Effects of various ballast water treatment methods on the survival of phytoplankton and bacteria

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

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Cover pictures by V. Liebich

ISBN: 978-90-367-8807-6

university of groningen

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PhD thesis

to obtain the degree of PhD at the
University of Groningen
on the authority of the
Rector Magnificus Prof. E. Sterken
and in accordance with
the decision by the College of Deans.

This thesis will be defended in public on

Friday 3 June 2016 at 11.00 hours

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1. Introduction

1.1 The problem of invasive species

An invasive species is a non-native species which was transported via a vector and by that experienced a human-mediated introduction outside its normal distribution followed by dominant abundance in the recipient ecosystem (Liebich 2013). To become invasive a non-native species has to survive introduction into the new ecosystem, establish itself and become dominant. This process of becoming invasive is influenced by a number of factors such as the number of introduced individuals with invasive potential and the frequency of introduction events, together referred to as propagule pressure (Colautti et al. 2006, Lawrence and Cordell 2010). The susceptibility of the ecosystem to invasion is referred to as invasibility (Lonsdale 1999) and the ability of species to establish in, spread, and become abundant in a recipient area is referred to as invasiveness (Colautti et al. 2006).

The spread of invasive species can be intentional, such as the introduction of new plant species for agriculture or gardens, or unintentional such as species being transported along with ships' cargo. In the marine environment, these invasive species form one of the greatest threats to biodiversity, next to pollution, habitat destruction and overexploitation. Aquatic invasive species can have varied negative effects; they can cause harm to the ecosystem by outcompeting native species; they can cause economic damage by fouling of hydroelectric power dams and inlets of power plants (Levine 2008); some can even cause harm to human health in case of pathogenic bacteria such as *Vibrio cholerae* (McCarthy and Khambaty 1994, Ruiz et al. 2000, Keesing et al. 2010). Well-known examples of aquatic invasive species include the European zebra mussel (*Dreissena polymorpha*) (Figure 1A) which causes ecosystem change and economic damage by extensive fouling in the North American Great Lakes and rivers (Mackie 1991; Connelly et al. 2007). Another example is the North American comb jelly (*Mnemiopsis leidyi*) (Figure 1B) which is a voracious plankton eater. By consuming large amounts of plankton, including eggs and larvae of fish it caused the population collapse of already overfished planktivorous fish species in the Caspian Sea, Sea of Asov and Black Sea. Because it keeps consuming the plankton and the fish larvae it also prevents the recovery of the fish populations (Ivanov et al. 2000; Shiganova 2002; Shiganova et al. 2001).

Smaller organisms such as phytoplankton can also cause problems; an example from Europe is the diatom *Coscinodiscus wailesii.* Originally from the North Pacific Ocean,it is invasive in the North Atlantic, North Sea and Celtic Sea (Edwards et al. 2001) where it has detrimental effects on fisheries due to mucus production that clogs fishing nets (Mahoney & Steimle 1980, Boalch 1987). Additionally it changes ecosystem functioning since it is inedible to the two common herbivorous copepods (Roy et al. 1989) and displaces native phytoplankton species (Dürselen & Rick 1999). There are also worldwide problems with the increased spread of toxic phytoplankton blooms, such as of the dinoflagellates *Alexandrium catenella* and *Gymnodinium catenatum* which cause paralytic shellfish poisoning in humans (Hallegraeff and Bolch 1991, Hallegraeff 1998, Van Dolah 2000, Hallegraeff 2010) (Figure 2). Aquatic invasive species are sometimes introduced as aquaculture species (~40 %), but the most important vector for the spread of aquatic invasive species is ships' ballast water (~60 %) (Gollasch 2006, Molnar et al. 2008).

Figure 1. Two of the most notorious aquatic invaders, the zebra mussel Dreissena polymorpha (A) and the comb jelly Mnemiopsis leidyi (B) (pictures used under Wikimedia Commons license).

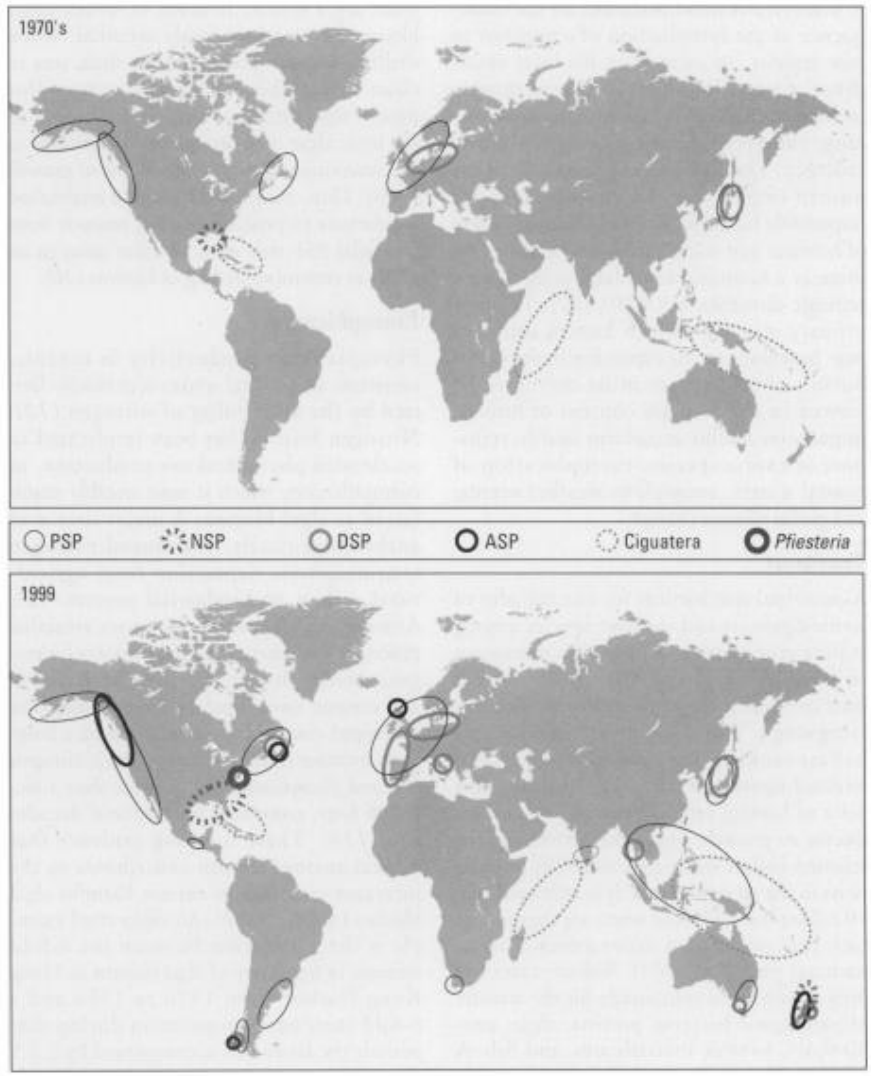

Figure 2. The spread of toxic algal blooms between 1970 and 1999, after Van Dolah (2000). PSP = Paralytic Shellfish Poisoning, NSP = Neurotoxic Shellfish Poisoning, DSP = Diarrhetic Shellfish Poisoning, ASP = Amnesic Shellfish Poisoning.

1.2 The problem of ballast water

Ballast water is the most important vector for the spread of aquatic invasive species, but it is also essential to the operation of ships. By filling or emptying the ballast tanks ships maintain stability, compensating for cargo weight and weather conditions. Shipping is the most important pathway for the worldwide distribution of goods (Figure 3) (Kaluza et al. 2010). The amount of shipped goods has steadily increased since the 1970s, although in recent years this increase has stopped (Figure 4) (UNCTAD 2011). Economic demands lead to the development of larger and faster ships. This means more potentially invasive species are transported and with the shorter transport time they have a higher chance of survival (Carlton 1996). The San Francisco estuary is a good example of this with an exponentially increasing number of invasive species from 1850 to 1990 (Cohen and Carlton 1998). Planktonic organisms are most commonly taken up in ballast water, although benthos and nekton are also sometimes taken up. Since many aquatic organisms have planktonic life stages, ballast water has the potential to spread almost any marine organism. Although a ballast water tank presents a hostile environment to most organisms, some are able to survive the travel (Hallegraeff and Bolch 1991, Hallegraeff 1998, Cordell et al. 2009).

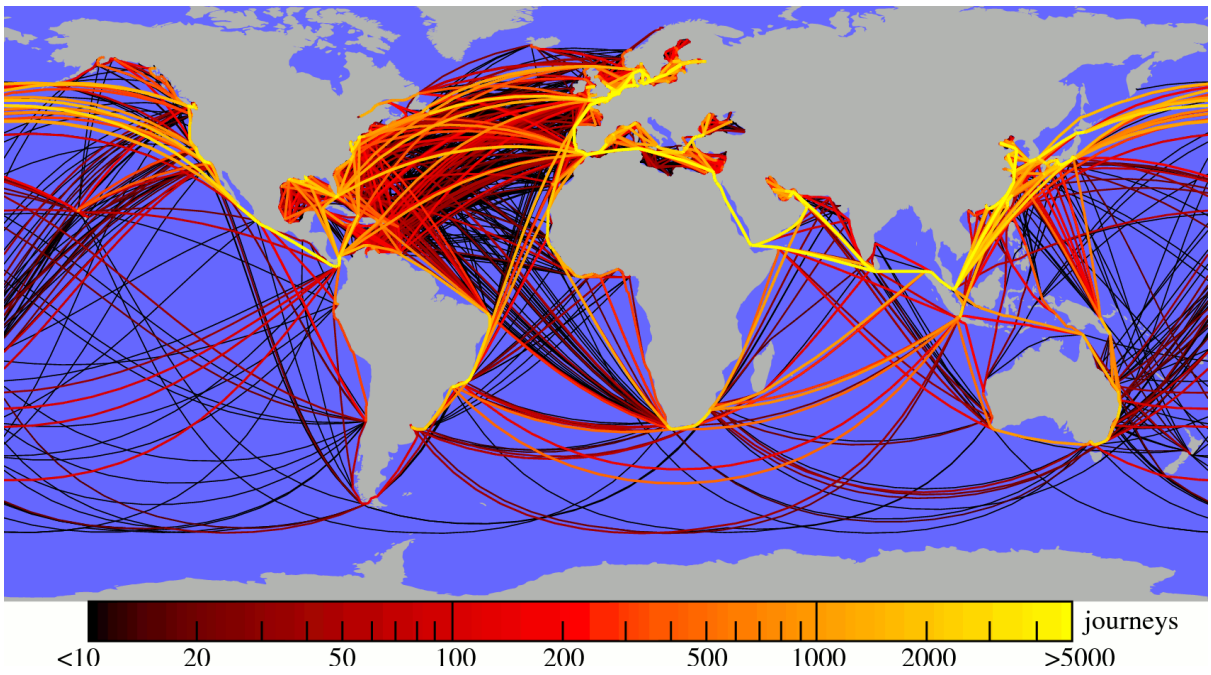

Figure 3. The complex network of global cargo ship movements (Kaluza et al. 2010) and thus ballast water transport.

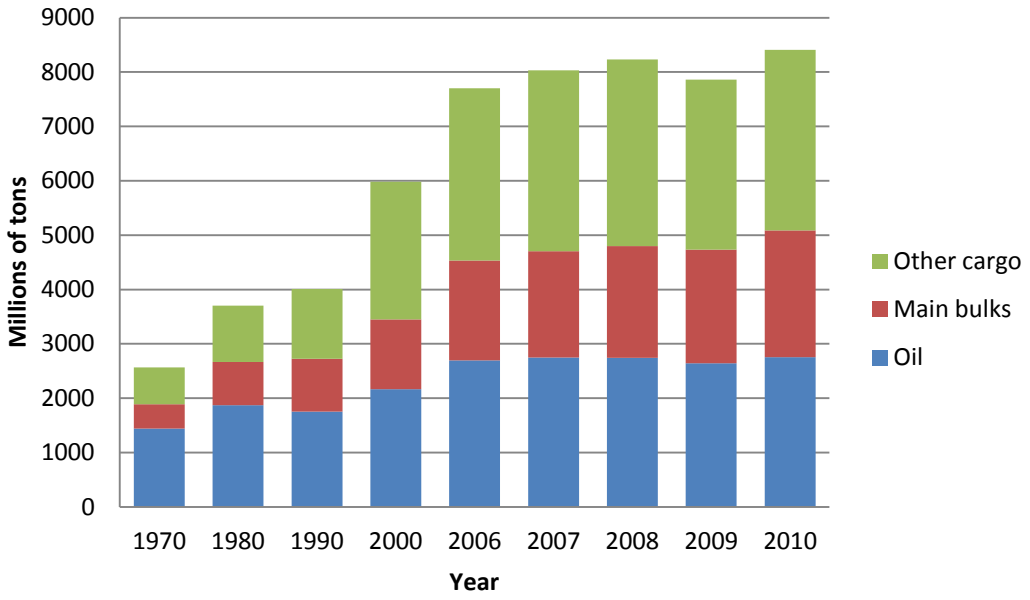

Figure 4. Increase in global shipping. (After UNCTAD review of maritime transport 2011)

1.3 The Ballast Water Convention

The threat of aquatic invasive species was brought to the attention of the International Maritime Organization (IMO) by the USA and Canada in the late 1980's. To this effect the IMO has assembled a list of the ten 'most unwanted' aquatic species. The species and groups mentioned earlier in this chapter, *Mnemiopis leidyi*, *Dreissena polymorpha*, *Vibrio cholerae* and toxic algae, are all on this list. The other six species are *Cercopagis pengoi* (a water flea), *Eiocheir sinensis* (mitten crab), *Neogobius melanostomus* (round goby), *Carcinus maenus* (European green crab), *Undaria pinnatifida* (Asian kelp) and *Asterias amurensis* (North Pacific seastar). Since the main vector for the spread of several of these invaders is ballast water, the IMO adopted the International Convention for the Control and Management of Ships' Ballast Water and Sediments (BWM). This convention was adopted on the 13th of February 2004 and will enter into force 12 months after ratification by 30 States, representing 35% of world merchant shipping tonnage. It has currently (19/03/2016) been ratified by 49 countries, representing 34.82 % of the world's merchant shipping tonnage. Several large shipping countries are postponing the signing of the convention.

Because ballast water treatment technologies needed to be developed, the convention included a transitional period in which vessels need to use ballast water exchange. This is described in regulation D-1, the Ballast Water Exchange Standard. This states that vessels need to have at least 95% volumetric exchange. This standard will be phased out in favour of regulation D-2, the Ballast Water Performance Standard. This describes the abundances of organisms allowed to be in ballast water upon discharge.

Regulation D-2 Ballast Water Performance Standard: Ships conducting ballast water management shall discharge less than 10 viable organisms per cubic metre greater than or equal to 50 micrometres in minimum dimension and less than 10 viable organisms per milliliter 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. The indicator microbes, as a human health standard, include, but are not be limited to:

a. Toxicogenic Vibrio cholerae (O1 and O139) with less than 1 colony forming unit (cfu) per 100 milliliters or less than 1 cfu per 1 gram (wet weight) zooplankton samples;
b. Escherichia coli less than 250 cfu per 100 milliliters;
c. Intestinal Enterococci less than 100 cfu per 100 milliliters.

The IMO regulations further define viable as: '*Viable Organisms are organisms and any life stages thereof that are living.*' Both the D-2 standard and the definition of viability are the subject of some controversy, but they represent a political compromise (Gollasch et al. 2007).

In order to meet this standard ships require a treatment system to reduce the amount of organisms in their discharged ballast water.

1.4 Ballast Water Treatment Systems

To meet the Ballast Water Performance Standard the development of ballast water treatment systems (BWTSs) was started right after the adoption of the IMO convention in 2004. These treatment systems are based on a variety of techniques. Some use physical methods, such as filtration, hydrocyclones, ultra-sound, heating or ultra-violet (UV) radiation. But also chemical methods are common such as the addition of hypochlorite by electrolytic chlorination, chlorine dioxide, ozone and a variety of other chemicals that are collectively known as "active substances" (Gregg et al. 2009, Tsolaki and Diamadopoulos 2009). Most BWTSs use a combination of methods to achieve an optimal treatment and filtration is almost always a part of this combination. Since filtration removes mostly the larger (≥ 50µm) zooplankton fraction, the focus of this thesis is on the smaller fractions: phytoplankton and bacteria. The term phytoplankton in this thesis includes all photosynthetic organisms, also the cyanobacteria. The term bacteria refers to heterotrophic bacteria, as well as archaea since the methods used do not distinguish between these two.

The development of a BWTS is difficult because many aspects need to be taken into account. The system needs to be small, because space on ships is limited. It needs to be simple; the crew needs to be able to operate it without special training. It needs to be affordable; ship owners will shop around for the best price. It needs to have reasonable running costs: systems that have a large power demand or that need large amounts of chemicals are not very economical. On the other hand, the system also needs to be robust and it has to meet the standards set by the IMO while not having any environmental effects upon discharge.

BWTSs have to be tested for compliance with the Ballast Water Performance Standard D-2. This has to be done according to the *Guidelines for the Approval of Ballast Water Management Systems* (G8, IMO 2008a) and the *Procedure for Approval of Ballast Water Management Systems that make use of Active Substances* (G9, IMO 2008b). The guidelines provide information on which variables should be measured and over which time periods tests should be performed. Land-based tests according to the IMO guidelines were performed at the harbour of the Royal Netherlands Institute for Sea Research (NIOZ) on Texel (Figure 5). These tests following IMO guidelines are the first comprehensive comparison of several ballast water disinfection methods, and are the basis of the present thesis.

Figure 5. Example of a ballast water treatment system inside a 20 foot container, tested at the NIOZ harbour. (Photo: P. P. Stehouwer)

A standard test according to IMO guidelines starts with the intake of ballast water. For the control this water was sent straight into the simulated ballast tank. For the treated samples the water was passed through all the steps of the BWTS being tested before being stored in the simulated ballast tank. To simulate a ships' journey the water was then kept in the simulated ballast tank for five days. After this five day period the water was discharged. For some BWTSs the water was discharged without additional treatment. Some systems with active substances added a neutralizing agent upon discharge. BWTSs that make use of ultraviolet (UV) radiation usually performed a second UV treatment during discharge. Filtration during discharge was not performed by any of the systems tested at NIOZ. Organism abundances in the discharge water were measured using various methods.

As part of this thesis additional experiments were performed which were not required by the IMO guidelines. These experiments were done to address the following questions:

1. Can phytoplankton and bacteria re-grow after ballast water treatment? And if yes, which species are re-growing?
2. Is there a difference in performance between different types of ballast water treatment systems?
3. Is there a possibility for adverse environmental effects due to ballast water treatment which is not covered by the IMO guidelines?

How these questions were tackled in the present thesis is explained in the following sections.

1.5 Incubation experiments as a tool for ballast water compliance testing

Question 1a. Can organisms re-grow after ballast water treatment? While testing according to the IMO guidelines gives a good impression of the immediate effects of ballast water treatment, the required five day holding time in a (simulated) ballast water tank does not offer much time for the

recovery of organisms. The circumstances in a ballast water tank are also not conducive for growth of especially phytoplankton. The most important point however is that after discharge recovery cannot be monitored although surviving organisms in this discharged water would still have the opportunity to be introduced in new environments and become invasive. Chapter 2 presents an incubation experiment (Figure 6) designed to simulate favourable conditions (using suitable conditions for the majority of the local plankton) for growth of surviving organisms after discharge of ballast water by BWTSs using UV radiation or active substances. In these incubations, the abundances of phytoplankton an bacteria were monitored for 20 days to study re-growth and species composition.

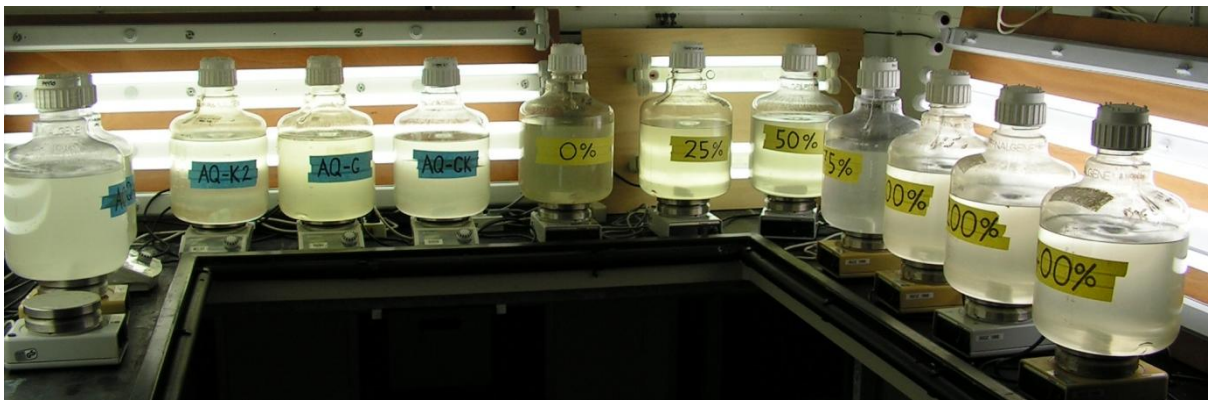

Figure 6. General set-up of the incubation experiment: 10 L bottles on magnetic stirrers in front of lights in a climate-controlled room. (Photo: P. P. Stehouwer)

1.6 Comparison of methods to enumerate and identify re-growing phytoplankton

Question 1b. Which species re-grow after ballast water treatment? Chapter 3 specifically focussed on the re-growth of phytoplankton in one ballast water treatment system using filtration and UV radiation. Microscopy was applied to identify if there was a difference in phytoplankton community structure after treatment with UV radiation. Therefore, the question was addressed if certain species of phytoplankton were more resistant to UV radiation than others, making it more likely that they would survive the treatment. In Chapter 4 various phytoplankton identification techniques were compared: flow cytometry, light microscopy (Figure 7) and denaturating gradient gel electrophoresis (DGGE) followed by sequencing , in order to assess their potential to enumerate the re-growing phytoplankton, to monitor changes in phytoplankton community structure and to identify surviving and re-growing phytoplankton species.

Figure 7. Re-growing phytoplankton after ballast water treatment: Pseudo-nitzschia sp. (A), Skeletonema sp. (A), Nitzschia sp. (A), Thalassiosira sp. (B). (Photo's: V. Liebich)

1.7 Comparison of ballast water treatment by UV radiation and active substances

Question 2. Is there a difference in performance and re-growing species between different types of ballast water treatment system? Different ballast water treatment systems have been developed, but commonly either filtration followed by UV radiation, or filtration followed by electrolytic chlorination are used. These two types of systems are very different from each other; they both have their own set of advantages and disadvantages. Chapter 5 summarizes results from incubation experiments of six different ballast water treatment systems which were tested at NIOZ. Three of these systems were based on UV radiation; two made use of electrolytic chlorination and one used chlorine dioxide. As a result the specific question addressed were: Are there differences in performance between UV radiation ballast water treatment and chlorine-based ballast water treatment and if so, what are these differences? Are there differences in performance between ballast water treatment systems using the same treatment technique? Data from re-growth experiments of all six systems were compared to answer these questions.

1.8 Testing for environmental effects of ballast water treatment using active substances

Question 3. Is there a possibility for adverse environmental effects due to ballast water treatment which is not covered by the IMO guidelines? Some ballast water treatment systems make use of active substances to deactivate organisms. Many BWTSs add a neutralizing agent on discharge to make sure that in turn the chemicals are deactivated on discharge. Others use active substances which are short-lived, or use low concentrations so the concentrations are negligible at discharge. However, even with neutralization or short-lived active substances, the water chemistry will be changed. The chemical mixture Peraclean® Ocean is different from most other active substances because it is used in much higher concentrations (150 mg/L, versus 5 - 15 mg/L) and it has residual by-products (acetate and phosphate) which can affect water quality and aquatic organisms. De Lafontaine et al. (2008) already expressed concerns on the effects of this chemical mixture, especially in low temperature waters where breakdown is slow. Therefore, the following specific questions were formulated: What are the long-term effects of Peraclean® Ocean addition to ballast water? What is the effect on the microbial community of the residual by-product acetate, particularly at lower temperatures? These concerns were addressed in Chapter 6 with an experiment set up to monitor the degradation of acetate at different temperatures and its impacts on the composition of the microbial community.

The various types of BWTS, their manufacturers and technical specifications are known with the author P.P. Stehouwer.

The large datasets that are the basis of this thesis have been archived at the NIOZ data centre.

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2. A novel approach to determine ballast water vitality and viability after treatment

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Published in: Emerging Ballast Water Management Systems – Proceedings of the IMO-WMU Research and Development Forum

Abstract

The spread of invasive species through ballast water is a major threat to the world's oceans. For that reason the International Maritime Organisation (IMO) has set rules for ballast water treatment. In response, many companies have developed ballast water treatment systems (BWTSs). Different techniques are used to reduce the numerical abundance of organisms; UV radiation, active substances, etc. To accurately measure the efficacy of different BWTSs, methods have to be developed that are applicable to each of the treatments. Two specific points are addressed in this paper. The first is if no re-growth of organisms is observed during the IMO tests, is re-growth on a longer time-scale possible? The second concerns the delayed effect of UV disinfection, does this lead to an underestimation of its performance? To answer these questions a set of incubation experiments was developed. Treated water is incubated up to three weeks under favorable conditions to stimulate growth of micro-organisms that survived the treatment. The timing of re-growth differed strongly, sometimes even within the same BWTS. The data from these incubations also allowed for calculating the estimated minimum number of organisms from the slope of survival, providing an accurate estimate of the number of organisms even when numbers were below the detection limit. These examples show that the incubation experiment is a useful method to get an accurate view of ballast water vitality and viability.

Key words: IMO, ballast water treatment, incubation, re-growth, micro-organisms, estimated minimum number

1. Introduction

The steady increase in size and speed of ships has led to more ballast water being transported and shorter holding times of ballast water (Carlton 1996). Ballast water contains organisms from the intake location. Ballast water therefore results in transport of non-indigenous organisms to other regions. More ballast water implies more organisms and shorter holding times mean more chance of surviving transit to the discharge location. When organisms survive and become a dominant species, they are recognised as invasive species. Invasive species can cause large ecological and economical damage (Ruiz et al. 1997, Pimentel et al. 2000). Some species can even form a threat to human health.

Because of the damage caused by invasive species the International Maritime Organization (IMO) adopted the Ballast Water Management Convention. The Convention Regulations specify the D-2 standard, which specifies the amount of organisms allowed to be present in ballast water upon discharge (Anonymous, 2008). One of the limits set in the D-2 standard is for organisms in the size

class between 10 and 50 micron; less than 10 viable organisms per mL of that size class are allowed to be in the ballast water on discharge. To meet these standards, different Ballast Water Treatment Systems (BWTSs) are developed and need to be tested according to IMO requirements. Different techniques are used for BWTSs, usually based on a mechanical step (filter, hydrocyclone) and a disinfection step (UV-radiation, active substances, heat, etc (Gregg et al. 2009)).

For land-based tests, the IMO requirements state that ballast water must be stored for five consecutive days in holding tanks (simulated ballast tanks) and sampled on intake (T0) and discharge (T5). This method of testing fails to answer some important questions. The first question concerns re-growth potential of the discharged ballast water. If no organisms can be detected on discharge, is this because there are no organisms left or are they only reduced to below detectable levels? Phytoplankton is especially difficult in this respect, capable of making cysts (resting stages) which can survive periods of physical stress (Hallegraeff 1998, Gregg and Hallegraeff 2007). Cysts are completely inactive and therefore almost impossible to detect, but when conditions improve they reactivate. The second question concerns systems with a delayed effect. Some BWTSs (UV-radiation based systems for example) have a delayed effect in their treatment, organisms are not dead immediately after treatment, but samples to determine the number of viable organisms are taken immediately after treatment. Are UV systems at a disadvantage because of this? To answer these questions, an incubation experiment was developed. In the incubation experiment samples from the holding tanks are incubated under favorable conditions and samples for determining vitality and viability of phytoplankton are taken daily for a period of up to 25 days.

2. Materials and Methods

Land-based testing. Water from the harbour of the Royal Netherlands Institute for Sea Research (NIOZ, Texel, The Netherlands) was pumped up through the treatment system (200 m^3/h) and stored in an underground holding tank. Control water was pumped straight into a separate holding tank, by-passing the treatment systems. Water was stored in the tanks for five consecutive days before being discharged. Depending on the treatment system, water was also treated on discharge. The two types of system tested are filter with UV-disinfection and filter with active substance disinfection.

Incubation experiment. Incubation samples were taken during land-based testing. Samples were collected in 10 L Nalgene bottles at both uptake (T0) and discharge (T5). Samples are transported to a climate-controlled room. This room is kept at a stable temperature of 15 ˚C (+/- 2˚C) and a 16:8 hour light/dark period is used. Bottles were placed on magnetic stirrers, which maintained the water movement (130 rotations/min.) that marine plankton is used to. Nutrients were added at concentrations typical for the Wadden Sea during winter (PO_4 1,6 µmol/L, NO_3 20 µmol/L, SiO_3 20 µmol/L).

