A COMPARISON OF VIABILITY AND VITALITY TESTING METHODS AFTER BENCH SCALE UV- AND HEAT-TREATMENT OF THE DIATOM *THALASSIOSIRA WEISSFLOGII*

by

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ABSTRACT

Thalassiosira weissflogii, a marine diatom, was inactivated using two bench-scale treatment methods: ultraviolet radiation (UV-C) and a water bath (50°C). After treatment with either UV-C or heat, and a 24-hour dark incubation, cultures were analyzed using a range of monitoring methods to assess viability and vitality: serial dilution culture most probable number (SDC-MPN), cellular ATP concentration, vital stains, and chlorophyll fluorescence. *T. weissflogii* viable cell concentration was reduced by 3-log after 6 minutes of heat exposure or 148 mJ/cm^2 of UV-C radiation. UV-C treatment is far more energy efficient, using less than 1% of the equivalent input energy required for heat treatment. Viability, as determined through a growth assay, is compared against the other monitoring methods. Vitality testing was demonstrated to produce false positive results, especially for the UV-C treated cells.

LIST OF ABBREVIATIONS USED

AIS	Aquatic Invasive Species
ATP	Adenosine triphosphate
DMSO	Dimethyl sulfoxide
ETV	Environmental Technology Verification Program
IMO	International Maritime Organization
RMSE	Root mean square error
SDC-MPN	Serial dilution culture – most probable number (assay)
T. weissflogii	*Thalassiosira weissflogii*
USCG	United Stated Coast Guard
USEPA	United States Environmental Protection Agency
UV	Ultraviolet
VIDA	Vessel Incidental Discharge Act

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CHAPTER 1 INTRODUCTION

1.1 EXPERIMENTAL RATIONALE

This thesis consists of a series of experiments using a marine diatom as a model organism for exploring the effects of ballast water treatment on healthy, actively growing *Thalassiosira weissflogii* (*T. weissflogii*) cells. Culture of *T. weissflogii* in balanced growth was harvested and treated with either ultraviolet (UV-C) radiation or heat stress to inactivate the cells. The goal was to develop dose-response curves for the two treatment methods, using a variety of monitoring or enumeration methodologies.

The dose response curves are necessary for helping aid in the understanding of how the plankton present in ballast water responds to treatment. Some of the tests that are being considered for ballast water management use vitality as a proxy for viability. The difference in terminology – viability vs. vitality – is significant and requires different criteria to be met in the context of ballast water treatment. Viability can be specifically defined as the ability to reproduce; which implies functional essential cellular components: intact membrane, undamaged genome, and operational organelles (Breeuwer and Abee, 2000). Vitality, in comparison, is simply the indication that a cell is functional to some extent. A cell could be injured to the point of no recovery, and slowly succumbing to the injury, but may still test positive as vital (Davey, 2011). Non-viable cells that remain vital after treatment do not pose a threat to the environment as an invasive species, as they are completely inactivated thus unable to divide and colonise.

Invasive species transported by ballast water are a rather diverse group of organisms (Gollasch *et al*., 2002). For the sake of simplicity and better understanding of treatment impact it is important to work with single-species cultures. For this thesis one diatom species was chosen as a model to

represent a potential strain of phytoplankton that falls in the regulatory fraction of 10-50µm (International Maritime Organization, 2004). The narrow scope restricts direct comparison to natural seawater plankton assemblages, but this restriction allows controlled comparisons of the treatment methods by maintaining the initial culture in consistent physiological state that allows replication of experiments over time (MacIntyre *et al*., 2018).

A range of monitoring methods was chosen to assess different aspects of cellular health as indicators of viability before and after treatment with either heat or UV-C. These included the vital staining coupled with flow cytometry, as well as chlorophyll a fluorescence, and extracted ATP concentration. Dose-response curves were developed from results using the serial dilution culture most-probable-number assay (SDC-MPN), as growth is a direct measurement of viability. The dose response curves were then compared to the vitality results for each treatment method, demonstrating the possibility for false positives with this type of testing.

1.2 Research Objectives

The overall goal of this thesis was to examine methods for assessing the disinfection of *Thalassiosira weissflogii* by heat or UV-C treatment. To meet this overall goal, the thesis was divided into several sub-objectives, namely;

- Review the literature to understand the disinfection process for ballast water
- Summarize and compare the current regulations
- Consider the distinction between vitality and viability
- Describe procedures for algal culture growth and characterization
- Develop dose curves to evaluate disinfection methods on algal culture

The main thesis is that heat and UV-C radiation are both effective disinfection processes, with differ responses from microbial monitoring and enumeration methods that test for viability and vitality of cells in culture. If there are no differences between the viability and viability test outcomes after lethal inactivation treatment, then it can be assumed that vitality testing is a suitable proxy for the validation of ballast water treatment. If there is a significant difference between the results of the two types of testing, then vitality testing should not be used as the main indicator of disinfection effectiveness.

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CHAPTER 2 LITERATURE REVIEW

Below is a broad overview of ballast water, from its role as a potential vector for invasive species, to the regulations in place to manage this issue, and the technology used to achieve this goal. The two technologies that are used in this thesis are heat treatment and UV-C radiation, and these technologies are discussed in greater detail. Four monitoring methods are employed to investigate culture viability and vitality after treatment: serial dilution culture most probable number (SDC-MPN), cellular ATP concentration, vital stains, and chlorophyll fluorescence. A background and general overview of each technology is presented, and then the gaps in the literature are discussed.

2.1 Ballast Water as a Vector

Trans-oceanic shipping is an essential part of the modern world, and the majority of the global economy depends on it (International Chamber of Shipping, 2019; National Research Council, 1996). Ships move between continents and connect many different ports, sometimes transferring ballast water (Kaluza *et al*., 2010). Ballast water is water pumped on board a vessel to offset changes in vessel buoyancy. When cargo is loaded onto a vessel, it must discharge ballast as to maintain the proper draft.

Ballast water often contains macro- and microscopic organisms that can slip through screens and filters and survive the trip through the pump into the tanks (Gollasch *et al*., 2002). These tiny plants, animals, fungi, and bacteria, have the potential to disrupt financially-important coastal ecosystems, if they can grow unchecked in the new environment (Lonsdale, 1999). Treatment of ballast water to inactivate plankton in ballast water prior to discharge is one of the main goals in managing the dispersal of nuisance organisms.

2.2 Aquatic Invasive Species

Aquatic invasive species (AIS) means any nuisance organism that lives in an aquatic environment, such as mussels, algae, and bacteria, that are non-indigenous and transported through human activity (Lovell *et al*., 2006). Due to the increasing movement of ballast water between harbours by the shipping industry, the numbers of aquatic invasive species events have been increasing (Hulme, 2009; Seebens *et al*., 2013).

Phytoplankton represent a group of AIS that are challenging to monitor and treat in ballast water (Havel *et al*., 2015). An increased effort to study and manage phytoplankton reflects an increasing awareness of the problems caused by the occurrence of harmful algal blooms (Hallegraeff, 2003). For instance, a study from Galveston Bay, Texas, USA, sampled coastal water and found 40 major genera of phytoplankton, demonstrating diverse assemblages of phytoplankton in coastal waters (Steichen *et al*., 2012).

Two different regions would have different risk level for damage from plankton in ballast water because of the variation in environmental tolerances of organisms present in the ballast tanks, receiving ecosystem characteristics (nutrient availability and temperature, primarily), and the propagule pressure (Lonsdale, 1999). Reducing propagule pressure, or colonisation pressure, may be the best way to prevent the risk of AIS (Lockwood *et al*., 2009). Propagule pressure is highest in areas that experience lots of traffic, especially in ports nations with high exports, as the cargo ships discharge ballast water when loading.

2.3 Regulations

To reduce the risk of damage to coastal ecosystems, a co-ordinated global effort is required to ensure all participants in the global shipping industry are attempting to keep ballast water as

clean as possible. This involves the development of robust regulations that are backed up with solid scientific methods and analyses. While ballast water is only one factor in the management of aquatic invasive species, it is the main area with mobilization and regulation in development (Bax *et al*., 2003).

2.3.1 Canadian Regulations

As Canada is a major participant in the global economy, many ships of foreign origin enter Canadian waters. The United Nations Conference on Trade and Development states that for Canada alone, over 2017 container port throughput increased from 4,780,790 to 6,298,590 Twenty foot Equivalent Units (UNCTAD, 2018). The majority of inbound ships are laden with ballast water from remote sources (Locke *et al*., 1993; Sutherland *et al*., 2001). Ships sailing partially laden or empty are moving large amounts of ballast water with them that is released in port when cargo is loaded (Brooks and Faust, 2018), but it is worth noting that many of the vessels in Canadian ports arrive fully loaded, and therefore are not carrying any ballast on board (Fisheries and Oceans Canada, 2004).

The Canada Shipping Act: Ballast Water Control and Management Regulations states that all ships carrying ballast of foreign origin in Canadian waters must either retain the ballast on board, exchange it in a designated open-ocean zone, or treat the ballast before discharge in order to "remove or render harmless harmful aquatic organisms or pathogens within the ballast water" (Government of Canada, 2017). The Canadian Government is a signatory body that helped ratify the ballast water treaty (International Maritime Organization, 2017). Canadian regulations defer to the guidelines set by the IMO and largely rely on the United States to set the processes in place for the approval of ballast water treatment systems.

2.3.2 International Regulations

The International Convention for the Control and Management of Ships' Ballast Water and Sediments was originally adopted 15 years ago (International Maritime Organization, 2004), but was more recently ratified by 52 member states in 2016 and entered into force in September 2017, (International Maritime Organization, 2016). The discharge limits for the regulations set out by the IMO are outlined below (Table 1) .The IMO defines "viable" as "organisms that have the ability to successfully generate new individuals in order to reproduce the species," and set discharge limits based on this definition.

Table 1 IMO Regulation D-2 Discharge Standards

Organism Category	**Size Fraction**	**Permissible Concentration**
Zooplankton	>50 µm	<10 viable organisms/ 1000L
Phytoplankton	10-50 µm	<10 viable organisms/ mL
Bacteria	<10 µm	
Toxigenic *Vibrio cholerae*		<1 cfu/ 100 mL
Escherichia coli		<250 cfu/ 100 mL
Intestinal *Enterococci*		<100 cfu/ 100 mL

As the discharge limits are defined in terms of permissible organism concentration, determining the concentration of viable organisms in a sample of ballast water must be achieved through microbiological techniques in the lab. The IMO has approved the use of the serial dilution culture most probable number (SDC-MPN) assay, as an appropriate test to determine the viability of phytoplankton cells present in ballast water (International Maritime Organization, 2017).

2.3.3 American Regulations

The other major regulatory body for ballast water management is the United States Coast Guard (USCG) which has requirements in terms of "living" cell concentrations using the same size fractions and limits as the IMO (US Coast Guard, 2012).

In addition to discharge limits, the USCG and the United States Environmental Protection Agency (USEPA) coordinated to publish a recommended protocol for approving treatment systems for use in the ballast water industry as part of the Environmental Verification Program (ETV). Type Approval is the specific process that ballast water treatment manufacturers must use to validate their systems as suitable for use before they are accepted for use on board ships in American water. The process involves the use of challenge water that has a living population in the 10-50µm size fraction of 10^3 organisms/m3, including 5 different species of phytoplankton across 3 phyla (ETV, 2010). A ballast water treatment system must be able to treat the challenge water to meet the requirements of the IMO D-2 standard. The protocol requires five sequential tests with the equipment that must all be successfully passed before equipment can be granted approval.

During the completion of this thesis, the discrepancy in language between the IMO and USCG regulations has been rectified, with the signing of the Vessel Incidental Discharge Act (VIDA) into law December 4, 2018. VIDA amended the language used in the original USCG regulation to explicitly define "living" cells to exclude non-viable organisms, where it was previously ambiguous whether living meant viable or vital (Cullen, 2019). The VIDA bill has also recommended that the SDC-MPN is used in conjunction with Stain-Motility assessment of culture to determine viability. Robust statistical evidence has been published defending the SDC-

MPN alone as a sufficient test for viability of treated cells in type approval of ballast water treatment systems (Cullen, 2018).

The USCG recommends a staining protocol, employing fluorescent dyes to aid in the counting of vital cells (US Coast Guard, 2010); however, published research has called into question the universal applicability of this kind of monitoring method (MacIntyre and Cullen, 2016). The protocol involves the stains fluorescein diacetate (FDA) and 5-chloromethylfluorescein diacetate (CMFDA), which are used to determine the concentration of vital cells in a sample using fluorescence microscopy or flow cytometry (Brookes *et al*., 2000). This method relies on an intact cellular membrane to concentrate the fluorescein as cellular enzymes cleave the acetyl groups off the stain molecule (Rotman and Papermaster, 1966). An intact membrane is one of the essential. requirements of a viable cell, but not enough on its own as a diagnostic of viability (Emerson *et al*., 2017).

2.4 Experimental Organism

The USEPA ETV recommends *Tetraselmis* sp. as a model organism at a concentration of at least 10^3 organisms/ mL to represent the protists in ballast water when verifying treatment technologies (ETV, 2010). However, there is no universally agreed-upon test organism for the validation of ballast water equipment (Liebich *et al*., 2012). While *Tetraselmis* is a very well-studied organism which grows well in the lab, it is probably not the best representative of a challenge organism that poses a threat as an invasive species in the context of ballast water management. Flagellated green algae were insignificant when compared to diatom abundance and survival in ballast tanks (Burkholder *et al*., 2007).

