a final density of 1,000 cells mL^{-1} (source culture: 94,970 cells mL^{-1}). The dilution was pumped (Aqua-Flow 50 pump, Aquadistri, Klundert, The Netherlands) at 20 mL s^{-1} through a low-pressure UV-C reactor (Van Gerven, Son, The Netherlands). The culture was treated with a calculated dose of 139 mJ cm^{-2} of monochromatic UV-C light (254 nm). As a control the culture was pumped through the UV-C reactor with the lamps turned off to compensate for the effects of the pump. Subsequently the cultures were incubated in the dark at 15°C for five days. On day 5, a second UV-C treatment was given to one part of the treated culture, simulating the usual UV treatment at ballast water discharge. The other half was pumped through the UV-C reactor with the lamps off serving as a secondary control. After five days the cultures, including the original control, were placed into a 15°C climate room under a 16:8 hour light:dark cycle (50 µmol photons m^{-2} s^{-1}). All cultures were sampled on day 0, day 5 and day 12. The cultures with the second UV treatment and second pump were also sampled on day 6. Samples were taken in triplicate for phytoplankton abundance, PSII efficiency (Walz PAM), FDA and ATP using CM2 and the BDK.

2.3.3. Test CME techniques during IMO G8 land-based verification testing

In the spring of 2012 land-based ballast water tests were performed using natural seawater and fresh water according to the IMO G8 guidelines (Anonymous 2005, Anonymous 2008). At uptake, the 200 m^3 h^{-1} treatment system utilized 40 µm filtration and polychromatic UV radiation of 200-400 nm using two medium pressure UV lamps. After 5 days the water was discharged, during which a second UV dose was delivered.

Many biotic and abiotic characteristics of the water were monitored during uptake and

discharge of the water (Peperzak 2013). ATP, FDA and DCMU analyses were carried out in triplicate using the same samples that were used for 10 to 50 µm organism abundance and PAM fluorometry analyses. ATP was analyzed using CM1 and ATP swabs. In total, 2 seawater control tanks, 4 freshwater control tanks, 3 seawater UV-treated tanks and 7 freshwater UV-treated tanks were included in the comparison.

2.3.4. Detection limit, concentration efficiency and salinity reduction of the concentration method

To investigate the lower limit of CM3 *T. rotula* was cultured at 15°C under a16:8 light:dark regime (50 µmol photons m^{-2} s^{-1}) in f/2 medium with silicate. When the culture was in the exponential growth phase a dilution series was made using sterile seawater as diluent. Concentrations of 10, 20, 50 and 100 cells mL^{-1} of the culture were made and verified using flow cytometry. The cell dilutions were concentrated in triplicate using CM3 and analyzed for ATP content using the BDK and the ATP swabs.

To increase the flushing efficiency of the filter, CM4 was developed. Fresh water from lake NIOZ, adjacent to the institute, was collected and pre-filtered over a 50 µm screen filter to remove large particles. A fractionation was made using subsequent filtration steps of 0.2 µm and 10 µm to determine the ATP content of the organisms in the 10-50 µm fraction. A freshwater sample of 3 L was placed in a polypropylene beaker and stirred using a magnetic stirrer at 160 rotations per minute (rpm). ATP measurements were made in 7-fold using either CM3 or CM4 and ATP swabs. The RLU level corresponding with 100% concentration efficiency was determined by multiplying the RLU in the 10-50 µm size fraction 20 times, since concentrating 100 mL of sample into 5 mL of milli-Q™ should ideally result in a 20-fold concentration.

To improve the salinity reduction factor, CM5 was developed. Natural seawater (salinity: 27,4 g kg^{-1}) was used for a salinity reduction comparison between CM4 and CM5 in 10-fold.

To test the precision of CM3 and CM5, seawater (salinity: 27 g kg^{-1}) from the Marsdiep inlet was collected at high tide, transferred to a 3 L polyethylene beaker and stirred using a magnetic stirrer at 160 rpm. ATP content was concentrated in 12-fold using CM3 or CM5 and analyzed with ATP swabs.

To investigate the lower limit of CM5 and possible interference of <10 µm cells with the concentration method, *T. rotula* and the prasinophyte *Micromonas pusilla* (CCMP 1545, NCMA) with a 2 µm diameter were cultured at 15°C under a 16:8 hour light:dark regime (50 µmol photons m^{-2} s^{-1}) in f/2 medium with silicate. When the cultures reached the exponential

growth phase, a dilution series was made using 0.2 µm filtered sterile seawater as diluent. A 1 L stock solution of ~160 cells mL^{-1} was quantified in 5-fold using flow cytometry. Subsequently, six consecutive *T. rotula* dilutions of 500 mL with sterile seawater were made using a glass cylinder (500 mL ± 0.5%, DURAN, Germany), resulting in solutions of 80, 40, 20, 10, 5 and 2.5 cells mL^{-1}. In addition, three *T. rotula/M. pusilla* mixtures were made containing 20/20,000; 10/10,000 and 5/5,000 cells mL^{-1} respectively. The respective CI's of cell concentrations were calculated using the confidence interval (CI) of the initial analysis of the ~160 cells mL dilution. For each dilution step 1% error was added since the glass cylinder was used twice per dilution. Cell dilutions/mixtures of 40 *T. rotula* cells mL^{-1} or lower, were concentrated in 5-fold using CM5 and analyzed for ATP content using ATP swabs. Following Box-Plot analysis, single outliers, exceeding 1.5x the interquartile range of the first or third quartile, were excluded from further analysis.

2.4. Statistical analysis

For all statistical test the null hypothesis was that there was no significant difference between treatment and control. As confidence level for statistical tests and CI's 95% was chosen (α = 0.05). When samples were analyzed in duplicate or more CI was calculated based on a Student's t-distribution using the MS Excel 2010 function CONFIDENCE.T. The Student's t-distribution was deemed more appropriate for small sample sizes than a normal distribution. Least-squares linear regression models, Analyses of Variance (ANOVA) and Box-Plot analyses were calculated in SYSTAT 13 (SYSTAT Software Inc. California, USA).

3. Results

3.1. Linearity and abiotic influences on the ATP assay

A regression analysis was made where the RLU signal was plotted against the chlorine concentration (data not shown). The slope of the model was not significantly different from zero (ANOVA: $P > 0.05$), indicating that chlorine levels of ≤10 mg L^{-1} did not significantly affect ATP measurements.

The least squares regression models of RLU as function of ATP concentration were: $y = 1{,}081x + 211$ (4°C); $y = 2{,}080x + 347$ (15°C) and $y = 2{,}104x + 150$ (26°C) (Figure 1a). The intercepts were not significantly different from zero which means that no blank subtraction was needed. However, at 4°C the RLU signal decreased 50% compared to the measurements at 15°C and 26°C.

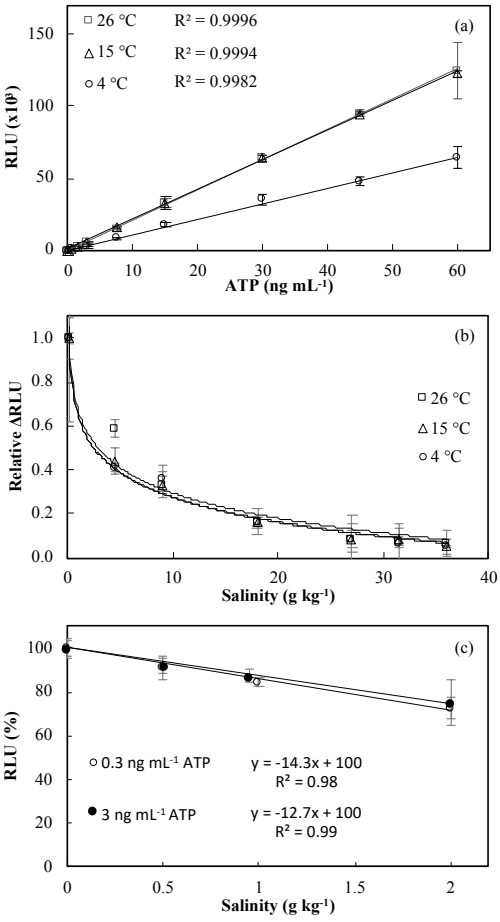

Fig. 1 a *ATP standard dilutions analyzed in triplicate with the biomass detection kit at 4, 15, and 26 °C.* ***b*** *ATP standard (6 ng mL^{-1}) analyzed in triplicate with the biomass detection kit at 4, 15, and 26 °C.* ***c*** *ATP standard (6 ng mL^{-1}) analyzed with the biomass detection kit. Error bars depict the 95 % confidence interval*

Increasing salinity caused the RLU signal to decline logarithmically (Figure 1b). At a salinity of 5 g kg^{-1} already 50% of the original RLU signal was lost. At the average salinity of seawater (35 g kg^{-1}) more than 90% of the original RLU signal was lost. The relative RLU decrease was similar for all three temperatures tested.

When the various types of concentration methods were applied, a residual level of salinity remained. The salinity usually ranged between 0.5-1.5 g kg^{-1} which had a significant effect on the resulting RLU signal. In Figure 1c the relative effect of the decrease in RLU signal resulting from a salinity of 0-2 g kg^{-1} is depicted.

When the measurements of 3 ng mL^{-1} ATP were divided by the measurements observed at 0.3 ng mL^{-1} a factor of ±10 was observed. To investigate whether this factor (y) was constant at all salinities tested (x) a least squares linear regression was carried out resulting in the model: y = 0.18x + 9.6. The slope had a P-value of 0.171, which exceeds α, so the salinity effect was similar at 0.3 and 3 ng mL^{-1} ATP for salinities of 0-2 g kg^{-1}. To correct for the percentage RLU loss (y) due to residual salinity in g kg^{-1} (x) the model: y = -12.7x was used in further experiments. This model was derived from the observed RLU losses at 3 ng mL^{-1} ATP (Figure 1c).