For every BWTS two long-term incubation experiments of up to 25 days were done where samples were taken daily. Samples were taken for phytoplankton abundance and viability. Phytoplankton is quantified by flow cytometry (Coulter Epics XL-MCL with a 488 nm argon laser). Samples were measured in triplicate, using red fluorescence to differentiate between phytoplankton and other particles. Phytoplankton viability, in terms of photosynthetic efficiency, is measured using Pulse

Amplitude Modulated (PAM) fluorometry (Water-PAM, Walz GmbH). Phytoplankton viability is expressed as a number between 0 and 1:

- ≥ 0.5: a healthy phytoplankton population
- 0.3 < r < 0.5: a phytoplankton population which is not under optimal conditions
- 0.1 < r < 0.3: a phytoplankton population which is dying
- ≤ 0.1: phytoplankton population is considered to be dead

3. Results

3.1 Incubation experiment

After five days of incubation, the amount of phytoplankton decreased in the control holding tanks, while the amount of phytoplankton increased in the control incubation (Figure 1). PAM viability values support this. At T0 phytoplankton viability values were usually between 0.51 and 0.66, after five days in the control holding tanks phytoplankton viability values were usually between 0.11 and 0.31 while phytoplankton viability values after five days in the control incubation were usually between 0.44 and 0.64. Figure 1 also shows that treated incubation samples had lower numbers of phytoplankton than control incubations. Treated holding tank samples and treated incubation samples did not show a clear pattern for phytoplankton abundance. Phytoplankton viability was similar after five days in both treated incubation and treated holding tank, both generally had values between 0,08 and 0.

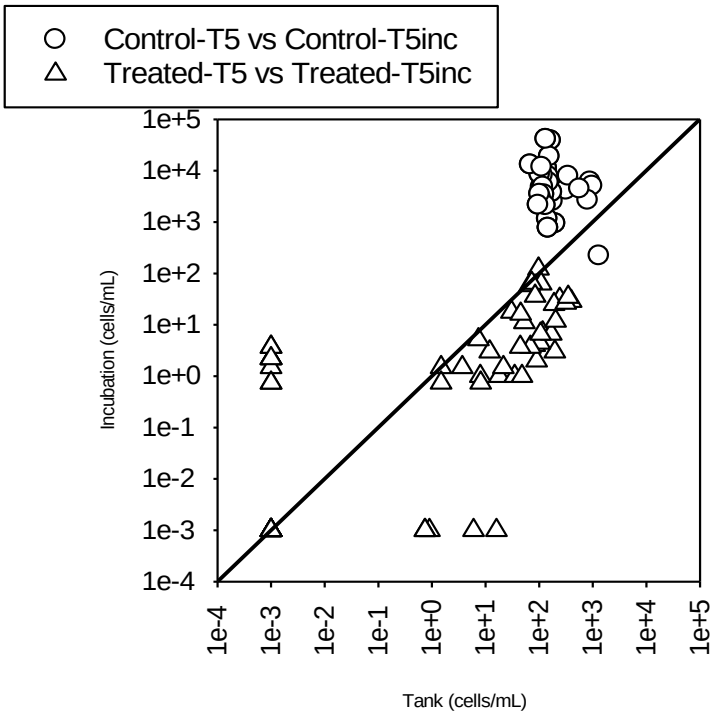

Figure 1. Phytoplankton abundance in incubated samples (vertical axis) plotted versus phytoplankton abundance in samples from the holding tanks (horizontal axis) for both control water (circle) and treated water (triangle).

3.2 Long-term incubation experiment

Active substance. Long-term incubation samples treated with an active substance showed re-growth of phytoplankton, but this may take a considerable period (Figure 2). Incidentally, for the same system (Ecochlor®, using chlorine dioxide), there were also samples (not shown here) where no re-growth of phytoplankton was found during the whole sampling period of 20 days.

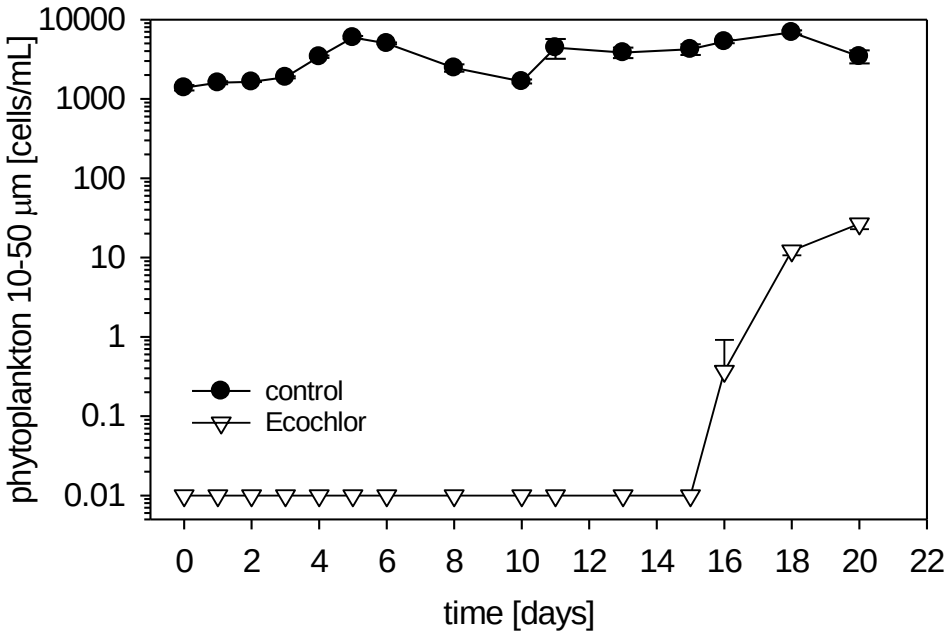

Figure 2. Phytoplankton abundance in a control long-term incubation and a long-term incubation treated with an active substance.

UV irradiation. Phytoplankton abundance showed a gradual decrease after UV treatment (Figure 3). Both single treated UV intake samples and twice treated UV discharge samples show this pattern. However the initial phytoplankton abundance is lower for UV discharge (at day T=5) than for UV intake (at day T=0). Phytoplankton abundance also decreases further for UV discharge than for UV intake (Figure 3). In both cases re-growth started around 7 or 8 days after start of the long-term incubation.

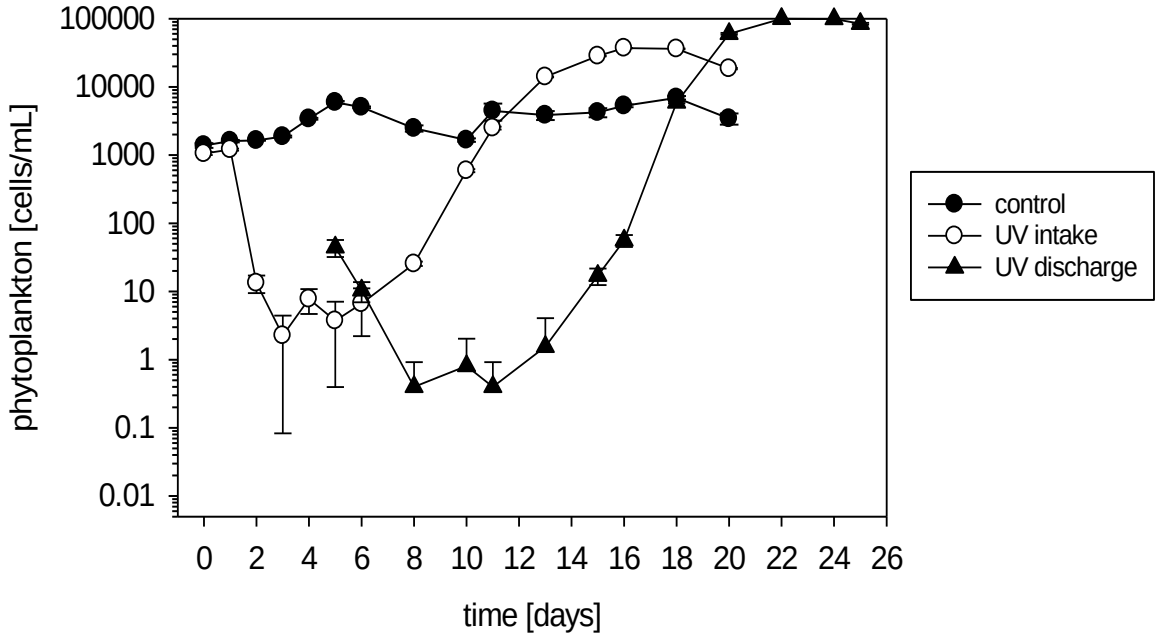

Figure 3. Phytoplankton abundance in a control, intake UV (UV treated once) and discharge UV (UV treated twice) long-term incubations. UV discharge samples were collected on T5 (discharge day), incubation and sampling for this experiment thus started on T5.

Estimated minimum number. In order to quantify the delayed effect seen in UV treatments, an extra experiment was performed using different UV intensities and an intensive sampling regime. This experiment showed that with a higher UV dose (400 %) phytoplankton abundance decreases faster than the normal (100%) dose (Figure 4). A lower UV dose (25%) showed an even slower decrease in phytoplankton abundance. From the slope of decrease (i.e. survival rate) in Figure 4 an estimated minimum number of phytoplankton can be calculated. This is an established method in cancer research and uses the formula: $y = a * e(bx)$. Where a is the initial value at T0 and b is the factor with which y changes. Using this formula the exact time can be calculated at which the number of phytoplankton is below 10 per mL. As shown in Table 1, this time differs considerably when comparing the UV doses used in this experiment.

Table 1. Estimated time before the number of phytoplankton drops below 10 per mL after treatment with different UV doses. 0 % UV shows a negative number, this means that the phytoplankton were actually growing.

UV dose (%)	0	25	100	400
Time (days)	-0.970	16.080	1.694	0.886

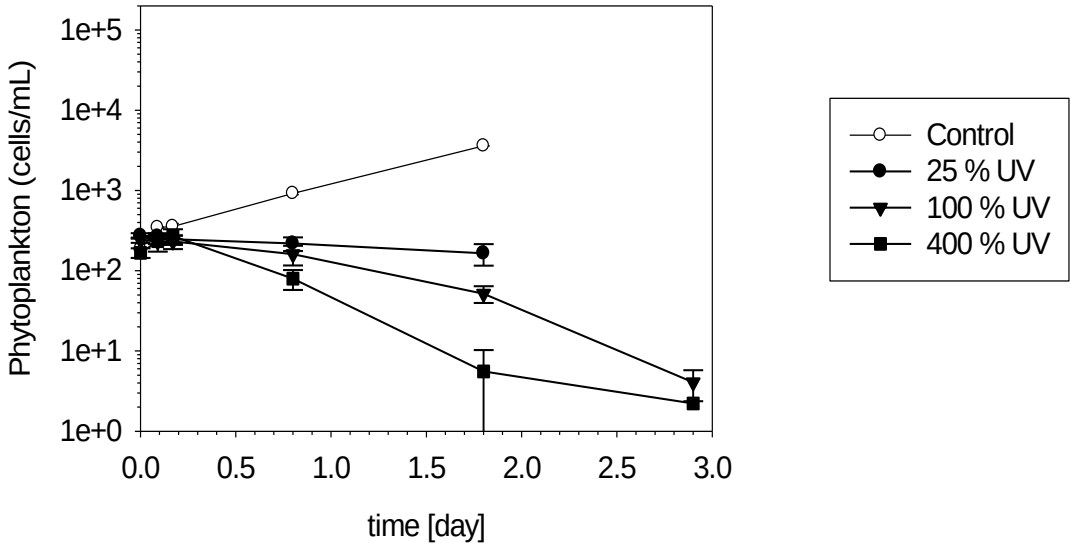

Figure 4. Phytoplankton abundance in samples subjected to different UV-intensities. UV-intensities expressed as % of normal treatment dose (exact dose not shared by manufacturer).

4. Discussion and Conclusion

Treatment of ballast water by BWTSs results in different vitality and viability of phytoplankton. BWTSs always reduce phytoplankton vitality and viability, however differences can be found when comparing different treatment systems. After treatment with an active substance, re-growth of phytoplankton can occur in ballast water. However, the timeperiod differs before re-growth occurs within one BWTS and when comparing several BWTSs (Figure 2). During some experiments no re-growth occurred. This re-growth might depend on the survival rate of phytoplankton cysts in the water, which can not be confirmed by flow cytometry. The species present in the water are also important, since cysts of some phytoplankton species are much more resistant to active substances or UV-radiation than those of other species (Gregg and Hallegraeff 2007). Phytoplankton are also known to have repair mechanisms that allow them to recover from damage. Further analysis is needed to confirm these assumptions.

Treatment with UV-radiation also resulted in a decreased abundance of phytoplankton, however a delayed effect was found. While treatment with active substance results in an immediate decrease in phytoplankton abundance, after UV treatment phytoplankton abundance shows a gradual decrease. Even the highest UV dose needs almost one day to meet the requirement of less than 10 cells per mL. Waite et al. (2007) observed a similar effect using the amount of chlorophyll *a* as indicator of phytoplankton survival. Immediately after UV treatment there were still detectable levels of chlorophyll *a*. The IMO requires the number of organisms in the size class between 10 and 50 micron (phytoplankton) to be below 10 per mL. The five-day storage period in ballast tanks provides sufficient time for the delayed effects of the first UV-treatment to occur, but values can be close to the IMO limit (Figure 3). The second UV-treatment further reduces phytoplankton abundance (Figure 3) and thus allows the UV-based BWTSs to meet the IMO requirements.

The incubation experiments, especially the long-term incubation experiment, provide data on phytoplankton survival and re-growth. This data can be compared with data from the holding tanks to gain a better understanding of how various BWTSs affect phytoplankton viability and vitality. Data from the incubation experiments can also be used to calculate the estimated minimum number of phytoplankton per mL of treated ballast water, even if those numbers are below detection limits of flow cytometry and PAM fluorometry. It is therefore recommended to include incubation experiments in BWTS tests.

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3. Re-growth of potential invasive phytoplankton following UV-based ballast water treatment

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Published in: Aquatic Invasions, Volume 7, pp 29-36

Abstract

Ballast water contains organisms which can survive the ship's journey and become established in the recipient water body when discharged. Phytoplankton species can become invasive and might be harmful by producing toxins or by leading to anoxic conditions following their blooms. Different technologies exist to treat ballast water in order to reduce the spread of invasive species. The effectiveness of a UV-based ballast water treatment system was tested in an incubation experiment over 20 days. After an initial decline in cell numbers, re-growth could be observed of certain phytoplankton taxa, namely the diatoms Thalassiosira, Skeletonema, Chaetoceros, Pseudo-nitzschia, and Nitzschia (order represents rank of abundance). The conclusion of this study is that a variety of taxa are able to survive UV-treatment. These may include harmful and potential invasive phytoplankton species. Long-term incubation experiments should be considered when testing the effectiveness of UV-based treatment systems. The dominant re-growing phytoplankton group was Thalassiosira which could be a suitable indicator organism for testing the efficiency of UV-units.

Keywords: UV-treatment, bioinvasion, *Thalassiosira*, *Skeletonema*, *Chaetoceros*, HAB

Introduction

Organisms are transported via the ballast water of ships (Carlton and Geller 1993;Williams et al. 1988). When non-indigenous species are released at the port of destination, they may become established in the recipient ecosystem and spread (Kolar and Lodge 2001). These invasive species can pose a risk to biodiversity (McGeoch et al. 2010) and, in some cases, also to human health (Ruiz et al. 2000). Presently, different methods exist to treat ballast water (Tsolaki and Diamadopoulos 2010) to reduce numbers of contained organisms in accordance with the Ballast Water Convention adopted by the International Maritime Organization (IMO) (IMO 2004). The convention includes requirements (D-2 standard) which refer to the discharge of certain concentrations and size classes of organisms. To reduce numbers of viable organisms in ballast water, one option is the use of certain wavelengths of ultraviolet light (UV-C). UV-radiation penetrates through cell membranes of organisms and damages deoxyribonucleic acids (Quek and Hu 2008). For this reason, UV-treatment is commonly used for disinfection of drinking water (Choi and Choi 2010). The lethal UV-dose is an important issue of research as phytoplankton and bacteria are able to recover. The marine diatom Cyclotella sp. for instance was able to repair the DNA damage caused by UV-B radiation within hours (Gieskes and Buma 1997). Even when UV-treatment (UV-C) reduced the viable count of microorganisms, remaining bacteria were able to grow again (Waite et al. 2003).

The effectiveness of UV-dosages depends largely on the organism, its size and pigments (Gregg et al. 2009). Potential survival and re-growth of (harmful) organisms after treatment should be considered when examining the effectiveness and efficiency of ballast water treatment systems (BWTSs), although this is not a standard requirement of IMO's guidelines for approval of Ballast Water Management Systems G8 (Anonymous 2008). However, only a few re-growth studies have been conducted so far. For example, Stehouwer et al. (2010) showed that after using different dosages of UV-radiation, several unidentified phytoplankton groups did survive UV-treatment and re-grew in long-term incubation experiments. However, no further taxa specification of re-growers was given.

The present study aimed at examining survival and re-growth of phytoplankton after UV-treatment in long-term incubation experiments over 20 days. Flow cytometry was applied to examine timing of re-growth and to indicate numbers and size of cells. Specifically, it was the aim to identify phytoplankton genera and species by using light microscopy. Special focus was drawn on diatoms due to their high ecological relevance as a major group of the phytoplankton, the presence of some invasive and harmful species (Nehring 1998), their ability to survive several weeks in the dark (Peters 1996), and the formation of resting stages (Sugie and Kuma 2008). Several studies confirm that diatoms are commonly found in ballast water (Olenin et al. 2000;McCarthy and Crowder 2000).

Re-growth after UV-treatment may occur related to quantitative or qualitative causes. Quantitative causes include a better chance of re-growth based on more surviving individuals of species with initial high numbers. Qualitative causes include physiological cell properties which support survival and re-growth. A comparison between species that survive and re-grow and those that do not may reveal especially UV-resistant species. These species could then be considered as indicator organisms for testing the effectiveness of UV-treatment. So far, a large diversity of phytoplankton organisms has been used (Tsolaki and Diamadopoulos 2010). Using different phytoplankton species makes comparison and compliance control complicated as differences in sensitivity to UV-dosage might affect test results. A standard phytoplankton species would therefore simplify the testing of UV-based BWTSs.

Phytoplankton species which are more resistant to UV-treatment and are faster to recover (repair potential damage) could re-grow and become invasive in their new environment after discharge. It is of special interest to examine the re-growth potential of harmful or invasive microalgae. To specify these re-growers and their functional aspects is essential for risk assessment and mitigation strategies. The identification of the re-growing phytoplankton groups is also crucial to determine effectiveness and efficiency of UV-treatment. For UV-units it might be more efficient to reduce the intensity if the required reduction of organism abundance is already achieved with lower dosages.

Methods

Ballast water treatment tests were conducted at the harbor of the Royal Netherlands Institute for Sea Research (NIOZ, Texel, The Netherlands). For further information on this land-based test facility for BWTSs see Veldhuis et al. (2006). The treatment system in the present study used a 20 µm mesh-size filter and low-pressure UV-radiation (fixed wavelength of 254 nm). Water from the Wadden Sea (a turbid estuary) was filtered and processed with UV-radiation at intake (ballasting) and discharge (deballasting). In between, the water was stored in holding tanks for five days simulating conditions

during a ship journey. Tanks had a size of 300 m^3 and were either located underground or at the surface. The temperature difference between the tanks was negligible (unpublished data). Experiments were conducted based on normal scheduled test runs according to the G8 guidelines (Anonymous 2008). They were carried out in duplicate resulting in two tanks (I & II). After filling tank I with treated water, the system was shut down and pipes were emptied. Then a control tank was filled and after another short shutdown to empty the pipes, water was treated and pumped into tank II. For both replicate tanks, the water was separately treated. Subsequently, for long-term incubations in pre-cleaned transparent polycarbonate bottles (Nalgene, Rochester, USA) of 10 L volume each, samples were taken from the water in the large tanks. The first incubation experiment started 1st of April 2010 and the second one 13th of May 2010, the latter with two bottles for each tank. For the control, harbor water was pumped (200 m^3/h) into a holding tank without passing through the treatment system. At day zero of the intake series, water was pumped up, filtered by the system and processed with UV-radiation. The water was treated a second time after five days which is day zero of the discharge series. Each series was incubated for 20 days. Samples were collected from the control C, from the duplicates Intake I (filter+UV) and Intake II (filter+UV) and from the other set of duplicates Discharge I (filter+UV+UV) and Discharge II (filter+UV+UV).

The samples were kept in a climate-controlled room with a temperature of 15 ˚C (+/- 2˚C) and a 16:8 hour light/dark period, similar to local, natural growth conditions. The bottles were placed on magnetic stirrers, which maintained gentle water movement to prevent the phytoplankton from settling. Nutrients were added at concentrations, which are typical for the Wadden Sea in early spring (PO_4 1,6 µmol/L, NO_3 20 µmol/L, SiO_3 20 µmol/L). Samples were taken daily for analyzing phytoplankton abundance and composition. Phytoplankton was quantified by flow cytometry (Coulter Epics XL-MCL with a 488 nm argon laser, Miami, USA). The flow cytometer measures various properties of individual cells including size and chlorophyll fluorescence (Veldhuis and Kraay 2004). Samples of one milliliter were measured in triplicate, using the red autofluorescence of the chlorophyll signal to differentiate between phytoplankton and other particles. Samples for species identification (Hoppenrath et al. 2009) were examined using an inverted light microscope (Zeiss Axiovert, 400x, Oberkochen, Germany). These samples had a volume of five milliliters, they were well-mixed, and the measurement was done immediately after sample collection and hence without any addition of preservative. All cells and particles in these samples were allowed to settle for at least 30 minutes.

Results

Flow cytometry:

In all cases the UV treatment (intake) or treatments (intake and discharge) did lead to a significant decrease of phytoplankton cell numbers (figure 8). The decline in total cell numbers occurred during the first week of the treated intake and discharge samples of both replicate tanks in April as well as in May. Re-growth, indicated by an increase of cell numbers, occurred comparably in all incubation bottles after day seven. The numerical trend over the first two weeks is comparable for all replicates in both experiments. In May's discharge samples, numbers in different bottles range in extreme

cases from 17200 cells per milliliter after three weeks in tank I bottle one to 300 cells per milliliter after three weeks in tank II bottle two, but in the series themselves the overall trend (first decline and re-growth after seven days) was again comparable. In both experiments, phytoplankton cell numbers in the control samples were considerably different from the treated samples.

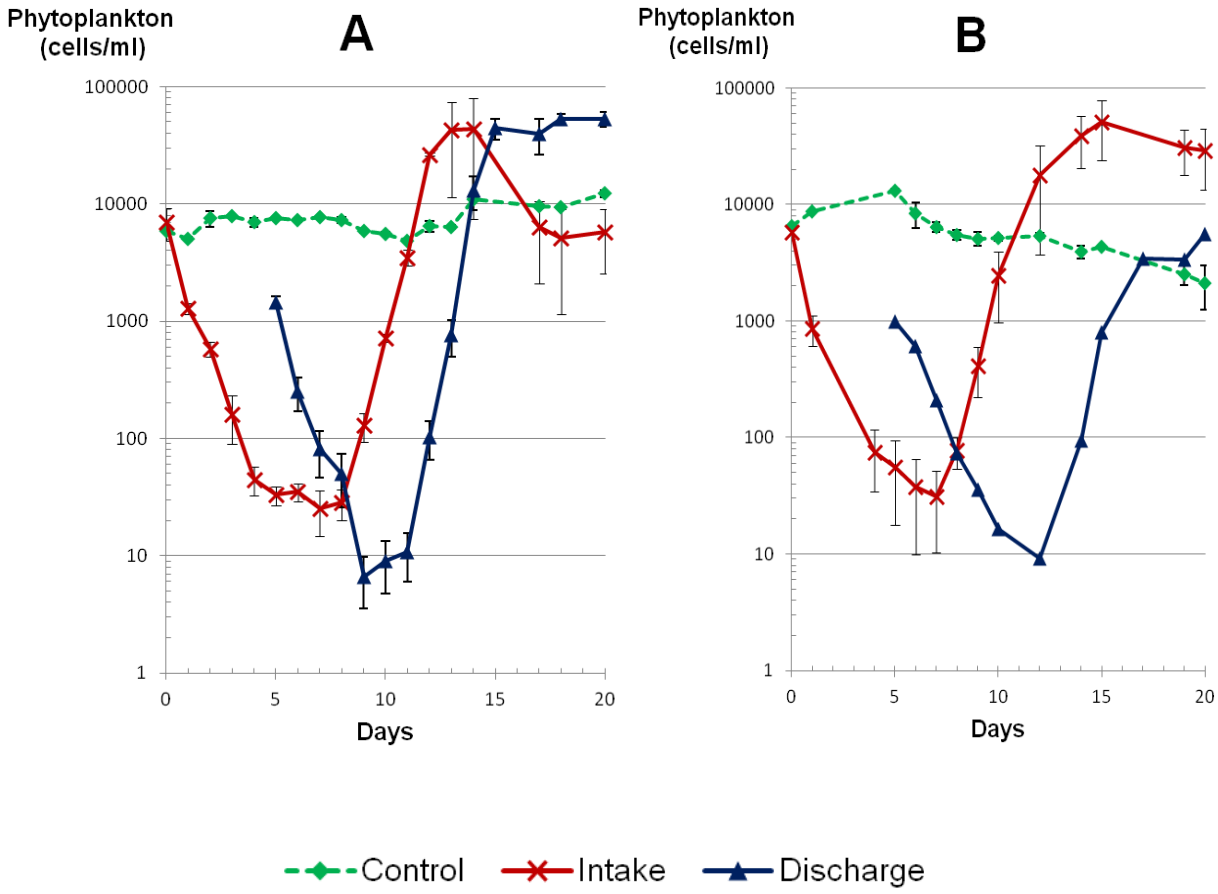

Figure 8. Phytoplankton cell abundances after UV-treatment only at intake (day 0) as well as after treatment at both intake and discharge (day 5), analyzed by flow cytometry. Incubation experiment one was performed in April (A) and experiment two in May (B). Data points show mean of incubation samples, error bars indicate standard deviation, no error bars are given for the discharge treated samples in May (B) due to distinct numerical differences (see text).

Light microscopy:

In April, *Thalassiosira* was the most abundant phytoplankton group in the control sample; additional phytoplankton included the diatoms: *Asterionellopsis*, *Chaetoceros*, *Coscinodiscus*, *Ditylum*, *Guinardia*, *Nitzschia*, *Pseudo-nitzschia*, and *Skeletonema* (figure 9). The control sample of May contained the above mentioned taxa as well as *Mediopyxis*, *Odontella*, and *Phaeocystis*. In May's control sample, *Mediopyxis* was the most abundant species.

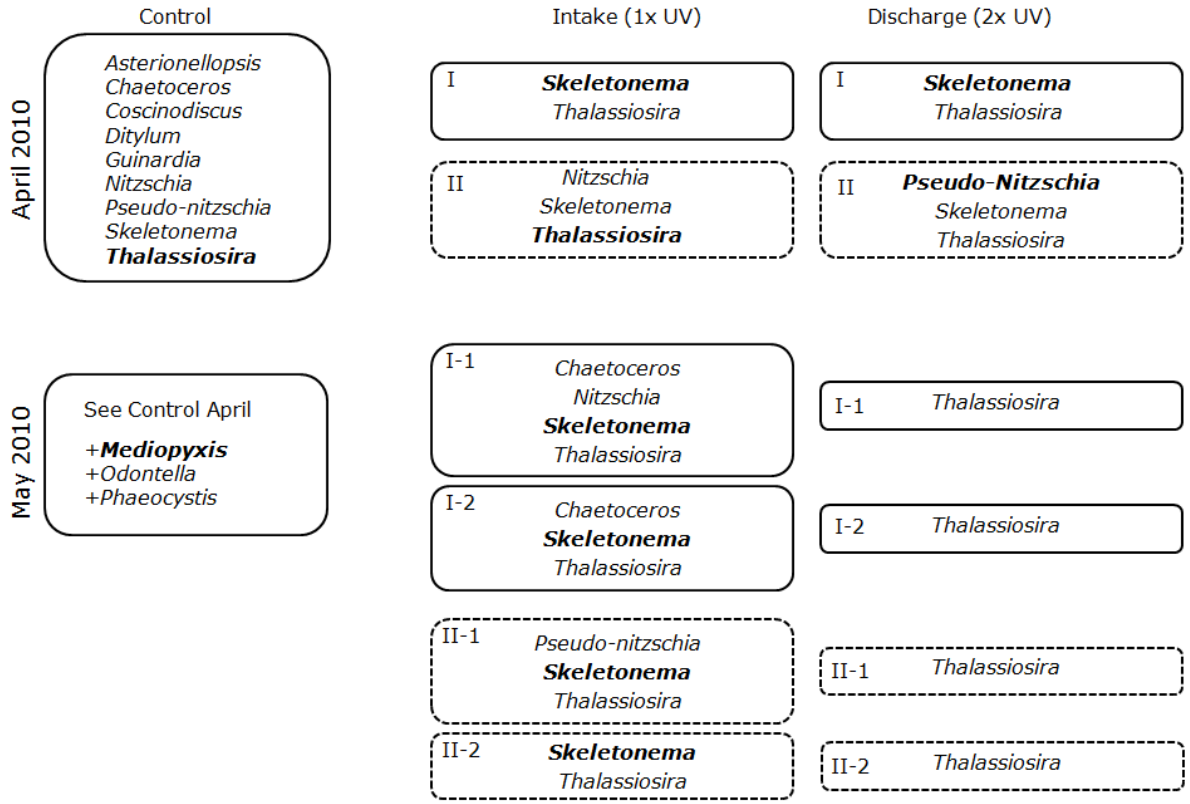

Figure 9. Overview of identified phytoplankton groups in re-growth experiments after UV-treatment. Control = untreated water, Intake = filtered and once UV-treated in replicate tanks I and II, Discharge = Intake with second UV-treatment after five days and two bottles for each tank in May. Taxa in bold letters mark the dominant group of this sample.