A study in the Pacific ocean found 132 diatom species in their ballast water out of 145 phytoplankton morphospecies identified (McCarthy and Crowder, 2000), demonstrating the broad diversity of phytoplankton in ballast water in just one category. A survey of United States military ship ballast water from the Atlantic, Pacific, and Indian oceans found high diversity in phytoplankton, with 59 of 100 phytoplankton species identified as diatoms, and described several *Thalassiosira* species as widespread in distribution (Burkholder *et al*., 2007). *Thalassiosira* was found to be the dominant taxa in several ballast water surveys (Hyun *et al*., 2018; Liebich *et al*., 2012; Sutherland *et al*., 2001).

Thalassiosira weissflogii was chosen for this study based on a few key characteristics. *T. weissflogii* is described as a cosmopolitan organism and is often found in ballast water (Steichen *et al*., 2012). It is a fast-growing diatom in the 10-50µm range; cells from this strain have an average diameter of 13µm (±1.4) and a generation time of 1.1-2.3 d^{-1} (MacIntyre *et al*., 2002). In past work done at Dalhousie University, the susceptibility of various phytoplankton to the fluorescent stains suggested by the EPA was investigated, and *T. weissflogii* was shown to respond well to staining protocols compared to other phytoplankton (MacIntyre and Cullen, 2016). Additionally, the silica frustule that encases a diatom cell poses a barrier which could potentially inhibit cell lysis during the extraction step of some laboratory tests, which makes it an interesting candidate for investigation into ATP extraction comparison.

2.5 Treatment Technologies

Rendering an organism harmless to prevent its colonisation of a new ecosystem requires that it be killed or injured enough to prevent their reproduction (Davey, 2011). This thesis evaluated two treatment technologies to render planktonic cells inactive: heat and ultraviolet radiation. Wastewater treatment technologies have been applied as ballast water management systems and

have been shown to successfully inactivate or kill planktonic organisms of various classifications from sea water (Tsolaki and Diamadopoulos, 2010). If damage to a cell is extensive, apoptosis as a form of programmed cell death may be triggered (Davey, 2011). Damage beyond a cell's ability to self-repair results in cell fragmentation.

A cell can be considered inactive (aka non-viable) if it is unable to make new protein, unable to replicate, does not have an intact membrane, or a combination of the three (Emerson *et al*., 2017). Most treatment methods attempt to disrupt one of these three factors to render algae inactivated, and thus not a threat as an invasive species.

Ideal treatment is on-board so that the water in ballast tanks can be discharged as soon as possible, versus docking and pumping water to an onshore water treatment facility. Many different techniques have been employed in the treatment of microorganisms that can be adapted to an on-board setting to inactivate any potentially invasive species prior to discharge (Davidson *et al*., 2017; Lloyd's Register Group, 2017; Tsolaki and Diamadopoulos, 2010).

The different ballast treatment methods can be broadly divided into three main categories: mechanical, chemical, and physical treatment. The primary method of mechanical treatment of ballast water is physical exclusion through screens and filters, which stops the larger organisms from being pumped into the tanks, but tangential flow cyclones are also commonly found (Pećarević *et al*., 2018). Chlorine and ozone are commonly-used chemical treatment methods, however due to disinfection by-products and the cost of consumable additives, this method of ballast water treatment has more downsides than others (Romanelli *et al*., 2018; Tsolaki and Diamadopoulos, 2010). Physical methods for inactivating organisms in ballast water include heating the water and UV-C irradiating it. Heat treatment can be achieved through microwaves

or using a heat exchanger set up with the engine. This thesis focuses on the physical methods for ballast water treatment, as expanded on in the sections below.

2.5.1 Heat Treatment

Heat treatment to kill microbial cells is a straightforward process to accomplish, however it is still uncertain what is the specific mechanism of action at work (Lepock, 2003). It can be difficult to determine exactly what intracellular target specifically is damaged from excess thermal energy in a phytoplankter, as elevated temperature affects all biological, chemical, and physical processes occurring in the cell (Drost-Hansen, 1969). The heat damages essential enzymes and other proteins which would be required to facilitate any repair, therefore heat treatment is an irreversible process, which is fatal in a single celled organism.

Unfortunately, there is not a wide breadth of published literature on the thermal tolerances of marine diatoms in the context of ballast water treatment, which can make developing methods challenging. In the context of growing phytoplankton, temperature interactions have been studied to some extent. It has been argued that mesophiles (such as those generally found in the ocean) have an upper thermal limit around 33°C that is unavoidable without modifications to cellular processes (Drost-Hansen, 1969). A model developed from phytoplankton growth rates after adaptation by the cultures indicates a maximum functional temperature of around 40°C (Eppley, 1972). Another study found that many single celled green algae and diatoms were unable to grow at temperatures higher than around 35°C, and also agreed with Eppley that 40°C is likely the upper limit for mesophilic phytoplankton (Goldman and Carpenter, 1974). As this is the upper limit for culturing of marine phytoplankton as demonstrated by previous studies, it was determined that 50°C is thus suitable as a treatment temperature. Treatment at lower

temperatures is possible, especially if the cells are experiencing thermal shock because the source water is significantly cooler than the treatment temperature.

One heat treatment system approved for use by the USCG uses a combination of deoxygenation and above-40°C temperatures (Davidson *et al*., 2017). Successful treatment of natural sea water organisms in the 10-50 µm size fraction was demonstrated with exposure to 35°C temperatures achieved using a heat exchanger set up (Rigby *et al*., 1999).

2.5.2 Ultraviolet Radiation

Electromagnetic radiation with a wavelength of 100 up to 400 nm is considered to be ultraviolet (UV) radiation by the International Organisation for Standardization, broken down further into light having a peak wavelength of between 315-400 nm (UV-A), 280-315nm (UV-B), and 100-280 nm (UV-C) (Tobiska and Nusinov, 2006). UV-C inhibits photosynthesis in phytoplankton, with damage increasing in severity as wavelength decreases (Cullen *et al*., 1992). UV-C is not experienced at the surface of the planet in significant amounts from sunlight and is only encountered when using germicidal lamps or UV-LEDs (Madronich *et al*., 1998).

Germicidal UV-C lamps are commonly employed in ballast water treatment systems. A recent review of USCG-accepted ballast water technologies found over 40% of treatment systems use filtration and UV-C for disinfection (Davidson *et al*., 2017; Dobroski *et al*., 2009). UV-C radiation is useful for disinfection treatment as it targets DNA, which is present inside all living cells, appropriate for inactivating an extremely wide range of microorganisms found in wastewater. Damage to the DNA within the cell occurs in two forms: 6’-4’ photoproducts (6-4PP) and cyclobutene-pyrimidine dimers (CPDs).This cross bonding of neighboring pyrimidine dimers interrupts DNA translation and replication. Proteins do not efficiently absorb light at 254-

nm, so are thought to be unaffected by UV-C treatment (Friedberg *et al*., 2005). Damage that is sufficient to change the physical conformation of the DNA preventing cell replication and the production of important metabolic proteins is likely to trigger apoptosis (Moharikar *et al*., 2006). Repair of DNA has been demonstrated after UV-C treatment, and occurs 6-12 hours after exposure to radiation (Hull et al., 2017).

UV sources used for disinfection are either low- or medium- pressure bulbs. Low-pressure mercury UV lamps emit radiation within a narrow range (254 nm), whereas the newer medium-pressure bulbs emit a broader spectrum of radiation and at a higher intensity (Bolton, 2001). The medium-pressure bulbs at higher doses probably achieve advanced oxidation as their mechanism of action, instead of exclusively DNA damage. At high doses of UV radiation from medium-pressure bulbs, cellular membranes and proteins are disrupted due to advanced oxidation production of free radicals, as well as direct damage to aromatic proteins at the 280 nm peak (Determann *et al*., 1998).

DNA damage plateaus at different UV-C doses depending on the neighboring nucleotide pair: cytosine dimers at 50 mJ/cm^2 and thiamine at 200 mJ/cm^2 (Friedberg *et al*., 2005). Eukaryotic cells have enzymes called photolyases which are capable of repairing the damage caused by ultraviolet radiation, which enables a process known as photoreactivation, resulting in repaired DNA and recovery of viability (Sancar, 2008; Sinha and Häder, 2002). This poses a significant challenge for wastewater treatment with UV-C treatment, as the capability of DNA repair can increase chance of viability. Treatment equipment manufactures must ensure their testing includes the potential for repair and ensure the treatment doses recommended to ships crews accounts for this potential.

2.5.3 Dark Incubation

Dark incubation is used in this thesis after treatment, for a duration of 24 hours. Researchers working with phytoplankton sometimes include a dark incubation step to simulate a hold time in a ballast water tank; the dark incubation does not seem to cause any harm to phytoplankton cultures itself but it does inhibit the cells' ability for photo repair (Sutherland *et al*., 2001). Other research has demonstrated month-long holds simulating ballast water tanks have minimal effect on phytoplankton cultureability (Flagella *et al*., 2007).

2.6 MONITORING TECHNOLOGIES

2.6.1 Growth Assay

The 'gold standard' in assessing true viability for microbes in liquid culture is currently the SDC-MPN method. Originally developed for determining cell concentrations of bacteria, it has been adapted for analysis of phytoplankton. Unfortunately, it is time consuming because of the number of replicates that must be monitored, and the time needed to reduce the likelihood of false negatives. When monitoring algae, the method recommended for distinguishing growing cells involves monitoring cultures non-invasively by measuring chlorophyll a fluorescence. The most direct and best method to determine true viability after treatment is to attempt to regrow any cells that may have survived. To test phytoplankton for viability cells are diluted into fresh culture medium and then monitored for increasing chlorophyll fluorescence as the positive indication of growth (Cullen and MacIntyre, 2016).

2.6.2 Adenosine Triphosphate Measurement

Phytoplankton, like many other microbes, use an energetic currency of adenosine triphosphate (ATP) which is present in all living cells, and the concentration of which represents a short-lived reservoir of potential cellular activity. ATP is a useful bioindicator because of this ability to distinguish between living cells and detritus or non-living matter (Holm-Hansen, 1973). Modern ATP protocols are capable of detecting ATP concentrations at extremely low per-cell levels (Hammes *et al*., 2010). ATP extraction and measurement has been developed for use in the ballast water industry, and has shown a high degree of agreement between ATP concentration and number of organisms, although physical extraction was used in addition to chemical lysing of cells (Curto *et al*., 2018).

The ATP concentration inside of cells in a sample can be measured by separating cells from the surrounding liquids using filtration. Particles retained on a filter can be extracted in parallel with a whole (unfiltered) sample, to distinguish the levels of ATP inside of cells (cellular ATP, or cATP) from the free ATP in solution which has been recently released from lysed cells. In this thesis, only cATP was measured as learning how cells respond to treatment is the main question being investigated.

2.6.3 Chlorophyll Fluorescence

Measuring chlorophyll a is a common practice in phytoplankton research due to the exclusivity of the molecule to autotrophic organisms (Cullen, 1982). This excludes a large range of other cells present in ballast water – such as most bacteria and zooplankton – helping to narrow down the information coming from a natural seawater sample without having to physically separate cells prior to investigation. While chlorophyll a fluorescence measurement is convenient, it is

important to keep in mind that the chlorophyll a to carbon biomass ratio is a highly dynamic value depending on cellular and environmental conditions (Geider *et al*., 1996). Fluorescence can be induced and measured with algal cells in culture to characterize their photophysiological responses (Huot and Babin, 2010), as well as in vitro after extraction of the pigment into solvent (Welschmeyer, 1994). Fluorescent detection takes advantage of cells' natural defence mechanisms for avoiding damage due to short-term exposure to higher intensity light (Maxwell and Johnson, 2000).

Energy absorbed by chlorophyll a has one of four options: photochemistry, dissipation as heat, re-emitted as a photon (fluorescence), or oxygen free radical production (Müller *et al*., 2001). As photons hit photosynthetic reaction centers in chloroplasts, the PSII protein passes the energy to the electron transport chain. There is a short period of time where a PSII protein is unable to accept any new photons, and this energy is usually released as either heat or fluorescence. Dissipation as heat is the primary route for the dissipation of excess energy but too small to alter the temperature of the culture during short interrogations. Free radical production only occurs if light levels are beyond the photosynthetic capacity and protective methods to dissipate the extra energy. When cultures are dark adapted, heat dissipation and free radical production are minimal and fluorescence, can be used as a first-order proxy for cell abundance and for assessing physiological status (Huot and Babin, 2010).

2.6.4 Flow Cytometry

Analysis of the 10-50 µm fraction of ballast water using flow cytometry has been recommended to the ballast water industry instead of manual microscopy (Burkill and Mantoura, 1990; Hoell *et al*., 2017). The advantages of flow cytometry over other fluorescent imaging options include the

speed of data collection, the large number of cells investigated, and the ability to standardize the fluorescent signal. Flow cytometry uses directional scattering of a laser beam to assess particle counts and size. Scattering is a general manifestation of the physical size and the refractive properties of each cell.

In addition to directional scattering, flow cytometers have detectors configured to monitor multiple wavelengths of re-emitted light (fluorescence). In phytoplankton research, the chloroplasts can be used to help identify cells from debris or other particles in the sample. The chlorophyll an autofluorescence can be measured using a blue laser in a similar fashion as the in-vivo chlorophyll fluorescence as described above. Forward/side light scattering by each cell can be used to approximate chlorophyll a to carbon ratios, with carbon being a proxy for biomass.