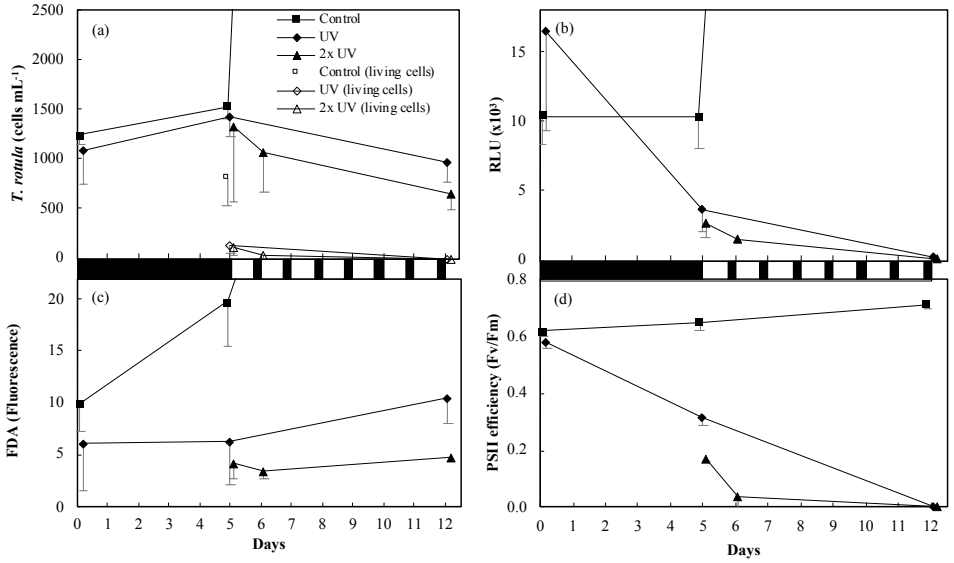

Fig.2 *T. rotula cells analyzed with* ***a*** *flow cytometry and SYTOX® Green. Living cells were not fluorescent after SYTOX® Green staining.* ***b*** *ATP assay using concentration method 2 and the biomass detection kit.* ***c*** *FDA and* ***d*** *PAM fluorometry. The black and white bars between the graphs indicate the dark (black) and illuminated (white) periods during the incubation. Error bars depict the 95 % confidence interval of triplicate measurements*

3.2. UV-C treatment of T. rotula

None of the compliance methods showed a significant change directly after UV treatment. (Figure 2). The abundance of UV-treated cells increased significantly after five days ($P < 0.05$; Figure 2a). ATP levels decreased significantly after five days ($P < 0.05$; Figure 2b), but FDA levels remained unchanged in the UV-treated incubation (Figure 2c). ATP levels were unchanged in the control incubation, but FDA levels in the control almost doubled. The PSII efficiency was strongly reduced, but still detectable in the UV-treated culture (Figure 2d). After the second UV treatment only the PSII efficiency was significantly lower than the pre-treatment value ($P < 0.005$). The other three compliance methods did not detect a significant change directly after the second UV treatment.

Both ATP levels ($P < 0.05$) and PSII efficiency ($P < 0.05$) were significantly reduced one day after the second UV treatment. Also, the PSII efficiency of the double pumped UV-treated culture showed a significant decline ($P < 0.01$) and was similar to the second UV-treated culture on day 6. The cell abundance and FDA fluorescence appeared unaffected by the second UV treatment.

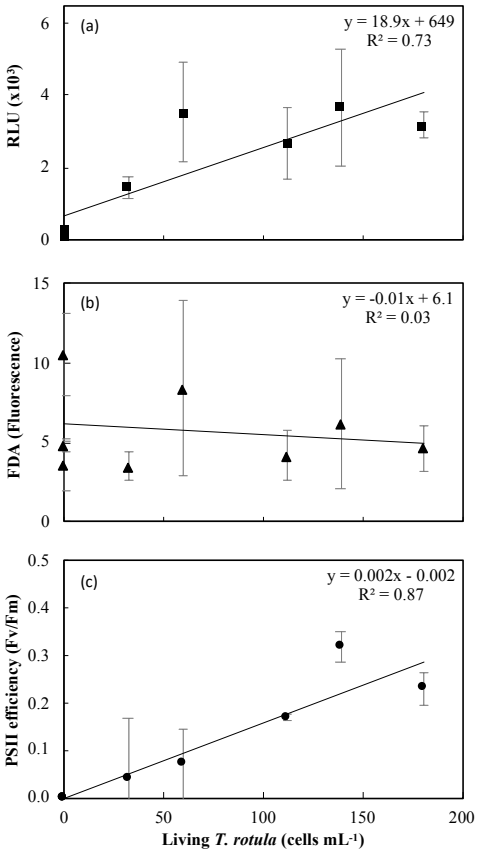

Fig. 3 *Correlation plots comparing living T. rotula cells to* ***a*** *ATP analysis using concentration method 2 and the biomass detection kit.* ***b*** *FDA and* ***c*** *PAM fluorometry. Error bars depict the 95 % confidence interval of triplicate measurements*

On day 12, following 7 days of light incubation, the cell abundance in the control incubation increased to >45,000 cells mL^{-1}. The cell abundance of the single and double UV- treated culture was significantly lower ($P < 0.005$; $P < 0.05$ respectively), but still well above 500 cells mL^{-1}. ATP levels decreased to 100-250 RLU, which represents 1-2% of the original RLU level. PSII efficiency was below the detection limit for all UV-treated cultures and remained at very high levels in the control. FDA levels did not significantly decrease between day 5 and day 12 in UV-treated incubations. In the control FDA and ATP levels increased 8-fold and 25-fold respectively between day 5 and day 12 coinciding with the increase in cell density.

At day 5 numbers of living cells were 100-200 cells mL^{-1} in the various UV-treated incubations, which was 10-20 times exceeding the D-2 standard (Anonymous 2004). At day 12 no living cells were detected in all UV-treated cultures. ATP showed a good correlation between living *T. rotula* cells and RLU levels with $R^2 = 0.73$ (Figure 3a). However, at cell numbers above 50 cells mL^{-1} a plateau appeared. FDA levels showed no correlation with the number of living cells (Figure 3b). Although PSII efficiency is not a quantitative indicator it showed the best correlation with living cells indicated by $R^2 = 0.87$ (Figure 3c).

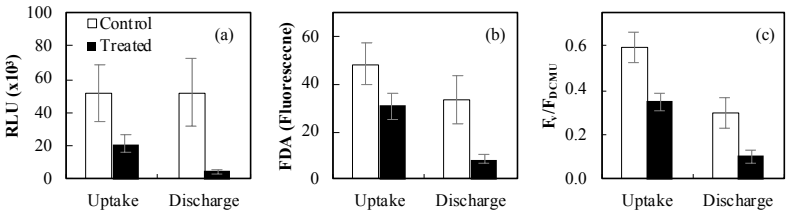

Fig. 4 *Three compliance tools used during the testing of a full-scale UV-based ballast water management system.* ***a*** *ATP analysis using concentration method 1 and ATP swabs,* ***b*** *FDA and* ***c*** *DCMU. Values represent the average of all tests carried out. Control: n=6. Treated: n=10. Error bars depict the 95% confidence interval*

3.3. Test CME techniques during IMO G8 land-based verification testing

The full-scale land-based test were successfully carried out according to the IMO G8 test guidelines using seawater and fresh water (Anonymous 2005, Anonymous 2008, Peperzak 2013). All three compliance tools showed a significant reduction in their respective signals between samples from the uptake before treatment and discharge after treatment (Figure 4). The largest reduction was recorded for ATP analysis (91%) between untreated uptake samples and treated discharge samples (Figure 4a). FDA fluorescence showed a decrease of 82% (Figure 4b). PSII efficiency levels derived from DCMU analysis resulted in decreases of 83% (Figure 4c). All compliance tools showed significant differences between untreated and treated water at uptake.

Official data for the G8 test protocol (10-50 µm cells mL^{-1} and PAM fluorometry derived PSII efficiencies) were compared with the three compliance tools (Figure 5). DCMU derived PSII efficiency data showed the highest correlation with cell concentrations (R^2 = 0.72; Figure 5c), followed by ATP (R^2 = 0.62; Figure 5a) and FDA (R^2 = 0.43; Figure 5e). DCMU derived PSII efficiency data showed the highest correlation with PAM fluorometry derived PSII efficiency data (R^2 = 0.75; Figure 5h), followed by FDA and ATP analysis (R^2 = 0.64 and 0.47, respectively).