In the incubation experiments, the following five taxa re-grew after UV-treatment: *Thalassiosira*, *Skeletonema*, *Chaetoceros*, *Pseudo-nitzschia*, and *Nitzschia* (this order represents rank of abundance estimated from all light microscopy samples).

Thalassiosira cells were re-growing in every series of the first and second experiment. In all four discharge samples of the May series, *Thalassiosira* was the only phytoplankton group coming back. *Skeletonema* was the most abundant re-growing phytoplankton group in the intake and discharge samples of April and in all four intake samples of May. *Pseudo-nitzschia* was the most abundant group in the April's discharge sample of the second tank. *Nitzschia* cells were re-growing in two intake samples, one from each experiment. In May, *Chaetoceros* re-grew in both bottles of tank I after being treated once with UV-radiation.

All intake samples contained, at day zero a few hours after UV-treatment, some intact *Thalassiosira* cells but rarely other phytoplankton. At day eight, all intake samples from April's and May's replicates looked comparably empty, containing single diatom cell walls without cell content. At day two or four, samples appeared in a similar way empty like samples at day eight. Ten and twelve days after UV-treatment, the April intake samples of tank I contained few *Thalassiosira* cells but more *Skeletonema*. Tank II samples at that time contained mostly *Thalassiosira* cells. In all of May's intake samples, *Skeletonema* was the most abundant phytoplankton but only occurred after day ten. In intake samples of tank I in May, *Chaetoceros* cells were nearly as abundant as *Skeletonema* cells.

Discharge samples out of tanks I and II, a few hours after the second treatment, showed no intact cells. Samples of the April series at day ten contained more *Skeletonema* than *Thalassiosira* cells (tank I) which was still the case at day 20. *Pseudo-nitzschia* was more abundant than *Skeletonema* (tank II), and by day 20 this incubation sample additionally contained some *Thalassiosira*. Discharge samples in May contained nearly no cells at days one and ten, but several *Thalassiosira* cells by day 15 and even more at day 21.

Discussion

Ballast water is the main vector for invasions in marine environments (Gollasch 2006). Phytoplankton is known to be transported via ballast water, to become invasive, and in some cases to pose a threat to ecosystem function of the recipient environment. The objectives of this study were (1) to identify if and which phytoplankton groups are re-growing after UV-treatment; (2) to find possible success factors for the survivorship of phytoplankton groups regarding usability as indicator organisms for treatment effectiveness; and (3) to evaluate if there is a risk through invasive (harmful) microalgae even though the ballast water is treated.

Re-growth of identified phytoplankton groups

Data of the flow cytometer indicate cell size and numbers but the various clusters could not refer to species level. A size range from 10 µm up to 50 µm is accurately detected by the flow cytometer. However, there is a chance that bigger and less common cells, chains or colonies are not in the measured volume which is only a part of the entire sample. This could explain that cell numbers in the treated samples outnumber cell counts of the control after approximately ten days. Control water was unfiltered, thus contained larger organisms like *Ditylum* cells, *Asterionellopsis*, and *Mediopyxis* chains. These were seen using the light microscope, but were not measured by the flow cytometer.

The main re-growing phytoplankton groups were: *Thalassiosira*, *Skeletonema*, and *Chaetoceros*. For *Thalasiosira* and *Skeletonema* it was not possible to identify at the species level (with only a light microscope). *Chaetoceros* could be identified as *C. socialis* due to its characteristic colony formation. *Skeletonema costatum* is a species mentioned in several ballast water (treatment) studies (e.g. Sutherland et al. 2001; Kang et al. 2010). There is however evidence that 'within the species complex

once perceived as '*Skeletonema costatum*,' there are cases of very clear distinction among species for morphological, phylogenetic, and ecological traits.' (Sarno et al. 2005 p. 174). For the exact species of *Skeletonema*, as well as for the other mentioned diatoms in our study, additional genetical studies or identification with an electron microscope would be needed.

In April, *Thalassiosira* was the dominant phytoplankton group in the control sample. It was also re-growing in every incubation sample. These results could lead to the assumption that this re-growth is only occurring as a matter of chance, resulting from high initial numbers. *Skeletonema* was found in the control sample in numbers comparable to species which did not re-grow. However, if it was present as a re-grower it was most often (six out of eight times) also dominant. These results could indicate certain advantages of *Skeletonema* over the other phytoplankton groups. *Pseudo-nitzschia* was present in only one discharge sample as most abundant taxa but was not found before the second treatment; maybe it was present as resting cells (Orlova and Morozova 2009). In May's control sample, *Mediopyxis helysia* is the most abundant species but it did not show re-growth at all. It was the largest species in April and May, with single cells having length measurements of 44-125 µm (apical axis or width of chain) and 27-78 µm (pervalvar axis) (Hoppenrath et al. 2009). It is therefore unlikely that *Mediopyxis helysia* was able to pass the 20 µm mesh sized filter lined in front of the UV-unit.

Success factors for the survivorship and usability as indicator organisms

The identified re-growers in the present study were all diatoms, which are ideal candidates for successful ballast water transport (McCarthy and Crowder 2000). This is because they are small, robust as vegetative cells or resting stages, and able to survive dark and unfavorable conditions in the tank. Most diatoms also have a broad temperature range; species of the genus *Chaetoceros*, *Skeletonema*, and *Thalassiosira* grew from -1,5ºC up to at least 20ºC (Baars 1979). Viable cultures of *Pseudo-nitzschia* were collected from ballast water tanks underlining the ability to survive darkness for days (Hallegraeff 1998). *Chaetoceros* and *Thalassiosira* species were not only found as vegetative cells in ballast water but also as resting stages (Klein et al. 2009). *Skeletonema* resting forms are also known (Durbin 1978). The formation of resting stages could facilitate survival of UV-treatment.

Re-growth of potential invasive organisms might be supported by favorable light and nutrient conditions and does not necessarily mean that re-growth occurs in dark ballast water tanks. Most invasive organisms fail also to establish after introduction (Williamson and Fitter 1996). For a successful establishment habitat invasibility and propagule pressure play an important role as well as invasiveness (Lonsdale 1999). Invasiveness is the ability to be successful in new environments and depends on species traits (Colautti et al. 2006). A high growth rate is considered to be a functional trait of a successful plant invader (van Kleunen et al. 2010). In general, smaller cells show higher growth rates than large ones (Kagami and Urabe 2001). *Chaetoceros*, *Skeletonema*, and *Thalassiosira* are small sized taxa and by their high growth rates could have an advantage when recovering and re-growing.

Species of the three re-growing genera have a broad temperature tolerance, resting forms, and high growth rates. Therefore, they appear to have greater potential to survive treatment and become invasive than the other identified microalgae. Some non-native *Thalassiosira* species are known to be already established in the North Sea (Reise et al. 1998). *Thalassiosira* cells were dominant as re-growers, from our own experience are easy to grow (unpublished data), and commonly found in the

marine environment. Therefore we consider them as suitable indicator organisms for testing the effectiveness and efficiency of UV-units.

Risk evaluation for (harmful) algae invasions - despite UV-treatment

Harmful diatoms like toxic *Pseudo-nitzschia* species causing Amnesic Shellfish Poisoning can be transported via ballast water (Zhang and Dickman 1999). However, harmful diatoms are not only those producing toxins. Species of the genus *Chaetoceros* have spines which are thought to cause mechanical damage to fish gills (Bell 1961). Ecological implications of phytoplankton invasions may include changes in the biodiversity of the food-web after successful establishment. Species of *Chaetoceros*, *Skeletonema*, and *Thalassiosira* are known to form blooms (Tiselius and Kuylenstierna 1996), thus may increase local blooming events leading to anoxic conditions following their decay. Species of the identified re-growing genera might not only get invasive but also cause negative effects on the recipient ecosystem.

Conclusion

It should be noted that the tested UV-treatment system in the present study caused a decline of phytoplankton numbers in compliance with the D-2 standard. Incubation experiments are not required for the G8 guidelines but help to evaluate effectiveness and efficiency of treatment systems. Other studies also examined plankton composition in incubation experiments after UV-treatment. Waite et al. (2003) showed the decline of phytoplankton after 18 hours. The present study proves however, that possible re-growth could only be seen after seven days. Sutherland et al. (2001) conducted incubation studies lasting for 16 days. They focused on the three dominant phytoplankton taxa *Chaetoceros gracile*, *Skeletonema costatum* and *Thalassiosira sp.*; our results validate the choice of the tested genera. If incubation experiments show that there is a chance of introducing invasive (harmful) species despite treatment, additional tests should be considered.

Acknowledgement

This work has been co-funded by the North Sea Region Programme under the ERDF of the European Union. The PhD scholarship for this work is gratefully appreciated and was provided by the Max Planck Institute for Comparative and International Private Law/International Max Planck Research School for Maritime Affairs. Two anonymous reviewers provided comments helping to improve the preceding article that is the basis of this Chapter 3.

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4. Flow cytometry, microscopy and DNA analysis as complementary phytoplankton screening methods in ballast water treatment studies

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Published in: Journal of Applied Phycology, Volume 25, Issue 4, pp 1047-1053

Abstract

Ballast water is the main vector for marine invasions. To minimize the spread of invasive species, the International Maritime Organization has adopted the Ballast Water Management Convention which requires the installation of shipboard ballast water treatment systems (BWTSs). During BWTS tests the phytoplankton abundance and species composition was followed after treatment with both filtration and ultraviolet (UV) radiation. Although the installation fulfilled the IMO criteria after a five day holding time in a model ballast tank, the ultimate effectiveness of the treatment was further tested in long-term (20 days) incubation experiments under favorable phytoplankton growth conditions. Application of flow cytometry, microscopy and DNA-sequencing to these incubation samples gave an indication of the phytoplankton species that might be introduced by ballast water discharge – despite treatment. Phytoplankton was reliably quantified using flow cytometry, while fast identification was best done using microscopy. Some groups that contained potentially toxic species could not be identified at species level using microscopy; for these species identification using genetic techniques was necessary. It is concluded that if long-term incubation experiments are used as an additional tool in testing BWTS effectiveness, a combination of phytoplankton screening methods can be applied depending on the detail of information that is required.

Keywords: ballast water, flow cytometry, microscopy, DNA-sequencing, micro-algae

1. Introduction

Ballast water, which is used for the stability of ships, is the main vector for the introduction of marine invasive species (Gollasch 2006; Molnar et al. 2008). Invasive species can pose a risk to marine ecosystem services (Levine 2008). They can negatively affect biodiversity (Molnar et al. 2008), increase the probability of disease transmission and be infectious for humans, animals, and plants (Keesing et al. 2010). The economic and ecologic impacts of marine invasive species can be enormous as shown by the examples of the Zebra mussel *Dreissena polymorpha* in North America (Mackie 1991; Connelly et al. 2007) and the comb jelly *Mnemiopsis leidyi* in the Black Sea, Sea of Asov and Caspian Sea (Ivanov et al. 2000; Shiganova 2002; Shiganova et al. 2001). Invasive phytoplankton, especially diatoms as primary producers providing the base of the marine food web, can cause changes with cascading effects up to higher trophic levels. Diatoms are able to survive ship journeys in ballast water (Klein et al. 2010) and may be translocated to new habitats.

Due to the negative effects of invasive species transported in ship's ballast water, the International Maritime Organization (IMO) has adopted the Ballast Water Management Convention in 2004. There are several regulations in this convention. The first set of regulations (G7) concerns ballast water exchange in open sea, but this is being phased out in favor of new regulations: Regulations G8 and G9 of this convention include the D-2 standard that describes limits on the numbers of viable organisms allowed in ballast water at discharge. To meet these regulations, ballast water treatment systems (BWTSs) were developed. BWTSs may use a variety of treatments that include filtration, hydro-cyclones, ultraviolet (UV) radiation and the addition of toxic chemicals (Tsolaki and Diamadopoulos 2010; Gregg et al. 2009). For a successful land-based test, according to the IMO, at least ten tests at two different salinity ranges should comply with D-2. In addition to the obligatory measurements required by the IMO, additional experiments such as long-term incubations to test for surviving and re-growing organisms are performed at the NIOZ BWTS test facility. These re-growth experiments provide important information about the effectiveness of BWTSs on phytoplankton and bacteria (Stehouwer et al. 2010). Additionally, identification of the re-growing species can provide important information about possible future invaders and indicator organisms for the testing of BWTSs (Liebich et al. 2012).

This paper aims to compare different analytical techniques for phytoplankton counting and identification as applied to incubation experiments used in ballast water testing. Flow cytometry and microscopy were compared both on counting and identification potential. Past studies compared flow cytometry and microscopy, but these focused on organism counts alone, not on identification (Monfort and Baleux 1992). Cluster analysis software was used as an objective method to assess the potential of the flow cytometer as possible tool for the identification of species or species groups, providing an objective measure of diversity. Genetic methods were also included to test their potential for species identification, especially when exact determination using flow cytometry or microscopy is more difficult.

These analytical methods were applied regarding three objectives: first, how well the technique could identify the different phytoplankton at the species level. Second, how suitable the technique would be for providing accurate numbers of phytoplankton. Third, how feasible the technique would be for future rapid phytoplankton screening in ballast water treatment systems.

2. Material and Methods

Sampling

Samples were collected at the NIOZ testing facility (Texel, The Netherlands) during tests of a combined filtration – UV BWTS. Four long term incubation experiments (I, II, III and IV) were performed over a period of 20 days. Intake samples for incubation experiment I and II were taken the 1st of April 2010. Harbour water passed through a pump at 200 m^3/h, a 20 µm mesh filter, and a UV treatment unit (254 nm wavelength) before sampling. Water for the control only passed through the pump. Following the G8 guidelines, the control and intake water was then stored in tanks of 300 m^3 for five days. After this holding time the water was discharged using the pump. Control samples were taken after the pump, samples from the treated tanks were taken directly after a second treatment by the UV unit (no filtration at discharge).

The intake samples for experiment III and IV were taken the 13th of May 2010. Sampling and treatment at intake and discharge were performed the same way as in experiment I and II, but duplicate samples were taken for each tank: resulting in four samples for the intake and discharge series each.

To ensure independent tests (replicates), first one holding tank was filled with treated water, then the system was shut down. Next, the control tank was filled. After this the BWTS was started again and the second holding tank was filled with treated water.

Post-treatment incubations

Samples from the BWTS were collected in 10 liter transparent polycarbonate bottles (Nalgene, Rochester, USA) and placed in a climate-controlled room on magnetic stirrers with 130 rotations per minute to prevent sedimentation. The room was kept at ambient seawater temperature (11 °C for the first set of tests, 15 °C for the second set of tests) at the time of sampling and a 16:8 (h:h) light : dark regime (100 µmol quanta m-2s-1, Philips TL-D Super 80 58W 865 daylight lamps). Nutrients were added on day zero of the incubation at concentrations which are typical for the Wadden Sea (nitrate 20 µmol/L, phosphate 1,6 µmol/L, silicate 20 µmol/L). Samples for flow cytometry and genetic analysis were taken daily, samples for microscopy were taken twice per week. Each incubation experiment was monitored for a period of 20 days.

Flow cytometry

Three samples of two mL from each incubation bottle were analyzed. Flow cytometric measurements were performed with a Beckman Coulter Epics XL MCL (488 nm laser) (Beckman Coulter, CA, USA). Phytoplankton cell numbers were measured by triggering on the FL4 parameter (red fluorescence, 675 nm, chlorophyll a) which allowed distinction from other particles than phytoplankton (Veldhuis and Kraay 2000). Other important parameters are FS (Forward Scatter, an indication of size), FL1 (green fluorescence, 525 nm), FL2 (yellow/orange fluorescence, 575 nm) and FL3 (red fluorescence, 620 nm). The flow cytometer data were presented in two-dimensional graphs in which particles with analogous properties showed up as clusters. An indication of diversity was obtained by visually determining the number of clusters of different size and fluorescence signal.

In addition, the Easyclus© software (v1.16, Thomas Rutten Projects, NL) was used as an objective (without training on data) method to assess diversity. Easyclus© uses bivariate scatterplot combinations to distinguish clusters of data that have similar optical properties. The principle of clustering is based on the density number, the 2-dimensional neighbouring distance between events and the similarity between clusters using all multivariate optical cytometric data (n-dimensional). The number of observed clusters is dependent on the chosen resolution, neighbouring distance length, similarity cluster fusing factor and the chosen bivariate scatterplot combinations. Visualization of clusters is done in scatterplot graphs showing the possible clusters, which may also overlap in multiple dimensions. This gives greater resolution in cluster identification than the human eye. The analysis was performed using ‘Auto-Clustering Easyclus Method 2’.

Microscopy

Settling chambers were filled with five mL sample from the incubation bottles. These samples were fresh and unpreserved. Before taking the samples, the bottles were well mixed to allow for a

representative phytoplankton distribution. These samples were then left in the settling chamber to settle for at least 30 minutes and examined with an inverted light microscope (Zeiss Axiovert, 400x, Germany). Phytoplankton species identification was achieved using Hoppenrath et al. (2009).

DNA analysis

For each sample 30 mL was filtered over a 0.2 μm filter (GTTP, Millipore) and stored at -80 °C. DNA was extracted from the filters with the UltraClean Soil DNA Isolation Kit (MO BIO Laboratories, Inc., CA, USA). DNA was amplified using primers specific for 16S rRNA gene segments of cyanobacteria and plastids (Nübel et al. 1997). Forward primer was CYA359F-GC, reverse primers were CYA781R(a) and CYA781R(b). The PCR program was set to five minutes at 94 °C, 35 cycles of one minute at 94 °C, one minute at 60 °C and one minute at 72 °C, and a last extension of five minutes at 72 °C. At the end of the PCR cycle temperature was reduced to 4 °C.

Denaturing Gradient Gel Electrophoresis (DGGE) was used to identify gene fragment diversity (Muyzer et al. 1993). A 6% acrylamide/bisacrylamide gel with a 20-80% urea/formamide gradient was used. An amount of 100 nanogram of quantified PCR product was loaded on the gel. The gel was stained using SYBR Gold (Molecular Probes, Inc. OR, USA) and analysed using a blue light converter. DNA bands were numbered and extracted from the gel for sequencing. Samples were re-amplified and cleaned using QuickClean 5M PCR Purification Kit (Genscript). Selected DGGE bands were sequenced twice, with primer 359F and with a mix of primers 781RA and 781RB. Samples were sequenced using a ABI PRISM 310 Genetic Analyzer. Results of forward and reverse sequences were combined in Autoassembler (ABI) and compared with sequences in Genbank using BLAST. Along with their blast hits, sequences were imported into Silva database nr. 102 (Pruesse et al. 2007), aligned accordingly and added to the tree sequences of photo-autotrophes using the ARB Parsimony algorhythm (Ludwig et al. 2004).

Statistical analysis

Statistical analysis was performed in Systat 13. An ANOVA model was constructed with the number of species or clusters as dependent variable and the methods of analysis as factor. The null hypothesis was that there are no differences between the analytical methods. Results of the ANOVA model were used in a Dwass-Steel-Chritchlow-Fligner pair-wise comparison of methods test.

3. Results

Flow cytometry

Analysis of diversity was performed using both classic manual cluster identification and Easyclus© cluster analysis. Distinction between phytoplankton and debris was done by visual analysis of the Easyclus© output (Figure 1). Detritus can be identified by its low red fluorescence compared to its size. Control samples were much more diverse (12-13 clusters) than treated samples (1-4 clusters) (Table 1, Figure 1 and 2). Easyclus identification resulted in a larger number of clusters than manual cluster identification (Table 1). Identification at the species level was not possible using the Coulter Epics XL flow cytometer.

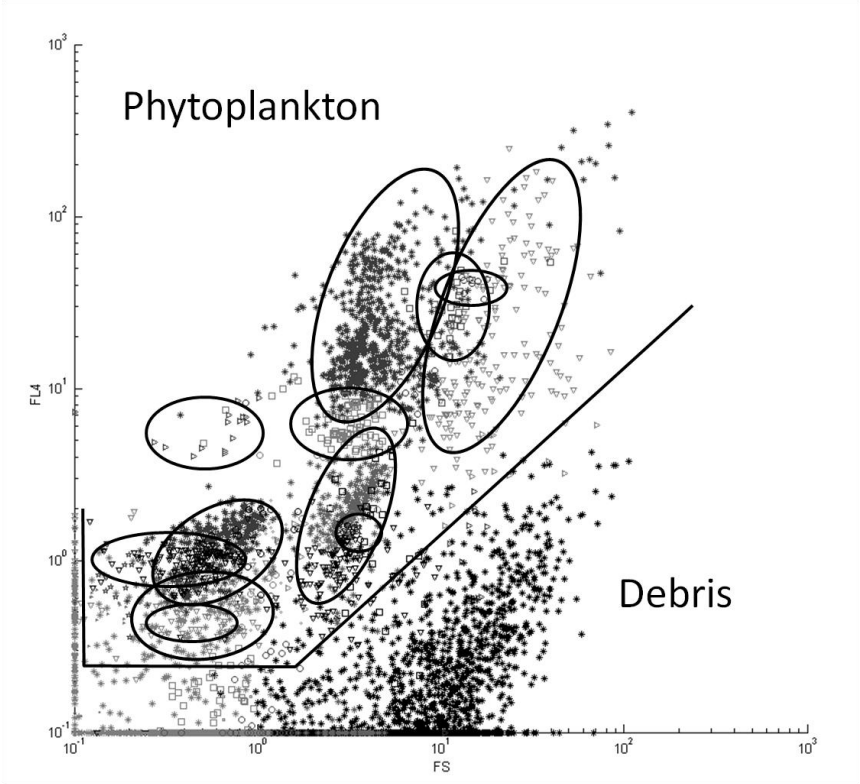

Figure 1. Example of an Easyclus® cluster analysis of an untreated incubation sample (experiment III, control 2). FL4 is chlorophyll fluorescence; FS is Forward Scatter (an indication of size). Every cluster is assigned a different shade of grey and symbol by Easyclus®. Clusters of symbols have been circled for ease of interpretation. The black line indicates the cut-off between the area which is considered phytoplankton and the area which is considered debris.

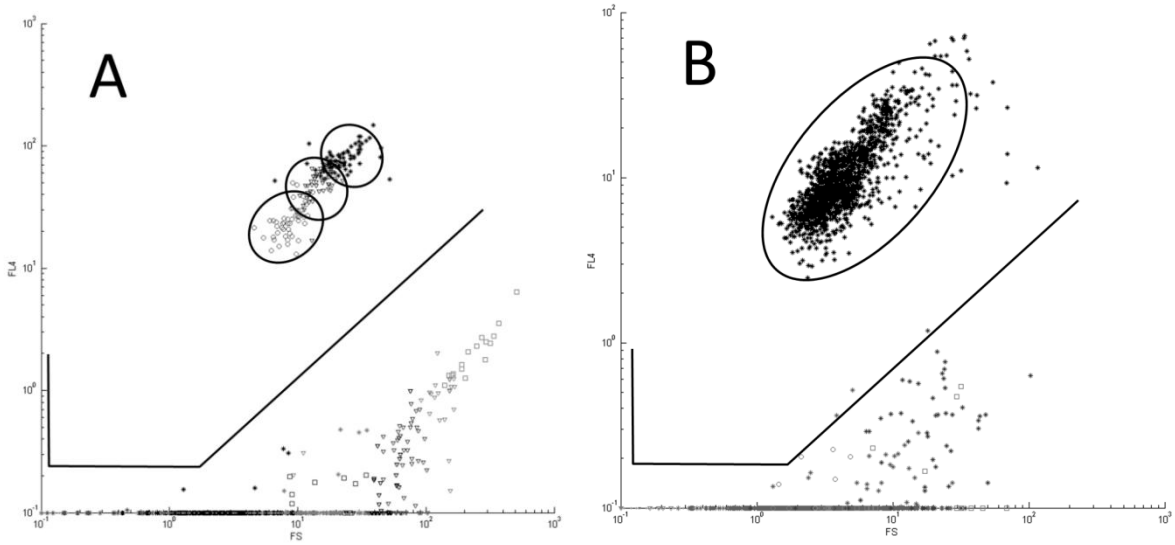

Figure 2. Examples of Easyclus® analyses of treated and incubated water. Experiment I treated (A) and experiment III treated (B). Every cluster is assigned a different color and symbol by Easyclus®. Clusters of symbols have been circled for ease of interpretation. The black line indicates the cut-off between the area which is considered phytoplankton and the area which is considered debris.

Microscopy

Control samples showed a large diversity of phytoplankton species of up to 12 different taxa in experiments III and IV (Table 1). After the first UV treatment a maximum of four different taxa in one sample survived and showed re-growth. *Thalassiosira*, *Skeletonema* and *Chaetoceros* were the genera found most often to survive UV treatment. *Chaetoceros* and *Nitzschia* were identified in two different incubation samples each after intake. *Thalassiosira*, *Skeletonema*, and *Pseudo-nitzschia* survived the first and also re-grew after the second UV treatment at discharge. Liebich et al. (2012) provide extensive results of the microscopic analyses.

DNA analysis

DNA analysis only identified one or two species per sample, in both control and treated water (Table 1 and 2). The sequences of the control samples of experiment I and II matched most closely with *Thalassiosira pseudonana*. Intake treated samples of experiment I and II matched most closely with *Coscinodiscus radiatus* and *Thalassiosira weissflogii*. Discharge treated samples of experiment I and II matched most closely with *Stephanopyxis nipponica* and *Thalassiosira weissflogii*.

The control samples of experiment III and IV most closely matched *Ditylum brightwellii*. Intake treated samples of experiment III and IV matched most closely with *Thalassiosira weissflogii* and *Skeletonema costatum*. Discharge treated samples for experiment III and IV were not taken.

Table 1. Comparison of different phytoplankton analysis methods on control and post-treatment incubation samples. Roman numerals indicate different experiments. "Treated" means samples incubated after filtration and UV on day 0. "Treated discharge" are incubated samples after a five day holding period and a second UV treatment at discharge. Flow cytometer analyses did not provide species identification, so only the number of clusters is given for both manual and Easyclus® methods. For microscopy and 16S rRNA the number of species is given as well as the number of species in common between both methods. 'nd' means no data were available.

	Flow Cytometer	**Flow Cytometer**	**Microscopy**	**16S rRNA**	**Microscope vs. 16S rRNA**
	Number of clusters Manual	Number of clusters Easyclus	Number of species	Number of genotypes	Number of species in common
I-Control	4-6	13	9	1	0
I-Treated	1-2	1	2	2	1
II-Treated	1-2	2	3	1	1
I-Treated discharge	1-2	2	2	2	1
II-Treated discharge	2	4	3	1	1
III-Control 1	6-9	12	12	1	1
III-Control 2	6-9	12	12	1	1
III-Treated 1	2-3	1	4	nd	nd
III-Treated 2	1-2	3	3	nd	nd
IV-Treated 1	1-2	3	3	2	2
IV-Treated 2	1-2	1	2	nd	nd
III-Treated discharge 1	1-2	2	1	nd	nd
III-Treated discharge 2	1	2	1	nd	nd
IV-Treated discharge 1	1	2	1	nd	nd
IV-Treated discharge 2	2	2	1	nd	nd

Table 2. Species or groups identified using DNA analysis. Species/groups shown in bold were also found using microscopy. 'nd' means no data were available.

