

## Research Article

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

Viola Liebich\*, Peter Paul Stehouwer and Marcel Veldhuis

Department of Biological Oceanography, Royal Netherlands Institute for Sea Research, P.O.Box 59, NL-1790 AB, Den Burg, Texel, The Netherlands

E-mail: [Viola.Liebich@nioz.nl](mailto:Viola.Liebich@nioz.nl) (VL), [PeterPaul.Stehouwer@nioz.nl](mailto:PeterPaul.Stehouwer@nioz.nl) (PPS), [Marcel.Veldhuis@nioz.nl](mailto:Marcel.Veldhuis@nioz.nl) (MV)

\*Corresponding author

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### Editor's note:

This special issue of *Aquatic Invasions* includes papers from the 17th International Conference on Aquatic Invasive Species held in San Diego, California, USA, on August 29 to September 2, 2010. This conference has provided a venue for the exchange of information on various aspects of aquatic invasive species since its inception in 1990. The conference continues to provide an opportunity for dialog between academia, industry and environmental regulators within North America and from abroad.

### 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 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.

**Key words:** 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 (D2 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 (BWTS), 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 BWTS.

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 concentration 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 BWTS 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<sup>3</sup> 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 and 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 temporary shutdown, water was treated and pumped into tank II. For both replicate tanks, the water was newly treated. The first incubation experiment started 1st of April 2010 and the second one 13th of May 2010, latter with two bottles for each tank. For the control, harbor water was pumped (200 m<sup>3</sup>/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, I Intake (filter+UV), II Intake (filter+UV), I Discharge (filter+UV+UV), and II Discharge (filter+UV+UV).

The samples were incubated in clean 10 Liter Nalgene (Rochester, USA) bottles and 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<sub>4</sub> 1.6 µmol/L, NO<sub>3</sub> 20 µmol/L, SiO<sub>3</sub> 20 µmol/L). Samples were taken daily for analyzing phytoplankton concentration 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, 400×, Oberkochen, Germany). These samples had a volume of five milliliters, they were well-mixed, and not preserved. All cells and particles in these samples were allowed to settle for at least 30 minutes.

## Results

### Flow cytometry

UV-treatment decreased phytoplankton cell numbers (Figure 1). 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

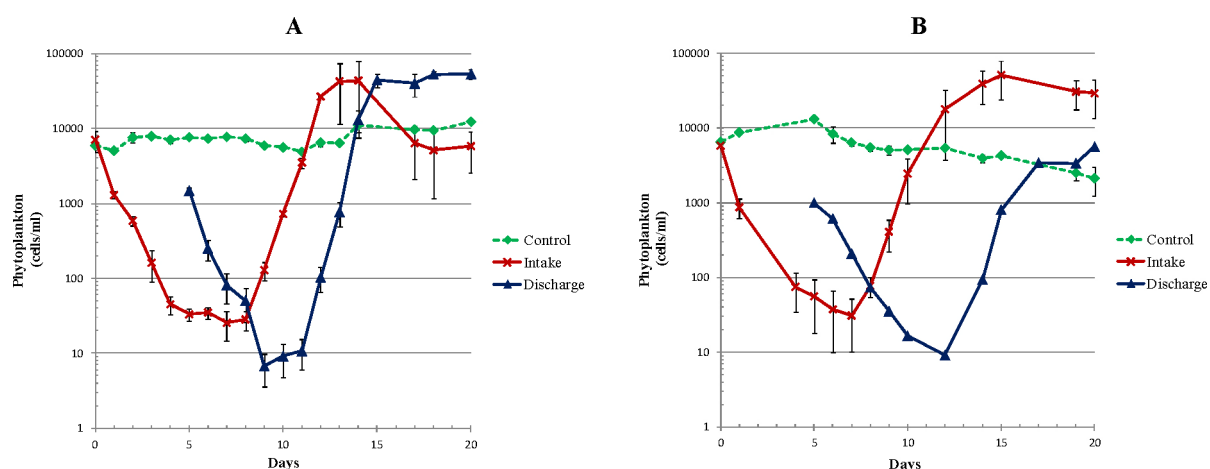
(first decline and re-growth after seven days) was again comparable. In both experiments, phytoplankton cell numbers in the control samples were considerably different from the treated samples.

### Light microscopy

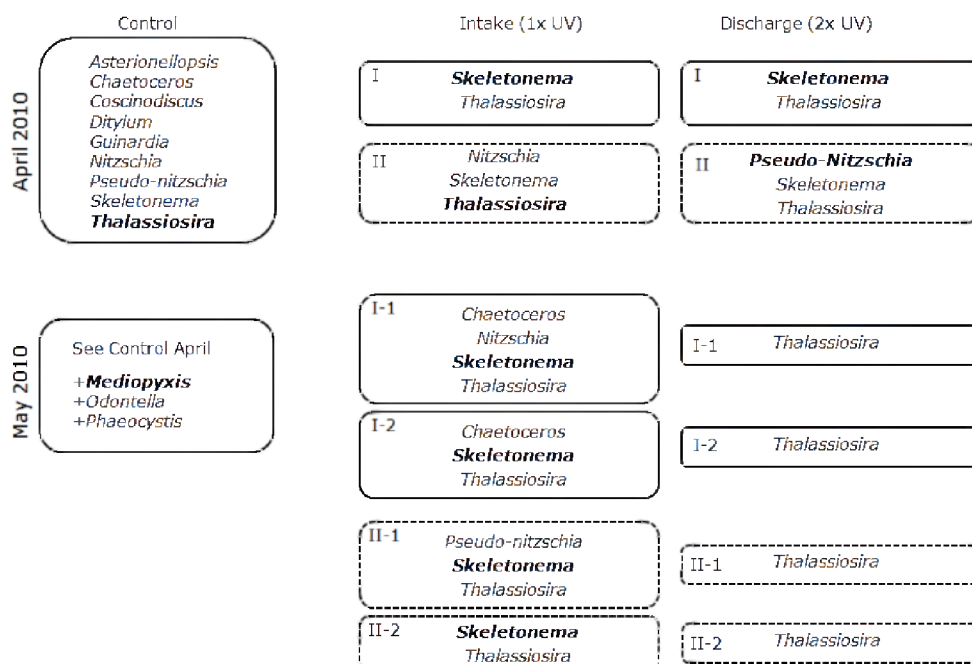
In April, *Thalassiosira* was the most abundant phytoplankton group in the control sample; additional phytoplankton included the diatoms: *Asterionellopsis*, *Chaetoceros*, *Coscinodiscus*, *Ditylum*, *Guinardia*, *Nitzschia*, *Pseudo-nitzschia*, and *Skeletonema* (Figure 2). The control sample of May contained the above mentioned taxa as well as *Mediopyxis*, *Odontella*, and *Phaeocystis*. In May's control sample, *Mediopyxis* was the most abundant species. 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.



**Figure 1.** Phytoplankton cell concentrations after UV-treatment at intake (day 0) 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 May's discharge samples due to distinct numerical differences (see text).



**Figure 2.** 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.

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 *Thalassiosira* 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 (perivalvar 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 -1.5°C up to at least 20°C (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 optimal 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, are easy to culture (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 D2 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.

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