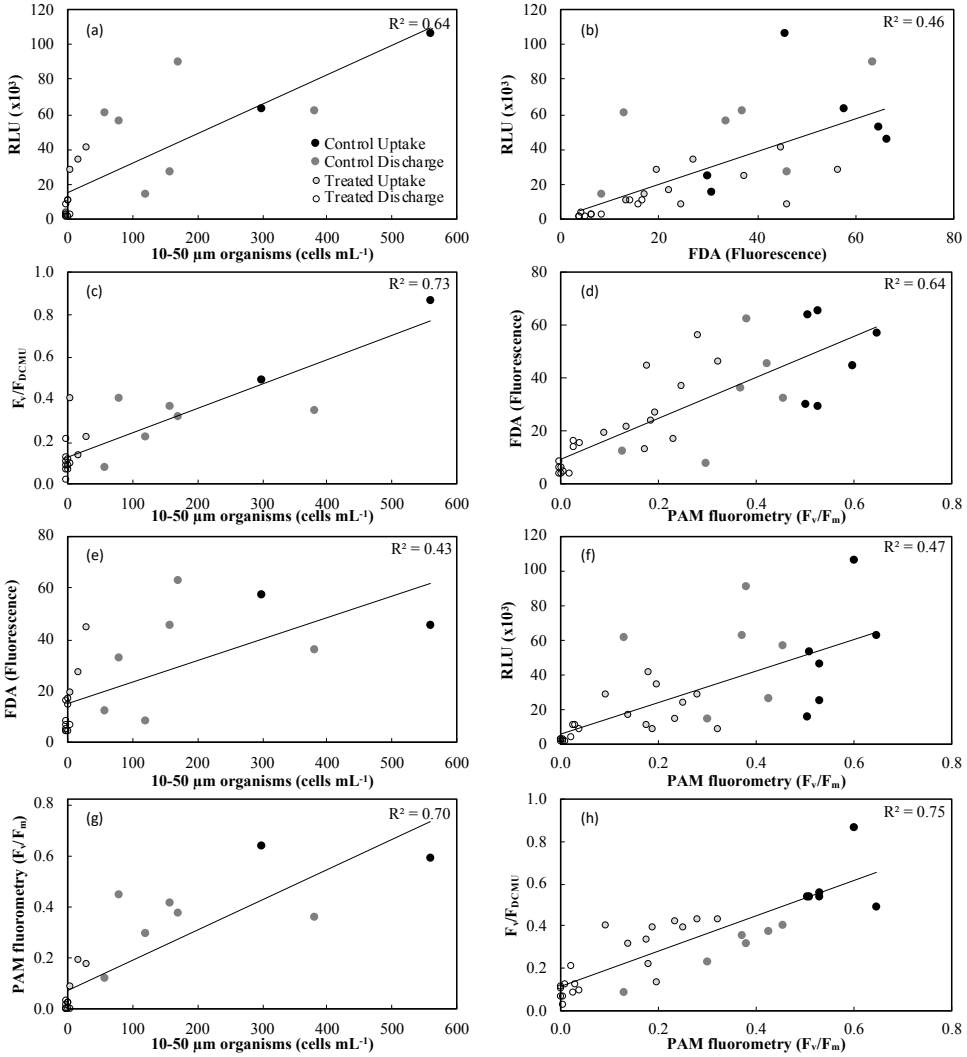

Fig. 5 *Correlation plots between the official IMO G8 test results and CME techniques. 10-50µm organism concentrations are based on phytoplankton and micro-zooplankton enumerations, obtained from the Cathelco test report (Peperzak 2013). Relative Luminescence Units (RLU) depict the results of the ATP assay using concentration method 1 and ATP swabs. F_v/F_{DCMU} indicates the PSII efficiency estimation based on DCMU. F_v/F_m indicates the PSII determination based on PAM fluorometry FDA indicated the results of the fluorescein diacetate assay. The graphs represent the correlation between:* ***a*** *ATP assay and 10–50 µm organisms,* ***b*** *ATP assay and FDA assay,* ***c*** *DCMU assay and 10–50 µm organisms,* ***d*** *FDA assay and PAM fluorometry,* ***e***
FDA assay and 10–50 µm organisms, ***f*** *ATP assay and PAM fluorometry,* ***g*** *PAM fluorometry and 10–50 µm organisms, and* ***h*** *DCMU assay and PAM fluorometry*

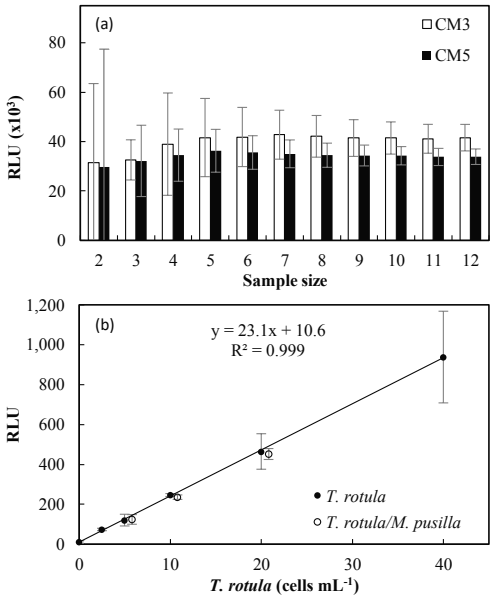

Fig. 6 a *Precision test comparing concentration method 3 and 5 using ATP swabs and natural seawater. RLU: relative luminescence units.* ***b*** *RLU: relative luminesce units resulting from concentration method 5 using ATP swabs. Closed circles indicate results of only T. rotula cells. Open circles represent solutions containing T. rotula and M. pusilla in a 1:1000 ratio. Open circles were moved to the right by 0.8 cells mL^{-1} to enhance visibility. Error bars depict the 95% confidence interval*

3.4. Detection limit, concentration efficiency and salinity reduction of the concentration method

During the detection limit test of CM3, the BDK produced statistically different RLU values between all dilutions except between 10 and 20 cells mL^{-1}. When the ATP swabs were used no significant difference was observed between 20 and 50 cells mL^{-1} (Data not shown). So, CM3 in combination with ATP swabs was not sensitive enough to distinguish *T. rotula* concentrations <50 cells mL^{-1}.

The concentration efficiency of CM3 and CM4 was 63% ± 12% and 85% ± 25%, respectively (average ± CI). Due to variability in the measurements the difference was not statistically significant (p = 0.15). However, CM4 was not statistically different from 100% concentration efficiency.

CM4 was detrimental to the salinity reduction factor due to mixing the milli-Q™ water with the residual sample in the 100 mL syringe. Using CM5 the salinity reduction factor was increased significantly from 17 to 33 times (P = <0.001). This meant that a seawater sample containing 35 g kg^{-1} salts, after concentration typically contained 1.1 g kg^{-1} salts (35/33 = 1.1). This salinity reduction was deemed sufficient for typical seawater samples, since RLU signal loss is likely to be ~15% or less, at a residual salinity of 1.1 g kg^{-1}.

Results of the precision test showed that at two replicates the 95% CI was larger than the average RLU signal observed for both concentration methods (Figure 6a). At five

replicates, the average RLU levels of CM5 stabilized and the CI was 24%, while the CI of CM3 still was 38% of the average. The average RLU values obtained using CM5 were not significantly different from CM3, whilst the concentration factor was 10 instead of 20 which illustrated the improved flushing efficiency of CM5. The variability among measurements using CM5 appeared to be lower than using CM3, which might be attributed to the improved resuspension efficiency of five times back and forth flushing.

The initial 1 L *T. rotula* solution for the detection limit test of CM5, contained 176 ± 15% cells mL^{-1} (average ± %CI). Following 6 dilutions steps the error had increased to 21% (15+6). So, the final dilution had a concentration of 2.5 ± 0.5 cells mL^{-1} (average ± CI). Significantly different RLU signals were observed for all *T. rotula* dilutions tested using CM5 and ATP swabs (Figure 6b). This indicated that the detection limit of CM5 is at least 2.5 ± 0.5 cells mL^{-1}. The improvement of the detection limit compared to CM3 was mainly attributed to a reduction in variability among the replicates presumably due to the improved flushing of the filter. Adding *M. pusilla* cells to the dilutions did not result in significantly different RLU levels. This was a strong indication that the concentration method was highly effective in disregarding cells <10 µm whilst concentrating cells >10 µm.

Using the regression model from Figure 6b, it is possible to estimate pass/fail levels for the ATP assay using CM5 and ATP swabs. According to the regression model (RLU = 23.1 * cell concentration + 10.6), the RLU level of 10 *T. rotula* cells is 241.6 RLU. *T. rotula* is a cylindrical cell of 15 µm in diameter and height. So, the volume of 10 cells is 26.507 μL^3 ($10 * Volume = \pi * 7.5^2 * 15$). Assuming that ATP levels remain constant among organism species and sizes, this translates to 0.009 RLU μL^{-1} cell volume (241.6 / 26.507). Using this value, it is possible to estimate the lower and upper limit of ATP assay at which ballast water is either D-2 compliant or likely non-compliant. In further calculations, cells are assumed to be spherical. A spherical cell of 10 µm would have a volume of 524 µL ($Volume = 4/3 * \pi * 7.5^3$). So, 10 cells of 10 µm would result in 48 RLU (10 * 524 * 0.009), which is significantly higher than the blank measurement: 11 ± 6 RLU (average ± CI). The upper limit would be when 10 cells of 50µm are present in the sample. This would result in a RLU level of 5,951 RLU (10 * 65,450 µL * 0.009). So, assuming constant ATP levels per cell volume, if the ATP assay yields a result of less than ~50 RLU, the ballast water sample is most likely D-2 compliant. If the ATP assay yields result of more than ~6,000 RLU the ballast water sample is most likely non-compliant. RLU levels between these two numbers are ambiguous, because a high abundance of small cells can give the same RLU signal as a few large cells.

4. Discussion

4.1. Data quality

Several aspects have been considered to assess the data quality obtained from the compliance tools. Firstly, it was shown that the ATP assay was not affected by chlorine conditions typically encountered in chlorine-treated ballast water. Moreover, the incorporation of the pre-concentration procedure tackled three major challenges at once. First, salinity interference was sufficiently eliminated by reducing the salinity 33 times (97%). Second, non-target dissolved ATP and ATP derived from <10 µm organisms were effectively removed from the concentrate, shown by the lack of RLU signal increase after the addition of *M. pusilla*. Third, the detection limit was decreased to 2.5 ± 0.5 cells mL^{-1}. These developments contribute to ATP having a high potential to become a viable ballast water compliance tool. It should be noted that the ATP assay is affected by ambient temperature. So, in order to obtain reliable results, all analyses should be carried out at room temperature. In Arctic regions, where ballast water temperatures are around freezing point, no problems are expected as long as the ATP-free water to flush the filter and other reagents and equipment are kept at room temperature.

In laboratory tests, of the three compliance techniques tested, ATP and PAM fluorometry showed the most promising results, since both demonstrated a reasonable to good correlation with the amount of living *T. rotula* cells (R^2 = 0.73 and 0.87, respectively). The correlation of PSII efficiency and cell concentration was considered to be indirect because water disinfection both decreased cell densities as well as PSII efficiency simultaneously. In principle, high PSII efficiency can be detected both at low and high cell densities since it is a relative measurement. However, due to the high correlations observed between PSII efficiency and cell density it can be of value for indicative testing.

The absence of a correlation (R^2 = 0.03) between FDA and living cells could be caused by intact enzymes still residing in the permeable cells. FDA fluorescence was based on esterase activity. However, UV-treatment of *T. rotula* did not appear to inhibit esterase enzymes. The concentration method used for ATP analysis appeared to effectively discard the ATP content of permeable and dead cells, indicated by the relatively high correlation with living cells and RLU signal (R^2 = 0.73). The living cells on day 5 in the UV-treated incubations were no longer viable, indicated by the absence of living cells on day 12 after 7 days of light incubation. The detection of living cells at day 5 clearly demonstrated the delayed effect of UV disinfection often observed after UV treatment (Stehouwer, Fuhr et al. 2010). Most compliance tools are designed to detect living cells instead of viable cells,

whereas viability is the variable that is needed to establish whether ballast water discharge is in compliance with IMO and USCG regulations (Anonymous 2004, Anonymous 2012).