	Species/groups identified
I-Control	***Thalassiosira pseudonana***
I-Treated	*Coscinodiscus radiatus,* ***Thalassiosira weisflogii***
II-Treated	***Thalassiosira weisflogii***
I-Treated discharge	***Thalassiosira weisflogii****, Stephanopyxis nipponica*
II-Treated discharge	***Thalassiosira weisflogii***
III-Control 1	***Ditylum brightwellii***
III-Control 2	***Ditylum brightwellii***
III-Treated 1	nd
III-Treated 2	nd
IV-Treated 1	***Thalassiosira weisflogii, Skeletonema costatum***
IV-Treated 2	nd
III-Treated discharge 1	nd
III-Treated discharge 2	nd
IV-Treated discharge 1	nd
IV-Treated discharge 2	nd

4. Discussion

Post-treatment incubation experiments are an extension of the measurements that are required by the G8 guideline. These incubations provide valuable information on the re-growth potential of phytoplankton treated with a ballast water system: which species can potentially survive certain types of treatment and are therefore more likely to become invasive?

Species identification proved to be impossible using the Coulter Epics XL Flow cytometer (Hofstraat et al. 1994). Cluster analysis based on Easyclus made it easy to determine the level of diversity in the samples, it also eliminated user bias associated with identifying clusters by hand. However, it was impossible to link the clusters to groups or species. Clusters identified in the control samples were not recognized in the treated samples and also between treated samples no identical clusters were identified. Physiological changes of one species over time, for example changes in size and fluorescence under nutrient limitation, were often sufficient to no longer classify as the same species in the cluster software. This may also be due to the limited number of variables generated by the Coulter Epics flow cytometer. A larger number of variables would allow for an easier and more accurate distinction between clusters. The Coulter Epics flow cytometer has only one laser and generates six scatter and fluorescence variables for every particle counted. Newer types of flow cytometers can have three or four different lasers and generate as much as 30 variables. This increase in variables will increase the possibility of species identification. Other options for species identification using flow cytometry are the FlowCAM®, CytoSense® and ImageStream® flow cytometers. Such instruments combine the techniques of flow cytometry with digital photographs of the counted particles (Sieracki et al. 1998; Takabayashi et al. 2006).

There are other options for species identification using the flow cytometer. This requires fluorescent stains which are specific for specific groups of phytoplankton or even certain species. An example of

this is immuno flow cytometry, where species-specific fluorescent antibodies are used to make specific species fluorescent (Peperzak et al. 2000). Another method is fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes (Simon et al. 1995). This method can distinguish between groups of phytoplankton but could also be adapted to identify species (Scholin et al. 2003). Both of these methods require knowledge of the species expected in the sample, since the probes need to be designed for a specific species or group.

The number of clusters found using the cluster analysis software was not significantly different to the number of species identified using microscopy (Table 3). Genetic analysis only found one or two (dominant) species in each sample. In treated samples this was comparable to cluster analysis and microscopy, but in control samples this was a clear underestimate of species diversity. A Kruskall-Wallis one-way analysis of variance showed there were significant differences between methods ($P < 0.05$). A Dwass-Steel-Chritchlow-Fligner test for pairwise comparisons showed that these differences resulted from significant differences between the genetic analysis and manual clustering, Easyclus clustering and microscopy (Table 3). Manual clustering, Easyclus clustering and microscopy were not significantly different to each other (Table 3).

Table 3. Results of Dwass-Steel-Chritchlow-Fligner test for all pairwise comparisons. n.s. is not significant.

	p-value
Manual clustering vs. Easyclus clustering	n.s
Manual clustering vs. Microscopy	n.s
Manual clustering vs. DNA analysis	<0.001
Easyclus clustering vs. Microscopy	n.s
Easyclus clustering vs. DNA analysis	<0.001
Microscopy vs. DNA analysis	<0.001

Most of the identified species or groups were diatoms. This is not unexpected, since during April and May in the Wadden Sea there is usually a diatom bloom (Cadee 1986; Hofstraat et al. 1994).

Cluster analysis of the flow cytometry results did not provide species identification, but microscopy was also limited in this aspect. Three of the main re-growing groups, *Skeletonema*, *Pseudo-nitzschia* and *Thalassiosira*, could not be identified at the species level using just light microscopy. This limitation has been recognized previously (Sarno et al. 2005; Kooistra et al. 2008; Amato et al. 2007; Park and Lee 2010). However, as pointed out in Park and Lee (2010), this can be solved for *Thalassiosira* using electron microscopy. Genetic analysis was performed as a third method to identify species in the samples. Samples were identified at species level. Identified species match groups found with microcopy with two exceptions (Table 2). Not all groups found with microscopy were identified using genetics (Table 1 and 2), e.g. *Pseudo-nitzschia*. Certain species in the *Pseudo-nitzschia* group are toxic. Another type of analysis aimed more specifically at these possibly dangerous species could be used. There are many other types of molecular analysis available which show promising results for phytoplankton identification such as 5.8S + ITS-2 (Moniz and Kaczmarska 2009), inter simple sequence repeats (Bornet et al. 2004) and diatom specific primers. There also

exist analysis techniques specifically for toxic phytoplankton (Scholin and Anderson 1998; Scholin et al. 2003). For future analyses these other techniques should be considered.

5. Conclusions

Microscopy appears to be the best method for species identification; it is faster than genetic analysis and a more able to assess the diversity of a sample in terms of number of species. However, light microscopy is not always sufficient to identify at species level, such as potentially toxic *Pseudo-nitzschia* spp.. If groups with potentially toxic species are identified using microscopy it is advisable to use additional genetic identification techniques to prove whether a toxic species is actually present or not. When only diversity is needed and the actual groups or species do not matter, using cluster software on flow cytometry data offers a good alternative.

For measuring the number of phytoplankton cells flow cytometry is the best method, it provides comparable numbers to microscopy (Monfort and Baleux 1992). Flow cytometry is preferable to microscopy, since counting time is much longer when using microscopy (Table 4) and because the volume counted by FCM is usually much higher, providing more precise abundance estimates (Hofstraat et al. 1994).

Costs are another major issue when choosing a technique (Table 4). While microscopy has very little costs per sample, the equipment is relatively expensive and requires a high level of expertise. Flow cytometry has high equipment costs, but not very high costs per sample and requires a lower level of expertise. Finally, genetic techniques have fairly low equipment costs, but costs per sample are high.

*Table 4. Comparison of time and costs required for each method. Costs are in euros, costs/sample are material costs only. The large spread in equipment costs for microscopy and flow cytometry is due to the large variety of available equipment. The large spread in costs/sample for genetics is largely dependent on the number of genotypes per sample. * = costs only include equipment for PCR and DGGE, sequencing was performed at an external facility.*

	Time/sample	Costs/sample	Equipment costs	Expertise required
Microscopy	15-30 minutes	< 1 ,-	10.000/50.000	High
Flow cytometry	1-5 minutes	2,-/3,-	40.000/200.000	Low-medium
16S rRNA	2-3 days	14,-/84,-	6000*	medium

Species identification after ballast water treatment offers another perspective; the identification of resistant species can be used to develop more thorough testing protocols for the treatment system. By testing a system with the most resistant organisms, the system can be exposed to a worst case scenario and can be better evaluated and calibrated. *Thalassiosira weissflogii* could be a candidate for this approach.

Acknowledgements

The authors thank Dr. Judith van Bleijswijk and Julie Ogier for their help with the DNA analysis and Thomas Rutten for his help with the Easyclus© analysis.

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

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5. A comparison of six different ballast water treatment systems based on UV radiation, electrochlorination and chlorine dioxide

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Accepted by: Environmental Technology (27-01-2015) DOI 10.1080/09593330.2015.1021858

Abstract

The spread of aquatic invasive species through ballast water is a major ecological and economical threat. Because of this the International Maritime Organization (IMO) set limits to the concentrations of organisms allowed in ballast water. To meet these limits ballast water treatment systems (BWTSs) were developed. The main techniques used for ballast water treatment are Ultraviolet radiation (UV) and electrochlorination (EC). A third technique based on chlorine dioxide (CD), is less commonly used. Here we tested three UV based systems of different designs, two EC based systems and one CD system. In this study, phytoplankton re-growth after treatment was followed for all six of these BWTSs. Natural plankton communities were treated and incubated for 20 days. Growth, PSII efficiency and species composition were followed. The three UV systems all showed similar patterns of decrease in phytoplankton abundances followed by re-growth. The three chlorine based systems (two EC and one CD) showed comparable results. However, significant different responses were observed for on the one hand the group of three UV systems versus on the other hand the group of three chlorine based systems. Overall, all BWTSs reduced phytoplankton abundances to below the IMO limits, which demonstrates a reduced risk of aquatic invasions through ballast water.

Keywords: ballast water treatment, UV radiation, electrochlorination, chlorine dioxide, phytoplankton

1. Introduction

Invasive species are one of the greatest threats to biodiversity. In the aquatic environment there are many invasive species causing great economic and ecologic harm. Examples of this are the American comb jelly (*Mnemiopsis leidyi*) which is partly responsible for ecosystem shifts and the reduction of fisheries in the Caspian Sea, Sea of Azov and Black Sea (Mackie 1991, Connelly et al. 2007). Another is the European zebra mussel (*Dreissena polymorpha*) which is causing fouling problems in North American lakes and rivers (Ivanov et al. 2000, Shiganova 2002, Shiganova et al. 2001). Smaller organisms can also cause problems; the diatom *Coscinodiscus wailesii* is invasive in the North Atlantic, North Sea and Celtic Sea (Edwards et al. 2001) where it has detrimental effects on fisheries due to mucus production that clogs fishing nets (Mahoney & Steimle 1980, Boalch 1987). In addition, it changes ecosystem functioning since it is undigestible to the two common herbivorous copepods (Roy et al. 1989) and displaces native phytoplankton species (Dürselen & Rick 1999). Another example is the increased spread of toxic phytoplankton blooms, such as of the dinoflagellates *Alexandrium catenella* or *Gymnodinium catenatum* which cause paralytic shellfish poisoning in humans (Hallegraeff & Bolch 1991, Hallegraeff 1998, Van Dolah 2000, Hallegraeff 2010). The most important vector for the spread of aquatic invasive species is ballast water (Molnar et al. 2008). Because of this the International Maritime Organization (IMO) created the D-2 ballast water performance standard that set limits on the concentration of organisms allowed to be in ballast water at discharge. For organisms ≥50 micron less than 10 per m3 are allowed to be discharged. For organisms <50 and ≥10 micron less than 10 per mL are allowed to be discharged. All sizes should be measured as the minimum dimension, meaning the smallest diameter of the organism. The standard also includes three indicator microbes; toxigenic *Vibrio cholerae* (less than 1 cfu (colony forming units) per 100 mL), *Escherichia coli* (less than 250 cfu per 100 mL) and intestinal *Enterococci* (less than 100 cfu per 100 mL). To meet these standards a number of companies started developing ballast water treatment systems (BWTSs). These BWTSs are based on a variety of techniques (Gregg et al. 2009, Tsolaki & Diamadopoulos 2010), but most common are a combination of a filter to remove organisms > 50 µm followed by disinfection by UV-radiation (UV) or electrolytic generation of hypochlorite (EC). Less commonly used are systems based on chlorine dioxide (CD). At the Royal Netherlands Institute for Sea Research (NIOZ) these BWTSs are tested according to IMO regulations G8 and G9 (IMO 2008a, 2008b).

Phytoplankton forms the basis of the marine food web and is known to survive transport in ballast water (Dickman & Zhang 1999, Zhang & Dickman 1999, Klein et al. 2010), which is why it was chosen as focus of this paper. Re-growth is defined as increase in phytoplankton abundance and viability after a BWTS treatment. Re-growth also provides an indication of risk of introducing non-native species even after ballast water treatment according to the IMO standards.

In earlier studies, the performance of the BWTSs was measured by its effect on phytoplankton survival and re-growth during incubation experiments (Stehouwer et al. 2010, Liebich et al. 2012). Stehouwer et al. (2010) showed that both a UV BWTS and a chemical treatment system have phytoplankton re-growth after treatment, but no statistical comparison was made.

Liebich et al. (2012) and Stehouwer et al. (2012) identified several re-growing phytoplankton species after UV treatment by one specific BWTS. All re-growing species were diatoms, most notably *Thalassiosira weissflogii*. However these studies did not investigate the possible differences in

species responses between BWTSs. Successful tests of UV and EC treatment systems have been evaluated previously, but always as one system per paper (Sutherland et al. 2001, Matousek et al. 2006, Wright et al. 2007, Jung et al. 2012, Zhang et al. 2012). Of these studies only Sutherland et al. (2001) addresses re-growth. This is the first time that multiple systems using multiple treatment types were directly compared.

It was the aim of the present study to compare re-growth in six BWTSs. Three BWTSs used UV, but differ in the number of UV reactors, number of lamps per reactor and type of UV source used. Two BWTSs used EC, generating hypochlorite by electrolyzing seawater, one system generated chlorine dioxide (CD) by adding sulfuric acid (H_2SO_4) and Purate™ ($NaClO_2$ and H_2O_2) together.

The six systems tested in this paper can be grouped into two general categories, disinfection by UV radiation and disinfection by chlorine. The main research question was: is there a difference in performance between these two types of ballast water treatment? In order to answer this question first a comparison was made between the BWTSs that use the same method to see if different systems using similar methods also have similar performance. Additionally, for both UV radiation and chlorine chemistry a dosage experiment was performed to investigate the effects that an increased or decreased dosage has on the organisms in ballast water. Finally the performance of all systems was compared. Phytoplankton response investigated by following cell abundance, PSII efficiency (as indicator of physiological status of the photosynthetic machinery) and species composition during and after BWTS treatments.

2. Methods

Ballast water treatment systems. Tests on ballast water treatment systems were performed in spring and early summer of 2008, 2009 and 2010. Six different treatment systems were tested, two in 2008 (UV1 and CD), two in 2009 (UV2 and EC1) and two in 2010 (UV3 and EC2) (Table 1). The UV1 BWTS used a 50 µm disk filter and one UV reactor with medium pressure (broad wavelength) UV. The UV2 BWTS used a pre-filtration over a 200 µm mesh filter, followed by a 50 µm mesh filter and two UV reactors with low pressure (254 nm) UV. The UV3 BWTS used a 20 µm mesh filter and three UV reactors with low pressure (254 nm) UV radiation. Pressure, in the context of UV, refers to the pressure of the mercury gas inside the UV lamp. The CD treatment system used a 40 µm mesh filter followed by an addition of chlorine dioxide. The EC1 system used a 40 µm mesh filter and electrolytic chlorination to generate hypochlorite, which is subsequently added to the ballast water and neutralized on discharge using sodium bisulfite. The EC2 system used a 200 µm filter, a cyclone to separate particles down to 20 µm and electrolytic chlorination to generate hypochlorite, which is subsequently added to the ballast water and neutralized on discharge using sodium bisulfite. The tests for UV1, UV2, CD, EC1 and EC2 consisted of filling two 250 m^3 simulated ballast water tanks (one treated and one control for each treatment system) at a speed of 200 m^3 per hour. Water was pumped up from the NIOZ harbor, passed through the pump and the treatment system after which intake samples were taken. For the control tank water also went through the pump but by-passed the entire treatment system. Thus control samples were not filtered. Intake samples of the controls were taken after the pump. The tests for UV3 were performed with 3 tanks of 250 m^3, one control and two treated. For tests of all six systems, both control and treated water was kept in the simulated ballast tanks for five days (as described in IMO guidelines). After this five day period the

water was discharged. All three UV BWTSs applied a second UV-treatment at discharge. Water from the treated tanks thus passed again through pump and treatment system, after which discharge samples were taken. Both EC BWTSs added a neutralizing agent on discharge. Here, discharge samples were taken far enough downstream of the point of entry of this neutralizing agent to allow for mixing of the neutralizing agent into the water stream.

Table 1. Overview of treatment details for all systems.

System	Pre-treatment	Treatment
UV1	50 µm disc filter	One UV reactor, medium pressure. Treatment at both intake and discharge
UV2	200 µm mesh filter and 50 µm mesh filter	Two UV reactors, low pressure. Treatment at both intake and discharge
UV3	20 µm mesh filter	Three UV reactors, low pressure. Treatment at both intake and discharge
CD	40 µm mesh filter	Chlorine dioxide addition through mixing of two chemicals
EC1	40 µm mesh filter	Hypochlorite addition through electrolysis, neutralized by sodium bisulfite on discharge
EC2	200 µm filter and hydrocyclone	Hypochlorite addition through electrolysis, neutralized by sodium bisulfite on discharge

Re-growth experiments. To investigate re-growth, samples of control and treated water were taken at both intake and discharge in 10 liter carboys. These samples were transported to a climate room (16:8 light:dark regime, 100 µmol quanta m-2s-1, Philips TL-D Super 80 58W 865 daylight lamps) which was set at the temperature of the seawater at the time of sampling. Nutrients were added on day zero of the incubation at concentrations which are typical for the Wadden Sea (nitrate 20 µmol/L, phosphate 1,6 µmol/L, silicate 20 µmol/L). Bottles were sampled daily or every other day for phytoplankton abundances (using flow cytometry), phytoplankton fitness (using PAM fluorometry) and species composition (using molecular fingerprinting). For the UV1 and CD systems 3 separate incubations were performed. For the EC1 and UV2 systems 2 separate incubations were performed. For the EC2 system 1 incubation was performed and for the UV3 system 6 (4 separate of which 2 were performed in duplicate) total incubations were performed. For the CD BWTS and control tanks no discharge incubations were performed. Incubations were monitored for 20 days.

Additional UV and EC dose response experiments. A UV dose response experiment was performed during the UV2 test. Water was pumped from the NIOZ harbor and passed through the pump and the treatment system (filter and two UV reactors), all samples were taken behind the treatment system. Different doses of UV were applied, expressed as percentages of the normal treatment dose. The normal treatment dose (100 %) consisted of water passing through both UV reactors at 200 m^3 per hour. 200 % and 400 % doses were achieved by passing water through the system at 100 m^3/h and 50 m^3/h, respectively. A 75 % UV dose was achieved by turning off half the lamps in one reactor, switching off every second lamp to keep an even spread of UV radiation throughout the reactor. The 50 % was achieved by turning one reactor off completely. The 25 % was achieved by turning one reactor off and turning off half the lamps of the second reactor, switching off every second lamp to keep an even spread of UV radiation throughout the reactor. The 0% was achieved by turning off both UV reactors. Samples were treated as described for the re-growth experiments.

An EC dose response experiment was performed during the EC2 test. Water was treated with a normal dose of hypochlorite, but was only stored in the simulated ballast water tank for 2 hours before neutralizing. Seven doses were used; a control, Cl3 = full dose of hypochlorite, Cl2 = about half neutralized, Cl1 = small fraction of hypochlorite left, BS1 = small excess of bisulfite, BS2 = excess of bisulfite equivalent to half the hypochlorite dose, BS3 = excess of bisulfite equivalent to full hypochlorite dose.

Analytical procedures:

Flow cytometry. Living phytoplankton samples were counted in triplicate on a Coulter Epics™ XL-MCL flow cytometer (Beckmann Coulter) with a 488 nm excitation laser, triggering on red fluorescence (620 +/- 15 nm). Samples were counted for 300 seconds at high flow. With these settings all intact cells with chlorophyll were detected. Phytoplankton abundances reported are for a size range of 1 micron up to 100 micron.

PAM fluorometry. Photosystem II (PSII) efficiency as a measure of phytoplankton fitness was measured in triplicate using a Pulse Amplitude Modulated (PAM) Fluorometer (Water-PAM, Walz, Germany). Settings used were: measuring light frequency = 5, out-gain = 2, actinic light intensity = 6, actinic light width = 0.10, saturation pulse intensity = 10, saturation pulse width = 0.8.

Molecular characterization of phytoplankton composition. Samples were taken for the UV2 and UV3 incubation experiments, as well as for the EC dose experiment. For each sample 30 mL was filtered over a 0.2 µm filter (GTTP, Millipore) and stored at -80 °C. DNA was extracted from the filters with the UltraClean Soil DNA Isolation Kit (MO BIO Laboratories, Inc., CA, USA). DNA was amplified using primers specific for 16S rRNA gene segments of cyanobacteria and plastids (Nübel et al. 1997). Forward primer was CYA359F-GC, reverse primers were CYA781R(a) and CYA781R(b). The PCR program was set to five minutes at 94 °C, 35 cycles of one minute at 94 °C, one minute at 60 °C and one minute at 72 °C, and a last extension of five minutes at 72 °C. At the end of the PCR cycle temperature was reduced to 4 °C.

Denaturing Gradient Gel Electrophoresis (DGGE) was used to identify gene fragment diversity (Muyzer et al. 1993). A 6% acrylamide/bisacrylamide gel with a 20-80% urea/formamide gradient was used. 100 nanogram of quantified PCR product was loaded on the gel. The gel was stained using SYBR Gold (Molecular Probes, Inc. OR, USA) and analysed using a blue light converter. DNA bands were numbered and extracted from the gel for sequencing. Samples were re-amplified and cleaned using QuickClean 5M PCR Purification Kit (Genscript). Selected DGGE bands were sequenced twice, with primer 359F and with a mix of primers 781RA and 781RB. Samples were sequenced using a ABI PRISM 310 Genetic Analyzer. Results of forward and reverse sequences were combined in Autoassembler (ABI) and compared with sequences in Genbank using BLAST. Along with their blast hits, sequences were imported into Silva database nr. 102 (Pruesse et al. 2007), aligned accordingly and added to the tree sequences of photo-autotrophes using the ARB Parsimony algorhythm (Ludwig et al. 2004).

Statistics. In order to compare all re-growth results simultaneously, five variables, representative of the phytoplankton dynamics in the re-growth experiments, were normalized. Next these five normalized variables were used to compute a Euclidean resemblance matrix. The latter matrix was then used for a Non-metric Multi-Dimensional Scaling (NMS) diagram and an analysis of similarities

(ANOSIM) for differences between groups of samples (treatments) using permutation/randomization methods in the statististical software package Primer version 6.1.13 (PRIMER-E Ltd., Plymouth, UK). The null-hypothesis tested was that there were no differences in re-growth between treatments. Five parameters were used (Figure 1).

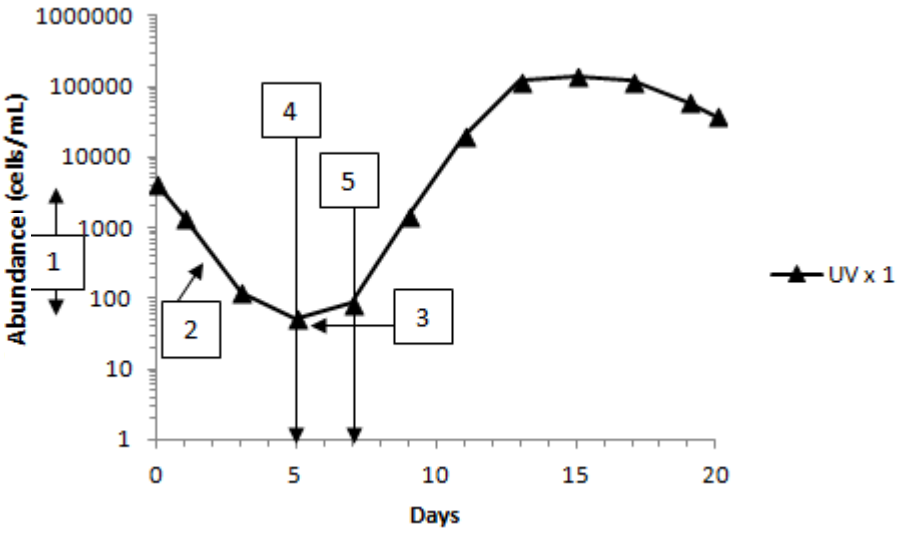

Figure 1. Parameters used for NMS analysis for all experiments with all treatment systems. 1. Percentage decrease, 2. Slope of decrease, 3. Minimum value, 4. Day of minimum value. 5. Day of re-growth.

In four cases no value could be assigned to the parameter "Day of re-growth". In order to still be able to perform the analysis, the Day of re-growth parameters were assigned the maximum value of 20 for these four cases. This was done for UV2 experiment 2 2xUV, CD experiment 2, EC1 experiment 2 (no neutralization) and EC2 experiment 1 (no neutralization).

3. Results

3.1 Comparison of BWTSs using UV radiation

There were common patterns between the three UV radiation based BWTSs. During the first days after treatment, abundances of phytoplankton only gradually decreased, both at intake (single UV treatment) and discharge (second UV treatment) (Figure 2). Abundances of phytoplankton in control samples stayed relatively stable while the PSII efficiency went down over the 20 day incubation period (Figure 2, 3). In treated samples the PSII efficiency was close to zero during the first days, but increased after 4-8 days to a peak value after which it decreased. (Figure 3).

All UV BWTSs showed re-growth after treatment, with the exception of the UV2 BWTS where there was no re-growth after the second UV treatment. For all three UV systems, re-growth occurred in 6-10 days after the first UV treatment and in 7-12 days after the second UV treatment (Table 2). Abundances of phytoplankton after re-growth were sometimes much higher than initial control abundances (Figure 2 A, C).

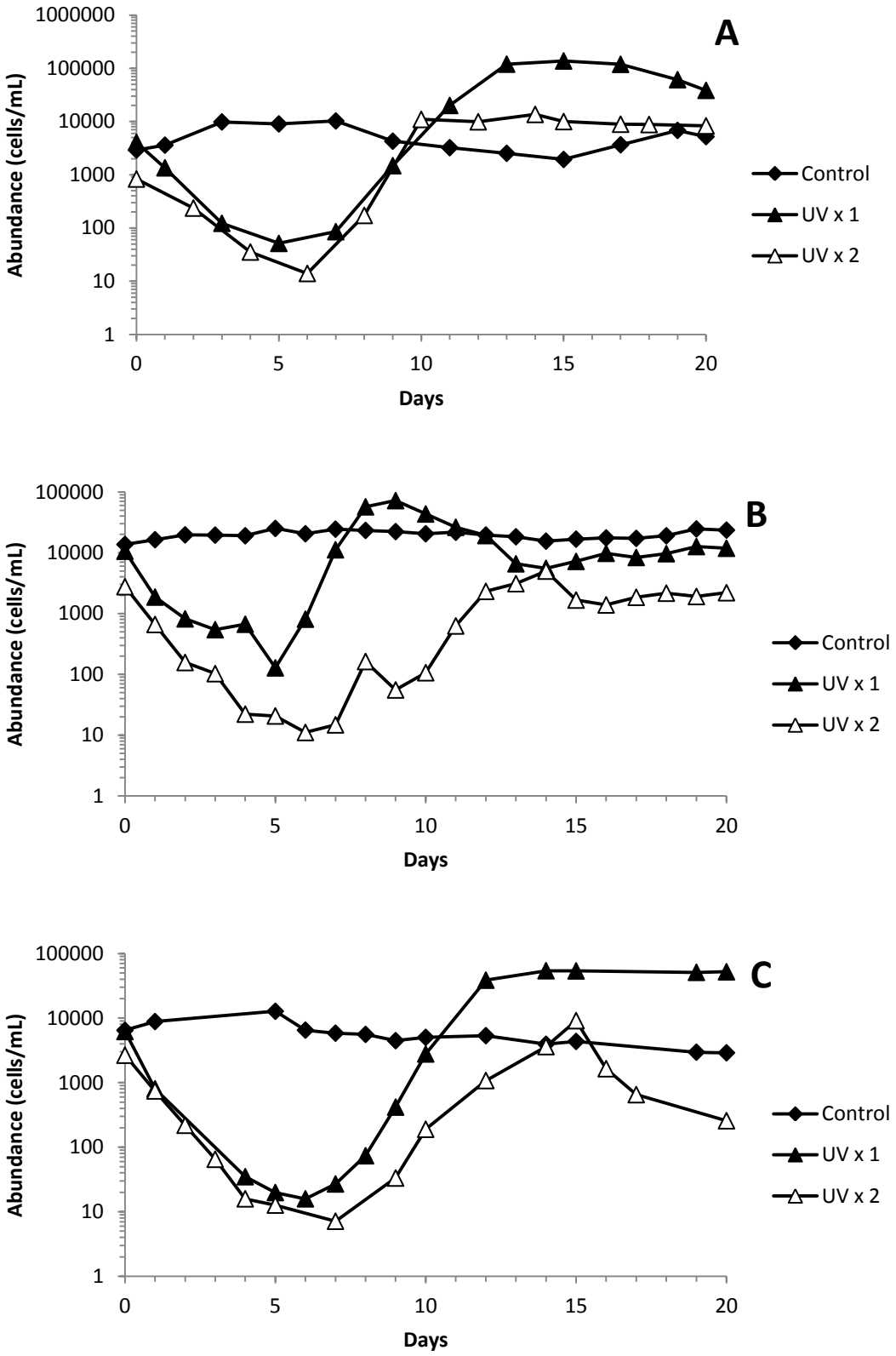

Figure 2. Abundances of phytoplankton in the re-growth experiment of the UV1 system (A), the UV2 system (B) and the UV3 system (CF). The UV x 1 is the ballast water at intake when it passed through the UV reactor once; the UV x 2 is the ballast water at discharge when it has passed through the UV reactor twice. Treatment was applied at T=0 and the incubations were monitored for 20 days.