In addition to scattering and chlorophyll a autofluorescence, there is also the ability to stain a sample with a contrasting fluorophore to help in the determination of cell number and vitality or investigate additional cell parameters (Veldhuis and Kraay, 2000). In this experiment a combination of two vitality stains were used; FDA and CMFDA as recommended by the USCG for ballast water testing (ETV, 2010), however the method suggests using fluorescence microscopy instead of flow cytometry. FDA is membrane permeable so may leak out of cells even after being cleaved by esterase activity, so a secondary stain is used, CMFDA which is considered to be membrane-permeable while in the complex, but after cleavage it is membrane-impermeable and cannot diffuse back out through the membrane (Peperzak and Brussaard, 2011). This accumulation of cleaved products is taken as a signal for cell vitality.

2.7 Literature Gaps

One of the major literature gaps found was the lack of data regarding thermal and UV-C radiation dose curves for phytoplankton, specifically the diatoms. Most research into algae conditions are related to growth and culturing, instead of inactivation of mixed assemblages or pure cultures. For treatment systems to be developed and tested, there needs to be a greater understanding of how treatment affects these organisms.

There is also a lack of research regarding ATP as a monitoring technology using the 2nd generation ATP test kits. While there are a few papers using this technology, it is relatively new to the ballast water world. It is valuable as it offers rapid test results on biological activity and load in ballast water, and when coupled with size fractionation, gives a good indication of potential phytoplankton load, which can then inform treatment or discharge decisions. Some of the plankton in the ocean pose a challenge for this type of monitoring due to the limited ability of the rapid test kits to fully lyse cell walls and membranes, due to the presence of the silica frustule in the case of the diatoms.

CHAPTER 3 MATERIALS AND METHODS

3.1 CULTURE MAINTENANCE

Thalassiosira weissflogii (*T. weissflogii*) culture was grown in an f/2-enriched 0.2µm sterile-filtered coastal seawater (Guillard 1975). Cultures were incubated at 18°C ±0.5°C with 12/12 light/dark cycle and illuminated at 130µmol photons·m^{-2}·s^{-1} of PAR using Osram FL40SS-W/37 fluorescent bulbs, as per the method described by MacIntyre *et al.* (2016). *T. weissflogii* (CCMP1050) culture was obtained from the National Center for Marine Algae and Microbiota (NCMA, East Boothbay, ME, USA). Algae cultures were grown on a semi-continuous basis through monitoring on a 2 to 3-day basis. Cultures were monitored using active fluorescence to establish balanced growth conditions (MacIntyre and Cullen, 2005).

Chlorophyll a fluorescence was measured using a Turner 10AU fluorometer (Turner Designs, Sunnyvale, CA, USA) and a FIRe benchtop fluorometer (Satlantic, Halifax, NS, Canada). Both instruments are designed to hold a 50 mL screw-top culture tube, and the smaller 7 mL screw-top tubes that were used for the SDC-MPN assay. All measurements and extractions were done after thoroughly mixing the cultures, because cells fall out of suspension quickly and it is important to ensure homogenous samples. Tubes and flasks of culture were inverted 10 times to ensure cells were as evenly distributed as possible.

Culture was grown to larger volumes by sub-culturing from the stock culture held in balanced growth. This experimental culture was then diluted in stages with a day or two of growth in between each addition of media, until the final volume required for experimentation was reached and culture had grown to the appropriate density (10^5 cells/ mL) . This maintained a consistent growth rate and ensured that cultures were in balanced growth, i.e. were fully acclimated to the growth conditions.

3.2 INACTIVATION TREATMENT

Flasks of culture were grown under controlled conditions to maintain balanced growth rates as described in MacIntyre *et al.* (2016). The contents of the large flask of culture were subdivided into multiple 50-mL samples that were exposed to either a heat or UV-C treatment. One treated tube for each dose was subdivided further after incubation for each viability- and vitality-based test. From each flask of culture used for experimentation a control sample was reserved that was not exposed to heat or UV-C radiation. Each experimental dose was tested in triplicate.

3.2.1 Heat treatment

Samples were transferred to a sterile screw-capped culture tube, which were capped securely and wrapped in parafilm. Each tube contained 50mL of culture and were filled with very minimal head space to prevent them from floating. Tubes were submerged in a preheated water bath (Isotemp 205, ThermoFisher) set to 50°C ± 0.1°C. Cultures were submerged in the bath for treatment times ranging from 1 to 10 minutes. After heat treatment, the tubes were promptly removed from the water bath, and the parafilm was removed. Tubes were dried with a paper towel and then wrapped in aluminum foil to begin the dark incubation.

3.2.2 Ultraviolet Treatment

UV-C dose was provided by a custom-built low-pressure collimated beam (Bolton and Linden, 2003). The intensity of the UV lamp was measured with an annually calibrated, NIST-traceable ILT1700 radiometer (International Light Technologies, USA) prior to treating samples. The UV_{254} transmittance value was measured on the culture before UV-C treatments were performed using a P100 UV254 meter (Real Tech Inc., Canada). Treatment dose times were calculated using the lamp fluence and the culture UV_{254} transmittance value following the approach defined

by Bolton and Linden (2003). Algal culture was transferred with a pipette to a 60 x 30 mm borosilicate glass petri dish with a 5 x 2 mm stir bar. The stir bar was set at a medium speed, c. 60 rpm, to ensure even treatment of cells within the dish. After samples were treated the culture was aseptically transferred by pipette to sterile 50 mL screw -top borosilicate test tubes.

3.2.3 Dark Incubation

After treatment the tubes were individually wrapped in aluminum foil to ensure the culture received no light from inside the incubator. Samples were incubated at18°C for 24 hours prior to analysis. The 24-hour hold was intended to simulate a ballast water tank during a short voyage as well as to allow cells to succumb to any latent damage induced by treatment. The incubation temperature is the same for growing cultures, which was chosen as a control to reduce the possible sources of treatment effects. This differs slightly from the IMO protocol for type approval of ballast water treatment systems includes a 5-day dark incubation in a ballast tank prior to treatment, followed by discharge (International Maritime Organization, 2004). The shorter protocol was used for practicality as well as a more direct investigation of treatment versus the effects of dark incubation as well as treatment.

3.3 VITALITY AND VIABILITY INVESTIGATION

The monitoring methods chosen in this study to investigate the cultures after treatment are described below. The direct method measuring true viability is the growth assay, and the rest of the methods are considered proxy indicators because they measure some aspect of vitality: mainly cellular ATP concentration (metabolic activity), chlorophyll a fluorescence (biomass), and membrane integrity (vital staining).

3.3.1 MPN Assay

A dilution series was prepared by pipetting 5 mL of well-mixed treated culture into 45 mL of f/2 seawater media in 70 mL screw-capped glass dilution tubes (Guillard, 1975). Tubes were inverted 10 times before removing a 5-mL aliquot with a sterile pipette and transferring it to another 45 mL of culture in second dilution tube. Five subsamples of 5 mL were taken from each dilution level chosen for monitoring and transferred into 10 mL sterile glass screw-top culture tubes. Tubes were placed in a test tube rack along the front and edge in an 'L' shaped configuration so that the tubes were evenly lit by the lights in the sides and door of the incubator.

The dilutions chosen for each SDC-MPN assay were determined using range-finding experiment and previous work done by Macintyre *et al.* (2018). The tenfold dilutions chosen ranged from 10^{0} to 10^{-7} depending on the estimated recovery from preliminary testing at a range of UV-C and heat doses. Dilution ranges were chosen to ensure that the estimate of MPN was within the up per and lower bounds of the assay and the growth responses from the top tier of dilutions was used to determine the end point for monitoring the experiment (MacIntyre *et al*., 2018).

Fluorescence in the MPN tubes was measured daily in a 10AU fluorometer (Turner Designs). Tubes were inverted ten times and the outside of the tube is wiped before being inserted into the fluorometer to ensure homogeneity of the culture and to prevent loss by absorption or scattering of contamination on the outside of the tube. Tubes were read daily or every other day to monitor fluorescence values. After tubes were read for their fluorescence value, they were returned to the rack but in a randomized order to ensure any variances in illumination were minimized.

Samples were monitored for up to three weeks, ensuring any cells with the potential for recovery were able to grow. Tubes with no visible growth were deemed appropriate for disposal at a time

(t_{end}) which affords a wide safety margin to avoid false negatives (MacIntyre *et al*., 2018). This method extrapolates the linear portion of the growth curve back to the origin to estimate a starting cell concentration. Based on this estimated initial cell concentration, the growth rate of the top dilution tier of tubes, and a safety factor, a tube was deemed to show no growth after this calculated length of time. A safety factor of 5x was used in this experiment, meaning tubes were monitored for an additional 5 generations after the growth should have been detected based on the growth in the least-diluted tubes.

At the end of the experiment, each tube was assigned a score 1 (growth) or 0 (no growth), depending on whether or not it exhibited at least three days of continuous growth above the lower limit of detection of the fluorometer (MacIntyre *et al*., 2018). The scoring of positive tubes at each dilution level was converted to an estimate of the most probable number of viable cells, after correction for the dilution range using a published calculator (Jarvis *et al*., 2010, 2015).

3.3.2 ATP Extraction

Samples were analysed using QGA extraction kits (LuminUltra), following the directions included. Ten mL of algal culture were gently pushed through the syringe filter, which was detached before removing the syringe plunger and reattaching the loaded filter back onto the syringe body. One mL of UltraLyse™ was pipetted into the syringe, pushed through the filter, and the extract was collected in the pre-prepared UltraLute™ dilution tubes containing 9 mL of diluent. Luminase activity was standardized using the Lumicheck™ assay before each set of samples was analysed. 10x diluted samples were measured using a Kikkoman photoluminometer. In a disposable plastic test tube, 100 mL of sample was mixed with 100 mL of room temperature Luminase enzyme. The tube was gently mixed by flicking the bottom of the

tube lightly and inserted into the photoluminometer for measurement within 30 seconds of adding the enzyme.

3.3.3 Chlorophyll Extraction

Chlorophyll was extracted using a 2:3 volume ratio of dimethyl sulfoxide (DMSO) to acetone solvent solution. In a darkened room, 200 µl of culture was direct injected into 6 mL of prepared solvent in a screw-top glass vial, capped, and left to incubate for 10 minutes in the dark. Tubes were inverted several times and then fluorescence was read using a Turner fluorometer that was configured for the Welschmeyer protocol (Welschmeyer, 1994) and calibrated with purified chlorophyll a (Sigma Aldrich C6144).

3.3.4 Flow Cytometry

Cells were counted using flow cytometry – differentiated from cell fragments and other debris based on the side scattering and the auto-fluorescence from chlorophyll a. Two 1 mL samples were taken from each treatment tube and transferred to 2-mL cryovials. To one of the vials 10 µL of FDA (5µM final concentration) (FDA, Molecular Probes-Invitrogen Carlsbad, CA) and 10 µL of CMFDA (2.5 µM final concentration) (CMFDA, CellTracker^{TM} Green; Molecular Probes-Invitrogen Carlsbad, CA) were added, and the tube was wrapped in aluminum foil and transferred to the 18°C growth incubator for 10 minutes (MacIntyre and Cullen, 2016). "Statistically-not-unstained" determination was made by comparing the intensity of fluorescence of the background signal to the stained cells. The median intensity of fluorescence from the stained *T. weissflogii* cells must be greater than the 95th percentile value of the background signal.

3.4 Data Normalisation and Regression

Data points (such as cell count, chlorophyll a concentration, ATP concentration) were normalised by dividing the treated sample's value by the corresponding value for the control sample from the same initial culture. These values were then log-transformed and plotted to display the dose-response curves based on each type of monitoring assay. Linear regression was used when a significant trend was assessed, and where appropriate, a first order Chick-Watson kinetics model was applied to the log normalised data (Chick, 1908; Watson, 1908). The root mean square error (RMSE) was calculated for the linear regression and the Chick-Watson regression when either fit had statistically significant ($\alpha < 0.05$) correlation. The regression with the lowest RMSE was chosen for analysis of that data.

The general equation for the Chick-Watson (first order) kinetics model (Watson, 1908) is:

$$Relative\ viability/vitality = (Dose\ -\ M3)\ \times\ M2, \text{when Dose} > \text{M3} \qquad \text{Eq. 1}$$

Where

Relative viability/vitality – log normalized response variable for each monitoring method tested in this thesis,

M2 – slope of the line after the shoulder,

M3 – threshold log-normalized dose below which there appears to be no affect from treatment in either minutes or mJ/cm^2 for heat and UV-C treatment respectively.

The shoulder in the data is a modified version of the basic Chick-Watson model to include a delay before response (Marugán *et al*., 2008).

CHAPTER 4 RESULTS

T. weissflogii culture was treated with a range of heat and UV-C exposures to investigate the responses from various viability- and vitality-based testing methods. The results from these analyses are presented below. Four methods tested included growth assay (SDC-MPN), ATP concentration, chlorophyll fluorescence, and vital stains. The different monitoring methods are presented together as a direct comparison, which provides an interesting comparison of the two treatment methods.

4.1 VIABILITY

The results of SDC-MPN assays of treated cultures are displayed in Figure 1 below.