In full-scale tests, major ATP differences between treated and untreated water were observed, both in seawater and fresh water. Correlation plots revealed that ATP correlates well with the concentration of 10-50 µm organisms. The strong correlation between DCMU and PAM fluorometry derived PSII efficiencies was expected, since both methods essentially aim to measure the same variable. It was surprising that DCMU showed a higher correlation with cell concentration than ATP or FDA. The latter two methods aim to quantify total metabolic activity and enzymatic activity, which is presumably a good indication for cell concentration. In contrast, DCMU aims to measure PSII efficiency which is independent of concentration. Previous studies however, have indicated that PSII efficiency was a poor predictor for phytoplankton regrowth potential (Van Slooten, Peperzak et al. 2014). Of the two quantitative methods, ATP was considered superior to FDA since ATP results correlated better with cell concentrations.

A major limitation of relying on the presence of PSII efficiency as compliance tool is that it only targets autotrophic organisms. Heterotrophic organisms such as ciliates, protozoa and many dinoflagellates cannot be detected using DCMU, Walz PAM or any other PSII-based method. Coastal ecosystems can rapidly shift from phytoplankton dominated to zooplankton dominated states in a matter of weeks (Peperzak, Colijn et al. 1998) so the need for a compliance tool capable of detecting all types or organisms is evident. Both ATP and FDA are capable of detecting all types of organisms, however ATP analysis is much less time-consuming than FDA analysis.

Differences in delayed disinfection effect between laboratory studies and full-scale land-based studies could be caused by the use of different UV technologies. In the laboratory, a low pressure UV-C reactor was used which produced monochromatic UV-C radiation at 254 nm. The medium pressure UV reactor in the full-scale land-based test produced a broad range of UV-C and UV-B radiation, ranging from 200-400 nm (personal communication M. Voigt, Cathelco, UK). Although disinfection efficiency is highest at a radiation of 254 nm, this wavelength is often quickly absorbed in natural freshwater due to dissolved organic matter. Each wavelength exhibits its own absorption rate which also tends to vary with various water qualities (Carter, Tipping et al. 2012). Thus, it could be preferable to apply medium pressure UV systems to account for varying water qualities a ship encounters at different moments and locations.

4.2. Sources of false-positive and -negative results

Leaking filters might produce false-negative results. However, the five-fold replicates should ensure the detection of such events. Risks of damaged filters are relatively small, since the filters used in the ATP assay are contained in sturdy plastic capsules and are intended for single use only. As mentioned earlier, the risk of filter damage or leakage is considerable using the FDA method. Moreover, similar risks for false-positive and -negative results are present when using the FDA method. Using DCMU however, no risk of bacteria induced false-positives are present, since DCMU specifically target PSII efficiency which is exclusively present in phototrophic organisms. On the other hand, DCMU can lead to false-positive results when the phytoplankton present in a sample comprises mainly of <10 µm cells since no separation between large and small cells was made beforehand. In addition, false-negative results are also possible when using DCMU, since the absence PSII activity does not guarantee phytoplankton's loss of regrowth potential. Moreover, even when phytoplankton is totally absent, micro-zooplankton might still be present in the ballast water, undetected, leading to false-negative results.

4.3. Validation recommendations

Despite a first attempt to calculate pass/fail levels for the ATP assay, several factors could pose additional challenges. During the growth cycle of phytoplankton, cellular ATP concentrations may vary (Holm-Hansen 1970). During the exponential growth phase, ATP levels are expected to be elevated compared to phases where cells are no longer dividing e.g., under nutrient limited conditions. Also, different species can exhibit different ATP levels depending on size and species-related metabolic states. However, a decrease of ATP during 5-day dark incubations was not observed in full-scale tests (Figure 4a). So, it is recommended to measure the ATP levels of a wide variety of 10-50 µm organisms in various stages of their growth cycle between 5 and 50 cells mL^{-1} to obtain an expected ATP level of D-2 compliant ballast water. In addition, to corroborate the excellent separation capacity of the filtration method in *T. rotula* and *M. pusilla* culture mixes, species of more similar cell sizes could be tested as well. ATP measurements should be carried out alongside full-scale land-based and shipboard trials of various BWMS techniques to examine the typical ATP concentration of D-2 compliant test water. It can be expected that chlorine-treated ballast water contains different ATP concentrations than UV-treated ballast water due to inherently different disinfection mechanisms. It is recommended that the ATP assay is tested using a representative number of available ballast water treatment techniques to investigate expected differences in typical ATP concentrations of D-2 compliant discharge water.

When ballast water with a high sediment load is taken up, bacteria adhered to the surface of sediment particles could end up in the concentrate and interfere with the ATP analysis, leading to false-positive results (First and Drake 2013). Sediment interference is only expected after short voyages since the larger particles will quickly sink out to the bottom of ballast tanks and typically will remain in the tank during ballast water discharge. It is recommended to investigate the effect of high sediment loads with and without bacteria on the performance of the ATP assay.

4.4. Comparison with previous ballast water-ATP studies

Quantifying ATP to estimate living biomass after ballast water treatment has been attempted before. In all studies a pre-filtration procedure was performed using 10 µm or 0.2-0.7 µm filters to differentiate between microbial and >10 µm organisms. In congruence with the current findings, all studies reported a strong (-90% to -99%) decline in ATP content after ballast water disinfection using full-scale systems applying peracetic acid, peroxide and electro-chlorination (de Lafontaine, Despatie et al. 2008, Welschmeyer and Davidson 2011). A delay in ATP degradation was observed in a laboratory study using UV radiation (First and Drake 2013), which was also observed in the current UV-based laboratory study. The delay was most likely caused by the delayed cell death caused by UV disinfection. Cells do not die right after treatment, but DNA damage inflicted by the radiation eventually leads to cell death. However, in the current research, the full-scale UV-based treatment test, ATP levels had strongly declined, leading to the suspicion that differences between low pressure and medium pressure UV systems could be of more significance than earlier expected.

4.5. Usability and time

The DCMU-based method was the easiest to use since the procedure involved very little equipment and sample handling which ensures an analysis time of <5 minutes. In stark contrast, the FDA-based method required at least 40 minutes to acquire a single measurement. During field tests, triplicates usually took one hour to obtain, since incubations could be run in parallel. Clogging of filters was a common issue with the FDA method, due to the large volume required to filter (200 mL) relative to the filter diameter (25 mm). The provided manifold required manual replacement of individual filters from the manifold, creating many opportunities for contamination and damaging of the filter before and after the filtration process.

Concerning the ATP assay, the concentration procedure to remove dissolved ATP and <10 µm organisms from the sample proved straightforward and easy to use. Syringes and filters were provided in sealed packages which proved clean due to consistently low blank

measurements. It is of importance that a blank measurement is made using only ATP-free elution water to ensure cleanliness of the procedure. Contamination is unlikely if the operator uses a clean beaker to acquire the sample and any contact with the sample is limited to the syringes and filters. Variation among measurements can be considerable though, so it is advisable that at least five replicates are made for each ballast water sample. All equipment needed to use the ATP compliance tool can be transported in a lightweight briefcase. Setting up the equipment and carrying out the concentration and analysis steps is done in a matter of minutes. In practice, the most time-consuming aspect of the procedure most likely will be the proper collection of a ballast water sample.

5. Conclusions

- The concentration procedure solved three problems: Interference of high salinity. Interference of dissolved ATP and <10 µm organisms. The detection limit was sufficiently decreased.
- Reagents for ATP analysis should be kept at room temperature.
- ATP and DCMU results correlate well with living *T. rotula* cells (R^2 = 0.73 and 0.87, respectively) but fail to predict viability.
- ATP and DCMU analysis exhibited reasonable correlations with 10-50 µm cells mL^{-1} (R^2 = 0.64 and 0.73, respectively).
- FDA analysis was considered too time-consuming (>40 minutes per analysis) to be an effective compliance method.
- When assessing ballast water for D-2 compliance, the estimated pass level of the ATP assay using concentration method 5 is ~50 RLU and the estimated fail level is ~6,000 RLU.
- Additional lab- and field-tests, incorporating phytoplankton monocultures, high sediment load and different treatment methods, are required to validate the ATP assay.

Acknowledgements

The authors would like to thank all personnel of the plankton laboratory of the Royal Netherlands Institute for Sea Research for their invaluable assistance in carrying out the research. This work has been co-funded by the North Sea Region Program under the European Regional Development Fund of the European Union.

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Summary

The spread of aquatic invasive species is considered one of the main threats to marine biodiversity. Through ballast water transport, many aquatic species are transported around the world and discharged into foreign ecosystems. These newly introduced species may become invasive and outcompete local species for habitat and food availability. To address this problem, in 2004, the International Maritime Organization (IMO) adopted the Convention on ballast water and sediments (the Convention). The Convention aims to address the risk of ballast water mediated invasions by introducing a Ballast Water Discharge Standard (BWDS) which limits the number of livings organisms permitted in the discharged ballast water. Independently, in 2012, the United States Coast Guard (USCG) adopted the Standards for Living Organisms in Ships' Ballast Water Discharged in U.S. Waters (Final Rule), introducing a similar BWDS for U.S. waters. Ballast water plays a critical function in a ship's stability, trim, draft, and structural integrity. In most cases therefore, a shipboard ballast water management system (BWMS) is needed to ensure compliance with the BWDS. Every BWMS must obtain IMO and/or USCG type approval by undergoing rigorous testing to be allowed to discharge its treated ballast water. Mandatory test protocols were issued by the IMO (BWMS Code) and USCG (ETV Protocol) which must be conducted by independent test facilities. Among other things, the BWMS must be subjected to a standardized test by pumping five times marine, brackish or freshwater, respectively, through the BWMS and temporarily store the treated water in designated test tanks until discharge. During the filling and discharging of the water, samples are taken for analysis of (in)organic substances and living organisms such as zooplankton, phytoplankton and bacteria to (1) quantify the challenge posed by the water to the BWMS and (2) to assess the BWMS efficacy in reaching the BWDS. The procedures for sampling and analysis are prescribed in detail by the IMO and USCG, aiming to improve the comparability of test results between separate test facilities. Finally, Port State Control (PSC) officers are responsible for inspecting individual ships for compliance monitoring and enforcement of the Convention.