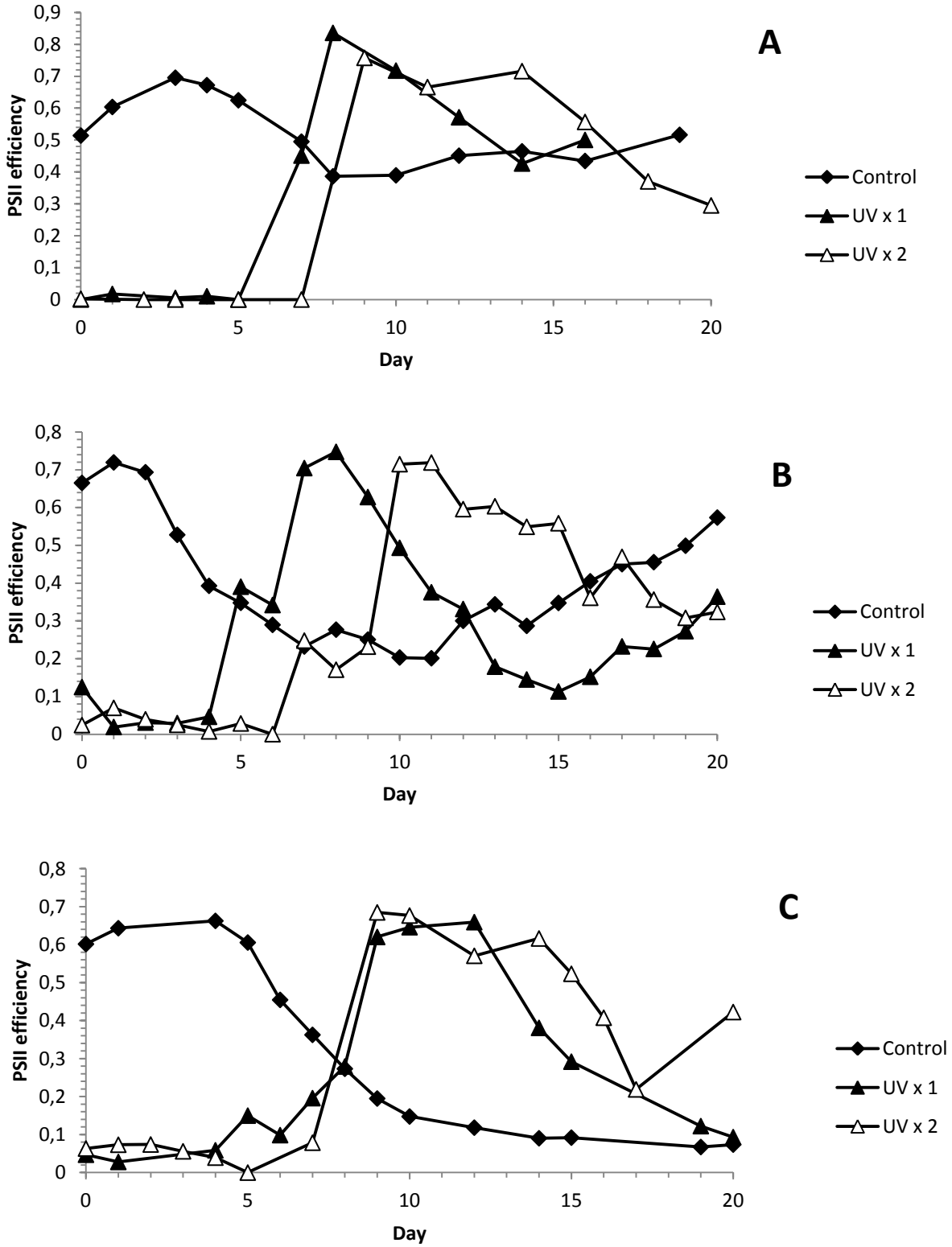

Figure 3. PSII efficiency in the re-growth experiment of the UV1 system (A), the UV2 system (B) and the UV3 system (C). The UV x 1 is the ballast water at intake when it passed through the UV reactor once; the UV x 2 is the ballast water at discharge when it has passed through the UV reactor twice. Treatment was applied at T=0 and the incubations were monitored for 20 days.

Table 2. Days that re-growth was first observed for all incubation experiments of the three UV BWTSs after both single and double treatment. The UV x 1 is the ballast water at intake when it passed through the UV reactor once, the UV x 2 is the ballast water at discharge when it has passed through the UV reactor twice. N = no re-growth.

System	Experiment	1 x UV	2 x UV
UV1	1	8	7
UV1	2	9	8
UV1	3	10	10
UV2	1	6	10
UV2	2	7	N
UV3	1	9	7
UV3	2	9	7
UV3	3	9	12
UV3	4	8	9
UV3	5	8	9
UV3	6	8	12

Genetic analysis of re-growing phytoplankton species was only performed on the UV2 and UV3 BWTSs (Table 3). Always only one species was found in the control sample, but this is a known limitation of the analytical method (Stehouwer et al. (2012)). The species found in the control sample did not match any of the species found after treatment for any of the experiments. While *Thalassiosira pseudonana* survived and re-grew after treatment with the UV2 system, this species apparently did not survive treatment with the UV3 system where it was detected in the control, but after treatment only *Thalassiosira weissflogii* was found.

Table 3. Phytoplankton species identified using genetic analysis during the re-growth experiments of the UV systems.

System	Experiment	Control	1xUV	2xUV
UV2	1	*Rhizosolenia setigera*	*Skeletonema costatum, Thalassiosira* sp., *Thalassiosira pseudonana*	*Thalassiosira* sp.
UV2	2	*Dinophyceae* sp.	*Chaetoceros calcitrans*	No re-growth
UV3	1	*Thalassiosira pseudonana*	*Coscinodiscus radiatus, Thalassiosira weissflogii*	*Stephanopyxis* sp., *Thalassiosira weissflogii*
UV3	2	*Thalassiosira pseudonana*	*Thalassiosira weissflogii*	*Thalassiosira weissflogii*
UV3	5	*Ditylum brightwellii*	*Skeletonema costatum, Thalassiosira weissflogii*	No sample

3.2 UV dose response experiment

The UV dose response experiment of the UV2 BWTS showed reduced abundances of phytoplankton with increasing UV dose. The control showed a gradual decrease in abundance of phytoplankton.

The gradual decrease in abundances of phytoplankton, which was observed during normal incubation experiments, was visible at all dosages; at higher dosages the decrease was more pronounced. Performance at a dosage of 75 % was similar to performance at a dosage of 100 %, but at 50 % the drop in abundance of phytoplankton was much less (Figure 4A).

All UV doses immediately reduced PSII efficiency to below 0.1, except for the 25% dosage. The PSII recovery occurred fastest at 50 % and slowest at 400 % (Figure 4B). The PSII efficiency in the control sample showed a gradual decrease while in treated samples it showed a peak after re-growth after which it decreased.

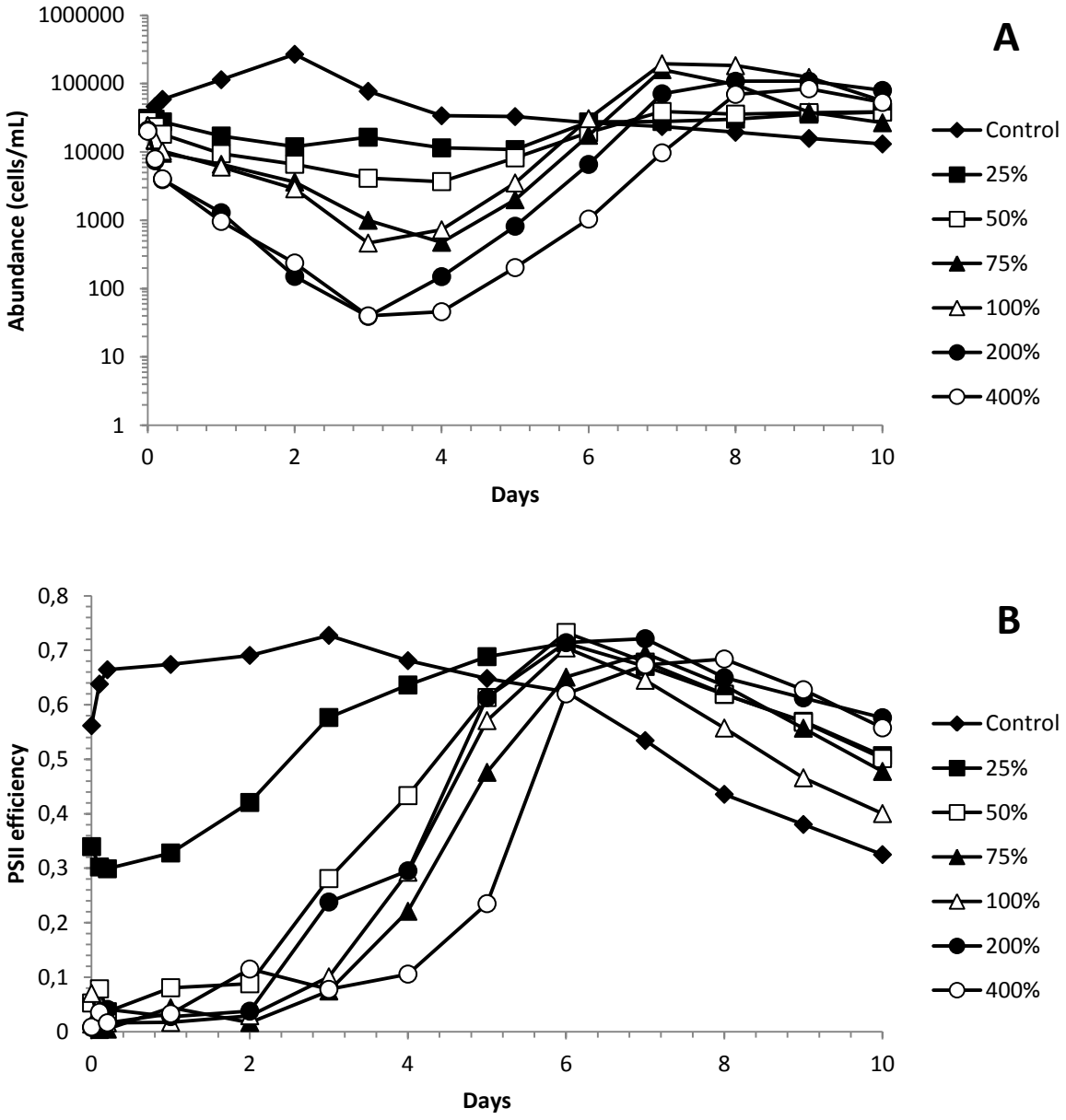

Figure 4. Abundance of phytoplankton (A) and PSII efficiency (B) in the dosage experiment of the UV2 BWTS. Dosage expressed in percentage of normal treatment dosage.

3.3 Comparison of chlorine-based treatment systems

The CD BWTS reduced abundances of phytoplankton and PSII efficiency immediately. This decrease continued during the first three incubation days, while abundances of phytoplankton in the control stayed relatively constant (Figure 5, 6). Of the three incubation experiments with the CD BWTS re-growth was observed twice, once at T12 and once at T20 (Table 4). Abundances of phytoplankton in the treated sample after re-growth were higher than abundances of phytoplankton in the control (Figure 5 A).

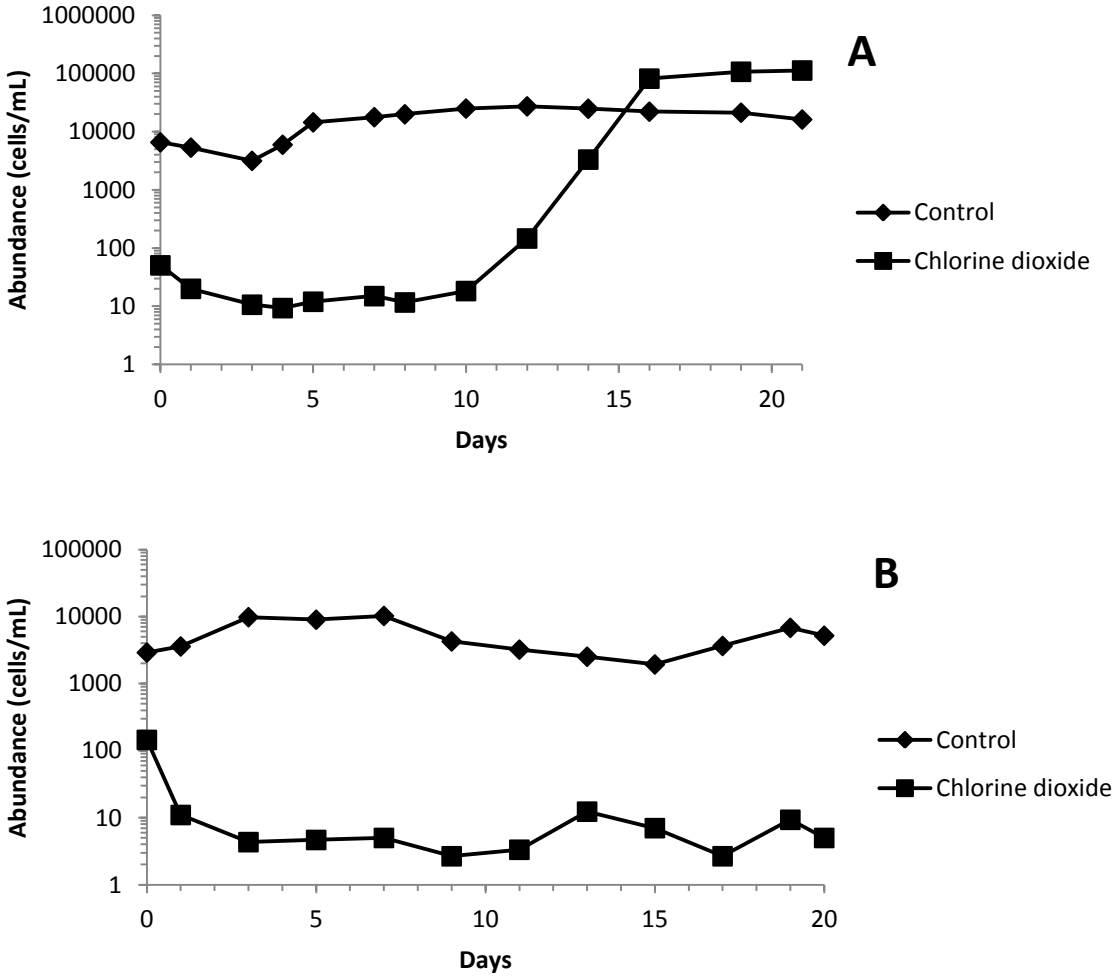

Figure 5. Abundances of phytoplankton in the re-growth experiment of the CD BWTS. The first re-growth experiment showed re-growth of phytoplankton after treatment (A), the second experiment showed no re-growth (B).

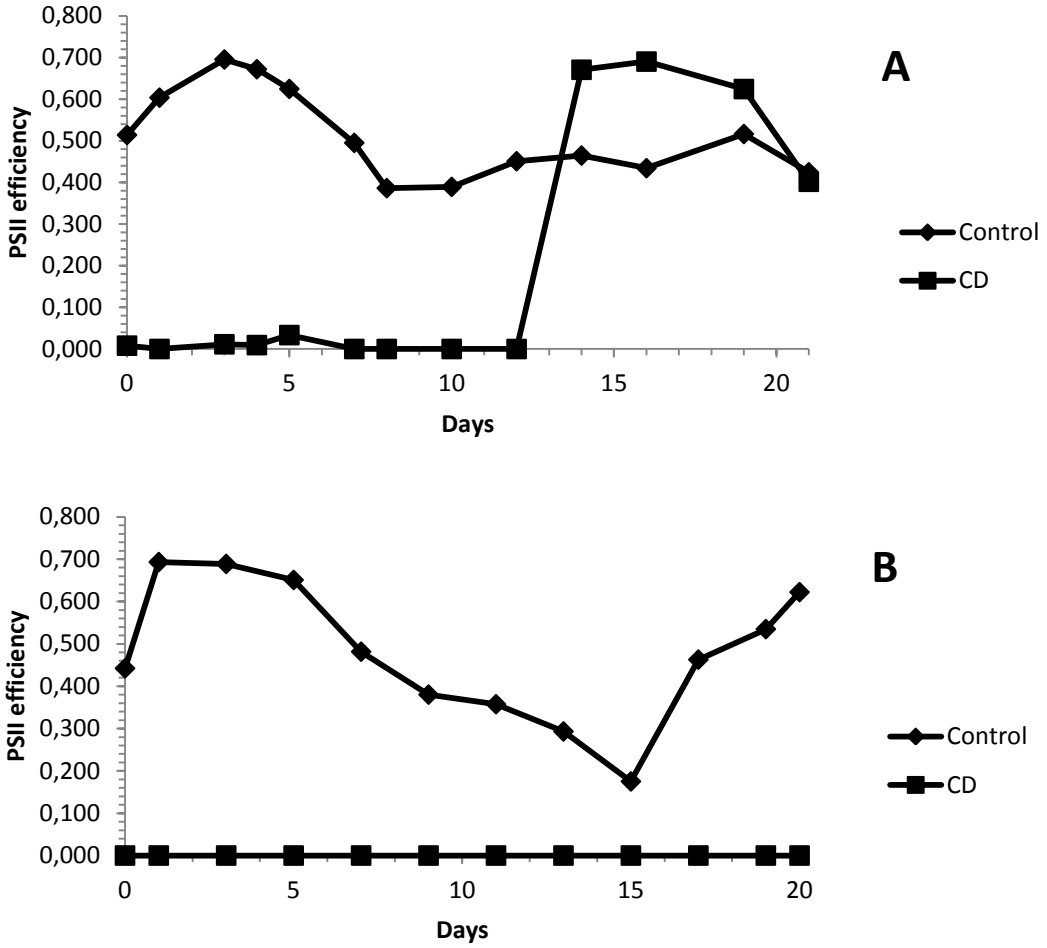

Figure 6. PSII efficiency in the re-growth experiment of the CD BWTS. The first re-growth experiment showed re-growth of phytoplankton after treatment (A), the second experiment showed no re-growth (B).

Table 4. Days that re-growth was first observed for all incubation experiments of the CD BWTS and the two EC BWTSs after treatment and neutralization.

System	Experiment	Treatment	Neutralization
CD	1	12	
CD	2	N	
CD	3	20	
EC1	1	19	11
EC1	2	N	18
EC2	1	N	7

In the first incubation experiment of the EC1 BWTS there was re-growth after 19 days without neutralization and after 11 days with neutralization (Table 4). The second incubation experiment of EC1 showed no re-growth without neutralization and re-growth after 18 days with neutralization (Figure 7A, 8A). The first incubation experiment of the EC2 BWTS did not show re-growth without neutralization, but with neutralization re-growth occurred after 7 days (Figure 7B, 8B). All three EC

incubation experiments showed similar patterns with an immediate and strong reduction in abundance of phytoplankton while the controls stayed relatively constant. PSII efficiency also showed an immediate and strong reduction, but PSII efficiency in the controls was also strongly reduced after about 10 days.

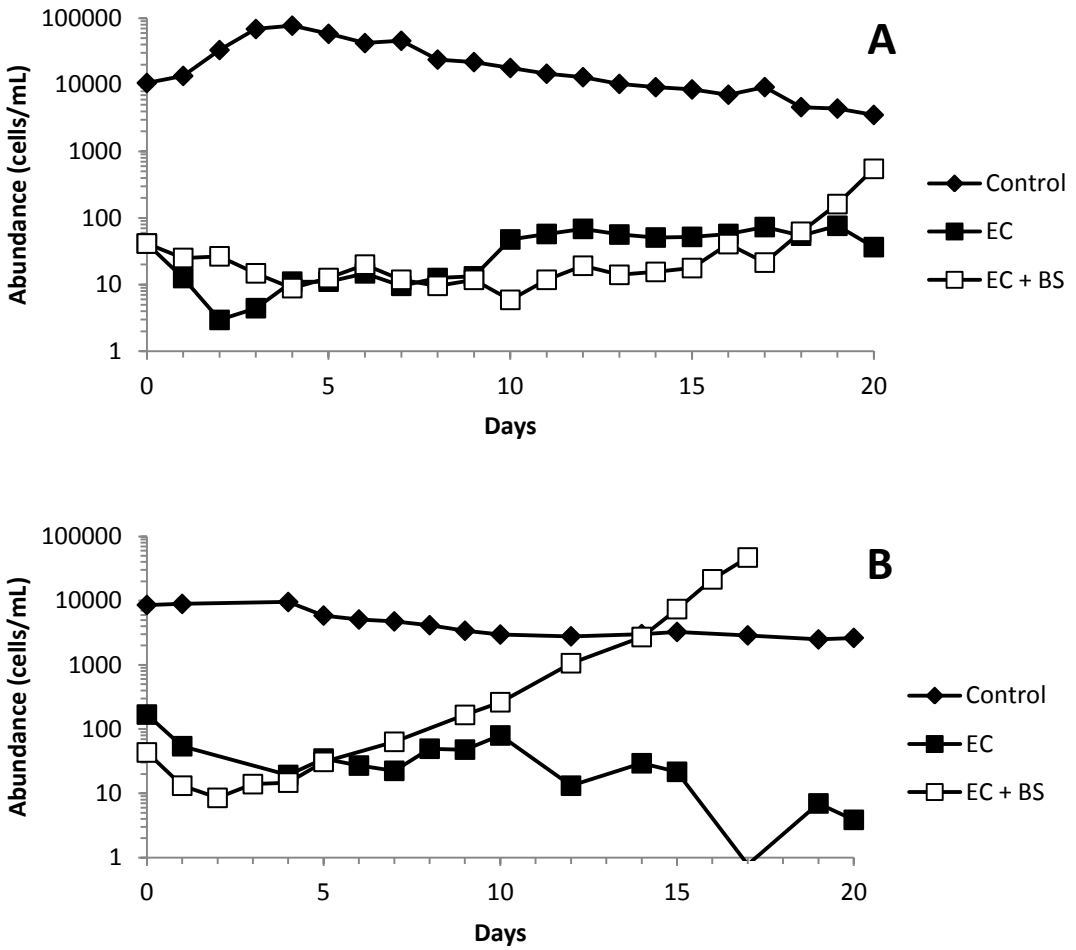

Figure 7. Abundances of phytoplankton in the re-growth experiment of the EC1 BWTS (A) and the EC2 BWTS (B).

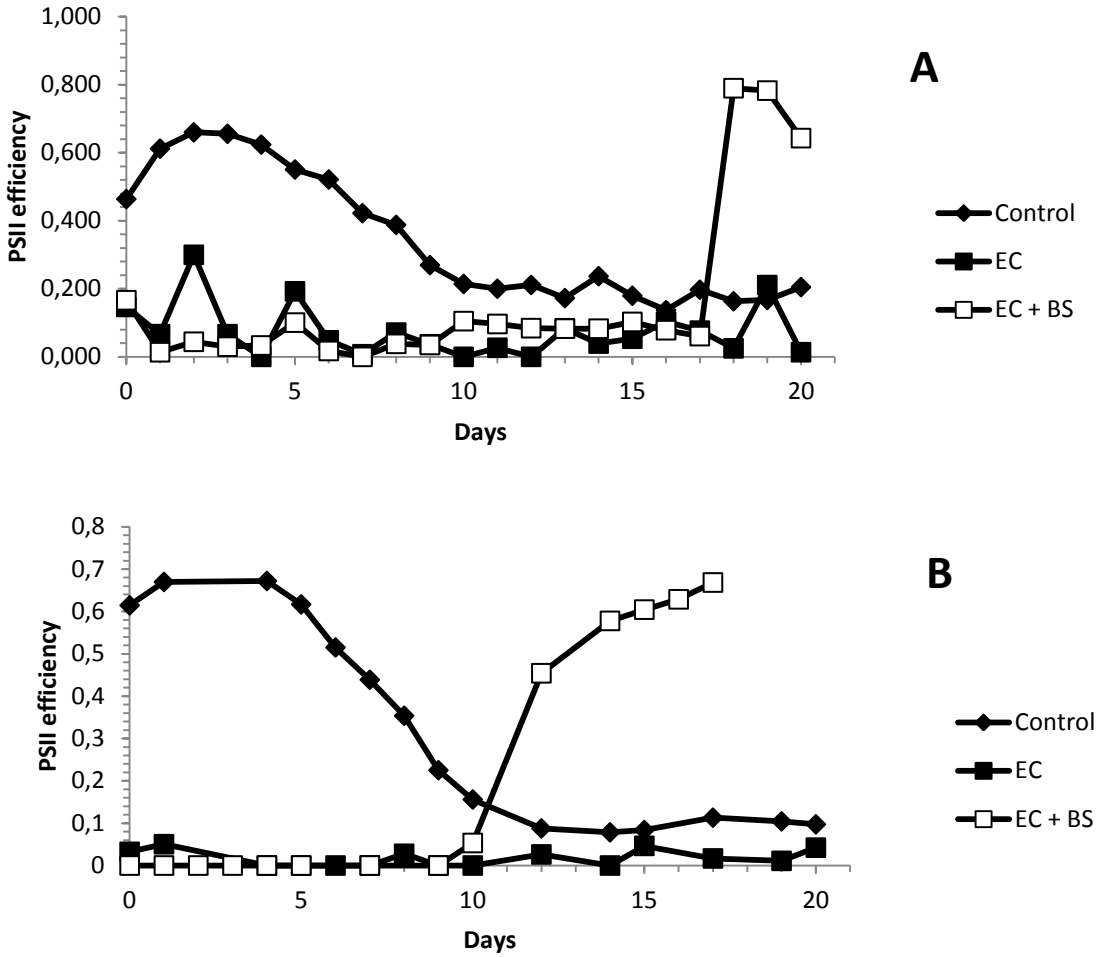

Figure 8. PSII efficiency (C, D) in the re-growth experiment of the EC1 BWTS (A) and the EC2 BWTS (B).

3.4 EC dosage experiment

Abundances of phytoplankton in the control showed a strong peak followed by a reduction to below the starting abundance, after which the abundance of phytoplankton remained stable (Figure 9A). The PSII efficiency showed a similar pattern, starting high but afterwards decreasing to a low but stable level (Figure 9B). Without neutralization of the hypochlorite no re-growth occurred. With partial neutralization of the hypochlorite with sodium bisulfite and with sodium bisulfite excess re-growth occurred (Figure 9). Abundances of phytoplankton after re-growth were higher than initial control abundances.

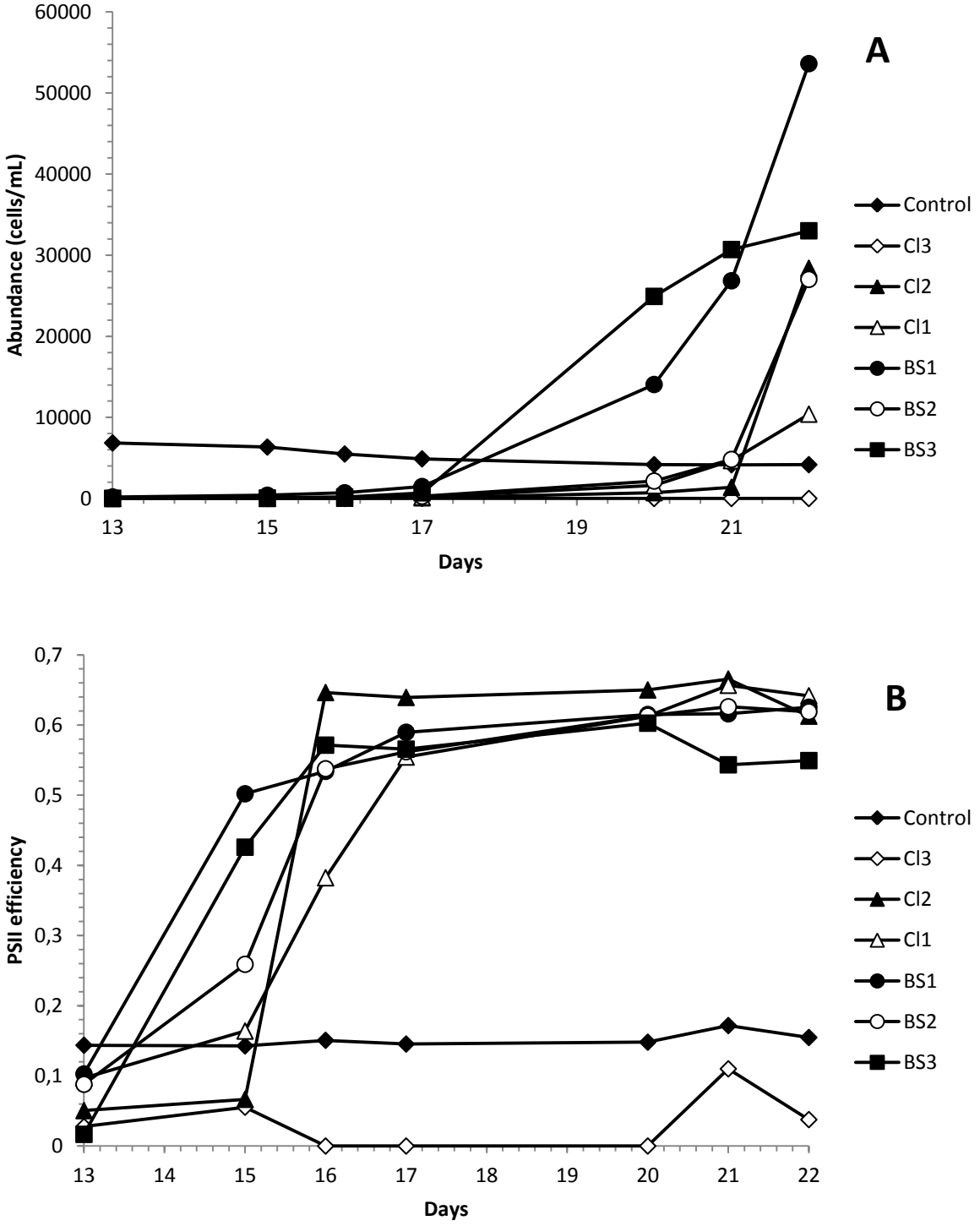

Figure 9. Abundances of phytoplankton (A) and PSII efficiency (B) after treatment with different dosages of hypochlorite and bisulfite in the EC2 system.