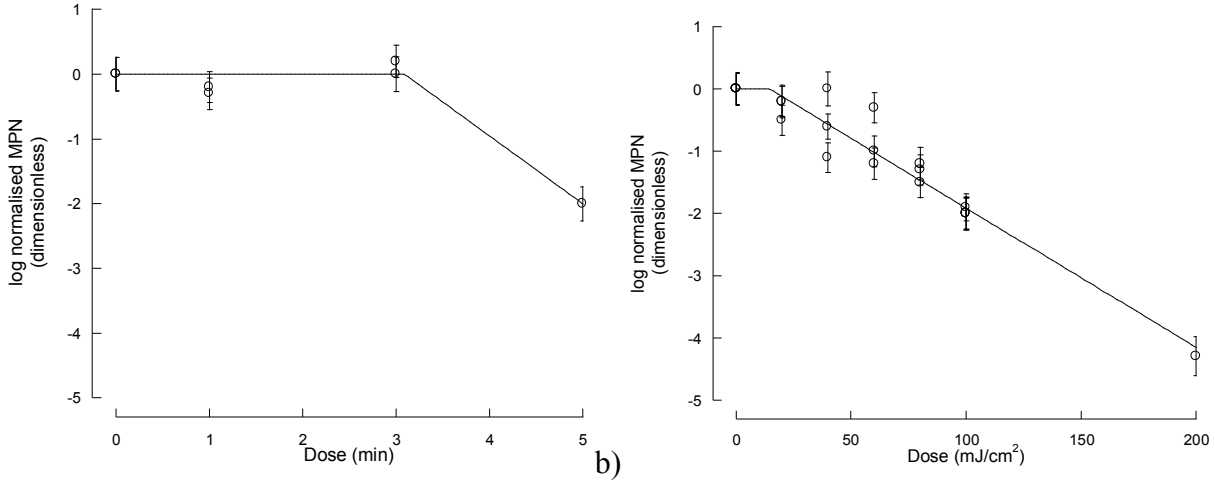


Figure 1 Relative viability of *T. weissflogii* determined through SDC-MPN growth assay.

T. weissflogii cells were successfully regrown from culture treated with lower doses of heat and UV-C. For the heat treated culture, any cells that were viable after treatment of 10 minute exposure at 50°C or longer were below the limit of detection of the SDC-MPN assay (Figure 1a). For the UV-C-treated cultures, any viable cells in treatments of 300 mJ cm^2 or higher were also below the limit of detection (Figure 1b). The lowest scoreable MPN result has a concentration of

0.036 cells/mL, tabulated as 0-1-0 or 0-0-1 for 5 mL volumes in which the upper tier is undiluted culture (Blodgett, 2010). This concentration is far below one whole cell, so this minimum detection limit for the SDC-MPN assay satisfies the regulations requiring fewer than 10 individuals per mL of treated water.

The Chick-Watson model was applied to dose-response curves to both heat and UV-C treatment (R^2 of over 0.90; see Table 2).

Table 2 Relative viability fit parameters.

Fit Parameter	Heat Treated		UV-C Treated	
	Value	Error	Value	Error
M2	-1.05	6.21×10^{6}	-0.0224	1.52×10^{-3}
M3	3.09	1.13×10^{7}	14.7	4.78
χ^2	0.170	-	1.452	-
R^2	0.952	-	0.938	-

The calculated dose required to inactivate the culture as determined from the dose-response curve is determined by the following equation:

$$\boldsymbol{Dose\ required} = [(\boldsymbol{Log\ Reduction}) \times (-\boldsymbol{M2})] + \boldsymbol{M3} \qquad \text{Eq. 2}$$

Where

M2 – slope,

M3 – threshold dose, below which there is no response in the y variable.

The two treatments can be compared based on the viable cell concentration reduction in the *T. weissflogii* culture using the previous equation. The treatments can be compared in terms of their energetic costs for the equivalent treatment log reduction. A 3-log reduction in *T. weissflogii* cells requires 6 minutes at 50°C, which is about 10 kJ of input energy to change the temperature of the sample water, however the equivalent for UV-C treatment requires less than 1% of the input energy, just over 3.5 J for the equivalent reduction in culture viability (Table 3).

Table 3 Decimal reduction for *T. weissflogii* cells treated with heat or UV-C

Log Reduction	Heat		UV-C		Comparison
	(min)	(J)	(mJ/cm^2)	(J)	(kJ_{UV-C} / kJ_{Heat})
2	5.00	9.83×10^3	103.92	2.47	2.5×10^{-4}
3	5.94	9.97×10^3	148.52	3.53	3.5×10^{-4}
4	6.88	10.05×10^3	193.12	4.60	4.5×10^{-4}

The calculated energy required for the equivalent log reduction in viability for the two treatment methods was based on measured temperature changes inside the treatment tube submerged in the water bath (see Appendix A, Figure 9).

Lower energy for treatment is important for economic and sustainability reasons. Fuel must be burned to provide for the treatment energy, which is a non-renewable resource, so any reduction in energy use is beneficial. This is reflected in the industry as many of the ballast water treatment systems available involve the use of UV-C radiation (Lloyd's Register Group, 2017)

4.2 VITALITY

4.2.1 Cellular ATP

Cellular ATP (cATP) concentration was reduced after heat treatment, with a 1-log reduction in concentration after about 13 minutes of treatment (Figure 2). Initial and 10-minute heat-treated culture average cATP concentrations were 1.09×10^4 pg/mL and 1.96×10^3 pg/mL respectively. These correspond to average cATP per cell concentrations of 0.79 and 0.23 pg/ cell for the control and 10-minute treated cultures. UV-C treatment did not affect the cellular concentration of ATP, in terms of a decimal reduction in cATP concentration. The data presented below in Figure 2 are individual measurements for each treated sample, and are presented without any

qualification of uncertainty. Chick-Watson kinetics were applied to the heat treated data, and the fit parameters are listed in Table 4.

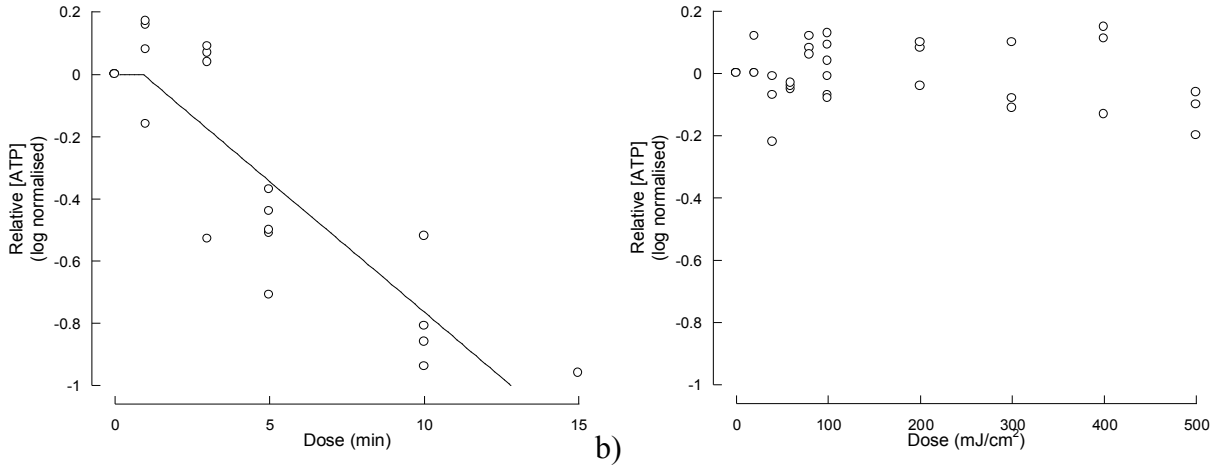


Figure 2 Relative vitality of *T. weissflogii* as cellular ATP concentration.

There was a fairly strong correlation between cATP concentration and heat treatment, with a linear response after a few minutes of treatment once the culture began to heat up (Figure 2). The fit parameters are displayed below in Table 4.

Table 4 Relative cellular ATP concentration fit parameters.

Fit Parameter	Heat Treated	
	Value	Error
M2	11.90	1.64
M3	0.90	0.81
R^2	0.76	-
χ^2	0.74	-

Cellular ATP concentration has been demonstrated to be fairly consistent between day and night cycles for algae, but nutrient stress does have an effect (Holm-Hansen, 1970). In this experiment cultures were grown in nutrient replete media and only held for one day without being refreshed after treatment. Due to this most of the response in ATP concentration can be credited to the effects of treatment, not the dark incubation.

Samples of ballast water sometimes need to be concentrated before enough individuals can be assessed for treatment, but recommendations have been made to preserve cellular ATP (van Slooten *et al*., 2015). This may be more of an issue with sampling from ballast water tanks and getting an underrepresented sample, than problems with ATP as a monitoring method specifically (Carney *et al*., 2013). This problem was avoided by using lab grown cultures for treatment, but the potentially sparse population in some ballast water should be considered when sampling plans are developed.

4.2.2 In-Vivo Chlorophyll A Fluorescence

In-vivo chlorophyll variable fluorescence (F_v) did not show a strong response to heat or UV-C treatment, even at doses that result in over 3 log reduction in viable cells (Figure 3 a). Data points represent individual readings of in-vivo chlorophyll a fluorescence.

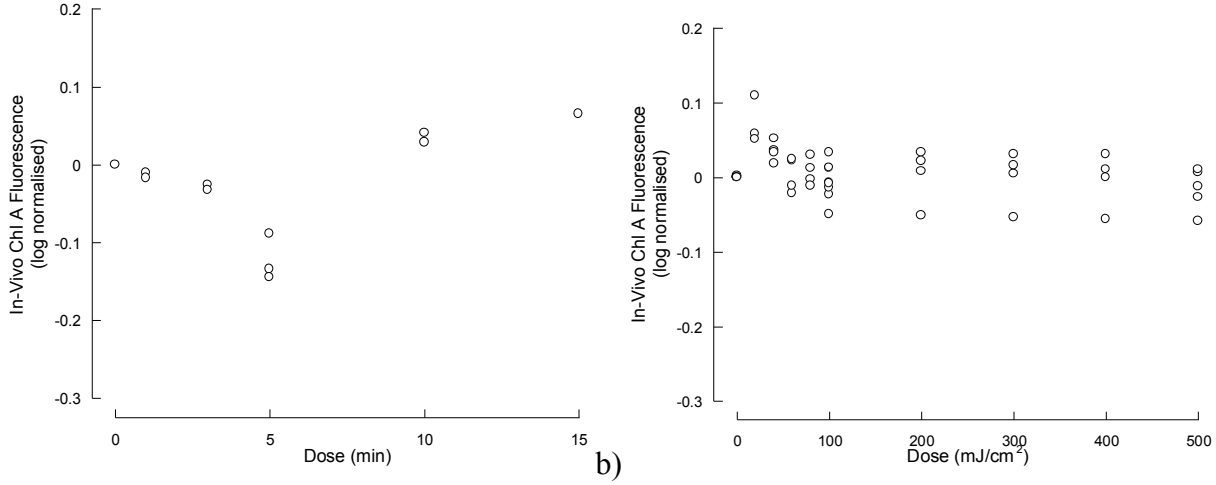


Figure 3 Relative vitality of *T. weissflogii* as in-vivo chlorophyll a fluorescence.

There appears to be some trend in the heat-treated data, with a low fluorescence around 5 minutes, but the signal increasing after that, even though the trend in relative viable cells does not appear to have the same pattern.

4.2.3 Chlorophyll A Concentration

Chlorophyll a was extracted and analysed, and the results are displayed below in Figure 4.

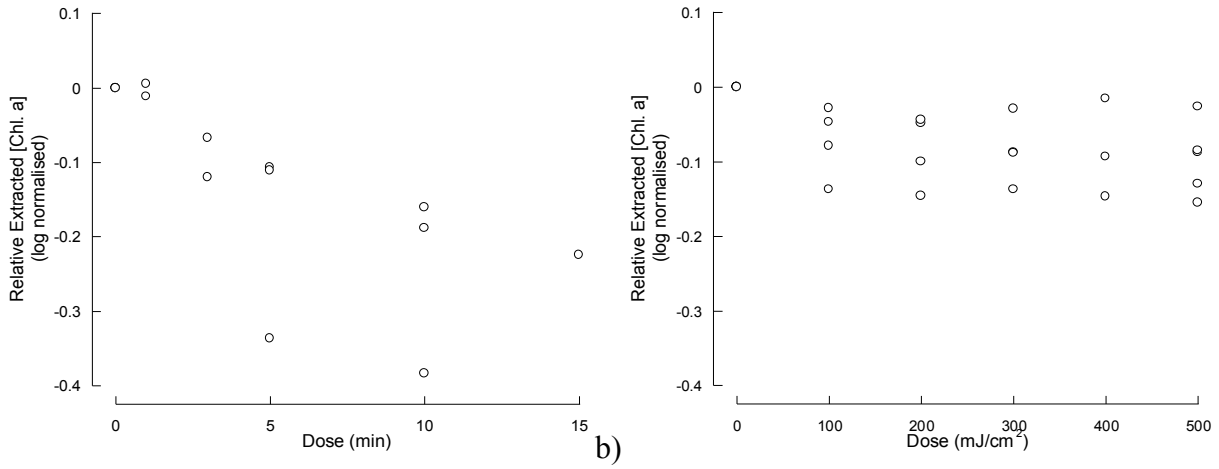


Figure 4 Relative vitality of *T. weissflogii* as extracted chlorophyll a fluorescence.

Chlorophyll a was determined fluorometrically after extraction with DMSO and 90% acetone (Welschmeyer 1994). Each point in the figure above (Figure 4) represents an individual estimate and is presented without an estimate of the uncertainty.

The extracted chlorophyll a did not show a significant response to UV-C treatment. After a dose of 500 mJ/cm^2 chlorophyll was only reduced by 0.1 log. For heat treatment, there was some decrease in chlorophyll a concentration; treatment of 15 minutes at 50°C reduced chlorophyll by about 0.3 log units (50% reduction) relative to the control. The decrease in chlorophyll was not as dramatic as the viability results, but still detectable in the culture after treatment and incubation.

4.2.4 Cell Counts

The flow cytometer was used to determine cell counts of the algal culture. These results are displayed below in Figure 5. Because of instrument breakdown, many samples could not be analysed; unfortunately, this included all of the lower doses of UV-C treatment (20-100 mJ/cm^2). There was a similar trend in the heat-treated cell counts to the results from in-vivo fluorescence, where the counts decreased for 0-5 minutes of heat treatment but then increased with longer exposure (Figure 3).