At the start of this PhD project many new BWMSs were in development and several disinfectants (hereafter called active substances) needed to be investigated for their applicability in ballast water disinfection. Also, the type approval test protocols were being criticized for prescribing labor-intensive bacterial plating methods whilst more efficient technologies were available. At the same time, it was unclear to port state control (PSC) officers and other stakeholders how to sample and analyze ballast water for compliance monitoring in a cost effective, timely and easy manner. To tackle this, the following more

specific research topics were addressed in this thesis, corresponding to various stages in the development, testing and compliance monitoring of ballast water management systems:

1. Is a quaternary ammonium compound suitable as active substance to disinfect ballast water? (Chapter 2)
2. To enumerate heterotrophic bacteria, how do agar growth media compare to automated cell counting and molecular techniques? (Chapter 3)
3. Is the FlowCAM a suitable device to conduct indicative ballast water discharge analysis? (Chapter 4)
4. How do several proxy measurements perform in indicative ballast water compliance testing? (Chapter 5)

In **Chapter 2**, didecyldimethylammonium chloride (DDAC) was tested for its applicability as a ballast water treatment method. The treatment of the marine phytoplankton species *Tetraselmis suecica*, *Isochrysis galbana* and *Chaetoceros calcitrans* showed that at 2.5 µL L^{-1} DDAC was able to disintegrate the cells after 5 days of dark incubation. The treatment of natural marine plankton communities with 2.5 µL L^{-1} DDAC did not sufficiently decrease zooplankton abundance to comply with the Ballast Water Discharge Standard (BWDS). Bivalve larvae were most resistant to the treatment. Although Photosystem II (PSII) efficiency was inactivated within 5 days, indicating the (temporary) loss of photosynthetic ability, the phytoplankton cells remained intact. Moreover, regrowth occurred within 2 days of incubation in the light. These findings highlight the importance of studying novel active substance applications both in lab-scale monocultures and natural communities. In terms of indicative compliance tools, the regrowth results indicate that the popular and widely used fluorescence-based PSII efficiency tools may result in false negatives. The Most Probable Number (MPN) method often used during IMO type approval testing would probably have detected regrowth similar to the protocol used in this study. By conducting pilot testing using IMO- and USCG-prescribed challenge conditions, limitations of the treatment method are detected at an early stage. For example, the resistance of the bivalve larvae to treatment can be addressed by the addition of a physical separation process to the BWMS. Indeed, the vast majority of type approved BWMS incorporate filtration prior to disinfection. The critical importance to evaluate residual toxicity in the treated water, as detailed in IMO Guideline G9, was also highlighted. Namely, after the 5-day incubation, untreated phytoplankton exposed to the residual DDAC (~0.3 µL L^{-1}) showed delayed cell growth and reduced PSII

efficiency, indicating toxicity in the treated water. Considering the over-the-counter sale of DDAC as a popular hard surface cleaner it is noteworthy that even commonly accepted household products may pose substantial environmental risks in novel applications. For the general public, this observation may provide some perspective when assessing the risk of discharging treated ballast water into the local environment. Like most active substances, treatment with DDAC required a neutralization process, and monitoring thereof, upon discharge. The study highlighted the challenges faced to successfully implement such processes. The colorimetric measurement of DDAC in grab samples was time-consuming and non-automated. As demonstrated by the availability of inline, automated TRO sensors, it is possible to automate colorimetric methods for inline shipboard use. However, the neutralization process itself posed additional challenges. Two successive neutralization cycles of 50 mg L^{-1} bentonite were necessary to inactivate residual DDAC upon discharge. The discharge of high amounts of suspended solids is likely prohibited in many harbors around the world. Also, the dosing device to suspend the bentonite would have to be subjected to a stringent testing regime to ensure its effectiveness in shipboard conditions. Lastly, the bentonite injection adds cost to the treatment process, making the system commercially less attractive.

In **Chapter 3,** heterotrophic plate counting (HPC) was compared with Flow Cytometry (FCM) and quantitative Polymerase Chain Reaction (qPCR). Per the USCG and IMO regulations, during BWMS type approval testing the number of culturable heterotrophic bacteria must be quantified., There are no performance standards on discharge associated with bacteria, except for a number of indicator microbes. Nevertheless, their presence in the testwater may pose an indirect challenge to disinfect the ballast water by causing (for example) degradation of active substances or UV-attenuation. The USCG-prescribed ETV protocol requires HPC techniques to quantify culturable bacteria. Yet, in the scientific community it is well established that HPC techniques may vastly underestimate the number of living non-culturable bacteria present in natural water samples. It is not expected that culturable or non-culturable bacteria would differ in their contribution to any indirect challenge posed to a BWMS to reach the BWDS. Therefore, it was important to determine if HPC bears any correlation to the total bacterial number as measured via alternate techniques. Samples were collected at a stationary natural fresh water and seawater location over a consecutive 30-week period. Analysis of bacterial abundance using HPC, FCM and qPCR generally yielded concentrations in the range of 10^4, 10^6 and 10^7 cells mL^{-1}, respectively. Substantial differences in abundance patterns were observed among the three techniques over

time. With respect to FCM, glutaraldehyde-fixed samples yielded similar results as samples fixed with formalin/hexamine. The absence of a correlation between FCM and qPCR in freshwater samples was potentially caused by variation in gene copy numbers among various bacterial species. In contrast, significant correlations were observed when a monoculture of *E. coli* was quantified using FCM and qPCR. In conclusion, FCM appears more reliable than qPCR to detect heterotrophic bacterial abundance in natural water samples. Most importantly, there was no correlation between HPC and FCM results in bacterial trends over time. Therefore, these results support the notion that the prescribed HPC techniques are not predictive of the actual challenge posed by bacteria in the landbased type approval test water. More fundamentally, it is unclear whether the challenge posed by bacteria can be distinguished from other organic matter sources contained in the Dissolved and Particulate Organic Carbon (DOC, POC) pools. Organic matter poses a challenge to oxidant-based systems by reacting with hypochlorite, thus (potentially) forming disinfection byproducts and lowering the Total Residual Oxidants (TRO) available to kill the organisms in the treated water. This chemical process supposedly does not discriminate between organic matter originating from dead or living material. In UV-systems, the main challenge is low ultraviolet-transmission (UV-transmission) in the water caused by attenuating substances. The main contributors to UV attenuation are humic and fulvic acids as part of the DOC fraction. It is unclear how living bacteria contribute to the challenge posed to UV-based BWMS other than being part of the POC fraction, which has its own minimum required concentration in the challenge water. Therefore, in the absence of discharge limits, the relevance of the heterotrophic bacterial challenge requirements to the type approval process should be reconsidered. At the same time, it should be noted that the absence of regulation may lead to unnaturally high bacterial growth in treated ballast water as a side-effect of disinfecting ≥50 µm and 10-50µm organisms, which impact should also be assessed.

Many stakeholders need access to simple, reliable and quick methods to inspect treated ballast water discharge. At installation an indicative commissioning test of treated ballast water is required to obtain the BWMS type approval certificate. Also, during routine usage, the crew may wish to monitor the performance of their BWMS by periodically testing the treated effluent. Thirdly, PSC officers require easy access to indicative compliance monitoring tools to enforce the Convention. Assessing compliance with the BWDS requires determining the size, viability, and concentration of planktonic organisms. The Flow Cytometer and CAMERA (FlowCAM) is an Imaging Flow Cytometer designed to obtain the concentration of particles in water along with their images and quantitative morphologic

information. In **Chapter 4**, the performance of the FlowCAM was evaluated by analyzing artificial microbeads, UV-treated *Prorocentrum minimum* cultures and seawater samples from a UV-treated BWMS discharge. Microbead analyses yielded high accuracy and precision in size and concentration measurements. However, contaminations prohibited the automated processing of ballast water samples at the <10 per mL BWDS limit for 10-50 µm organisms. Using *P. minimum,* automated FlowCAM analysis was able to detect UV-treatment in cell appearance and growth. However, in natural seawater the low concentration and heterogeneity of particles still necessitated the manual observation of images by experts. In terms of shipboard usability for compliance testing, some physical characteristics of the device must be improved. For example, background de-calibration may occur due to ambient vibrations. Moreover, bubble formation and clogging occurred frequently when processing natural seawater samples. In terms of data transfer, the optimization of device configuration enables the quick transferring of files and information between stakeholders. However, one of FlowCAM's limitations is its inability to assess movement in zooplankton organisms of the ≥50µm fraction. Interestingly, other promising flow-through image-processing tools have recently been developed that should also be considered, such as the BallastWISE (MicroWISE). For ≥50 µm organisms it uses a motility assay and for 10-50µm organisms a motility + fluorescence assay. In contrast to organism detection in a flow, BallastWISE employs a stationary cell to enable detection of individual organism movement and fluorescence via video-tracking. Motility is the cardinal viability indicator for organisms ≥50µm which until now required manual detection using microscopy. Furthermore, it's detection limit is sufficient to reach the BWDS limit and results correlate well with traditional microscopy in natural water samples. By providing the simultaneous analysis of fluorescence and motility in 10-50µm and ≥50µm organisms the BallastWISE may have solved many of FlowCAM's limitations.