Genetic identification of phytoplankton species was performed on the dosage experiment of the EC2 BWTS (Table 5). None of the species detected in the control sample were found after treatment but neither were the species detected after treatment found in the control. The phytoplankton species *Navicula phyllepta* and *Chaetoceros socialis* were both found in samples with excess hypochlorite

and excess sodium bisulfite. The phytoplankter *Emiliania huxleyii* was only found in samples with excess sodium bisulfite.

Table 5. Phytoplankton species identified using genetic analysis in the samples of the EC dosage experiment. Cl3 = full dose of hypochlorite, Cl2 = about half neutralized, Cl1 = small fraction of hypochlorite left, BS1 = small excess of bisulfite, BS2 = excess of bisulfite equivalent to half the hypochlorite dose, BS3 = excess of bisulfite equivalent to full hypochlorite dose.

Dose	Species identified
Control	*Rhizosolenia setigera, Stephanopyxis* sp.*, Thalassiosira weissflogii*
Cl3	No re-growth
Cl2	*Navicula phyllepta*
Cl1	*Chaetoceros socialis*
BS1	*Emiliania huxleyi*
BS2	*Navicula phyllepta, Chaetoceros socialis, Emiliania huxleyii*
BS3	*Chaetoceros socialis*

3.5 Comparison of UV and EC treatment systems

The NMS analysis of the comparison between all treatment types revealed a difference between treatments ($R = 0.33$, $P<0.01$). Two different groups were found: the first group consisted of 1xUV and EC, the second group of EC + BS, CD and 2xUV (figure 10). However, 1xUV can be considered an incomplete treatment, since in all UV systems water is also treated on discharge. When an ANOSIM analysis was conducted excluding the 1xUV data, the result was different. A significant difference was found between 2xUV and CD ($P<0.05$) and between 2xUV and EC, including EC+BS ($P<0.05$). No significant difference was found between CD and EC.

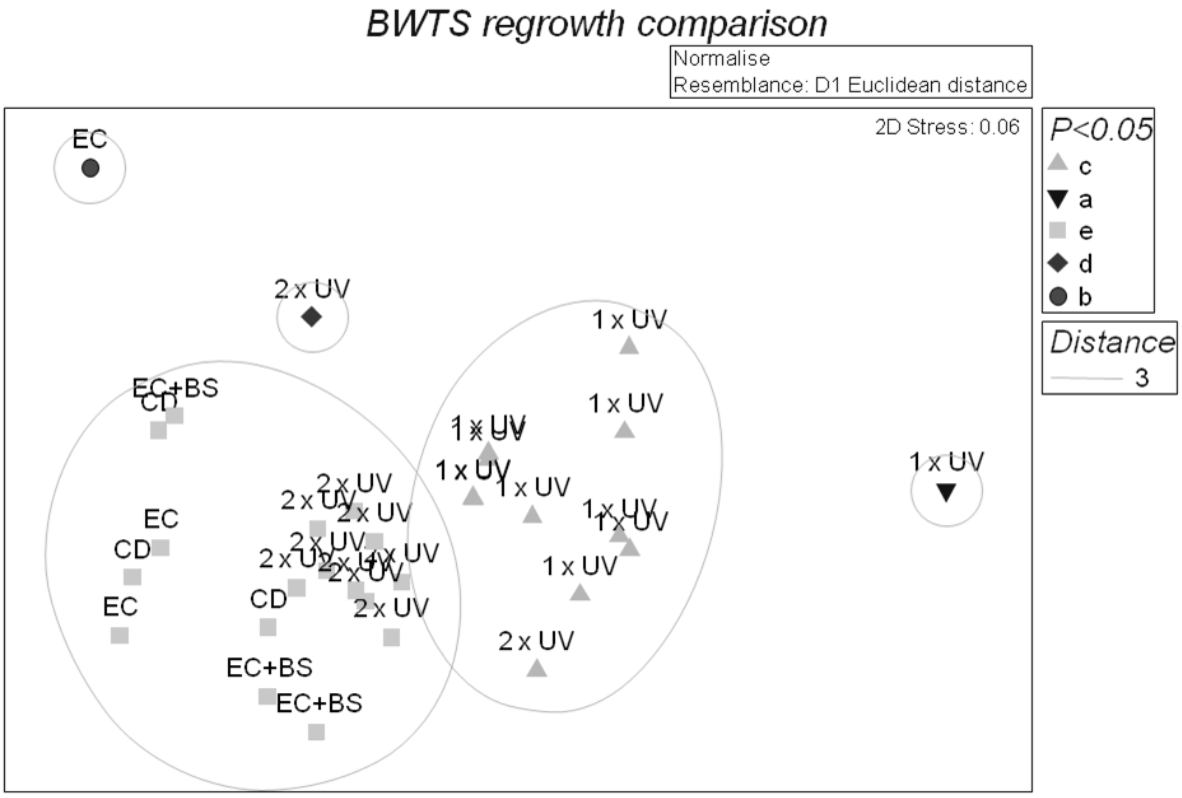

Figure 10. NMS of all incubation experiment data. Two main groups are significantly different, 1xUV (light gray triangles) and 2xUV, CD, EC and EC+BS (light gray squares). There are also three outliers. (The EC outlier is the EC experiment with the earliest re-growth (EC2, EC+BS). The 2xUV outlier is the only UV experiment with no re-growth (UV2 experiment 2), set to re-growth on day 20. The 1xUV outlier is an experiment with a low initial reduction, high minimum value and high slope of decrease (UV1, experiment 2).)

4. Discussion/Conclusion

Comparison of UV. Despite their differences in configuration (the filters as well as the reactors) and UV wavelength (Table 1), the three UV radiation based treatment systems produced similar results. All of them showed a gradual decrease in phytoplankton numbers after treatment. This 'delayed effect' emphasizes the importance of PSII efficiency measurements for phytoplankton, since abundances of phytoplankton (as measured by flow cytometry) are higher than IMO standards immediately after treatment. However, the phytoplankton have very low PSII efficiency and disintegrate over time, reaching abundances below the IMO standards (Figure 2, 3). Re-growth occurred in all systems, and in all systems around the same time, between 6 and 12 days (Table 2) which is similar to the 6 to 8 days observed by Martínez et al. (2012). Buma et al. (2009) also showed the ability of phytoplankton to recover after PSII efficiency had been reduced. On discharge initial abundances of phytoplankton are lower than at intake and minimum abundances of phytoplankton are also lower. The results of genetic identification of phytoplankton were also similar for the two UV BWTSs tested (UV2 and UV3). For both systems *Skeletonema costatum* was a re-growing species, but the most frequent re-grower were species belonging to the genus *Thalassiosira*. After treatment

with the UV2 BWTS *Thalassiosira pseudonana* was the main re-grower while after treatment with the UV3 BWTS it was *Thalassiosira weissflogii*. This indicates that *Thalassiosira* is more resistant to UV radiation than other phytoplankton genera, matching the conclusions of Sutherland et al. (2001), Liebich et al. (2012) and Stehouwer et al. (2012). It is interesting however that *Thalassiosira pseudonana* was found in the control samples for the UV3 system, but after treatment *Thalassiosira weissflogii* was the re-growing species. It is unknown if this is really a species-shift or a misidentification by the genetic analysis method.

UV dosage. The UV dosage experiment of the UV2 BWTS showed that even with treatment dosage reduced to 75% (by disconnecting half the lamps of the second UV reactor), the abundance of phytoplankton and viability behaved similar to 100% treatment (Figure 4A and B). This suggests that even when treatment effectiveness is reduced, the system will still perform up to IMO standards. Additionally, the 200% and 400% dosage treatments (achieved by lowering flow speed so water spent more time in the reactors) showed a stronger reduction in abundance of phytoplankton than the 100% treatment, but re-growth occurred around the same time. This suggests that an increase in UV radiation dose will not eliminate the possibility of re-growth.
It should be noted that all UV doses in the UV dosage experiment were from a single treatment; in normal UV ballast water treatment the water would get a second treatment before discharge which would further lower organism numbers and possibly delay re-growth.

Comparison of CD and EC. Both EC BWTSs and the CD BWTS showed an immediate decrease of the abundance of phytoplankton after addition of chemicals. The main difference of the CD BWTS compared to the EC BWTSs is that the CD BWTS does not add a neutralizing agent upon discharge. For both normal CD treatment and EC treatment without neutralization re-growth occurred in part of the experiments (re-growth in 2 out of 3 experiments for CD and 1 out of 3 experiments for EC) (Table 4). In two of the three re-growing experiments, re-growth only occurred just before the end of the experiment (day 20 for the third CD experiment, day 19 for the first EC1 experiment) (Table 4). It is therefore recommended to conduct re-growth studies on thess types of systems for longer than 20 days. The EC results with neutralization were very different; all three showed re-growth suggesting that neutralization of the residual hypochlorite (and possible by-products) accelerates the re-growth process.

EC dosage. Without neutralization no re-growth occurred, but even partial neutralization of the hypochlorite with sodium bisulfite resulted in re-growth within 20 days. Re-growth also occurred when excess sodium bisulfite was added. Neutralization is therefore an important part of making the treated ballast water safe for discharge since even partial neutralization apparently mitigates the harmful effect of hypochlorite. While the major re-grower from the UV experiments, *Thalassiosira weissflogii*, was present in the control samples it was not detected after treatment. The re-growing species for the EC experiments were *Navicula phyllepta*, *Chaetoceros socialis* and *Emiliania huxleyii* of which *Emiliania huxleyii* only occurred in samples that were completely neutralized or had an excess of neutralizing agent.

Comparison of UV and CD/EC. As expected, statistical analysis showed that there was a difference between treatment types. Unexpectedly, 2xUV grouped together with EC and CD, with 1xUV grouping separately (figure 10). With 1xUV excluded from the analysis the data showed the expected pattern with no significant difference between CD and EC but significant difference

between UV and chemical treatments. The most important contributing factors to this difference were the lower initial reduction in phytoplankton numbers, lower slope of decrease and earlier start of re-growth of the UV systems.

In both UV and CD/EC BWTSs abundances of phytoplankton after re-growth were higher than initial abundances in the control. This is probably due to the fact that most re-growing species are small (10 micron or smaller) while the control consisted of a mixture of species of different sizes. Since small organisms need less nutrients per individual than larger organisms, this would explain the differences in abundance.

All BWTSs compared used a filtration step and a self-cleaning filter, but the mesh size of these filters varied between 20 and 200 micron. However, when comparing the results of UV2 (50 micron filter mesh) and UV3 (20 micron filter mesh) re-growing species were similar. Re-growing species of the EC2 BWTS, which used a 200 micron filter mesh, were all below 10 micron in minimum dimension. Since all re-growing species were smaller than the smallest filter mesh used, it is suggested that the size of the filter mesh did not significantly affect the re-growing species.

Re-growing phytoplankton species differed between UV and chemical systems. This indicates there is not one 'super plankton' resistant to all treatments. Different ballast water treatment techniques have different challenge species, whether it is because of a built-in resistance or because of a life history which allows such species to escape the effects of the treatment.

When comparing UV and EC BWTSs from a ship owner perspective, UV systems have the advantage that no chemicals need to be carried aboard. In case of emergency, the ballast water can be discharged at any point without environmental problems. As a disadvantage, when scaling up the system both extra filtration and UV units need to be installed, and this requires more space. Additionally, UV reactors are large energy-consumers, especially in low UV-transmittance waters (such as coastal waters with high turbidity due to suspended solids) where the ballast water flow rate might even have to be reduced in order to treat the ballast water with the required minimum dose. The EC systems are easier to scale up since the reactor needs only a minimal size increase to increase hypochlorite production; only extra filtration units are required. However, these systems need to have chemicals onboard. In the case of the chlorine dioxide system these chemicals are the two components of the reactor mixture, whereas with the EC systems the chemical sodium bisulfite is required to neutralize the hypochlorite before discharge. Additionally, EC systems require salt water to produce the active substance. When operating in fresh water a supply of salt water will have to be carried onboard.

All six systems described in this paper did meet the IMO D-2 standards for ballast water treatment systems. The IMO standards do not ask for ballast water free of organisms, but set a strict and low maximum standard. As the present experiments have shown, organisms can re-grow after treatment by each of the six BWTSs when provided with favorable growth conditions. This means that the risk of invasive species is not eliminated by ballast water treatment. On the other hand, the abundance of organisms introduced is strongly reduced, which results in a reduction in propagule pressure. Propagule pressure is a key factor in the success of non-native species in a new environment (Holle & Simberloff 2005, Lockwood et al. 2005, Colautti et al. 2006). Even though ballast water treatment is not 100% effective it still greatly reduces the threat of invasive species spread through ballast water.

Acknowledgements

Special thanks to the NIOZ Ballast Water team for their assistance during the ballast water treatment system tests and to Dr. Judith van Bleijswijk and Julie Ogier for their assistance with the genetic analyses.

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

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6. Microbial dynamics in acetate-enriched ballast water at different temperatures

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Published in: Ecotoxicology and Environmental Safety, Volume 96, Pages 93–98

Abstract

The spread of invasive species through ships' ballast water is considered a major ecological threat to the world's oceans. For that reason, the International Maritime Organisation (IMO) has set performance standards for ballast water discharge. Ballast water treatment systems have been developed that employ either UV-radiation or 'active substances' to reduce the concentration of living cells to below the IMOs standards. One such active substance is a chemical mixture known as Peraclean® Ocean. The rest product of Peraclean® Ocean is acetate that might be present at high concentrations in discharged ballast water. In cold coastal waters the breakdown of acetate might be slow, causing a buildup of acetate concentrations in the water if regularly discharged by ships. To study the potential environmental impact, microbial dynamics and acetate degradation were measured in discharge water from a Peraclean® Ocean treatment system in illuminated microcosms. In addition, microbial dynamics and acetate degradation were studied at -1°, 4°, 10°, 15° and 25°C in dark microcosms that simulated enclosed ballast water tanks.

Acetate breakdown indeed occurred faster at higher temperatures. At 25°C the highest bacteria growth, fastest nutrient and oxygen consumption and highest DOC reduction occurred. On the other hand, at -1 ˚C the bacterial growth was strongly delayed, only starting to increase at the end of the experiment. Furthermore, at 25°C the acetate pool was not depleted, probably due to nutrient and oxygen limitation. This means that not all acetate will be broken down in ballast water tanks, even during long voyages in warm waters. In addition, at low temperatures acetate breakdown in ballast water tanks and in discharged water will be extremely slow. Therefore, regular discharge of acetate-containing ballast water in harbors and bays may cause eutrophication and changes in the microbial community, especially in colder regions.

Keywords: ballast water, Peraclean® Ocean, bacteria, acetate

1. Introduction

Invasive species are a major ecological and economical problem worldwide. In the marine environment ship's ballast water is one of the major sources of invasive species (Gollasch, 2006; Molnar et al., 2008). Because of this, the International Maritime Organisation (IMO) has set up regulation G8 for the management of ballast water to minimize the transfer of harmful aquatic organisms (IMO, 2008). Regulation G8 includes the D-2 ballast water performance standard, which states the limits on organisms larger than 50 micron, organisms between 10 and 50 micron and

certain 'indicator microbes' (*Vibrio cholerae*, *Escherichia coli* and *Enterococci*) in ballast water; no standards are included for smaller organisms or heterotrophic bacteria abundances. In order to meet the D-2 standard ships will have to be equipped with ballast water treatment systems (BWTSs). Several companies developed BWTSs, using a variety of treatments such as UV-radiation or electrolytic chlorination (Gregg et al., 2009; Tsolaki and Diamadopoulos, 2010).

The SEDNA ballast water treatment system used for this paper uses a combination of hydrocyclone, filter and a chemical mixture called Peraclean® Ocean. This mixture consists of hydrogen peroxide, a bacteriostatic, that comprises an equilibrium between peroxyacetic acid, which generates radicals that damage cell structures, and acetate (Fuchs and de Wilde, 2004). Pilot studies on this system were performed at the Royal Netherlands Institute for Sea Research (NIOZ) between 2004 and 2006 (Veldhuis et al., 2006). Land-based testing according to IMO G8 regulations was performed in 2007 at the Dutch island Texel, located at the border of the North Sea and the Wadden Sea. Land-based tests showed that the SEDNA system meets the D-2 standards as set by the IMO.

The effectiveness of Peraclean® Ocean as a biocide on different types of zooplankton and phytoplankton has been proven (Fuchs and de Wilde, 2004; Gregg and Hallegraeff, 2007; Veldhuis et al., 2006). Peraclean® Ocean is, however, not biocidal to bacteria. It only inhibits bacterial re-growth. Gregg and Hallegraeff (2007) showed that Peraclean® Ocean is biodegradable, but that degradation is slower than claimed by the manufacturer. At treatment concentration it required 3-6 weeks to degrade to non-toxic level in filtered seawater. Sediments and biological matter would sometimes speed up the degradation and light also caused it to degrade faster.

Low temperatures reduced the activity of Peraclean® Ocean. On the other hand, De Lafontaine et al. (2008) found Peraclean® Ocean to be effective down to 1 °C. They also show that it is effective in both fresh and salt water, although degradation was much faster in salt water. They express concern that discharged water might still be toxic, especially when used in fresh water. It is known that the degradation of Peraclean® Ocean is inversely related to temperature (Kunigk et al., 2001). Additionally, De Lafontaine et al. (2008) indicated the possible risk of eutrophication by large amounts of acetate.

Because of concerns about residual toxicity and eutrophication two different experiments were set up in addition to the standard land-based BWTS tests. The first experiment consisted of incubation experiments with Peraclean® Ocean treated water during land-based testing to examine the response of phytoplankton and bacteria to Peraclean® Ocean during simulated ballasting procedures. The second experiment consisted of acetate enrichment of natural seawater. This enriched seawater was then incubated at different temperatures to monitor the response of the bacterial community and the degradation of the acetate.

This paper focuses on the bacterial component of ballast water, since bacteria are frequently overlooked in ballast water research. Ruiz et al. (2000) stated that bacteria are more abundant, reproduce faster and have higher environmental tolerances than invertebrates, which are already known to be successful invaders of coastal habitats. Drake et al. (2007) added, that bacterial pathogenicity or toxicity could cause detrimental effects on the ecosystem and that delivery of bacteria to the world's ports is expected to escalate. Finally, bacteria are important to get studied because they are more likely to survive Peraclean® Ocean treatment compared to otic plankton (Fuchs and de Wilde, 2004; Gregg and Hallegraeff, 2007; Veldhuis et al., 2006).

2. Materials and Methods

2.1 Chemical analyses.

Dissolved Organic Carbon (DOC), serving as an indication of the concentration of acetate, was measured using a TOC-V Total Organic Carbon Analyzer (Shimadzu) after filtration of 20 ml sample over an Acrodisc® 25 mm syringe filter with a 0.2 µm HT Tuffryn® membrane (Pall® Life Sciences, NY, USA) to remove particulate carbon.

For Dissolved Oxygen (DO) 120 ml Winkler oxygen bottles with no headspace were incubated under in situ circumstances. Manganese sulfide and potassium iodine were added to precipitate the oxygen. Sulfuric acid was then added and DO concentration was determined using a Hitachi U-1100 spectrophotometer set at 456 nm.

Samples for the inorganic nutrients nitrate and phosphate were filtered through 0.2 µm Acrodisk filters (Pall® Life Sciences, NY, USA), frozen, and stored at –80°C. Nutrient analyses were performed according to state of the art NIOZ protocols as described in Peperzak et al. (2011).

2.2 Bacteria enumeration, phytoplankton viability and enumeration.

Living phytoplankton samples were counted on a Coulter Epics™ XL-MCL flow cytometer (Beckmann Coulter) with a 488 nm excitation laser, triggering on red fluorescence (620 +/- 15 nm). This count comprises all autotrophs, both eukaryotic as well as prokaryotic phytoplankton. Phytoplankton viability was measured using a Pulse Amplitude Modulated (PAM) Fluorometer, which measures the photosynthetic efficiency. Efficiency (yield) of zero indicates that there is no living phytoplankton in the sample. Settings used were: measuring light frequency = 5, out-gain = 2, actinic light intensity = 6, actinic light width = 0.10, saturation pulse intensity = 10, saturation pulse width = 0.8.

For heterotrophic bacteria enumeration 1,5 ml sample was fixed with 150 µl of formaldehyde/hexamine (1,8 %/1 % final concentration). The samples were then snapfrozen in liquid nitrogen and stored at -80 °C until analysis. After thawing, 100 µl of sample was added to 400 µl of TE buffer (10 mM Tris, 1 mM EDTA) and 10 µl of PicoGreen (Invitrogen) (10 times diluted from factory stock with milliQ) was added. It was left to stain for 12 minutes. The bacteria were then counted on a Coulter Epics™ XL-MCL flow cytometer (Beckmann Coulter) with a 488 nm excitation laser, triggering on green fluorescence (525 +/- 20 nm).

2.3 Bacterial species composition.

For genetic analysis of the bacteria population 30 ml of sample was filtered over a 0.2 µm GTTP filter (Millipore) and stored at -80 °C. DNA was extracted from the filters with the UltraClean Soil DNA Isolation Kit (MO BIO Laboratories, Inc., CA, USA). The forward primer was 341F-GC, reverse primers were 907RA(a) and 907RC(b). The PCR program was set to five minutes at 94 °C, 35 cycles of one minute at 94 °C, one minute at 60 °C and one minute at 72 °C, and a last extension of five minutes at 72 °C. At the end of the PCR cycle, the temperature was reduced to 4 °C. Denaturing Gradient Gel Electrophoresis (DGGE) was used to identify gene fragment diversity (Muyzer et al., 1993). A 6%

acrylamide/bisacrylamide gel with a 20-80% urea/formamide gradient was used. 100 nanogram of quantified PCR product was loaded on the gel. The gel was stained using SYBR Gold (Molecular Probes, Inc. OR, USA) and analyzed using a blue light converter. DNA bands were numbered and extracted from the gel for sequencing. Samples were re-amplified and cleaned using QuickClean 5M PCR Purification Kit (Genscript). DGGE bands were sequenced twice, with primer 341F and with a mix of primers 907RA and 907RC. Samples were sequenced using an ABI PRISM 310 Genetic Analyzer. Results of forward and reverse sequences were combined in Autoassembler (ABI) and compared with sequences in Genbank using BLAST. Along with their blast hits, sequences were imported into Silva database nr. 102 (Pruesse et al., 2007), aligned accordingly and added to the tree sequences of photo-autotrophs using the ARB Parsimony algorithm (Ludwig et al., 2004).

2.4 Statistics

Averages, 95 % confidence intervals and paired one-tailed t-tests were calculated using the AVERAGE, CONFIDENCE.NORM and T.TEST functions of Microsoft Excel. Linear correlation was performed by linear regression analysis in Microsoft Excel .

Non-metric Multi-Dimensional Scaling (NMS) diagrams were performed in Primer version 6.1.13 after calculation of a Kulczynski presence/absence resemblance matrix . The Kulczynski matrix was also used to investigate the null hypothesis that there are no differences in bacterial composition between treatments using one- or two way analyses of similarities (ANOSIM). The significance of the ANOSIM test statistic R was computed by a permutation (n = 999) test.

2.5 Tests and Experiments

2.5.1 Peraclean® Ocean treatment system tests. During spring and early summer of 2007 twelve land-based IMO certification tests were performed at the NIOZ test facility on a BWTS consisting of hydrocyclones, filters and Peraclean® Ocean addition (150 mg/L). NIOZ harbor water originating from the Wadden Sea was taken in and passed through the BWTS (T0), stored in 200 m^3 simulated ballast water tanks and discharged after five days (T5). Both at T0 and T5 samples were taken for phytoplankton and bacteria abundances, oxygen concentration, dissolved organic carbon (DOC) and nutrients (PO_4^{3-}, NO_3^-).

2.5.2 Peraclean® Ocean incubation experiment. In order to study the effects of Peraclean® Ocean over a longer time period, 10 L samples from the second Peraclean® Ocean treatment system test were taken at intake (T0) and stored in a climate controlled room (15°C, 16:8 light:dark regime, 100 µmol quanta m-2s-1, Philips TL-D Super 80 58W 865 daylight lamps). These samples were incubated 20 days and sampled for phytoplankton and bacteria abundances every other day.

2.5.3 Acetate addition experiment. To examine the effect of temperature on microbial dynamics in acetate-enriched simulated ballast water, water samples were incubated for twelve days at different temperatures. Wadden Sea water was first filtered into two-liter glass bottles with plastic caps using 1 µm polysulfone groundwater filter capsules (GWSC10001-1.0 µm, Millipore) to remove the algae but to keep most of the bacteria. Next, 50 mg/L acetate (1677 µM labile DOC) was added, mimicking the acetate concentration immediately after treatment with Peraclean® Ocean. In addition, several 120 ml Winkler bottles were incubated for the determination of Dissolved Oxygen (DO). The five incubation temperatures were: <-1, 4, 10, 15 and 25 ^{o}C. The lowest temperature was kept

consistently below -1 °C, but not colder than -1.8 °C because that is the freezing point of seawater. This temperature was chosen to keep metabolic activity as low as possible and to mimic water temperatures in Polar Regions. The 25 °C incubation was used to mimic water temperatures in tropical regions. The 4, 10 and 15 °C incubations approximately represent the winter, spring/autumn and summer temperatures in the North Sea and Wadden Sea. Bacteria numbers and DO were monitored every other day for the <-1, 4 and 10 °C incubations. In the 15 and 25 °C incubations, bacteria numbers and DO were measured daily. Nutrient concentrations were measured every other day; samples for DGGE were also taken every other day. DOC was measured at T1 and at the end of the experiment (T12).

3. Results

3.1 Peraclean® Ocean treatment system tests

Addition of Peraclean® Ocean significantly increased the concentrations of DOC ($P < 0.01$) and PO_4^{3-} ($P < 0.01$). Bacteria abundances were higher in treated samples and the decline in bacteria numbers over the five day period was greater in control samples. Over the five day period not only bacteria abundances, but also phytoplankton abundances and DOC concentration decreased in both the treated and control. Phytoplankton was still present in treated samples but it was not viable (Table 1).

Table 1. Average values and 95 % confidence intervals over all twelve land-based tests for bacteria abundance (cells/mL), phytoplankton abundance (cells/mL), viability of phytoplankton (PAM), DOC concentration (µmol/l), NO_3^- concentration (µmol/l) and PO_4^{3-} concentration (µmol/l). C = Control, T = Treated, T0 = day of intake, T5 = day of discharge.

	C T0		C T5		T T0		T T5	
Bacteria	30898	± 6521	16094	± 7830	48091	± 6515	38855	± 8874
Phytoplankton	8397	± 3029	1459	± 443	6938	± 3270	1011	± 803
PAM	0.53	± 0.07	0.25	± 0.09	0.01	± 0.01	0.00	± 0.00
DOC	343	± 71	295	± 42	1680	± 53	1524	± 227
NO_3^-	19.3	± 9.1	19.4	± 9.2	21.2	± 9.2	14.8	± 9.9
PO_4^{3-}	0.9	± 0.3	0.7	± 0.2	6.7	± 0.6	5.7	± 0.4

3.2 Peraclean® Ocean incubation experiment

Abundances of bacteria in the control remained stable during the whole experiment. In the treated incubation, the bacteria numbers declined until day 10, at which point they increased to 10 times the control level (Figure 1A). Phytoplankton numbers in the control incubation gradually decreased (Figure 1B), but after 20 days they were still viable (Figure 1C). On the other hand, phytoplankton abundances in the treated incubation were below detectable levels from the first day onwards (Figure 1B).