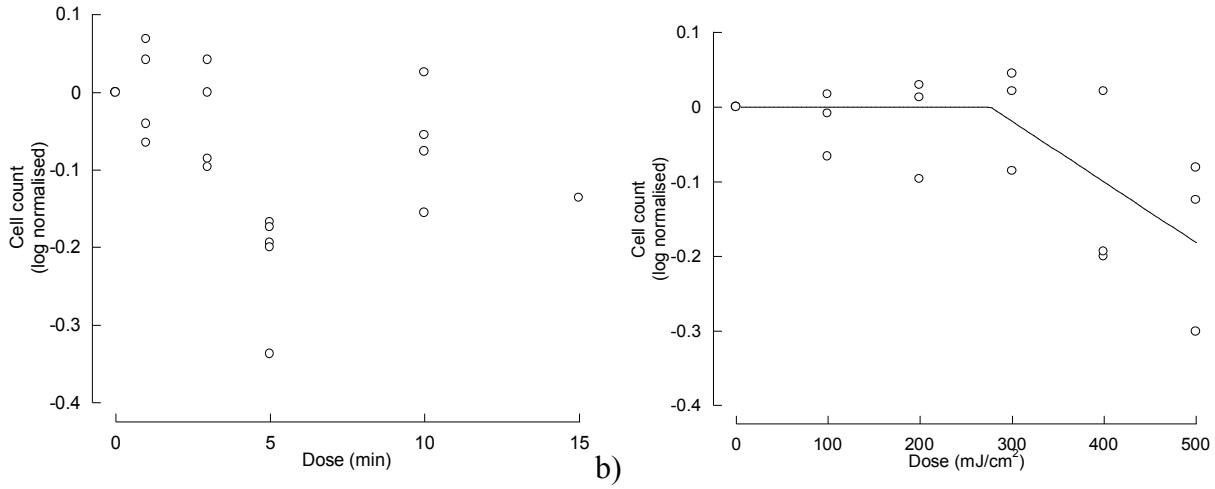


Figure 5 Relative vitality of *T. weissflogii* as flow cytometric cell counts.

Table 5 Relative flow cytometric cell count fit parameters.

Fit Parameter	UV-C Treated	
	Value	Error
M2	1231.5	455.54
M3	276.87	54.623
R^2	0.086	-
χ^2	0.456	-

There is a very broad shoulder in the UV-C treated samples, with no effect of UV-C at doses below c. 300 mJ/cm^2 and 0.2-log reduction between 300 and 500 mJ/cm^2 (Figure 5).

4.2.5 Vital Staining Assay

The results from cell staining and analysis with flow cytometry are displayed in Figure 6 below.

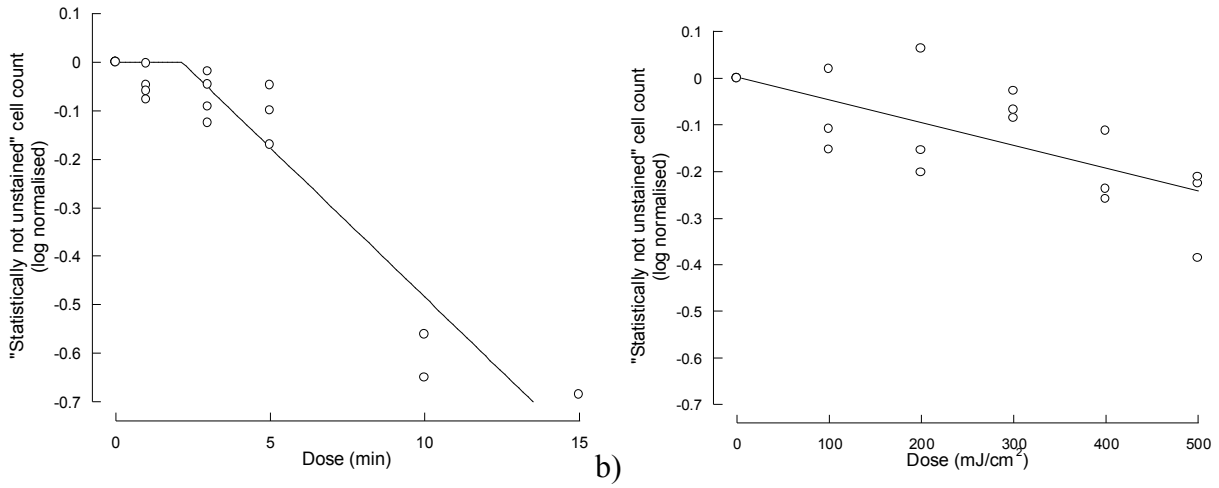


Figure 6 Relative vitality of *T. weissflogii* as "statistically not unstained" cell counts (FDA/CMFDA staining).

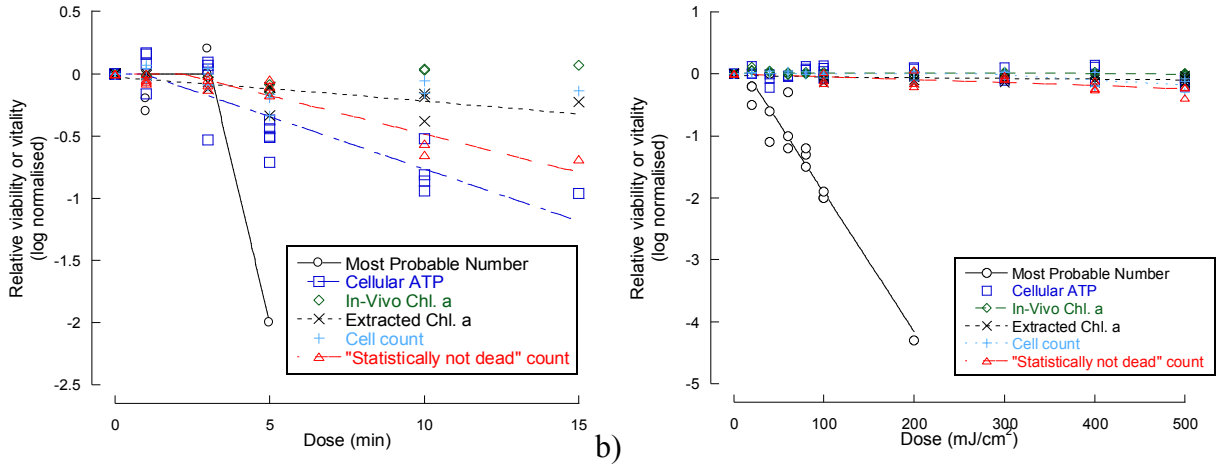
Cells treated with heat demonstrated some shouldering before about a 0.5 log reduction relative cell counts after 10 minutes of treatment. UV-C treated cells did not show a shouldering trend, but had a log-linear decline of 0.2 log decrease at 500 mJ/cm^2 (Figure 6). UV-C treated samples had an R^2 of 0.515. The heat treated samples are quite strongly correlated to the shouldered Chick-Watson model, with an R^2 of 0.901, (Table 6).

Table 6 Relative "statistically not unstained" cell count fit parameters.

Fit Parameter	UV-C Treated	
	Value	Error
M2	-0.0617	0.00601
M3	2.15	0.548
R^2	0.0877	-
χ^2	0.901	-

4.3 Comparison of Viability and Vitality Monitoring

A direct comparison between the monitoring methods used in this thesis is displayed in Figure 7 below. A remarkable difference between the response in viability and vitality assessment is apparent when plotted on the same axis.



a) b)

Figure 7 Direct comparison of relative viability and vitality assessments of *T. weissflogii*.

For the heat-treated cells, there starts to be some ability to determine heat damage in some of the vitality tests; ATP being the most sensitive, followed by the cell staining with flow cytometry. However, even these two most sensitive vitality tests only showed at most a 1-log decrease in relative signal, even after treatment had rendered all cells in the culture non-viable. UV-C treatment did not affect any of the vitality tests, although there was a 4-log reduction in viability.

CHAPTER 5 DISCUSSION

5.1 VIABILITY

Viability was successfully reduced through application of heat and UV-C radiation, methods often used in ballast water treatment. Treatment performed on *T. weissflogii* cultures with a starting concentration of 1.2 x 10^4 viable cells/mL. Treatments above threshold doses (see below), reduced cell concentrations by less than an order of magnitude but reduced viable cell concentrations by as much as 4 orders of magnitude. Further reductions could have been detected with confidence. At the assay volumes and level of replication used, the lower limit of detection for the growth assay is 0.036 cells/mL, a score of one positive tube from 5 x 5-mL volumes of undiluted culture, with no growth in the serially diluted tubes. The MPN assay is therefore sensitive to a 5.4-5.7 order-of-magnitude reduction.

5.1.1 Heat Treatment

Heat treatment was demonstrated to be effective at reducing cell viability. Samples treated for 10 minutes and longer showed no detectable viability in the MPN assay, nor did two out of the three samples treated for 5 minutes. The fit to data above the limit of detection of the method (Figure 1) suggests that an exposure of 6 minutes or longer is required to reduce viability beyond the 3-log reduction required for the Type Approval of a ballast water treatment system.

These results are supported by similar studies using high temperature treatment for phytoplankton inactivation. Treatment of at least 50°C for at least 10 minutes has been demonstrated to be successful at inactivating phytoplankton cells (Balaji and Yaakob, 2012; Durand *et al*., 2011).

It has also been demonstrated in other studies that it is possible to inactivate phytoplankton using temperatures lower than 50°C, but the lower temperature is applied for a longer exposure time for similar treatment effects. Treatment at 40°C on algal culture was effective after 4 hours of exposure (Jiménez *et al*., 2009). At 35°C, treatment is effective when phytoplankton cultures are exposed for at least 24 hours (Rigby *et al*., 1999). These results from other studies demonstrate that there is the possibility for energy savings if using heat as a treatment method, due to the successful inactivation of phytoplankton at lower temperatures, but for a longer time. Considering that most voyages between ports take longer than a few hours, using a lower temperature is a possibility for the inactivation of phytoplankton in ballast water with a reduced cost of heating.

Treatment by exposure to heat is effective at inactivating phytoplankton cells in the laboratory on a small scale, and full-scale ballast water heating methods have been demonstrated in the field. The major drawback that prevents its widespread use is the energy required to heat the ballast water, so most vessels use other methods. As previously mentioned the popular technology for ballast water treatment in the industry is ultraviolet radiation as a disinfection method, usually coupled with some form of pre-filtration (Dobroski *et al*., 2009).

5.1.2 Ultraviolet Treatment

UV-C radiation was demonstrated to be effective as an inactivation treatment in bench-scale lab work on healthy cultured phytoplankton. The dose required for a 3-log reduction in viable cell concentration of *T. weissflogii* was 148 mJ/cm^2 of UV-C radiation using a medium-pressure mercury bulb (Figure 1). Mixed samples treated with UV-C radiation of doses above 200 mJ/cm^2 were inactivated without potential for regrowth (Cullen and MacIntyre, 2016). Natural ballast

water samples that were treated with UV-C had some *Thalassiosira sp.* cells survive treatment unscathed (Liebich *et al*., 2012), providing further evidence that this strain of diatom may be a good pick as a challenge organism for ballast water treatment studies.

Some of the commercially available ballast water treatment systems employ a hydrocyclone filtration step prior to UV-C radiation, which helps reduce the shading effect from larger particles ensuring better exposure of the plankton in the water (Pećarević *et al*., 2018). In Vancouver, Canada a team worked with a hydrocyclone tangential filter + UV-C treatment system (Sutherland *et al*., 2001). The use of a combined treatment system helps to ensure a proper dose is applied to all of the plankton in the ballast water that are unable to be easily filtered out first, potentially reducing the total energy needed to treat the same volume of water.

5.2 VITALITY

The dose-response curves for heat and UV-C treatment were elucidated using the direct method of a growth assay (Figure 1). While growth assays are a direct method for investigating viability, they can take days to weeks to run. In contrast, the biochemical and physiological techniques offer rapid results, on the order of minutes to hours. Chlorophyll fluorescence, ATP concentration and vital stains were used as the indirect proxy methods in this study. These rapid methods measure some aspect of viability as an indicator of living cells. A discussion of the results from the vitality-based monitoring methods is presented below.

5.2.1 Cellular ATP Concentration

There was some reduction in cellular ATP concentration with increasing heat treatment, but it was not possible to distinguish between UV-C damaged and undamaged cultures. Heat-treated samples demonstrated a reduction after extremely lethal heat treatment. This is a small reduction

in response signal when compared to the 2-log reduction in viability at moderate doses of heat and UV-C.

ATP has been demonstrated to be an excellent characteristic to detect the presence of living cells, but it is not suitable for detecting non-lethal damage immediately post-treatment. In support of this idea, other researchers have not been able distinguish damaged phytoplankton from untreated when UV treatment was used (Bradie *et al*., 2018; First and Drake, 2014). A study using a combined UV +TiO_2 treatment system on natural sea water found that ATP was significantly reduced, suggesting that at high doses of UV-C radiation with a catalyst, advanced oxidation was occurring and extremely effective at reducing cell vitality immediately after treatment (Hyun *et al*., 2018).

Another model organism was demonstrated to be able to survive, and sometimes reproduce after disinfection treatment with UV-C. *E. coli* cultures treated with UV-C retained some ability to synthesize ATP, and if incubated in favorable conditions could proliferate (Rauch *et al*., 2019). The favourable conditions that may be found in a harbour where ballast water is discharged could be suitable for treated organisms to grow and pose a threat. Taking the possibility of regrowth into consideration is important for the effective management of ballast water.