Compliance with regulations of the International Maritime Organization (IMO) and the United States Coast Guard (USCG) will have to be achieved by onboard ballast water management systems. To monitor the treatment system performance, rapid and easy compliance techniques are required. **Chapter 5** reports on the suitability of adenosine triphosphate (ATP) to quantify living 10-50 µm organisms at <10 cells mL^{-1}, which is the upper limit of the BWDS. Initial tests revealed that the commercially available ATP assays lacked sensitivity to monitor ATP in treated ballast water due to salt-interference and non-specificity to the target organisms. To resolve these issues, a rapid and easy concentration method was developed to (1) increase sensitivity, (2) remove interfering salts, (3) remove

non-target organisms and (4) remove extracellular ATP. Laboratory experiments showed that salinity was reduced 33 times and concentration efficiencies reached 85 %. The ATP assay was tested in a UV-based full-scale BWMS, treating seawater and fresh water. Compared to the alternative compliance tools Fluorescein Diacetate (FDA) and PSII efficiency the ATP assay performed better in discriminating treated and untreated samples. Following refinements, the ATP assay's detection limit reached well below the BWDS in a *T. rotula* monoculture. In recent years commercial ballast water compliance tools have been developed and deployed successfully such as the B-QUA Plus (Luminultra). Notably, additional sample processing using microbeads to break up sturdy cell walls has helped the release of intracellular ATP to improve detection. Therefore, the B-QUA Plus kit is also able to detect ATP in the fraction of ≥50µm organisms dominated by sturdy copepods. It's main advantages over fluorescence-based tools, such as PSII efficiency, is the ability to detect ATP from both size classes of the BWDS and from heterotrophic bacteria. In contrast, fluorescence-based tools solely target a subset of organisms, namely the 10-50 µm phytoplankton cells. When applied correctly, ATP assays are therefore expected to report more reliable results than fluorescence-based tools.

In conclusion, the research presented in this PhD thesis has contributed to several important topics in the field of ballast water treatment. (1) DDAC was effectively assessed for its (un)suitability in ballast water treatment. (2) The applicability of HPC was compared to alternate techniques and the contribution of heterotrophic bacteria to BMWS type approval testing was discussed. (3) The suitability of the FlowCAM to analyze treated ballast water was investigated and important deficiencies were identified. (4) A simple, quick and reliable sample processing solution was developed for ATP-analysis

Finally, the following recommendations are made to continue this research.

1. When assessing active substances for type approval, do not ignore the necessity for reliable automated inline sensors for dosing control and residual discharge monitoring.
2. Considering the BWDS does not regulate discharged heterotrophic bacteria, the impact of living heterotrophic bacteria, relative to dead organic matter (DOC, POC), on the formation of toxicity, disinfection byproducts, TRO-degradation and UV-transmission should be investigated. At the same time, the absence of regulating heterotrophic bacteria may lead to unnaturally high bacterial growth in

treated ballast water as a side-effect of disinfecting ≥50 µm and 10-50 µm organisms, which impact should also be assessed.

3. As the FlowCAM is neither able to simultaneously analyze the ≥50 µm and 10-50 µm organisms, nor assess organism motility, it is recommended to continue development of alternative imaging devices for compliance monitoring.
4. It is recommended to measure the ATP levels of a wide variety of 10–50-µm organisms in various stages of their growth cycle between 5 and 50 cells mL^{-1} to obtain an improved pass/fail ATP level for BWDS compliant ballast water.

Samenvatting

De verspreiding van aquatische invasieve soorten wordt beschouwd als een van de belangrijkste bedreigingen voor de mariene biodiversiteit. Via transport van ballastwater worden veel aquatische soorten over de hele wereld vervoerd en geloosd in exotische ecosystemen. Deze nieuw geïntroduceerde soorten kunnen invasief worden en de lokale soorten verdringen door te concurreren op habitat en hun voedsel te eten. Om dit probleem aan te pakken, heeft de Internationale Maritieme Organisatie (IMO) in 2004 het Verdrag inzake ballastwater en sediment (het Verdrag) aangenomen. Het Verdrag heeft tot doel het risico van door ballastwater veroorzaakte invasies te verminderen. Dit doel wordt bereikt door een Ballast Water Lozingsnorm (Lozingsnorm) in te voeren, die limieten stelt aan het aantal levende organismen dat via ballastwater geloosd mag worden. Onafhankelijk van de IMO heeft de Amerikaanse kustwacht (United States Coast Guard, USCG) in 2012 soortgelijke regels ingevoerd genaamd (vrij vertaald): “Standaarden voor Levende Organismen in Ballast Water via Schepen Geloosd in Amerikaanse Wateren (Final Rule)”. Ballastwater speelt een cruciale rol in de stabiliteit, trim, diepgang en structurele integriteit van een zeegaand schip. Voor de meeste schepen is daarom een BallastWater BehandelingsSysteem (BWBS) aan boord nodig om ervoor te zorgen dat de Lozingsnorm niet overschreden wordt. Elk BWBS moet IMO- en/of USCG-typegoedkeuring behalen om het behandelde ballastwater te mogen lozen. Bindende testprotocollen zijn gepubliceerd door de IMO (BWMS Code) en USCG (ETV-protocol) die moeten worden uitgevoerd door onafhankelijke testfaciliteiten. Het BWBS moet (onder andere) in een gestandaardiseerde testopstelling getest worden door respectievelijk vijfmaal zoet, brak of zout water door het systeem te pompen waarna het tijdelijk wordt opgeslagen alvorens het weer wordt geloosd. Tijdens het oppompen en lozen wordt het water bemonsterd en geanalyseerd op (an)organische stoffen en levende organismen zoals zoöplankton, fytoplankton en bacteriën om enerzijds te meten of het water uitdagend genoeg voor betrouwbare test is en anderzijds of na behandeling de Lozingsnorm wordt gehaald door het BWBS. De monstername- en analyseprocedures zijn exact voorgeschreven door de IMO en USCG wat de vergelijkbaarheid van testresultaten ten goede komt. Tenslotte is de havenstaatcontrole (Port State Control, PSC) verantwoordelijk voor het inspecteren van individuele schepen voor toezicht op de naleving en handhaving van het Verdrag en de Lozingsnorm.

Bij de start van dit PhD project waren veel nieuwe BWBS’en nog in ontwikkeling en moesten verschillende desinfectiemiddelen (hierna actieve stoffen genoemd) worden onderzocht of ze geschikt waren voor ballastwater behandeling. Ook was er kritiek op de verplichte testprotocollen voor typegoedkeuring omdat ze arbeidsintensieve bacteriële agar-

plaat methoden voorschreven terwijl er veel efficiëntere methoden beschikbaar waren. Tegelijkertijd was het een vraag voor PSC-functionarissen en andere belanghebbenden hoe ze ballastwater op een kosteneffectieve, snelle en gemakkelijke manier konden bemonsteren en analyseren voor de nalevingscontrole. Om deze onderwerpen aan te pakken, werden in dit proefschrift de volgende onderzoeksvragen geformuleerd, corresponderend met verschillende stadia in ontwikkeling, testen en nalevingscontrole van BWBS'en:

1. Is een quaternaire ammoniumverbinding geschikt als actieve stof om ballastwater te desinfecteren? (Hoofdstuk 2)
2. Hoe verhouden agar-groeimedia zich tot geautomatiseerde cel-tellingen en moleculaire technieken om heterotrofe bacteriën te kwantificeren? (Hoofdstuk 3)
3. Is de FlowCAM een geschikt apparaat om indicatieve ballastwater lozingsanalyses uit te voeren? (Hoofdstuk 4)
4. Hoe presteren verschillende proxy-metingen als methode voor indicatieve nalevingscontrole (Hoofdstuk 5)

In Hoofdstuk 2 werd didecyldimethylammoniumchloride (DDAC) getest op zijn toepasbaarheid als ballastwaterbehandelingsmethode. De behandeling van de eencellige mariene fytoplankton-soorten *Tetraselmis suecica, Isochrysis galbana* en *Chaetoceros calcitrans* liet zien dat bij 2,5 µL L^{-1} DDAC de cellen desintegreerden na 5 dagen incubatie in het donker. De behandeling van natuurlijk marien plankton met 2,5 µL L-1 DDAC verminderde de hoeveelheid zoöplankton niet genoeg om te voldoen aan de Lozingsnorm. Schelpdierlarfjes bleken het meest resistent tegen de behandeling. Hoewel de efficiëntie van Fotosysteem II (Photosystem II, PSII) binnen 5 dagen werd geïnactiveerd, wat wijst op het (tijdelijke) verlies van fotosynthetisch vermogen, bleven de fytoplanktoncellen intact. Bovendien vond hergroei plaats binnen 2 dagen nadat het behandelde water was teruggeplaatst in het licht. Deze bevindingen onderstrepen het belang van testen van actieve stoffen in zowel monoculturen op laboratoriumschaal als in natuurlijk water. De snelle hergroei na 2 dagen geeft aan dat de PSII-efficiëntie meting kan resulteren in vals-negatieven, dus dat ten onrechte de conclusie wordt getrokken dat het water voldoet aan de Lozingsnorm. De Meest Waarschijnlijke Aantal (MWA) -methode die wordt gebruikt tijdens veel IMO-typegoedkeuringstests, zou naar verwachting een vergelijkbare hergroei hebben gedetecteerd. Verder is het noemenswaardig op te merken dat PSII-efficiëntie analyses (of afgeleiden daarvan) erg populair zijn als snelle, simpele indicatieve ballastwater

