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Ballast water treatment system testing

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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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