Studies correlating ATP to cell concentration demonstrated that diatoms had lower ATP content than other phytoplankton, with per-cell concentration ranging over two orders of magnitude (Hyun *et al*., 2018). This variation in ATP concentration among many kinds of algae suggests that wide safety margins should be employed if using ATP as a proxy for biomass, especially if it's the only aspect of ballast water quality that is being measured. However, modern test kits are capable of sensitive detection of minute concentrations (Hammes *et al*., 2010). With all this

considered, ATP is recommended as an accessory for characterizing ballast water but should not be relied on as the only metric for analysis.

5.2.2 In-Vivo fluorescence

In general in-vivo chlorophyll a fluorescence was not a strong indicator of treatment by heat exposure, although there was a minor linear decrease with UV-C exposure (Figure 3). The signal comes from an excess of light energy, so a functional chloroplast offers a lower fluorescent signal compared to a damaged one (Maxwell and Johnson, 2000). In-vivo fluorescence is best suited for non-destructive measurement of cultures that are known to be in balanced growth, rather than a tool for determining damage to phytoplankton after exposure to wastewater treatment technologies.

Other researchers found similar results as those found in this thesis for measuring in-vivo chlorophyll a after UV-C treatment. Doses of up to 200 mJ/cm^2 on mixed algal culture were demonstrated to have no effect on in-vivo fluorescence (First and Drake, 2014). UV-C treatment doses of 750 mJ/cm^2 of on *P. globosa* culture were inactivated photosynthetically, but were able to be recultured in recovery experiments (Martínez *et al*., 2013).

Experiments using green algae cultures revealed that a UV-C dose of 3200 mJ/cm^2 completely destroyed photosynthetic activity of *Chlorella autotrophica* (Martínez *et al*., 2012), indicating that it takes a very large dose of energy to create advanced oxidation products which eventually damage the cell enough to interfere with photosynthetic yield. A dose of 3.2 J/cm^2 is slightly over 30x the dose required for a 2-log reduction in *Thalassiosira weissflogii* viability (Table 1). While this comparison is not completely fair because it is a green alga vs. a diatom, this offers a good example of how overtreatment may be required by UV systems to meet vitality test limits.

5.2.3 Extracted Chlorophyll Fluorescence

Extracted chlorophyll a fluorescence had a strong response to heat treatment, which demonstrates the denaturation and degradation of the chloroplast proteins as the cell succumbs to the damage. This relationship is not as strong when it comes to UV-C treatment (Figure 4). Heat treatment for longer than 5 minutes resulted in the degradation of chlorophyll pigments. In this set of experiments, chlorophyll a concentration did not show a significant response to either treatment on *T. weissflogii* cells, which makes it unsuitable for the determination of heat and treatment effectiveness.

Chlorophyll a is a useful proxy for abundance when cells are known to be growing and healthy but is a poor indicator of mortality. Chlorophyll a molecules can remain intact in cells that have been dead for weeks (Wright, 2012). In seawater samples, phytoplankton treated with low doses of UV-C (25, 40, and 60 mJ/cm^2) did not cause significant decreases in chlorophyll a concentration (Waite *et al*., 2003).

5.2.4 Flow Cytometry

The speed of sample measurement, and amount of data that can be captured in that short time makes flow cytometry a valuable tool for the ballast water industry. Flow cytometry offers very fast results for cell counts but has no ability to distinguish between viable and non-viable cells. This is not completely surprising as the threshold for rendering a cell non-viable is surpassed before physical cell damage is obvious, for both heat and UV-C treatment.

Cells determined to be "statistically not unstained" are considered to be alive because they have an intact membrane that is capable of accumulating fluorescein and functional enzymes to cleave the molecule (MacIntyre and Cullen, 2016).

The vital staining does show a small response to the level of physical damage once treatment levels reach extreme levels. For the UV-C treated samples, it is presumed that somewhere beyond 300 mJ/cm^2 the energy applied is so intense that advanced oxidation is occurring, in addition to the DNA damage. This is because of the response in the vital stain testing around this level of treatment, whereas the cultures were demonstrating a 2-3 log reduction in viable cell number at energy levels below this threshold. This threshold seems to be echoed in the other vitality testing. The vital staining correlating to advanced oxidation damage makes sense, as the stain works by accumulating inside of the cell membranes to the point where they become intensely fluorescent compared to the background signal. The stain would be unable to accumulate inside of a cell that had been damaged by advanced oxidation, but before this threshold the cell integrity would be unaffected even though the DNA had sustained enough damage for many of the individuals in the culture to fail to reproduce.

The heat-treated cells demonstrated a larger response in the staining assay than the UV-C treated samples. The lack of response in cell staining at low doses of UV-C was found in study that used a range of 0-100 mJ/cm^2 and demonstrated no change in living cell concentration, even after 5 days of incubation post-treatment (First *et al*., 2016). This study's conclusions for the 10-50 µm fraction agree with what was found in this thesis, as a dose of 100 mJ/cm^2 was found to be insufficient for the inactivation of phytoplankton cells. Another team testing an onboard UV-C treatment system found that vital staining was unable to demonstrate a treatment effect (Bradie *et al*., 2018).

What is left of cells after being treated with heat or UV-C can be anything from whole intact, viable cells down to disintegrated fragments of destroyed cells, as well as on a secondary level with individual cell deaths continuing up to mass die-offs (Berges and Choi, 2014; Moharikar *et al*., 2006). Interestingly UV-A, -B or -C at low doses (70mJ/cm^2) caused cell swelling and organelle membrane disruption in what is described as, so this physical indicator of minor exposure to UV-C might be detectable by a sensitive instrument that includes imaging.

It should be noted that not all phytoplankton stain equally and that even though this organism was chosen for it's demonstrated ability to stain there are many species for which this technique is unable to differentiate between live and dead (MacIntyre *et al*., 2017). *T. weissflogii* was chosen because of previous work demonstrating that the FDA stain works, but this does not truly represent all diatoms, let alone phytoplankton as a group.

Another detail that should be considered when using flow cytometry to monitor biomass in ballast water is the length of time to sample as well as the buoyancy of the plankton in the sample. Some cells in a sample may float or sink out of reach of the sample inlet and not be measured. In this thesis each sample was analysed by flow cytometry three times and the sample tubes were inverted between each run to attempt to minimise loss from cells sinking. If cells are floating or falling out of the sample before they can be pumped through the instrument, they will be excluded from analysis, potentially creating a false negative.

Researchers working with mixed populations of algae and flow cytometry have begun to develop protocol for differentiating between cell types and classes, but the different populations are not always easily differentiated in analysis (Peniuk *et al*., 2016). The addition of vital stains allows researchers to discrimination between vital and non-vital populations post treatment which lends

to more reliable and trustworthy flow cytometry analysis (Brussaard *et al*., 2001; Veldhuis and Kraay, 2000). The use of Fluorescence In-Situ Hybridization in combination with flow cytometry for the targeting of phytoplankton on a more specific taxonomic level could be possible in combination with a well designed vital staining protocol (Medlin and Orozco, 2017). Molecular biology has many new tools that can be paired with flow cytometry to help identify problematic species using DNA barcoding (Stehouwer *et al*., 2013).

5.3 Model Outcomes to Aid in Understanding of Disinfection Processes

The (modified) Chick-Watson model of applying a kinetic model to disinfection of microbes is a commonly used practice in sanitation and water treatment (Cho and Yoon, 2008; Cho *et al*., 2004; Lambert, 2003). This model demonstrates that as cultures are exposed to disinfection treatment, there is a log-linear response in the survivorship (Chick, 1908; Watson, 1908). Many of the figures demonstrating the effects of heat and UV-C treatment on *T. weissflogii* cells have a shoulder in the fit, indicative of doses that do not affect viability or vitality. This shouldering has been investigated in the literature (Lambert, 2003; Marugán *et al*., 2008). Above this threshold dose, which is different for each treatment (see Section 4.2), the data conform to a log-linear (Chick-Watson) model. Treatment of *T. weissflogii* culture below the threshold dose (see Figure 1) may result in the release of propagules.

There seems to be a trend between some of the viability and vitality testing with an anomalous data point at 5 minutes of heat treatment. The ATP, chlorophyll, and flow cytometry cell counts all have a low data point for this treatment relative to the other doses. This may be due to the low number of replicates (triplicate) used in this study, as the general trend was being investigated. Further studies investigating treatment between 5 and 10 minutes would help determine if there are any trends that a finer range of temperatures would reveal.

5.4 Energy Considerations and Other Treatment Challenges

Appendix B outlines some of the additional work done to quantify the amount of energy applied for each heat treatment dose. Even though the calculations in this side experiment are most likely underestimates, the amount of energy required for an equivalent log-reduction in *T. weissflogii* cells by heating is an order of magnitude more than if you treated by UV-C. Water is difficult to heat because of the specific heat capacity, and it takes a long time as well. These two factors alone make it a poor choice for ballast water treatment when considering the volumes that would be required to be treated.

Waste heat could be used from the engine, but the system would have to be constantly maintained as the increased heat would also add to increased corrosion rates. Most of the components in a heat exchanger are made of steel or other metal that are vulnerable to corrosion by sea water. The best way to exchange heat is through a large surface area, which is problematic for this type of water. Corrosion can become a big issue and may change flow rates as surfaces are slowly dissolved, resulting in potentially reduced treatment of any plankton present.

One of the main weaknesses of UV-C treatment is radiation being the source of activity, which is limited to the effects of photons hitting particles in the water. This is greatly affected by particle shading and the transmittance of the water. Most treatment systems use some form of mercury bulb, under varying pressures, to produce the radiation. These bulbs have service lives that are not limitless, so monitoring energy output and replacing bulbs as necessary is very important to ensure an effective treatment system. This adds operational costs, maintenance and inspection tasks, in addition to the initial equipment purchasing. Regardless of these drawbacks, UV-C treatment is a promising because of its lower energetic costs.

5.5 Implications for Environmental Regulations

In the laboratory we have the luxury of a reference culture as a control to compare the response after treatment. In the field, any compliance testing would only have the treated sample as a data point (Wright, 2012). There is usually little to no information about the initial water quality. ATP test kits are able to provide quick results for a general idea of bio load in the ballast water prior to treatment, alerting operators to increase the treatment dose if there is an unusually high level of plankton present in the water.

Most research involves microscopy to identify phytoplankton, but a group using genomic data found an enormous amount of diversity in oceanic plankton globally, many of which would not be included in the 10-50 µm fraction including the majority of the photosynthetic plankton (De Vargas *et al*., 2015). As samples are fractionated to meet the designations in the regulatory guidelines, they are usually passed through a 50-µm mesh to trap any zooplankton or larger organisms, and then a 10-µm mesh to trap phytoplankton. The remaining sample is the smallest portion which includes anything <10 µm in diameter or is often inaccurately referred to as the "bacterial" fraction; data on the phytoplankton size spectra (Maraóón et al., 2007) indicate that <99% of phytoplankton in both oligotrophic and mesotrophic waters are <3 µm in diameter. As this smallest fraction is assumed to be mostly bacteria after filtration, samples are often enumerated using plate counts and most of the picophytoplankton are not culturable on agar (which are typically incubated in the dark) when so we have been missing them from our accounting of ocean diversity.

5.6 Aquatic Invasive Species Prevention

These data points are for one species of diatom, with a limited range of treatment methods and assessment technologies. While *T. weissflogii* can be found in association with problematic

phytoplankton in bloom communities, it is not necessarily is a threatening species on its own. While using a model organism can be helpful to elucidate the specific effects of the different methods for monitoring algal vitality and viability in treated water, the need to integrate this information into a broader context is important. A future meta-review of ATP concentrations of diatoms, or effectiveness of UV-C treatment on marine phytoplankton, as examples may include the data presented in this thesis in order to develop a better understanding of these phenomena. This effort has been started for other organisms found in ballast water, but there is a lack of phytoplankton data (Malayeri *et al*., 2016). Work with mixed cultures is important as there is probably a large community effect. There may be interactions between different species of phytoplankton that may stimulate each other in a cascade effect as one example of how a bloom may form suddenly. Unfortunately, working with mixed cultures tends to be more complicated than single cultured species, so tools such as flow cytometry paired with fluorescent DNA tags would be helpful to tease apart the effects of treatment on each individual cell type (Medlin and Orozco, 2017).

CHAPTER 6 CONCLUSION

This study was conducted to compare vitality and viability using four assay methods (growth assay, ATP concentration, chlorophyll fluorescence, and vital staining) after disinfection treatment. Dose-response curves were developed for *T. weissflogii* culture exposed to heat and UV-C as disinfection treatments. These treatments are used to inactivate microbes present in ballast water being transported between ports globally. Inactivated microbes are unable to present a threat to aquatic ecosystems due to their inability to reproduce post-treatment.

6.1 CONCLUSION

Heat and UV-C treatment were found to be very effective at inactivating *T. weissflogii* cells. Treatment was effective at reducing cultures by over 3-log after at least 6 minutes at 50°C or 148 mJ/cm^2 of UV-C radiation. There were significant differences found between the true viability of *T. weissflogii* cells and the estimates provided by the vitality-based tests used in this thesis after treatment with heat and UV-C.

Overall none of the vitality tests offered a comparable response to actual cell damage. The most responsive of the indicative tests to vitality was ATP extraction, followed by vital staining and flow cytometry, but both over-estimated viability by orders of magnitude at treatment levels required for regulatory testing (a 2-log reduction in viability). The response was very small in comparison to viable cell concentration reduction. These proxies for cell concentration were far less sensitive to algal health than the MPN results.