controlemethode. Door kleinschalige studies uit te voeren gebruikmakend van door IMO en USCG voorgeschreven testwater, worden tekortkomingen van de experimentele behandelmethode in een vroeg stadium ontdekt. De resistentie van de schelpdierlarfjes tegen behandeling kan bijvoorbeeld worden omzeild door een filtratiestap aan het BWBS ontwerp toe te voegen en zodoende grotere organismen direct uit te filteren. Inderdaad heeft de overgrote meerderheid van de typegoedgekeurde BWBS'en filtratie als eerste stap voorafgaand aan de desinfectie-stap. In IMO-richtlijn G9 staat beschreven dat het geloosde water dat behandeld is met een actieve stof moet worden beoordeeld op eventuele giftigheid voor het ontvangende water. Het cruciale belang hiervan werd benadrukt door deze studie. Namelijk, na de incubatie van 5 dagen vertoonde onbehandeld fytoplankton dat was blootgesteld aan de resterende DDAC (~ 0,3 $\mu L\ L^{-1}$) een vertraagde celgroei en verminderde PSII-efficiëntie, wat wijst op giftigheid in het 'geloosde' water. Gezien de vrije verkoop van DDAC als groene aanslagreiniger, is het opmerkelijk om te zien dat algemeen aanvaarde huishoudelijke producten milieurisico's kunnen opleveren als ze een andere toepassing krijgen. Voor veel mensen kan dit wellicht enige context bieden bij de risico-inschatting van lozing van behandeld ballastwater in de lokale omgeving. De risico's voor het milieu worden nauwkeurig in kaart gebracht en beoordeeld alvorens een lozingsvergunning mag worden afgegeven. Zoals de meeste actieve stoffen, vereist behandeling met DDAC een neutralisatieproces en monitoring daarvan voor of tijdens het lozen. Deze studie bracht enkele knelpunten aan het licht om dergelijke processen met succes toe te passen. De colorimetrische meting van DDAC zoals gebruikt in deze studie was tijdrovend en niet-geautomatiseerd. Maar, het bestaan van inline, geautomatiseerde chloor-sensoren toont aan dat het wel degelijk mogelijk is om colorimetrische methoden voor inline gebruik aan boord te automatiseren. Echter, het neutralisatieproces zelf bracht extra uitdagingen met zich mee. Een dubbele dosering van elk 50 mg L^{-1} bentoniet klei was nodig om de resterende DDAC bij lozing te neutraliseren. Het lozen van grote hoeveelheden klei of andere zwevende stoffen is naar verwachting in veel havens verboden. Ook zou het doseer-apparaat om het bentoniet te injecteren aan een streng testregime moeten worden onderworpen om de doeltreffendheid ervan onder scheeps-omstandigheden te waarborgen. Ten slotte zorgt de bentoniet-injectie voor extra kosten voor het behandelingsproces, waardoor het systeem commercieel minder aantrekkelijk wordt.

In Hoofdstuk 3 werd de heterotrofe strijkplaat methode (Heterotrophic Plate-Count, HPC) vergeleken met FlowCytoMetrie (FCM) en kwantitatieve polymerasekettingreactie (quantitative Polymerase Chain Reaction, qPCR). De HPC-methode houdt in dat tijdens

BWBS-typegoedkeuringstests het kiemgetal van de heterotrofe bacteriën worden bepaald. Het kiemgetal is het aantal bacteriekolonies dat na een vijfdaagse incubatie is gegroeid op een kweekplaat. Behoudens een aantal humane pathogenen zijn er geen lozingsnormen voor deze voornoemde heterotrofe bacteriën. Desalniettemin kan hun aanwezigheid in het testwater een belemmering vormen voor het BWBS om het ballastwater goed te desinfecteren. Denk hierbij aan (bijvoorbeeld) versnelde afbraak van actieve stoffen of door verhoogde UV-absorptie te veroorzaken. Hierdoor blijft er minder actieve stof of UV licht over om de andere organismen te doden of inactiveren. Het door de USCG voorgeschreven ETV-protocol vereist HPC-technieken om kweekbare bacteriën te kwantificeren. Echter, het is in wetenschappelijke kringen algemeen bekend dat HPC-technieken het aantal levende niet-kweekbare bacteriën in natuurlijke watermonsters enorm kunnen onderschatten. Voorts ligt het niet in de lijn der verwachting dat kweekbare of niet-kweekbare bacteriën verschillen in hun bijdrage aan de uitdaging die ze vormen voor een BWBS om de Lozingsnorm te bereiken. Daarom was het belangrijk om te bepalen of HPC-resultaten enige correlatie vertoonden met het totale aantal bacteriën zoals gevonden via alternatieve technieken. Monsters werden verzameld op een vast monsterpunt voor natuurlijk zoetwater en zeewater gedurende een aaneengesloten periode van 30 weken. Analyse van bacteriële aantallen met behulp van HPC, FCM en qPCR leverde over het algemeen concentraties op in de ordegrootte van respectievelijk 10^4, 10^6 en 10^7 cellen mL^{-1}. Aanzienlijke verschillen in concentratie-trends werden in de loop van de tijd tussen de drie technieken waargenomen. Wat FCM betreft gaven glutaaraldehyde-gefixeerde monsters vergelijkbare resultaten als monsters gefixeerd met formaline/hexamine. De afwezigheid van een verband tussen FCM en qPCR in zoetwater monsters werd mede veroorzaakt door variatie in het aantal gen-kopieën tussen verschillende bacteriesoorten. Daarentegen werden dezelfde aantallen gevonden wanneer een monocultuur van de *Escherichia coli* bacterie werd geteld met behulp van FCM en qPCR. Concluderend lijkt FCM betrouwbaarder dan qPCR om heterotrofe bacterieconcentraties in natuurlijke watermonsters te meten. Meest opmerkelijk was dat er geen correlatie tussen HPC- en FCM-resultaten in bacteriële trends over tijd werd gezien. Daarom ondersteunen deze resultaten de kritiek dat de HPC-technieken weinig zeggen over de daadwerkelijke uitdaging die bacteriën vormen tijdens typegoedkeuringstesten. Daarbij komt dat het onduidelijk is of de uitdaging die bacteriën vormen kan worden onderscheiden van andere bronnen van organisch materiaal in de opgeloste en particulaire organische koolstofbronnen (Dissolved and Particulate Organic Carbon, DOC and POC). Bacteriën vormen slechts een klein deel van de totale organische koolstofbron. Organische koolstof

vormt een uitdaging voor oxiderende actieve stoffen door ermee te reageren, waardoor (mogelijk) schadelijke bijproducten worden gevormd en de beschikbare oxidanten (Total Residual Oxidants, TRO) worden verlaagd. Hierdoor blijft er minder TRO over om de organismen te doden. Dit chemische proces maakt naar verwachting geen onderscheid tussen organisch koolstof dat afkomstig is van dood materiaal of levende cellen. In UV-systemen is de belangrijkste uitdaging de lage ultraviolette doorzichtigheid (UV-transmissie) die in het water veroorzaakt wordt door licht-absorberende stoffen. De belangrijkste veroorzakers van UV-absorptie zijn humuszuren als onderdeel van de DOC-fractie. Het is onduidelijk hoe levende bacteriën bijdragen aan de uitdaging die wordt gesteld aan UV-gebaseerde BWMS, behalve dat ze deel uitmaken van de POC-fractie, die reeds zijn eigen minimaal vereiste concentratie in het test water heeft. Daarom moet de relevantie van de eisen voor heterotrofe bacteriële besmetting voor het typegoedkeuringsproces worden onderzocht, aangezien er geen lozingslimieten zijn. Tegelijkertijd moet worden opgemerkt dat het ontbreken van lozingslimieten kan leiden tot een onnatuurlijk hoge bacteriegroei in behandeld ballastwater als bijwerking van het desinfecteren van organismen van ≥50 µm en 10-50 µm. Dit risico moet ook nader worden beoordeeld.

Veel belanghebbenden vragen naar eenvoudige, betrouwbare en snelle methoden om de lozing van behandeld ballastwater te inspecteren. Bij installatie bijvoorbeeld, is een indicatieve inbedrijfstellingstest van behandeld ballastwater verplicht om het BWBS-typegoedkeuringscertificaat te krijgen. Ook kan de bemanning routinematig de prestaties van hun BWBS willen controleren door het behandelde water periodiek te testen. Ten derde hebben PSC-functionarissen toegang nodig tot eenvoudige indicatieve instrumenten om naleving van het Verdrag te kunnen handhaven. Om ballastwater goed te beoordelen, moet de grootte, levensvatbaarheid en concentratie van organismen worden bepaald. De Flow Cytometer-en-CAMERA (FlowCAM) is een FCM-techniek gecombineerd met een fotocamera. Deze is ontworpen om de concentratie en morfologische eigenschappen van deeltjes in water te bepalen, via geavanceerde software die iedere foto automatisch analyseert. In Hoofdstuk 4 werden de prestaties van de FlowCAM geëvalueerd met behulp van kunstmatige microbolletjes, UV-behandelde *Prorocentrum minimum* fytoplankton-culturen en natuurlijk zeewater behandeld door een BWBS op basis van UV licht. Analyse van microbolletjes leverde een hoge nauwkeurigheid en precisie op bij het meten van grootte en concentratie. Tijdens metingen van *P. minimum*, was de geautomatiseerde FlowCAM-analyse in staat om UV-effecten in cel-uiterlijk en celgroei te detecteren. In natuurlijk zeewater maakten de lage concentratie en heterogeniteit van deeltjes echter nog steeds

handmatige analyse van de afbeeldingen noodzakelijk. Om geschikt te worden als indicatief test-instrument moeten enkele eigenschappen van het apparaat worden verbeterd. Ten eerste is de camera-focus te gevoelig voor omgevings-trillingen. Bovendien kwamen luchtbelletjes enerzijds en verstoppingen anderzijds vaak voor tijdens het verwerken van natuurlijke zeewatermonsters. Wat goed functioneert is de mogelijkheid voor snelle overdracht van bestanden en informatie tussen partijen om de integriteit van de metingen te waarborgen. Echter, een van de fundamentele beperkingen van FlowCAM is dat het onmogelijk is om motiliteit te meten van zoöplankton organismen in de fractie van ≥50 µm. Motiliteit is de belangrijkste levensvatbaarheidsindicator voor organismen ≥50 µm wat tot nu toe handmatige detectie met behulp van microscopie vereiste. Opvallend genoeg is recentelijk de BallastWISE ontwikkeld, een ander veelbelovend instrument gebaseerd op een videosysteem. Voor organismen van ≥50 µm gebruikt dit instrument een motiliteitstest en voor organismen van 10-50 µm een motiliteits- plus fluorescentietest. BallastWISE gebruikt een stationaire cel om de beweging en fluorescentie van individuele organismen in stilstaand water te monitoren gedurende een aantal seconden. Bovendien is de detectielimiet voldoende om de Lozingsnorm te bereiken en komen de resultaten goed overeen met traditionele microscopie in natuurlijke watermonsters. Door de gelijktijdige analyse van fluorescentie en motiliteit van 10-50 µm en ≥50 µm organismen, heeft de BallastWISE mogelijk veel van FlowCAM's beperkingen opgelost.

