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.