This study demonstrates that UV-C treatment can be used successfully to inactivate *T. weissflogii* but depending on the method chosen for investigating treatment effectiveness, it may show up as a false positive. This is a potential problem especially for the UV-C-treatment industry as it

forces manufacturers to set the systems to overtreat the ballast water. The work presented in this thesis demonstrates that treatment with UV-C radiation is effective for reducing viable *T. weissflogii* culture concentration by over 4-log.

It is important to remember that ballast water contains a wide range of planktonic organisms, and treatment may affect each fraction differently. Treatment with heat is expected to be effective on all planktonic cells found in ballast water, including bacteria and zooplankton. UV-C is also expected to be effective due to the universal nature of DNA and the mechanism of action with this treatment technology. Doses may be different, so treatment should be tailored for the most challenging organism to inactivate that is present in the ballast water.

6.2 Recommendations

Quick results from indicative test kits that can inform an optimised treatment programme or reflect the true viability of phytoplankton present in ballast water after treatment is still something that needs to be developed. For now, equipment manufacturers would be advised to use the SDC-MPN method to assess the true viability of test culture after treatment to develop robust systems, and pair the equipment regulatory approval with other viability or vitality tests to ensure onboard performance and compliance.

There remains a large gap in the knowledge of phytoplankton regarding thermal and UV-C death curves, and the application of this data to ballast water treatment industry. Studying mixed cultures is difficult; however, experimenting with a large number of different strains of phytoplankton in monoculture may allow us to understand how best to treat our wastewater to reduce the risk of AIS problems in our ports, and help identify any especially challenging organisms that may resist treatment.

New technologies, such as microfluidics, may be able to overcome some of the disadvantages currently experienced with growth assays. A miniaturized version of the SDC-MPN assay in a "lab on a chip" format that could detect growth of small cell numbers to a very accurate degree, allowing for viability assessment but in a much shorter time and with fewer resources.

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APPENDIX A
ENERGETICS OF BENCH SCALE HEAT AND UV-C TREATMENT

When it comes to treatment of ballast water, two commonly-discussed non-chemical methods are ultraviolet radiation and heating (Tsolaki and Diamadopoulos, 2010). These methods were applied to a marine diatom in culture in the main work of this thesis. To compare the two methods directly, the amount of energy used for each dose was calculated and discussed in this appendix for discussion.

As an inactivation method for ballast water, heat has a large disadvantage in the specific heat capacity of water is high. The value used for the isobaric mass heat capacity for water at 25° is 4.181 kJ/kg (Engineering ToolBox, 2004). Meaning for every kilogram of water, over 4 kJ of energy must be absorbed to increase the temperature of the water by 1 K. This capacity to absorb energy with relatively small changes in temperature means that it takes a large amount of energy to heat water from environmental to treatment temperatures, which in the context of ballast water means a lot of additional cost.

UV-C treatment also involves an energy cost. Energy is needed to run the lamps, whether they are mercury lamps or lower-energy light emitting diode arrays. UV-C treatment targets the nucleic acids inside living cells, compared to heat treatment, which affects all the cellular components (Davey, 2011; Drost-Hansen, 1969; Sinha and Häder, 2002). A review of available treatment systems lists the power usage for many UV-based systems and they range from about 25 to 100 kW to treat 500m^3/h of ballast water (Lloyd's Register Group, 2017).

Heat Treatment

Using water as a proxy for cell culture, the temperature of water inside of sealed 50 mL glass test tubes was measured using a probe thermometer. Temperature was tracked from room- to

treatment-temperatures between 38 and 50°C. The thermometer logged the temperature every minute. The results are displayed below (Figure 8).

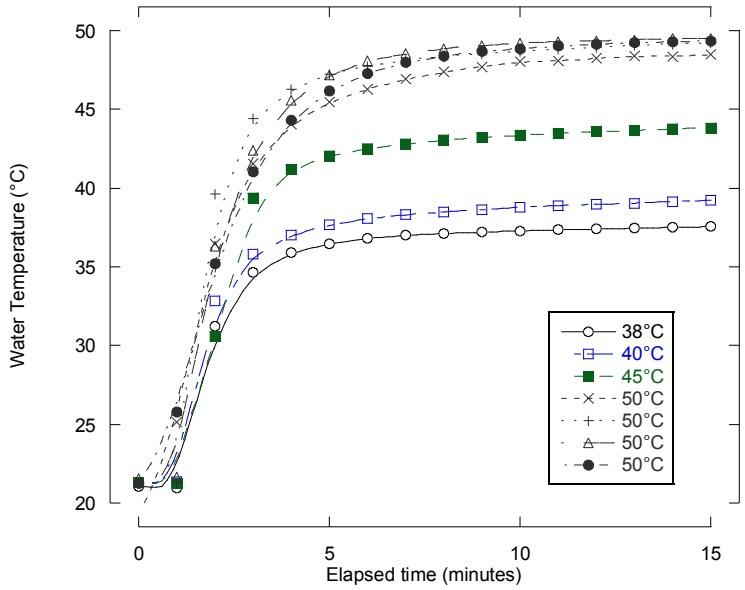

Figure 8 Heating curves for treatment temperatures of 38 to 50°C.

The samples started at room temperature (20°C) and it was assumed that 50 mL of water was equal to 0.05 kg. It was assumed that there was no energy lost to the surroundings. Additionally, the energy input was calculated using distilled water, whereas the samples were grown in coastal saltwater at with an average salinity of 30 (dimensionless). The specific heat capacity of water at this salinity is 4.007 kJ kg^{-1} K^{-1}. With this assumption the temperature of the culture would be about 4% warmer than the fresh water at the same time in the bath, up to the treatment temperature where the thermostat would turn the heater off. Due to these assumption actual energy input values are underestimates, as heat is lost to the surroundings but is unaccounted for, and the actual energy input is slightly higher with the salt present (Engineering ToolBox, 2003). Using these measurements, assumptions about treatment energetics of diatoms in liquid culture were made. Temperature increase was determined based on the heating curves measured for each

water bath setting and converted to an energy input for a direct comparison to other treatment methods used in this thesis.

50°C was used as lethal heat treatment of algal culture in this thesis. For this reason, the temperature measurement was replicated to develop a more precise model. The temperature of 50 mL water in glass culture tube submerged in 50°C water bath, mean temperature, n=4, error bars represent standard deviation, and sigmoidal line of best fit (Figure 9).

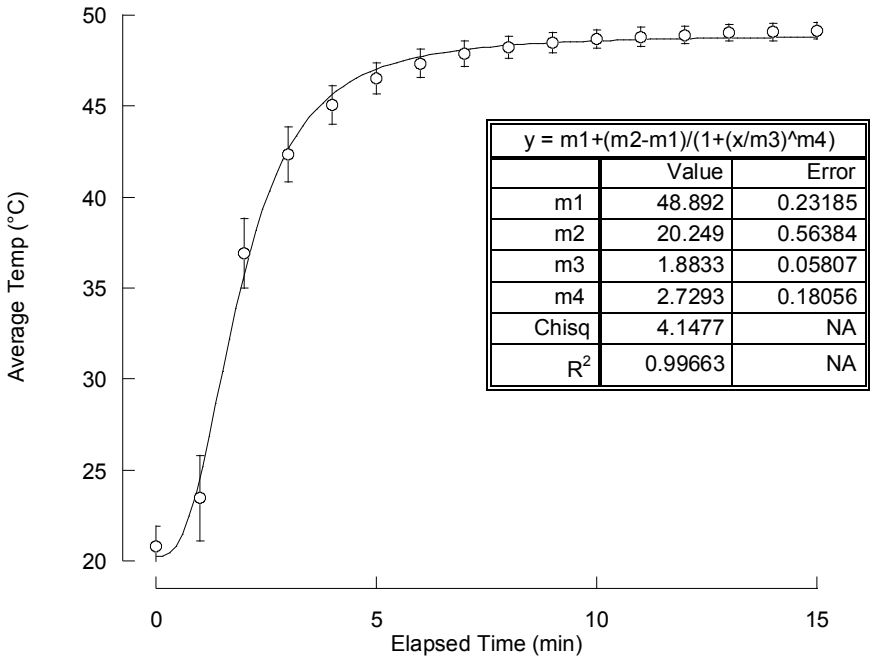

Figure 9 Temperature of water in culture tube in 50°C bath.

A range of temperature exposure times were used to stress the diatom cells. To compare the heat treatments of different temperatures and times, the amount of energy added to each sample was calculated. A model system using water as a proxy for phytoplankton culture was used to measure the change in temperature inside of the culture tubes, as measured using a probe thermometer recording data every minute. The data was used to fit sigmoidal curves to determine the amount of energy added for each treatment.

The model was applied to each dataset for the treatment temperatures that the water bath was set to in the various experiments. The model was used calculate the temperature that each tube would have been at for each dose used in this thesis. The equation used for this is

$$\text{Sample temperature (°C)} = y = m1+(m2-m1)/(1+(x/m3)^{m4})$$

where variables used depend on the treatment temperature as outlined in Table 1 below.

Table 7 The variable values for the model for experimental water bath temperatures

Temp. (°C)	Variable M1	M2	M3	M4
38	37.3	20.3	1.60	3.66
40	38.9	20.5	1.77	3.54
45	43.6	20.7	2.15	3.82
50	48.9	20.2	1.89	2.73

The formula used for the calculation of the energy input for each temperature change is:

Input energy (kJ) = mass of water (kg) · specific heat capacity (kJ/K/kg) · Δ temperature (K)

This formula was used to determine the energy input for each sample according to the exposure times used in this thesis as outlined below (Table 8).

Table 8 Calculated energy input for doses at 50°C.

Time (min)	Energy (J)
1	891.6
3	4664.1
5	5586.6
7	5814.4
10	5914.1
15	5955.6

At the maximum dose used in these series of experiments, the most energy that was applied to one 50mL sample was slightly less than 6 kJ.

Ultraviolet Treatment

A low-pressure mercury-bulb collimated-beam UV-C system was used to irradiate *T. weissflogii* culture. The calibrated fluence of the lamp was determined via radiometer alongside the UV_{254} transmittance of the culture, and these parameters were used to determine the exposure time required for a given dose. Exposure times were computed using the petri factor and the UV_{254} transmittance of the culture. Each dose was calculated to determine the energy input for the treatment. The illuminated area of the petri dish used was 23.8 cm^2. Thus,

$$\text{Input energy} = \text{Dose}(mJ/cm^2) \cdot (23.8\ cm^2)$$

and this formula was used to generate the table below based on the doses used in this thesis (Table 9). Each treatment time varied slightly based on the lamp intensity, but the times were only ever a few seconds longer or shorter than the time displayed in the table below.

Table 9 Energy input for UV-C doses on 50mL of *T. weissflogii* culture

Dose (mJ/cm^2)	Treatment Time (min)	Treatment energy (J)
20	1	0.476
40	2	0.952
60	3	1.428
80	4	1.904
100	5	2.38
200	10	4.76
300	15	7.14
400	20	9.52
500	25	11.9

The maximum dose applied to the UV-C treated samples was about 12 J.

Conclusion

Based on these calculations it is easy to recommend UV-C treatment for inactivation if energy costs are a concern. The specific heat capacity of water requires huge energy inputs to reach lethal temperatures, whereas the UV-C treatments use several orders of magnitude less energy to

inactivate the phytoplankton cells. Unless ballast water is heated to treatment temperatures using waste heat from the engines, it is too expensive to recommend heat treatment as an inactivation step before discharging ballast water

Energy input to reach treatment conditions for two methods of wastewater treatment was considered. Heating water from room temperature to a treatment temperature of 50°C requires 63 kJ. The highest dose used in this experiment was 500 mJ/cm^2 which used 11.9 J of energy. The lowest dose used in the heat treatment was 892 J, which is an order of magnitude larger than a UV-C dose that results in advanced oxidation, which is far beyond what is required for treatment of biological systems.

Part of the rationale for recommending one system over another must consider the cost of use, and in this case, there is a very big difference between the two methods which compels the recommendation of UV-C treatment as the logical choice. Heating large volumes of water is extremely energy intensive, which at sea means the burning of fuel to produce electricity. This means the cost is immense, so other ballast water treatment methods would be more economical.

APPENDIX B
COMPARISON OF ATP EXTRACTION METHODS

Three different methods for extracting adenosine triphosphate (ATP) from diatomaceous cells in a saltwater culture are examined. Two of the methods tested include commercially available ATP test kits, whereas the third is a lab-made extraction process that was replicated from previous research in the field of ballast water monitoring. Heat treatment was applied to *Thalassiosira weissflogii* culture in balanced growth in f/2 media. After treatment, culture was exposed to 24 hours of dark incubation at 18°C. ATP was extracted using each of the three methods from the samples and measured using a luciferase enzyme to quantify cellular concentration. After testing, the ATP test kit that resulted in the highest recovery was a method that involved a grinding step to fully lyse cells, after comparison to two chemical lysing methods.

Introduction

Adenosine triphosphate is a useful molecule for indicative testing as it is present in all living cells, and it's presence in detectable quantities implies that there is some level of a functional metabolism (van Slooten *et al*., 2015). One application for this investigation is in the assessment of phytoplankton presence in sea water, in instances sūch as ballast water monitoring, with focus on gross noncompliance with ballast water regulations. One of the challenges for this type of assay is the diversity of cell types present in ballast water samples, with a large range in the phytoplankton alone. An example of the challenge this diversity can pose for monitoring: the heterokonts can have silica frustules, and other groups can have other tough materials in their cell walls such as cellulose.