De ballastwater Lozingsnorm van het IMO Verdrag en de USCG zal in de meeste gevallen worden bereikt door installatie van een BWBS aan boord. Om de prestaties van het systeem te monitoren zijn snelle en eenvoudige test-technieken onmisbaar. Hoofdstuk 5 beschrijft of adenosinetrifosfaat (ATP) geschikt is om levende 10-50 µm organismen te kwantificeren rond <10 cellen mL^{-1}. ATP is het essentiële energiemolecuul wat alle levende organismen bevatten. Uit de eerste tests bleek dat commercieel beschikbare ATP-testen niet zonder meer geschikt waren voor ATP-metingen in behandeld ballastwater. Dit kwam door zout-verstoring van de analyse-reactie en gebrek aan specificiteit voor de doelorganismen. Om deze problemen op te lossen, werd een snelle en simpele concentratiemethode ontwikkeld om (1) de gevoeligheid te verhogen, (2) zouten te verwijderen, (3) niet-doelgroep organismen te verwijderen en (4) opgelost ATP te verwijderen. Laboratoriumexperimenten toonden aan dat het zoutgehalte 33 keer werd verminderd en de concentratie-efficiëntie 85% bedroeg. De ATP-methode werd vervolgens getest op natuurlijk zoet- en zout water behandeld door een BWBS op basis van UV. Vergeleken met de alternatieve technieken Fluoresceïne Diacetaat (FDA) en PSII -efficiëntie presteerde de ATP-test beter in het

onderscheiden van behandeld en onbehandeld water. Na verdere verfijningen was de detectielimiet van de ATP-test ruim onder de Lozingsnorm in een fytoplankton cultuur van *Thalassiosira rotula*. In de afgelopen jaren zijn commerciële ATP-instrumenten voor ballastwater controle met succes ontwikkeld en ingezet, zoals de B-QUA Plus (Luminultra). Met name aanvullende monsterverwerking met behulp van microbolletjes om stevige celwanden te breken, draagt bij aan het vrijkomen van intracellulair ATP wat de detectie ten goede komt. Daarom kan de B-QUA Plus-kit ook ATP detecteren in de fractie van ≥50 µm organismen die wordt gedomineerd door roeipootkreeftjes met harde exoskeletten. De microbolletjes breken de exoskeletten af waardoor de ATP vrijkomt voor detectie. De belangrijkste voordelen ten opzichte van op fluorescentie gebaseerde tools, zoals PSII-efficiëntie, is het vermogen om ATP te detecteren van beide grootteklassen van de Lozingsnorm en van de heterotrofe bacteriën. Op fluorescentie gebaseerde technieken daarentegen richten zich uitsluitend op een beperkt deel van de organismen, namelijk de 10-50 µm fytoplanktoncellen. Als ATP-testen correct worden toegepast, is het de verwachting dat ze betrouwbaardere resultaten rapporteren dan op fluorescentie gebaseerde technieken.

Concluderend, het onderzoek dat in dit proefschrift wordt gepresenteerd, heeft bijgedragen aan verschillende belangrijke onderwerpen op het gebied van ballastwaterbehandeling. (1) DDAC werd beoordeeld op zijn (on)geschiktheid voor ballastwaterbehandeling. (2) De toepasbaarheid van HPC werd vergeleken met alternatieve technieken en de bijdrage van heterotrofe bacteriën aan BWBS-typegoedkeuringstests werd besproken. (3) De geschiktheid van de FlowCAM om behandeld ballastwater te analyseren werd onderzocht en er werden belangrijke tekortkomingen vastgesteld. (4) Er werd een eenvoudige, snelle en betrouwbare oplossing voor monsterverwerking ontwikkeld voor ATP-analyse.

Ten slotte worden de volgende aanbevelingen gedaan om dit onderzoek voort te zetten.

1. Let bij het beoordelen van actieve stoffen voor typegoedkeuring op de noodzaak van betrouwbare geautomatiseerde inline sensoren voor doseercontrole en monitoring tijden lozen.
2. Aangezien de Lozingsnorm de heterotrofe bacteriën niet reguleert, moet de impact van levende heterotrofe bacteriën, in verhouding tot dood organisch materiaal (DOC, POC), op de vorming van giftigheid, desinfectiebijproducten, TRO-afbraak en UV-transmissie worden onderzocht. Tegelijkertijd kan het gebrek aan regulering van heterotrofe bacteriën leiden tot een onnatuurlijk hoge bacteriegroei in behandeld

ballastwater als bijwerking van het desinfecteren van organismen van ≥50 µm en 10-50 µm. Deze impact moet ook worden beoordeeld.

3. Aangezien de FlowCAM niet in staat is om gelijktijdig de organismen van ≥50 µm en 10-50 µm te analyseren, noch de beweeglijkheid van organismen kan beoordelen, wordt aanbevolen om door te gaan met de ontwikkeling van alternatieve apparaten voor ballastwater testen.
4. Het wordt aanbevolen om de ATP-concentraties van een grote verscheidenheid aan 10-50-µm-organismen in verschillende stadia van hun groeicyclus tussen 5 en 50 cellen mL^{-1} te meten om een betrouwbaar geslaagd/gefaald ATP-niveau te krijgen voor ballastwater dat wel of niet voldoet aan de Lozingsnorm.

Acknowledgements

Completing this thesis also completes my time at NIOZ, where all my research took place. Therefore, I would like to thank everyone at NIOZ starting from my internships onwards. In 2007, I started as intern for Henk Zemmelink, and vividly remember the great times we had together with Hans Slagter, measuring CO_2 fluxes in the Mokbaai on Texel, at Lutjewad in Groningen and, of course, our stay on the island Griend to install the CO_2-flux tower. Thank you, Henk, for introducing me to NIOZ and offering me this chance to prove myself as a researcher. Thank you, Hans, for the good times we shared back then and later aboard Polarstern. My first encounter with NIOZ apparently was deemed successful, as Hein de Baar offered me the rare privilege to join the ANT XXIV/3 expedition onboard the RV Polarstern to support Rob Middag in measuring dissolved aluminium and manganese in the Southern Ocean and the Weddell Sea. Thank you, Hein, for that unique opportunity. Our adventures over there are too numerous to summarize here. Save to say, I often think back to those days roaming the Southern Ocean and ploughing through the icy Weddell Sea together with Rob, Loes, Patrick, Charly, Claire, Anne-Carlijn, Hans, Steven, Maarten, Ika, Babette, Sven, Michael, Liz, Aurélie, Célia and all those other researchers and crewmembers joining that fateful journey. And those we had to leave behind: Willem Polman and Stefan Winter. To all participants, I'm deeply grateful for the life-changing experiences gained back then.

In late-2008, I became an intern in the NSBWO project headed by Marcel Veldhuis to help Peter Paul Stehouwer investigate the effects of Peraclean Ocean. The ballast water team immediately felt like family. Thank you, Peter Paul for supervising me and for our many lively discussions on ballast water and beyond. After the internship had concluded, I was offered to join the ballast water research team for a year, which ultimately culminated in a PhD-project from 2010-2014. Many adventures come to mind during those years. Joining the conference at the WMU in Malmö and participating in the ICAIS 2010 conference in San Diego I recall as great opportunities for the team to bond and connect with the wider field of ballast water research. To all members of the NSBWO ballast water team, thank you so much for the friendship, support, and good times we shared over the years together, Peter Paul, Viola. Eva, Marieke, Dörte, Felicia, Jaap and Andre. Also, Isabel, Frank, Cato and Etienne, I still remember the good times we had back then. Also, my great appreciation goes out to the NIOZ researchers and technicians, Judith, Harry, Josje, Evaline, Anna, Kirsten and Alma for your friendship and invaluable support to help me conduct the research presented herein.

I had the great privilege to supervise many students for internships on ballast water during my stay at NIOZ. I would like to thank all these students for their enthusiastic work ethic and great conversations we shared at the time. Thank you Biense, Dennis, Peter, Esther

and Valentijn. I would like to highlight the support of two students in particular that uniquely contributed to this thesis. For Chapter 3, the contribution of Merlina Kranenburg was essential to conduct this research. Merlina, thank you for all the hard work in conducting the sampling and data analysis and your tireless search, together with Judith and Harry, for the best bacteria-specific primers for qPCR. For Chapter 5, the contribution of Tom Wijers was essential to complete the research reported herein. Thank you for the good times we shared and our engaging discussions that truly elevated this research to a level I could not have done alone. Lastly, I'm grateful to Leonardo Romero-Martínez for our time together at NIOZ studying the BallastCAM and FlowCAM. Thank you for agreeing to include Chapter 4 into this thesis.

As many of you know, Marcel Veldhuis left NIOZ suddenly in 2011 and sadly passed away in 2019. Between 2008 and 2011, Marcel was my supervisor, co-promotor and mentor. He was the catalyst for starting my career in ballast water for which I'm grateful. I will forever cherish those precious years.

I am deeply grateful to Jan Boon and Louis Peperzak who stepped in to carry the NSBWO project forward in 2011. Louis, thank you for relentlessly pushing me to lift the quality of this thesis to a higher level. Thank you for your endless patience as co-promotor all these years it took me to finally complete this thesis. I couldn't have done it without you. Anita Buma, thank you for your patience as promotor and giving me the opportunity to complete my thesis. I am grateful for your tireless support all these years.

To my mother, brothers and sisters, thank you for believing in me and never doubting I could do this. Priscilla, my love, I couldn't have done this without you. You are the rock that keeps me grounded whenever I start drifting into over-confidence or doubt. Thank you for asking me over and over again: 'How is your PhD thesis going? Is it finished already?" To which I replied: "Don't worry, I'll finish it, but stop reminding me every 6 months, all right." Thank you for helping me find the time to complete this thesis amid our work and raising our beautiful boys, Dylan, Nolan and Bran.

And now finally, this thesis is finished. But the story isn't over. I have the great fortune that my career is still intimately involved in ballast water. I'm glad that to this day, the research presented here is directly relevant for my work at Control Union. I'm looking forward to the next chapter.