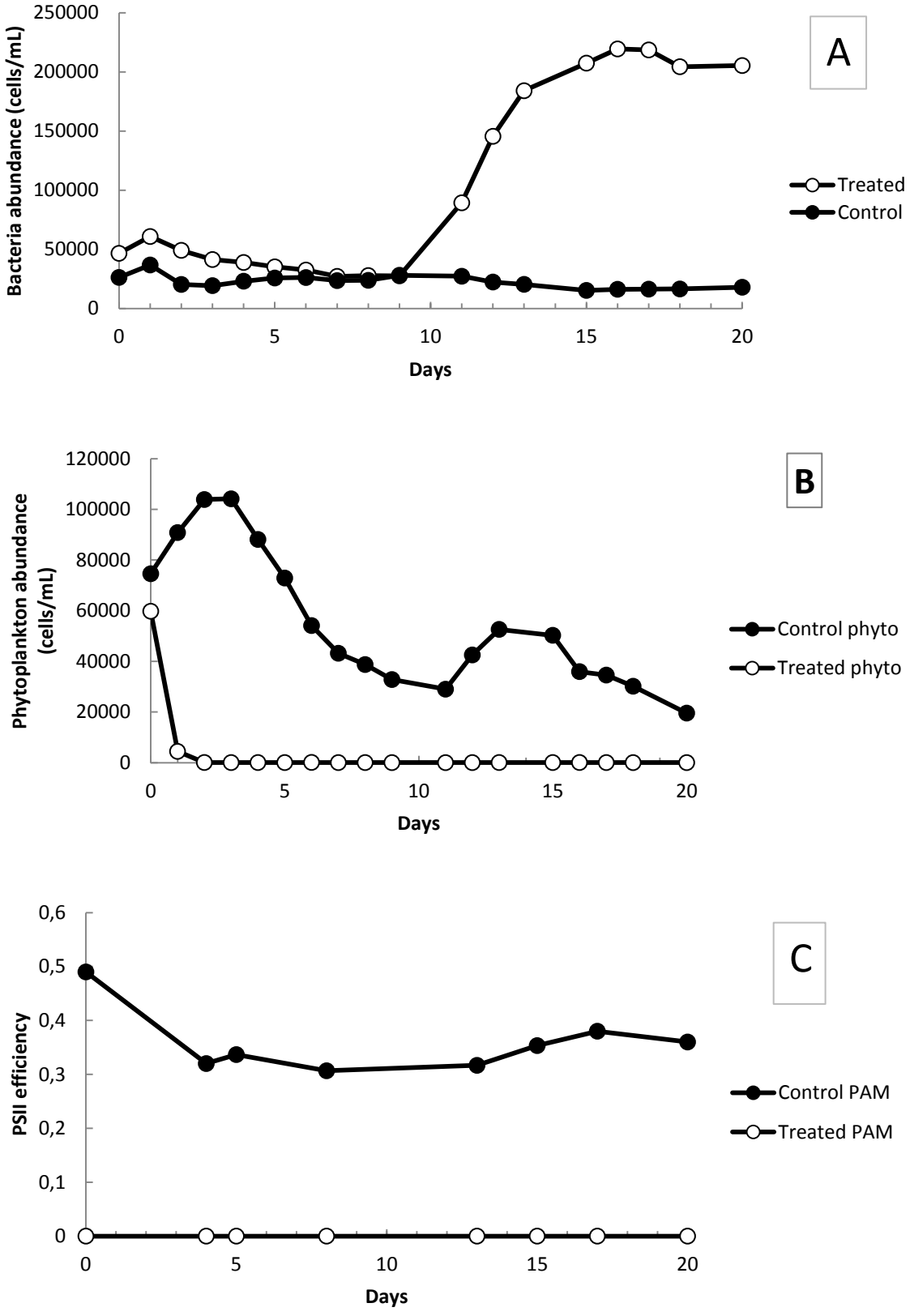

Figure 1. Bacteria abundances (A), phytoplankton abundances (B) and phytoplankton viability (C) in the control samples and the treated samples of the Peraclean® Ocean incubation experiment. Each point represents an average of three measurements.

3.3 Acetate addition experiment

NO_3^-, PO_4^{3-} and DO were not depleted in any of the control incubations. However, in the acetate enriched incubations PO_4^{3-} and DO were depleted before the end of the experiment at all temperatures except for <-1 °C (Table 2). The NO_3^- was only depleted at 25 °C.

Table 2. Day of depletion of PO_4^{3-}, NO_3^- and DO in the acetate enriched incubations of the acetate experiment. No depletion occurred in the control incubations.

°C	PO_4^{3-}	NO_3^-	DO
< -1	x	x	x
4	3	x	8
10	3	x	6
15	3	x	6
25	3	4	2

In the control incubations, the DOC increased over the time of the experiment, the increase was greatest at cold temperatures (Table 3). In the acetate enriched incubations, the DOC only increased at <-1 °C, all the other temperatures showed a DOC decrease. The DOC decrease was greater at higher temperatures (Table 3).

Table 3. Difference in DOC concentration (µmol/l) between T1 and end (T12) of the acetate enrichment experiment.

°C	Control	Acetate
< -1	114	364
4	27	-213
10	21	-611
15	21	-901
25	16	-1074

Abundances of bacteria in the control incubations increased over the twelve days of the experiment at all temperatures (Figure 2A), but the increase started the earliest and was the highest at 25 °C. At <-1 °C bacteria showed an exceptional trend, with very slow increase in numbers and stayed below

abundances observed at other temperatures throughout the experiment (Figure 3). In the acetate enriched incubations the increase in bacterial numbers started later than in the control, but the numbers increased to higher levels than in the control (Figure 2B).

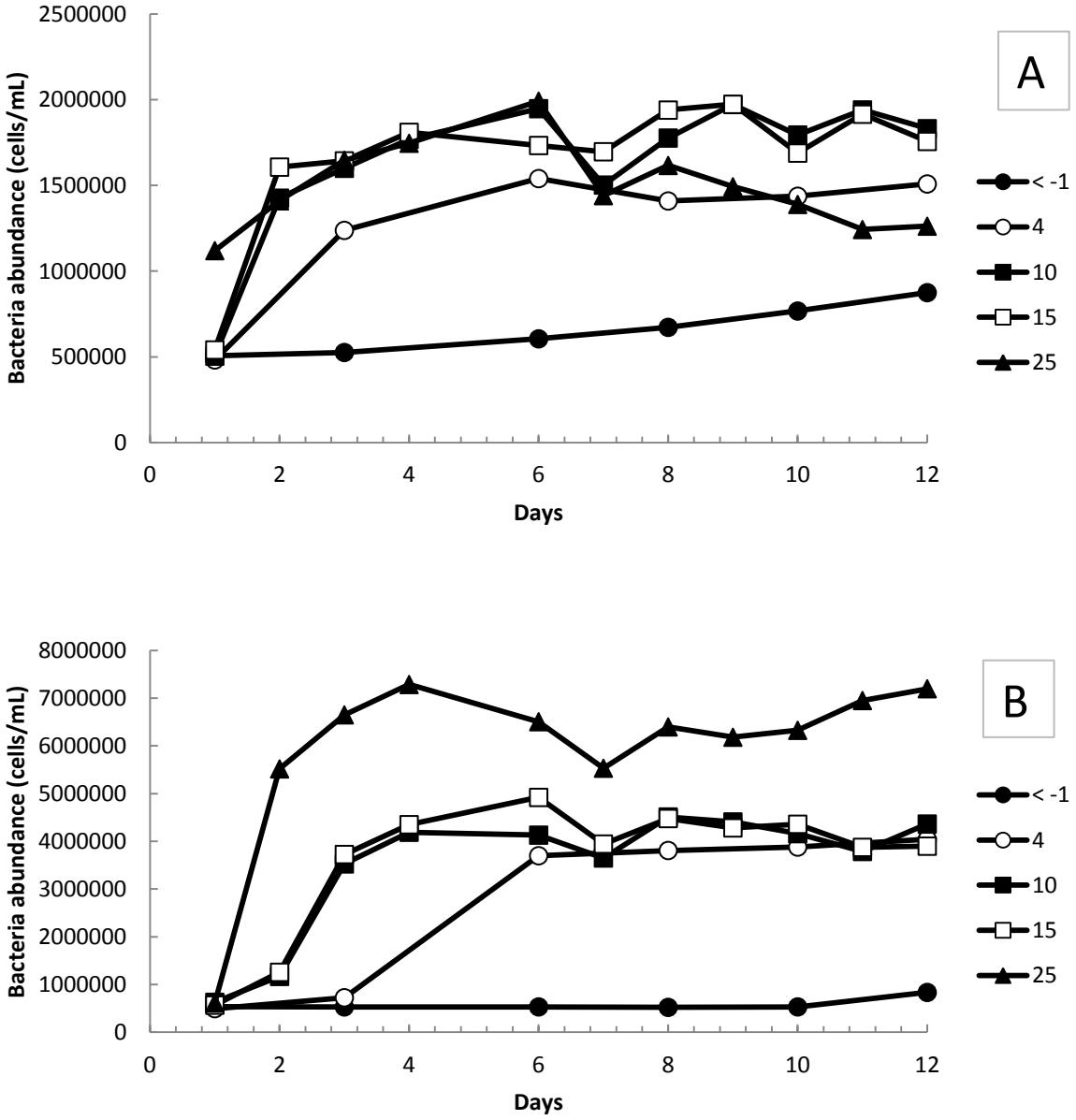

Figure 2. Development of bacteria abundances over time at all five different temperatures in the control (A) and acetate enriched (B) incubations of the acetate enrichment experiment. Each point represents an average of three values.

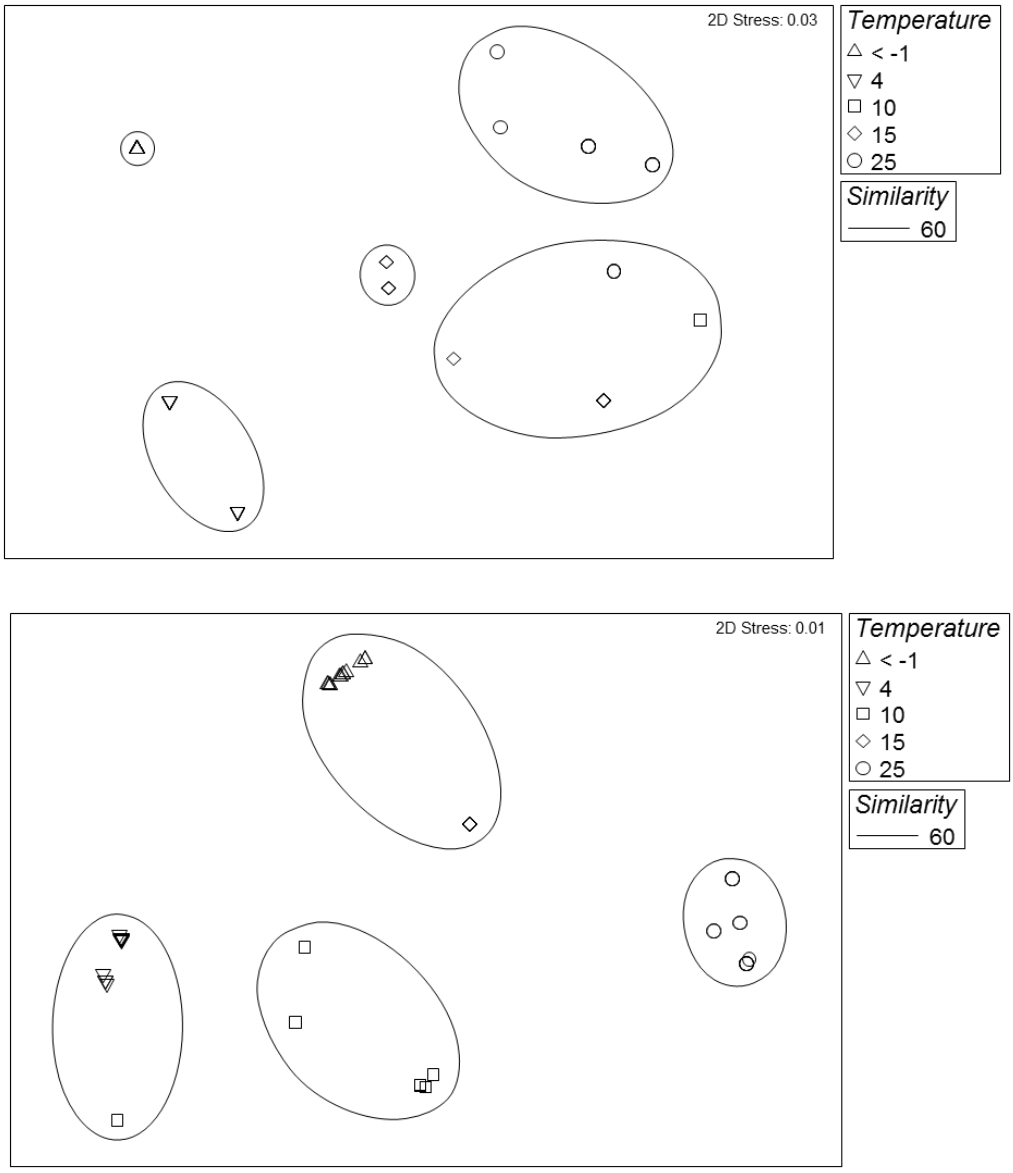

Figure 3. Non-metric Multidimensional Scaling diagrams showing the bacterial diversity at temperatures ranging from -1°C to 25°C in the acetate experiment. A = control, B = with acetate. Data inside the ellipses have a >60% similar diversity.

The bacteria genetics results showed at <-1 °C, both in the control and acetate enriched incubations, *Burkholderia* and *Methylobacterium* during the 12 days of the experiment. Additionally, the control showed *Arcobacter*, *Marimonas*, *Colwellia* and *Prevotella* throughout the whole period.

At 4 °C again *Burkholderia* was present throughout the whole period in the control. Additionally, the control showed *Polaribacter* from day 8 onwards. The acetate enriched incubation, however, only showed various types of *Owenweeksia* during the entire period.

At 10 °C in the control no bacteria were identified in the first 8 days. After this, *Vibrio* and *Marixanthomonas* were found which stayed until the end of the experiment. In the acetate enriched incubation *Owenweeksia* and a Rhodobacteraceae were present on day 1, but they were not identified after day 3. *Vibrio* was found on day 2 and stayed in the samples until the end of the

experiment. *Burkholderia* was present only on day 3. Finally, *Colwellia* was found on day 2 and stayed in the samples until the end of the experiment.

At 15 °C both control and acetate enriched incubations had *Vibrio* present on all days. In the acetate enriched incubations also *Pseudoalteromonas*, *Burkholderia* and *Methylobacterium* were found on all days. *Burkholderia* and *Methylobacterium* were also present in the control at day 1, but they were not in samples after day 6. The control had *Sulfitobacter* present on all days, *Roseivarius* from day 3 until the end, *Polaribacter* and *Marixanthomonas* from day 6 until the end.

At 25 °C in the control *Alcanivorax* and *Vibrio* were present from start to end, *Sulfitobacter* was present from day 2 to end. *Marixanthomonas* was present from day 3 to end, *Roseivivax* from day 3 to day 9, *Roseivarius* from day 10 to end and *Methylobacterium* from day 1 to day 6. In the acetate enriched incubations *Amphritea*, *Pseudoalteromonas* and *Marinomonas* were present from start to end, *Muricauda* was present from day 6 to end and *Nisaea* from day 8 to end.

Using ANOSIM analyses, the bacterial composition between all control and acetate addition samples at all temperatures was found to be significantly different ($R = 0.12$, $P < 0.001$). Significant differences were also found between temperatures in the control samples (Fig 3A, $R = 0.93$, $P < 0.001$) and in the acetate addition samples (Fig 3B, $R = 0.95$, $P < 0.001$).

4. Discussion

After breakdown of the active substances, the Peraclean® Ocean treatment leaves seawater enriched in both DOC and phosphate (Table 1). Natural DOC is 90% unusable by bacteria (Smith and Prairie, 2004), only the labile fraction of DOC can be used by bacteria. The large increase in DOC caused by Peraclean® Ocean not only greatly increases the DOC pool, but it specifically increases the labile fraction. Average DOC values in control water are 343 µM/L of which 10 % is labile. In the treated samples 1677 µM/L of labile DOC is added, an almost 50 times increase in the labile DOC fraction.

Bacteria abundances were higher in water with Peraclean® Ocean than in the control (Table 1). Only a small volume of Peraclean® Ocean was added to over 200 m^3 of ballast water. The original undiluted Peraclean chemical mixture presents an extremely hostile environment such that we can rule out that bacteria were present in this undiluted Peraclean. On the other hand, another possible explanation might be that the peracetic acid present in Peraclean® Ocean caused bacteria which are normally attached to a substrate (organic or inorganic larger particles in suspension) to be released in the water column (McEldowney and Fletcher, 1987).

Large numbers of phytoplankton cells were still present in treated samples of the twelve BWTS land based tests (Table 1), but not in treated samples of the incubation experiment (Figure 1B). This is most likely because treated BWTS samples come from simulated ballast tanks which are dark and have no water movement. The treated incubation samples were exposed to light, which could have degraded the pigments preventing detection of cells by flow cytometry. Additionally, the water was kept in motion, which could have disrupted the dead cells. This is supported by the fact that PAM measurements showed no photosynthetic activity in the treated samples (Table 1) which means the cells were most likely dead. Over the course of the incubation experiment there was no re-growth of

phytoplankton (Figure 1B). This distinguishes this method of ballast water treatment from others, which are known to have phytoplankton re-growth (Liebich et al., 2012; Stehouwer et al., 2010).

In the control of the acetate addition experiment, the DOC values were higher at the end of the experiment then at the start, most likely due to breakdown of particulate organic carbon. The increase in DOC was greater at lower temperatures (Table 3). In the acetate enriched incubations all temperatures except <-1 °C showed DOC decrease (Table 3). The amount of DOC consumption was significantly related to temperature ($R^2 = 0.89$).

Drake et al. (2001) stated that ballast water did not contain more bacteria and viruses, showed no higher bacterial productivity and showed less phytoplankton abundance compared to the Chesapeake Bay water in which it was discharged. In a follow-up study Drake et al. (2002) presented that ballast water holds do not act as incubators for microorganisms, but that microbial abundance actually decreased during a voyage from Israel to the USA. Quilez-Badia et al. (2007) performed a similar study but also included the effects of ballast water exchange. They indicated that the bacterial abundance decreased over time in both exchanged and control tanks, where exchanged tanks showed significantly greater decrease in abundance. Our study presented here shows that the addition of Peraclean® Ocean changes this situation. During short trips the bacteriostatic agent will suppress bacterial growth, but on longer voyages there will be a large increase in bacterial abundance (Figure 1A), which could also increase the risk of microbial invasions. The increase in bacterial abundance also shows that on longer voyages residual toxicity of discharged ballast water is not an issue, as the bacteria are clearly not negatively affected. On short voyages residual toxicity may still be problematic.

Bacterial diversity showed interesting patterns. In the <-1 °C and 15 °C incubations the bacterial diversity in the treated samples was strongly reduced compared to the control samples. However, in the 10 °C incubations treated samples show higher diversity than the control samples. In the 4 °C and 25 °C incubations there were no matches in the bacteria detected in control samples and treated samples. A multi-dimensional scaling analysis of all control versus all treated samples also showed that the bacterial species composition was significantly different. This shows that the high acetate concentrations not only affect bacteria numbers but also bacterial diversity and species composition.

The most important residual effect of Peraclean® Ocean on ballast water is acetate enrichment. This affects both bacterial numbers and bacterial composition. Carlson et al. (2002) found similar responses in abundance and composition in their DOC enrichment experiments. However, even in the 25 °C incubation when bacterial growth is strongest, not all acetate is used. This means the application of Peraclean® Ocean as a ballast water treatment will result in the discharge of acetate enriched ballast water, particularly in colder areas. Acetate is easily broken down by bacteria, but these experiments show that in cold water this breakdown only occurs very slowly. In harbours, which do not have a high rate of water exchange, this could lead to eutrophication due to acetate build-up; this eutrophication effect is compounded by the fact that Peraclean® Ocean also includes phosphate. But in all harbours it will mean a shift in the DOC availability towards more labile DOC. This could lead to shifts in bacterial composition which could have consequences for ecosystem functioning (Dobbs and Rogerson, 2005).

The tests and experiments on this BWTS show that it is possible to effectively neutralize phytoplankton but have no negative effect on bacteria. Over longer time the effects of Peraclean® Ocean treatment are even positive on microbial abundance. This demonstrates how important it is to include bacteria concentrations in the D-2 ballast water performance standard, not just the few pathogenic bacteria strains included now.

Acknowledgements

The authors thank Dr. Judith van Bleijswijk, Maaike Brink, and Julie Ogier for their help with the DNA analysis.

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

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7. Discussion

To prevent further spread of aquatic invasive species through ballast water the IMO established the ballast water management convention. Part of the convention is the D-2 ballast water performance standard which defines limit values on the amount of viable organisms allowed to be present in ballast water upon discharge (an overview of the size classes and limit values is given in below). To meet this standard, ballast water treatment systems were developed. These systems use various methods to disinfect ballast water. In this thesis the effects of various ballast water disinfection methods were evaluated regarding survival and re-growth potential of plankton after treatment, the potential of specific plankton species being discharged alive, methods for the detection of these species and the effects of disinfection chemicals on natural plankton communities. This thesis also gives the first comprehensive comparison of several ballast water disinfection methods.

This thesis focuses on the plankton size fraction ≥10 µm and <50 µm, which is part of the IMO D-2 standard. This size fraction consists mostly of phytoplankton, forming the basis of the marine food web and any changes to this group will impact the entire food web.

The IMO guidelines for type approval of ballast water treatment systems (IMO 2008a, 2008b), as base for all experiments analysed in this thesis, state that treated ballast water has to be stored for five days in simulated ballast water tanks and analysed on discharge. Since the IMO guidelines provide no information on possible re-growth after discharge, experiments were performed on treated ballast water, sampled upon discharge and subsequently incubated. Through these experiments valuable additional information was gained: by incubating the water for up to 20 days under favourable conditions for nutrients and irradiance, re-growth of phytoplankton occurred. This was observed for both the Ultraviolet radiation (UV) ballast water treatment system (BWTS) and the chlorine dioxide (CD) BWTS tested (Chapter 2). All BWTSs tested showed re-growth, but all also reduced plankton abundances to below the IMO D-2 standard. The D-2 standard does allow for low abundances of viable organisms to still be present after ballast water treatment. Even though the risk of spreading aquatic invasive species through ballast water was not eliminated, the reduction in abundance of plankton translates to a greatly reduced propagule pressure (Holle & Simberloff 2005, Lockwood et al. 2005, Colautti et al. 2006). Propagule pressure is a function of quantity, quality and introduction frequency of non-native species, and through this an important factor in the establishment of non-native species in new environments, the first step in becoming an invasive species. By reducing propagule pressure the likelihood of aquatic invasions is reduced.

Martinez et al. (2012b) also executed incubation experiments and reached a similar conclusion on the importance of incubation experiments over longer periods of time to assess the risk of re-growth. These experiments show that discharged ballast water still has the potential to spread species outside their native range and thus can potentially become invasive. Similar considerations that current ballast water treatment is not 100% sufficient to prevent the spread of non-indigenous species were expressed by Martinez et al. (2012a).

Re-growth of phytoplankton after ballast water treatment was found, but how was the species composition affected and how can that be detected? Samples treated by a UV BWTS were analysed using light microscopy to identify changes in phytoplankton composition (Chapter 3). Ballast water treatment reduced the diversity of the phytoplankton but certain diatom genera, namely ***Thalassiosira*, *Skeletonema*, *Chaetoceros, Pseudo-nitzschia* and *Nitzschia* grew back.** The dominance of specific species in these samples is also dependent on the initial plankton composition

and the competitive advantage compared to other surviving phytoplankton species or surviving grazers. However, identification to the species level was not possible for all re-growing phytoplankton, since determination to the species level for most of these genera would require sophisticated microscopic techniques such as scanning electron microscopy. Therefore the microscope results were compared with a DNA fingerprinting method, i.e. denaturing gradient gel electrophoresis (DGGE) followed by sequencing and flow cytometry for additional plankton diversity analysis (Chapter 4). DGGE was more time consuming than microscopy but allowed for identification of cryptic species in cases such as the genus *Thalassiosira* where the identifying characteristics for species cannot be observed using normal microscopy. In addition sequencing identified species not observed by microscopy. Flow cytometry was a faster semi-automated method for counting phytoplankton; this method is normally not able to unravel different sub-clusters in the phytoplankton assembly, but the possibility for identification of apparent sub-clusters based on six measured cell variables using automated cluster analysis was tested.

Flow cytometry allowed for phytoplankton enumeration while at the same time providing a rough indication of diversity in ballast water samples. Flow cytometry was as yet not able to identify in terms of taxonomy at the level of family or genus or species, even using automated cluster analysis. The flow cytometer used in this study is capable of measuring six cell variables (i.e. channels) and hence the maximum number of clusters is in that order. Newer model flow cytometers with larger numbers of channels would provide a better resolution in distinguishing clusters and may eventually be capable of identifying the genus or species of phytoplankton. Flow cytometry using both manual and automated cluster analysis confirmed the microscopy findings that ballast water treatment reduced phytoplankton diversity. Due to the multi-dimensional nature of automated cluster analysis it was able to identify clusters that are difficult to recognize using manual clustering, resulting in higher phytoplankton diversity estimates for automated cluster analysis although the trends were similar for both methods.

Fluorescent stains can be used to assist in species identification and viability or vitality assessment in flow cytometric analyses. FDA and CMFDA are common vitality stains, which can also make heterotropic organisms visible to flow cytometry. However, these stains vary in reliability based on the composition of the plankton (Steinberg et al. 2011). Species-specific fluorescent probes can also be used in species identification (Peperzak et al. 2000), but this requires prior knowledge of species composition. While not applied in the experiments described in the present thesis, this technique could be used in the future to screen for harmful species.

DGGE identified phytoplankton to the species level, but it only identified the dominant species in the sample. All three methods tested had their disadvantage and advantages. For analysis of ballast water a tiered approach is recommended, flow cytometry for fast enumeration, viability (by using fluorescent stains) and diversity assessment, microscopy as an intermediate step for species or group identification and sequencing for more precise species identification, especially in the case of cryptogenic (obscure) phytoplankton species. However, not all possible methods were tested. Identification of cryptogenic diatom species can be performed using electron microscopy and new genetic identification tools are available. In the future these other methods could be tested to establish an optimal tiered approach.

The most commonly re-growing phytoplankton species in our experiments was the diatom *Thalassiosira weissflogii,* this based on DGGE sequencing was. The fact that it re-grew in all treated samples which were analysed using the molecular approaches while not being detected in the control samples suggests that it is relatively resistant to the UV treatment. This raises the possibility

that the introduction of ballast water treatment may select for resistant species, because these species survive treatment after which they will still be able to spread through ballast water and potentially become invasive.

To assess if re-growing species differ among different ballast water treatments systems or disinfection methods, the results of the incubation experiments of six BWTSs were compared (Chapter 5). Three of the BWTSs were based on UV, two on electrochlorination (EC) and one on addition of chlorine dioxide (CD). The EC and CD treatments showed some similarity in that they are both based on chlorine chemistry. The BWTSs were compared on timing of re-growth of phytoplankton, phytoplankton abundances and re-growing phytoplankton species. EC and UV are the most common types of ballast water treatment. Out of 86 BWTSs listed by Gollasch and David (2012), 24 were based on UV and 25 on EC.

No significant differences in the performance of the three UV BWTSs were found. There were also no significant differences in the performance of the two EC systems and the CD system. However, significant differences were found between the three UV BWTSs and the three chlorine chemistry BWTSs. These two major types of treatment were also different in the phytoplankton species showing re-growth. For example, *Thalassiosira weissflogii*, the most common re-growing species after UV treatment, was present in the control samples of one of the EC systems but was not detected after EC treatment. Two of the re-growing species after EC treatment, *Emiliania huxleyi* and *Navicula phyllepta*, were never found in UV treated samples. In all cases re-growing phytoplankton species did not match the dominant species in the control samples, suggesting that it is not just the dominant species from the control which re-grow after treatment. Since these surviving species differed between types of treatment it is not likely that 'super phytoplankton' exists that can survive all types of treatment. The resistant species found in the present thesis may be good indicators to test effectiveness of ballast water treatment, but further tests are needed to identify the level of resistance of these organisms.

Although there are significant differences in performance between the different types of BWTSs, there is no perfect treatment system. Both UV and chlorine treatments reduced abundances of plankton according to the IMO D-2 standard but both types of treatment also showed re-growth, although chlorine treatment systems on average reduced plankton to lower abundances and had a longer lag phase before re-growth than UV treatment systems. Choosing which system is best for using on board will therefore depend on practical requirements such as the higher power consumption of UV BWTSs and the necessity to carry neutralizing chemicals on board for BWTSs using active substances.