Considering many of the commercially available ATP test kits are intended for the measurement of bacterial ATP, the lysing agents may not be strong enough to extract ATP from some of the

more resistant types of cells present in ballast water samples. Previous research performed on natural samples of phytoplankton collected from a range of water bodies suggested a reduced effectiveness for ATP extraction kits in the saline samples (Kuo, 2015). This inspired the development of an improved extraction method in the presence of salinity (Welschmeyer and Kuo, 2016).

The work done on ATP extraction efficiencies in saline samples inspired the work that started this thesis. An attempt to investigate the extraction efficiency of three different ATP kits is presented in this appendix.

The three extraction methods all involve some chemical extraction, but one method uses an additional grinding step as well. The first method is a commercially available extraction kit developed for drinking water applications. The second method is a modified version of the drinking water test kit, with a lysing agent that was optimised for saline environments and involves a small grinding step to aid in extraction. The third method uses solely chemical extraction but was developed as an optimised method in saline environmental samples.

The "Quench-Gone Aqueous" (QGA) kit from LuminUltra is designed for low-biomass drinking water applications. The kits use a proprietary lysing agent referred to as UltraLyse™ 7. Sample is collected on a proprietary membrane filter treated to reduce quenching of the ATP signal.

The product developed for ballast water applications is the "B-QUA" ATP extraction kit. This method features size fraction separation by filtration and a physical grinding step. In this experiment the protocol was modified to exclude the additional filtration steps, as phytoplankton culture was used in place of natural sea water samples. The samples were collected on a 10 μm

nylon mesh filter before being extracted in a bead tube with a modified lysing agent optimised for marine samples, modified UltraLyse™ 30.

The final method was developed as an optimised extraction method for seawater samples (Kuo, 2015; Welschmeyer and Kuo, 2016). This method uses benzalkonium chloride (BAC) and phosphoric acid diluted in tricine for the lysing agent, hence the nickname "P-BAC".

These extraction methods will be compared on lab grown algal cultures, with each extraction method being performed the same treated culture to develop a direct comparison of how effective the methods are.

Methods

Thalassiosira weissflogii (*T. weissflogii*) was used as a model organism. *T. weissflogii* culture was grown in f/2 seawater media using filtered seawater collected from a coastal location and grown at 18°C and 80μm·photons·cm^{-2} s^{-1} in semi-continuous culture to maintain balanced growth. The condition for balanced growth, as defined by MacIntyre and Cullen (2005), is met when culture growth rates are steady over a period of 10 generations to within a 10% variability. Experimental stock cultures were monitored on a frequent basis using in-vivo fluorescence as a proxy for biomass.

Samples were treated with heat by submerging parafilmed screw-top glass culture tubes in a 50°C (±5) water bath. Each tube held 50 mL of culture, which was treated and then wrapped in aluminum foil to exclude light and incubated in the same incubator as the cultures were grown in. After 24 hours treated culture was subjected to ATP testing.

Modifications and specific methods for each of the extraction methods are described in the following paragraphs. For the most part, methods were adhered to as strictly as possible, but

fractionation was skipped because lab grown cultures were used instead of natural samples requiring sorting.

Quench-gone Aqueous (QGA) Method was applied according to the test kit directions. 10 mL of culture was syringe filtered through the filters provided in the kit. The cells retained on the filter were lysed with 1 mL of UltraLyse™ into a 9mL UltraLute™ dilution tube.

The "B-QUA" test kit method (LuminUltra, Fredericton, NB, Canada[1]) was modified by excluding some of the filtration steps, thus ignoring the fractions other than the 10-50µm size-class extraction instructions. These steps were skipped as culture of *T. weissflogii* culture was used in these experiments, instead of a natural seawater sample. Filters were added to the bead extraction tube (Product code: AT010247, aqua-tools, France) along with 1 mL of UltraLute™.

The P-BAC method was followed as closely as possible. 10 mL of sample was passed through a syringe filter with a 10-µm nylon filter to capture the treated cells. The changes to the protocol were the exclusion of additional filtration steps to create size fractions of the sample, with the larger and smaller filtration steps omitted due to the use of cultured phytoplankton samples instead of seawater samples with a mixed population.

An overview of these methods is displayed below (Table 10)

[1] During the experiments the B-QUA kit was sold by aqua-tools, France (Product code AT010406), now is offered by LuminUltra, Fredericton NB Canada

Table 10 Comparison of ATP extraction methods

ATP Extraction Method Step	QGA	B-QUA	P-BAC
Filtration	Quench-Gone luer-lock cartridge (0.45µm), syringe filtered	10µm PTFE 47mm diam. Membrane, vacuum filtered	10µm nylon mesh filter 25mm diam., syringe filtered
Lysing agent	UltraLyse™7 (UL7-125mL)	Modified UltraLyse™30	P-BAC solution [5% phosphoric acid (w/w) and 1% BAC (w/w) in 25mM tricine buffer (pH set to 7.8)]
Lysing action	1mL UltraLyse™7 pushed through membrane directly into 15 mL falcon tube with UltraLute™	Filter ground in an AT*PREP*™ bead tube with 1mL UltraLute™ (as 'resuspension' vol.) and 5mL UltraLyse™ 30 according to recommended methods provided with the test kit	Filter transferred to bottom of vial and wet soaked in 1mL P-BAC solution for 20 minutes at room temperature
Dilution	Filtrate added directly from syringe filter, into 9mL UltraLute™ tube	100µL sample from bead tube transferred to 5mL UltraLute tube	100µL sample from wet soak vial to 9900µL tricine buffer

After extraction 100µL of diluted sample + 100µL of Luminase enzyme (Lu-3mL-FD, LuminUltra, Fredericton, NB, Canada) were pipetted into a disposable plastic cuvette and measured in a Kikkoman Lumitester C-100 (HACH, USA).

Data Analysis

Sample relative light units (RLU) were recorded along with a standard assay known as UltraCheck™ 1; this value is recorded as ATP1 to account for the enzyme activity (ULu-9mL-50R, LuminUltra, Fredericton, NB, Canada).

Cellular ATP concentration (cATP) was calculated using the following equation:

$$cATP\ \left(pg\frac{ATP}{mL}\right) = \frac{RLU_{cATP}}{RLU_{ATP1}} \times \frac{Dilution\ Factor\ (pg\ ATP)}{V_{Sample}(mL)}$$

Where RLU is the relative light units measured from the sample divided by a calibration standard and adjusted for dilution. The dilution factor is 10,000 for the QGA test kit, 306,000 for the B-QUA test kit, and 100,000 for the P-BAC method.

Results and Discussion

Heat treatment is effective for reducing ATP concentration in *T. weissflogii*) cells at 50°C as demonstrated by the data displayed in figure 1 below. Healthy *T. weissflogii* culture was reduced in cell concentration by 3-log after 6 minutes of exposure (Table 3).

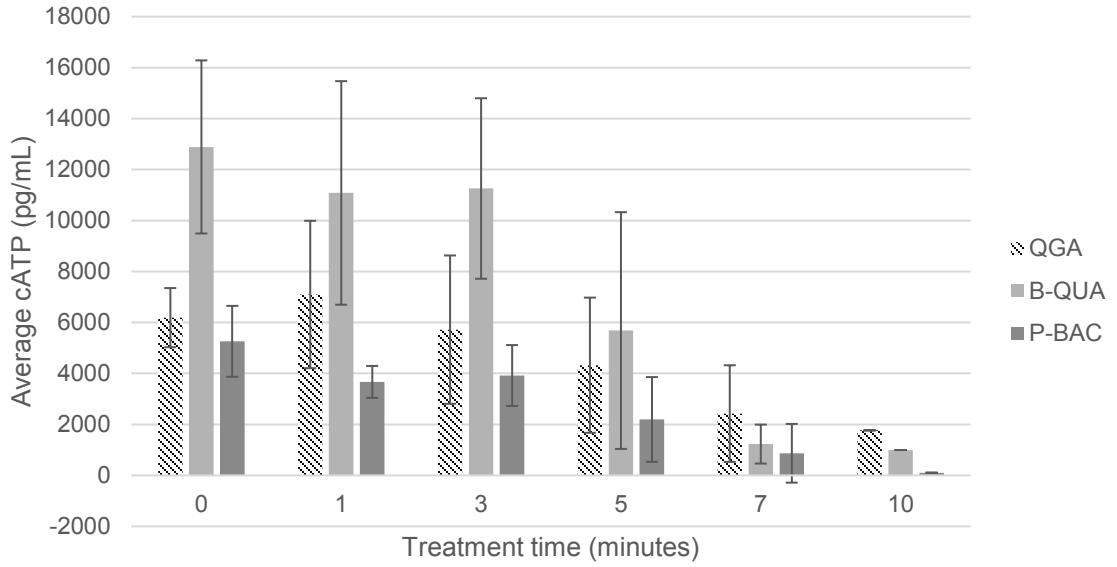

Figure 10 Extracted cellular ATP concentration.

For each treatment time, each of the three ATP extraction methods were used on the same treated culture. The modified B-qua method offered the highest extraction of cATP when compared to the QGA and P-BAC test kits. A dose of 7 minutes results in a 60% reduction in ATP signal, and after 10 minutes a 71% reduction of ATP. Considering a recommended ATP level for

dischargeable ballast water of <1000 pg/mL, a treatment of at least 10 minutes exposure to 50°C would be recommended for inactivating this type of phytoplankton (Lumin Ultra (previously aqua-tools, France), 2016).

The differences between each extraction method do tend to differ from each other. The paired ATP concentration results based on the aquatools B-qua and P-BAC extraction methods with the LuminUltra QGA test as a benchmark are displayed in Figure 11. The QGA test kit from LuminUltra was considered the benchmark because of the author's familiarity using the kit in the laboratory, and because it was the ATP extraction kit that was available for regular use in the data collected in the main part of this thesis.

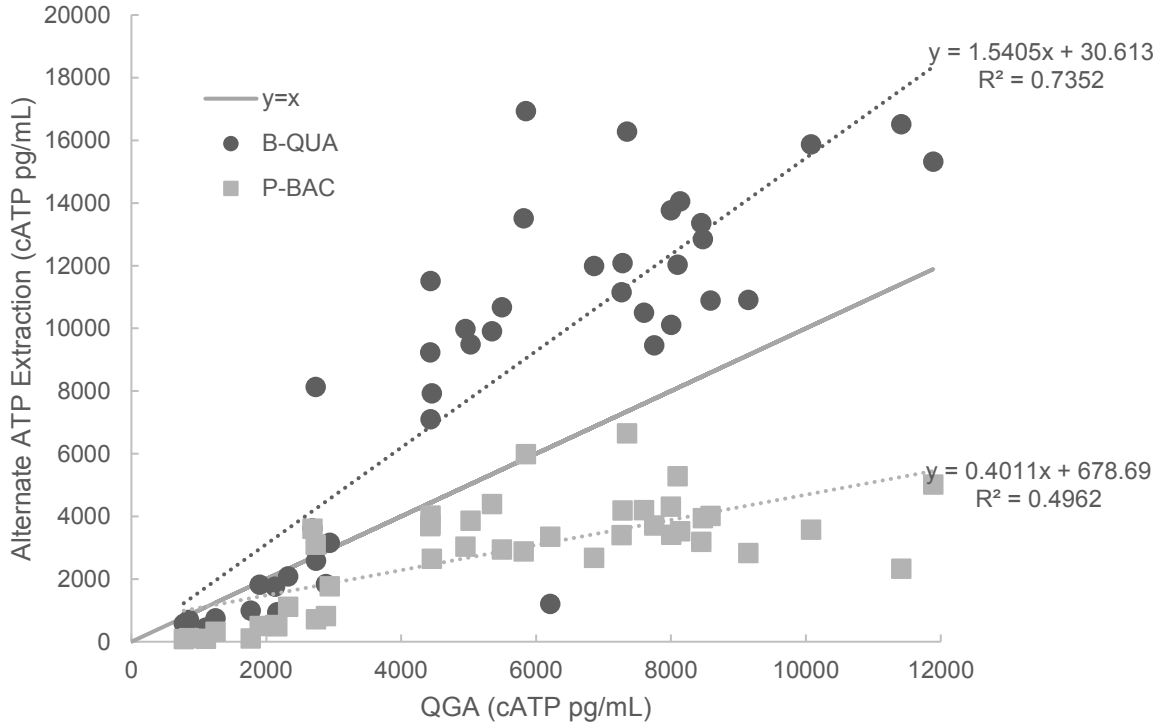

Figure 11 Relative cATP extraction efficiencies compared to QGA-ATP test kit.

The two alternate extraction tests demonstrated a deviation from the benchmark value in this experiment. The modified B-qua method tended to result in a higher ATP concentration reading.

This higher value may be due to the improved action of the modified UltraLyse 30™, as well as the mechanical grinding step using the vortex mixer and bead tube. The other data from the P-BAC extraction method tended to have a lower signal than the benchmark method. The lower signal from the P-BAC method may be due to the long incubation time, where released enzyme into the solution can use up any free ATP and reduce the measured concentration.

Conclusion

This experiment was designed to investigate the differences in ATP concentration of various extraction methods. The extraction methods differed in their filtration methods, lysing agent used, and dilution method (Table 1). After calculating the cellular concentration of ATP for each method it is possible to make the following suggestions about *T. weissflogii* culture grown in balanced growth at the specified conditions:

Heat treatment of 10 minutes or longer at 50°C reduces the cATP to a sufficient regulatory level of <1500pg/mL ATP. The B-QUA method resulted in a 154% increase in ATP extraction efficiency when compared to the QGA method. The P-BAC method resulted in a 60% decrease in ATP extraction efficiency when compared to the QGA method. Overall there is an increased extraction efficiency when physical extraction is added to chemical lysis for extracting ATP from phytoplankton in culture.