Chemical treatment systems, such as the EC and CD BWTSs discussed above, have a certain environmental risk associated with them because of residuals or by-products. This was a particular concern for another treatment system based on a chemical mixture called Peraclean® Ocean (Chapter 6). This mixture consists of peroxy-acetic acid and acetate in equilibrium and was added to the ballast water at a concentration of 150 mg/L. The peroxy-acetic acid serves as the disinfecting agent and it rapidly degrades to acetate, allowing for safe ballast water discharge. However, this leaves the ballast water enriched in acetate, which might have consequences for the environment in which the ballast water is discharged. Release of large amounts of acetate might lead to eutrophication, acidification and anoxia.

Acetate, the residual product of Peraclean® Ocean for ballast water treatment, caused massive increases in bacteria abundance in ballast water tanks through nutrient enrichment (Chapter 6). The

acetate was not completely metabolized by bacteria even after several weeks at high (25 °C) temperatures. This means that acetate may be discharged along with the ballast water, increasing the risk of rapid growth of heterotrophic bacteria also resulting in oxygen deficiency in harbours. In addition to the acetate, the ballast water would be enriched in bacteria. The bacterial community structure was also altered by the acetate enrichment, showing that similar changes in community structure might occur in semi-enclosed harbours when ballast water enriched with acetate is regularly discharged.

With respect to the bacteria, only the *Escherichia coli*, intestinal enterococci and toxigenic *Vibrio cholerae*, all pathogenic to humans, are included in the D-2 standard. *Escherichia coli* stops replicating if the salinity reaches levels above 0.4% (Hrenovic & Ivankovic 2009) and in diffusion culture experiments survival of both *Enterococcus* and *Escherichia coli* was negatively correlated to salinity (Lessard & Sieburth 1983). This makes them poor indicators for survival of bacteria in ballast water tanks and effectiveness of ballast water treatment, except when fresh water is used. *Vibrio cholerae* however is known to be spread through saline ballast water (McCarthy & Khambaty 1994). The results above show that ballast water treatment can cause increased bacteria abundances in ballast water, yet little is known of the effect these bacteria will have upon discharge (Ruiz et al. 2000).

Critical comments on the IMO ballast water regulations

The issues mentioned above concerning re-growth of phytoplankton and bacteria are not the only points of concerns on the IMO ballast water regulations. Both Gollasch et al. (2007) and Liebich (2013) pointed out that the limits set in the D-2 standard still cannot rule out the spread of (potentially invasive) species. Assuming a large vessel carrying 100,000 tonnes of ballast water, this vessel could transport 100,000 organisms ≥50 µm (based on 1 organisms/m^3, well below the D-2 standard) and 100,000,000,000 organisms in the size range between 10 and 50 µm (based on 1 organisms/mL, well below the D-2 standard). In an untreated ballast tank, these abundances would be much higher, but even with these reduced abundances some organisms will be discharged into new environments and some might be able to get established and become an invasive species.

Another concern about the IMO regulations is the lower limit of the size classes. The smallest size class (not considering the indicator microbes as a size class) of the IMO D-2 standard is ≥10 and <50 µm. It is defined that this size class applies to the minimum dimension of the organism, meaning the smallest observable dimension. This means that an organism which is 20 µm long but only 6 µm wide is regarded as an organism <10 µm. IMO guidelines further clarify that in the case of colony-forming organisms it is the size of the single organism which determines the size class to which it is assigned. Most re-growing phytoplankton found during the research presented in this thesis were <10 µm in minimum dimension (Table 1). This shows another fallacy of the D-2 standard: phytoplankton of this size class can be present in the ballast water in high Abundances but the discharged water would still be in compliance with the D-2 standard.

Table 1. Re-growing phytoplankton species and their minimum and maximum dimensions in µm.

Re-growing species	Min. Size	Max. Size	Reference
Thalassiosira pseudonana	3.0	7.0	Stramski et al. 2002
Skeletonema costatum	3.6	12.9	Harrison et al. 1977
Chaetoceros calcitrans	4.0	5.0	Tobias-Quinitio & Villegas 1982

Emiliania huxleyi	5.7	5.9	Engel et al. 2005
Chaetoceros socialis	6.0	10.0	Tomaru et al. 2009
Navicula phyllepta	7.0	30.0	DeTroch et al. 2006
Thalassiosira weissflogii	9.9	13.5	Bonnet & Carlotti 2001
Coscinodiscus radiatus	34.0	101.0	Alpine & Cloern 1985

While many harmful algal bloom species have a minimum dimension >10 µm and are thus covered by the D-2 standard, some species such as *Pseudo-nitzschia* sp., *Lyngbya* sp., *Karlodinium veneficum* and *Pfiesteria piscicida* are <10 µm in minimum dimension and are thus not covered by the D-2 standard (Table 2). This means they could still be transported in ballast water without violating the IMO convention. Similar concerns about organisms <10 µm were expressed by Gollasch et al. (2007), Van der Star et al. (2011) and Liebich (2013). Van der Star et al. (2011) further showed using flow cytometry that phytoplankton <10 µm is often numerically dominant in natural phytoplankton communities, comprising > 90% of total phytoplankton numbers. Gollasch et al. (2007) also underlined the danger of bloom forming harmful algal species in this size category, using *Phaeocystis* sp., *Pfiesteria* sp. and *Chrysochromulina* spp. as examples. Gollasch et al. (2012) provided an overview of harmful species <10 µm and included the threat posed by other eukaryotes such as fungi and slime moulds in addition to phytoplankton species. Liebich (2013) also suggested to put more emphasis on the detection of potentially harmful species in treated ballast water in addition to the IMO size standards. However, this would require specialized techniques such as fluorescent in-situ hybridization (FISH), or the above mentioned application of specific probed in combination with flow cytometry. Yet, generally monitoring for all potentially harmful species would be very labour-intensive.

Table 2. Examples of harmful phytoplankton species and their minimum and maximum dimensions in µm.

Harmful species	Min. Size	Max. Size	Reference
Protoperidinium crassipes	78	80	Latz & Jeong 1996
Coscinodiscus wailesii	44	500	Fernandes et al. 2001
Pyrodinium bahamense	33	47	Taylor & Fukuyo 1989
Gambierdiscus toxicus	30	90	Durand-Clement 1987
Prorocentrum lima	26	50	Aligizaki et al. 2009
Gymnodinium catenatum	22	46	Graham 1943
***Alexandrium* sp.**	22	27	Colin & Dam 2002
***Dinophysis* sp.**	21	56	Reguera & Gonzalez-Gil 2001
Karenia brevis	13	24	Kubanek et al. 2007
Karlodinium veneficum	7	18	Bergholtz et al. 2005
***Pseudo-nitzschia* sp.**	6	140	Davidovich & Bates 1998
Pfiesteria piscicida	5	8	Steidinger et al. 1996
***Lyngbya* sp.**	2	64	Speziale & Dyck 2004

Not only scientists are suggesting changes to the D-2 standard. Since the ballast water management convention has not been ratified yet, certain countries have been setting their own, sometimes stricter, ballast water discharge standards. The United States of America already adopted the IMO D-

2 standard for ships coming to their ports, but refer to this standard as 'phase 1'. The phase 2 standard is 1000 times more stringent than the IMO standard (Table 3). Additionally, the state of California has its own ballast water performance standard which is identical to the USCG phase 2 standard, except for the ≥50 µm fraction (Table 3). The California State Land Commission (CSLC) conducts regular studies if technologies are available to meet the standard or if the implementation of the standard has to be delayed. So far the conclusion has always been that there are no BWTSs that can meet the standard and measuring such low limit values is beyond the capabilities of current detection methods (CSLC 2013, 2014). CSLC (2014) provides a listing of 24 BWTSs for which performance data is publicly available. These BWTSs span a wide variety of available technologies, yet none of these 24 BWTSs is able to consistently meet the California Standard. However, as Van der Star et al. (2011) pointed out, while these standards do include bacteria and even viruses, they still do not include other organisms <10 µm.

Table 3. Overview of the different ballast water standards. cfu = colony-forming units.

	Organisms			Indicator microbes		
Standard	≥50 µm	≥10, <50 µm	<10 µm	Vibrio cholerae	Escherichia coli	Intestinal Enterococci
IMO D-2	<10/m^3	<10/mL	N/A	<1 cfu/ 100 mL	<250 cfu/ 100 mL	<100 cfu/ 100 mL
USCG phase 1	<10/m^3	<10/mL	N/A	<1 cfu/ 100 mL	<250 cfu/ 100 mL	<100 cfu/ 100 mL
USCG phase 2	<0.01/m^3	<0.01/mL	<1000 bacteria/100 mL, <10,000 viruses/100 mL	<1 cfu/ 100 mL	<126 cfu/ 100 mL	<33 cfu/ 100 mL
California	None detectable	<0.01/mL	<1000 bacteria/100 mL, <10,000 viruses/100 mL	<1 cfu/ 100 mL	<126 cfu/ 100 mL	<33 cfu/ 100 mL

There are also risks associated with ballast water treatment itself. Most ballast water treatment systems that use UV radiation are targeted to destroy the DNA of organisms. Survivors of this treatment are likely subject to UV-induced DNA damage which will impair their long-term survival. However, there is also the chance that this may cause unintended mutations in organisms with unexpected consequences. Especially when bacteria are considered, the environment of a ballast water tank provides opportunities for horizontal gene transfer i.e. transfer of genes between bacteria and sometimes between bacteria and other organisms (Dobbs and Rogerson 2005). This gene transfer is not just an issue for BWTSs using UV radiation, also with EC treatment there are survivors. If these survivors are resistant to the treatment, they can not only keep multiplying in the ballast tanks, but they can also pass on this resistance to other micro-organisms. An additional risk of EC treatment systems is in the toxic residuals, since many types of these systems use a neutralizing agent to prevent discharge of excess treatment chemicals. There are studies that show that at least one of these neutralizing agents, sodium bisulfite, has a negative impact on growth of certain phytoplankton species(Tamburri et al. 2006). How much effect sodium bisulfite has when discharged and diluted with the local water is not yet clear.

A topic which has gotten little attention so far is the possible accumulation of toxins, by-products of active substance treatment, in the sediments at the bottom of the ballast tanks. These sediments

are not discharged with the ballast water, but have to be periodically removed from the tanks. If toxins accumulate there the workers performing this task would need special protection and the sediments from the tanks would have to be disposed appropriately.

As mentioned above, in an effort to completely eliminate the risk of spreading aquatic invasive through ballast water some countries or states are suggesting a stricter ballast water performance standard. However, setting stricter standards for ballast water greatly increases the effort that needs to be put into its treatment. This creates greater costs for ship owners and more environmental risks because, for instance, higher concentrations of chlorine need to be used. In addition to creating a more stringent standard it would be important to also deal with the 'gaps' in the current standards. One gap is the organisms smaller than 10 µm, as explained above. To deal with this gap this organism size class would need to be considered in the D-2 standard. Liebich (2013) proposed to expand the ≥10 µm and <50 µm size range to ≥5 µm and <50 µm. Since this would mean that more organisms are covered by this size class the limit on organism abundance might have to be increased. An alternative suggested by Liebich (2013) is to create a new size range ≥5 µm and <10 µm. If either of these new standards was selected it would mean that most re-growing species in this study are covered by the new standard (Table 1) and all but one of the harmful species in Table 2. The lower limit of 5 µm was chosen because this size of organism can still be detected using microscopy. However, it would still leave a gap between organisms of 5 µm and a possible bacteria standard mentioned in the next paragraph. Gollasch et al. (2012) suggested extending this new size range even further, to ≥2µm and <10µm, which would eliminate this gap. In both cases the limit in organism abundances should be considered, since smaller organisms are present in higher abundances enforcing a <10 organisms/mL standard may require increased treatment effort.

Although the USCG phase 2 standard and the California standard include a limit value for heterotrophic bacteria and the US Vessel General Permit includes a monitoring requirement for total heterotrophic bacteria, the IMO D-2 standard only has limit values for three so-called 'indicator microbes', chosen for their negative effects on human health. However, there are many more bacteria species that can have an effect on human health or marine ecosystems. Chapter 6 also showed that it is possible to effectively neutralize phytoplankton without negatively affecting bacteria, and it is possible for heterotrophic bacteria to grow in ballast water tanks. Since it is not possible to include all bacteria species of interest in the standard, the recommendation is to include a limit value for heterotrophic bacteria in the IMO standard, as is already done for the USCG phase 2 and California standards. However, the limit value should be carefully considered regarding feasibility as the limit value of the USCG phase 2 and California standards of <10 bacteria (cfu)/mL is very stringent. As a comparison, drinking water standards commonly use a limit value of <100 cfu/mL (Bartram et al. 2003), 10 times higher than the California and USCG phase 2 standards.

Recently, in the IMO Marine Environment Protection Committee (MEPC) meeting 67, the subject of adjustment of the ballast water regulations was discussed. This immediately led to a debate that the situation of ship-owners who have already made a considerable investment to install ballast water treatment systems should be considered. Even though the prevention of further aquatic invasions is an urgent issue, it has taken more than 10 years for the ballast water management convention to be close to entering into force. A large part of this delay was due to challenges that had to be overcome to decrease resistance to signing the convention. One of the first challenges was the lack of approved ballast water management systems. If changes to the D-2 standard are made which may invalidate some or all of the currently available ballast water management systems, this could cause the convention to be further delayed. An example of this are the California State ballast water

regulations, which are delayed indefinitely because no current technologies are capable of meeting the stricter limits of organism numbers. It is therefore important that any changes to ballast water regulations and standards are realistic given current technology and economy.

To summarize: current ballast water treatment regulations, when in force, reduce the organism numbers before discharge, reducing propagule pressure and thus the risk of spreading aquatic invasive species. However, the re-growth experiments presented in this thesis showed that plankton species are able to survive approved treatments. Different identification methods confirmed that certain species were more likely to survive UV treatment. Using the same methods on chlorine treated samples, showing survival of different species, this indicated that survival of certain plankton organisms is specific to the disinfection method used. All but one of the re-growing species were smaller than 10 µm and thus not covered by the D-2 standard.

Experiments using the residual chemical acetate of Peraclean® Ocean showed that this supposedly harmless chemical causes strong growth of heterotrophic bacteria and changes in the bacteria species composition. These changes would also go undetected in the current D-2 standard.

As a conclusion it is recommended that the D-2 standard is amended to include limit values for phytoplankton and zooplankton organisms < 10 µm as well as total heterotrophic bacteria.

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8. Summary

Aquatic invasive species are among the worst threats to marine biodiversity. The main vector for the spread of these aquatic invasive species is ships' ballast water. Because of this, the International Maritime Organization (IMO) adopted the Ballast Water Convention. Part of this convention is the D-2 ballast water performance standard, which sets limits to the amount of viable organisms allowed to be in ballast water upon discharge. The limits of the D-2 standard are: 1. less than 10 viable organisms/m^3 in the size class ≥50 µm; 2. less than 10 viable organisms/mL in the size class ≥10 - <50 µm; 3. limits on the abundance of toxigenic *Vibrio cholera*, *Escherichia coli* and intestinal enterococci. In order to meet this standard, manufacturers developed different types of Ballast Water Treatment Systems (BWTSs). These BWTSs need to be approved according to IMO regulations by an independent party. Several approval tests were performed at the Royal Netherlands Institute of Sea Research (NIOZ). The focus of this thesis was to test the effects of various ballast water treatment methods on the survival of phytoplankton and bacteria.

Different methods are used to reduce the numerical abundance of organisms, most notably Ultraviolet-radiation (UV) and 'active substances' (chemicals). Both treatment methods were considered in this thesis. To measure the efficacy of different BWTSs, methods had to be developed that are applicable to all types of treatments. The standard IMO regulations state that treated ballast water has to be stored in the dark in simulated ballast water tanks for five days before being tested against the D-2 standard. However, it was questionable if this time period would be sufficient to account for delayed effects of the disinfectant and possible recovery of organisms. In other words, it was not known if re-growth of micro-organisms could occur after this standardized five day period. Therefore, in the present thesis, the possibility of re-growth was examined by executing long term incubation experiments under light-dark conditions simulating the post-discharge situation in the open sea. Phytoplankton and bacterial abundance, composition and diversity were monitored by a range of analytical techniques, including classical microscopy, flow cytometry and molecular fingerprinting.

In a first series of experiments, UV and chlorine dioxide (CD) treated water was incubated for 20 days under favorable conditions with respect to irradiance and nutrient availability to stimulate the growth of micro-organisms that had survived the treatment. After both treatments, re-growth of phytoplankton occurred (Chapter 2). This suggests that currently approved BWTSs meet the IMO D-2 standard, but do not completely eliminate the potential spread of aquatic organisms through ballast water.

To identify the species that re-grew after ballast water treatment, UV treated samples were incubated and monitored for phytoplankton abundance and species composition. Microscopy showed that ballast water treatment changed the species composition and that certain species were more likely to re-grow after treatment (Chapter 3). However, microscopy was not always able to identify the exact species. Because of this the application of flow cytometry, microscopy and DNA-sequencing as methods of species identification were investigated. Flow cytometry provided fast quantification of phytoplankton, but could only provide a rough indication of phytoplankton diversity. Microscopy provided a more qualitative method of identification, but could not always identify the phytoplankton to the species level. DNA-sequencing provided accurate species identification but proved to be time-consuming and only identified one or two of the most dominant species in the sample. The most common re-growing species after UV treatment proved to be *Thalassiosira weissflogii*. This indicates that some species are more likely to survive ballast water

treatment than others and that ballast water treatment may apply selective force to create resistant species (Chapter 4).

In the follow-up experiment, phytoplankton re-growth was monitored in six BWTSs; three systems were based on UV, two based on electrochlorination (EC) and one based on chlorine dioxide (CD). All BWTSs incubation experiments were performed for 20 days with treated ballast water, during which growth, photosynthetic efficiency and phytoplankton species composition were followed. The three UV systems all showed the same pattern after the initial UV exposure, notably a gradual decrease in phytoplankton abundances followed by re-growth. Treatments using 200 % or 400 % of the normal UV dose reduced phytoplankton numbers more strongly, but did not prevent their re-growth. Results of EC and CD BWTSs were comparable to each other. However, UV and active substance-based treatment systems showed significantly different responses. Both types of systems showed an immediate reduction in phytoplankton photosynthetic efficiency. However, for UV treatment systems phytoplankton abundances decreased over several days while for chlorine-based treatment systems the drop in phytoplankton abundance was immediate. The species composition of re-growing phytoplankton also differed between UV and EC treatment. Overall, all BWTSs reduced phytoplankton abundances to below the values of the D-2 standard, which represents a reduced risk of future aquatic invasions through ballast water. However, all (but one) re-growing species were smaller than 10 µm, which means they are not covered by the D-2 standard (Chapter 5).

To assess possible environmental risks associated with BWTSs that use 'active substances', a BWTS that uses a chemical mixture known as Peraclean® Ocean (PO) was evaluated. The residual of PO is acetate that might be present in concentrations exceeding 100 mg/L in discharged ballast water. To study the potential environmental impact of PO, microbial dynamics and acetate degradation were measured during incubation of discharge water following PO treatment. In addition, microbial dynamics and acetate degradation were studied at different temperatures in dark microcosms that simulated enclosed ballast water tanks. After about nine days bacteria abundances greatly increase in PO treated waters to almost ten times of initial control abundances. Furthermore, bacterial diversity was also altered by the changes in water chemistry. Breakdown of acetate occurred faster at higher temperatures. At the lowest temperatures almost no acetate breakdown occurred, but even at the highest temperature the acetate pool was not depleted. This implies that not all acetate will be broken down in ballast water tanks, even during long voyages in warm waters. It was concluded from this study that regular discharge of acetate-containing ballast water in harbors and bays may stimulate growth of heterotrophic bacteria, causing oxygen depletion and changes in the microbial community, especially in colder regions (Chapter 6). The D-2 standard does not consider total heterotrophic bacterial abundances. Increases in bacterial abundance as shown for this BWTS are allowed under current IMO regulations. The potential harmful effects on the ecosystem presented by the discharge of bacteria-rich ballast water demonstrate the necessity to include total heterotrophic bacteria in the D-2 standard.

In conclusion, the present thesis has revealed two major shortcomings in the ballast water regulations and particularly in the D-2 standard. It is recommended that the D-2 standard is amended to include limit values for viable phytoplankton and zooplankton organisms < 10 µm as well as total heterotrophic bacteria.

9. Samenvatting

Aquatische invasieve soorten vormen een van de grootste bedreigingen voor de mariene biodiversiteit. De voornaamste bron van verspreiding van deze aquatische invasieve soorten is het ballastwater van schepen. Dit is de reden dat de Internationale Maritieme Organisatie (IMO) de Ballast Water Conventie aannam. Een onderdeel van de conventie is de zogenaamde D-2 ballast water prestatie standaard, die grenzen stelt aan het aantal organismen in het ballastwater bij lozing. De grenzen van de D-2 standaard zijn: 1. minder dan 10 organismen/m^3 in de grootteklasse ≥50 µm; 2. minder dan 10 organismen/mL in de grootteklasse ≥10 - <50 µm; 3. vaststelling van maximaal toelaatbare concentraties van toxigene *Vibrio cholerae*, *Escherichia coli* en intestinale *Enterococci*. Om aan deze standaard te voldoen moeten schepen uitgerust worden met een ballastwater behandelingssysteem (BWBS). Deze BWBS moeten getest worden volgens de IMO regels door een onafhankelijk testinstituut. Een aantal van deze tests is uitgevoerd bij het Koninklijk Nederlands Instituut voor Onderzoek der Zee (NIOZ). Het doel van dit proefschrift is om de effecten van verschillende BWBS op het overleven van phytoplankton en bacterien te testen.

Verschillende behandelingsmethoden worden toegepast om de aantallen organismen te reduceren. De meest gebruikte behandelingsmethoden zijn blootstelling aan Ultraviolette straling (UV) en 'actieve substanties' (chemicaliën). Om de efficientie van deze verschillende behandelingsmethoden nauwkeuring te bepalen zijn tests nodig die bij ieder type behandeling werken. Volgens de IMO regels moet behandeld ballastwater vijf dagen in een donkere gesimuleerde ballastwatertank opgeslagen worden voordat getest word of het water aan de D-2 standaard voldoet. Het is echter niet duidelijk of deze vijf dagen voldoende zijn om te testen voor vertraagde effecten van de disinfectie methode en mogelijk herstel van de organismen. Kort samengevat, het was niet bekend of organismen zich weer zouden kunnen gaan vermenigvuldigen (vanaf hier hergroei genoemd) na deze periode van vijf dagen. Daarom werd in dit proefschrift het punt hergroei onderzocht aan de hand van incubatie experimenten onder licht-donker condities. Fytoplankton- en bacterie-aantallen, samenstelling en diversiteit werden gecontroleerd met verschillende analytische methoden, waaronder microscopie, flow cytometrie en genetische analyse.

In een eerste serie experimenten werd water dat met UV of chloordioxide (CD) behandeld was 20 dagen onder gunstige condities qua licht en nutriënten geïncubeerd om de groei van micro-organismen die de behandeling overleefd hadden te stimuleren. Bij beide behandelingen vond hergroei van phytoplankton plaats (Hoofdstuk 2). Dit betekent dat zelfs wanneer BWBS-en aan de IMO D-2 standaard voldoen, dit niet betekent dat er geen risico meer is van verspreiding van aquatische organismen via ballastwater.

Om de soorten te identificeren die na behandeling hergroeien werden UV-behandelde ballastwater monsters geïncubeerd en de fytoplankton aantallen en soortensamenstelling gevolgd. Met behulp van microscopie werd duidelijk dat ballastwater behandeling de soortensamenstelling verandert, daarnaast kwamen sommige soorten vaker terug na behandeling (Hoofdstuk 3). Omdat microscopie niet altijd de precieze soort kon identificeren werden flow cytometrie, microscopie en DNA-sequencing toegepast om te vergelijken welke methode het beste is voor de soortenidentificatie in ballastwater monsters. Flow cytometrie leverde snel fytoplankton aantallen, maar gaf slechts een ruwe indicatie van de soortensamenstelling. Microscopie leverde een meer kwalitatieve identificatiemethode, maar kon niet altijd tot op soortsniveau gaan. DNA-sequencing leverde een precieze identificatie op soortsniveau, maar bleek zeer tijdrovend en kon alleen de meest dominante soorten onderscheiden. De meest frequent hergroeiende soort na UV-

behandeling was *Thalassiosira weissflogii*. Dit wijst er op dat sommige soorten ballastwater behandeling beter kunnen overleven en dat ballastwaterbehandeling daardoor selectie-druk uit kan oefenen om resistente soorten te creëeren (Hoofdstuk 4).

In het aansluitende experiment werd de fytoplankton hergroei van zes BWBS-en vergeleken; drie systemen gebruikten UV, twee gebruikten electrochlorinatie (EC) en een gebruikte CD. Bij alle BWBS werden 20 dagen incubatie experimenten met behandeld ballastwater uitgevoerd. Tijdens deze incubatie experimenten werd groei, fotosynthese-efficiëntie en fytoplankton soortensamenstelling gevolgd. De drie UV BWBS-en hadden allemaal hetzelfde patroon van geleidelijke afname in fytoplanktonaantallen na behandeling gevolgd door hergroei. Behandelingen met 200 % of 400 % van de normale UV dosis reduceerden fytoplanktonaantallen sterker, maar er vond nog steeds hergroei plaats. De resultaten van EC en CD waren vergelijkbaar met elkaar. Daarentegen waren de resultaten van BWBS-en op basis van UV en chemicaliën significant verschillend. Beide types BWBS reduceerden de fotosynthese-efficiëntie onmiddelijk, maar bij de UV BWBS-en namen de fytoplankton aantallen geleidelijk over meerdere dagen af terwijl bij actieve substantie BWBS de afname in fytoplankton aantallen onmiddelijk plaatsvond. De soortsamenstelling van hergroeiend fytoplankton verschilde ook tussen UV en chemicaliën BWBS-en. Alle geteste BWBS-en reduceerden plankton aantallen tot onder de D-2 standaard, wat een verminderd risico van toekomstige aquatische invasies impliceert. Echter, alle (op één na) hergroeiende soorten waren kleiner dan 10 µm, wat betekent dat ze niet onder de D-2 standaard vallen (Hoofdstuk 5).

Om de mogelijke gevaren van BWBS die 'actieve substanties' gebruiken in te schatten werd een BWBS dat de chemische mix Peraclean® Ocean (PO) gebruikt geevalueerd. Het residu van PO is acetaat dat bij lozen in concentraties van meer dan 100 mg/L in het ballastwater kan voorkomen. Om de potentiële effecten van PO op het milieu te beoordelen werden microbiële dynamiek en acetaatafbraak tijdens incubatie van PO behandeld ballastwater gevolgd. Daarnaast werd de microbiële dynamiek en acetaat afbraak gevolgd bij verschillende temperaturen in donkere microcosmi die gesloten ballastwatertanks simuleerden. Na ongeveer negen dagen namen de bacterieconcentraties sterk toe in PO behandeld ballastwater, tot bijna 10 keer de beginwaarde van de controle. Daarnaast was de bacteriële diversiteit ook beïnvloed. De afbraak van acetaat was sneller bij hogere temperaturen. Bij de laagste temperaturen vond bijna geen afbraak van acetaat plaats, maar zelfs bij de hoogste temperaturen werd niet alle acetaat afgebroken binnen de duur van het experiment (20 dagen). Dit wijst er op dat niet alle acetaat in de ballastwater tanks afgebroken word, zelfs niet bij lange reizen in warm water. Dit betekent dat regelmatig lozen van acetaat-verrijkt ballastwater in havens en baaien eutrofiëring en veranderingen in de microbiële gemeenschap kan veroorzaken, vooral in koudere gebieden (Hoofdstuk 6). De D-2 standaard heeft geen grenzen voor heterotrofe bacterie-concentraties. Toenames in bacterieconcentraties zoals bij dit BWBS zijn toegestaan onder de huidige regelgeving. Echter, deze resultaten laten zien dat het nodig is om totale heterotrofe bacterien in de D-2 standaard op te nemen.

Dit proefschrift brengt twee belangrijke tekortkomingen van de ballastwater regelgeving en vooral de D-2 standaard aan het licht. De D-2 standaard moet aangepast worden door het toevoegen van grenswaardes voor phytoplankton en zooplankton < 10 µm en totale heterotrofe bacteriën.

10. Acknowledgements

I would like to thank Prof. Anita Buma and Dr. Louis Peperzak for their supervision and input, Dr. Marcel Veldhuis for giving me the chance to work on this subject and the entire NIOZ ballast water team for their assistance over the years. A special mention to Felicia Arenoe, Cees van Slooten, Josje Snoek, Eveline Garritsen, Dr. Judith van Bleijswijk and Julie Ogier for their help with analyses. I would like to thank everyone at NIOZ for the unique atmosphere they provided.

Very special thanks to Dr. Viola Liebich, without her support and encouragement I could not have finished this thesis.