Investigating the Mechanism Behind the Release of Microcystins in Freshwater Cyanobacteria

by

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ABSTRACT

The frequency and distribution of toxic cyanobacterial blooms are increasing globally, creating the need for a better understanding of the processes involved in toxic secondary metabolite production. Microcystins (MCs) are potent hepatotoxins produced by a wide range of bloom-forming cyanobacteria genera such as *Microcystis* and *Planktothrix*. Although the release of MCs to the extracellular environment has long been considered a by-product of cell lysis and death, several studies suggest the presence of a mechanism that actively transports these toxins outside the cell membrane. The aim of the present study was to find evidence for a link between cell lysis and concentrations of extracellular MCs.

A dual-fluorescence cell viability assay using the nucleic acid stain SYTOX Green was optimised for use on *Microcystis* and *Planktothrix*. A SYTOX Green concentration of 1 µM, and an incubation time of 30 minutes, yielded a bright and even fluorescent signal that readily identified lysed cells.

The improved staining technique, in conjunction with liquid chromatography-mass spectrometry analyses, was employed in a culturing experiment to track the transfer of MCs to the extracellular environment in relation to the amount of cell lysis. For *Microcystis*, there was a strong and significant positive relationship between cell lysis and the concentration of extracellular MC. When the extracellular MC was predicted according to cell lysis levels and the MC content per cell, lysed cells were a major contributor of MCs to the extracellular environment, although the model overestimated the concentrations. Relationships for

Planktothrix were significant but weaker, possibly due to reduced accuracy in the cell enumeration step, which would have altered the calculated MC content per cell.

Whilst these findings support the hypothesis that cell lysis is the main contributor of extracellular MCs, the results do not exclude a role of MCs as signalling molecules. The recent finding that programmed cell death may occur in *Microcystis* under various environmental conditions may explain the commonly observed increase in extracellular MCs. Understanding the mechanisms involved in the transfer of MCs to the extracellular environment will provide further clarification on the function of these secondary metabolites and lead to the improvement of water quality management strategies.

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"Apporcooooo!"

- Dad

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ABBREVIATIONS USED

ACN, acetonitrile;
Adda, 3 *S*-amino-9 *S*-methoxy-2 *S*,6,8 *S*-trimethyl-10-phenyl-4,6-dienoic acid;
ATP, adenosine-5'-triphosphate;
ABC, adenosine triphosphate binding cassette;
ANOVA, analysis of variance;
DMSO; dimethyl sulfoxide;
DNA, deoxyribonucleic acid;
ELISA, enzyme-linked immunosorbent assay;
FA, formic acid;
FDA, fluorescein diacetate;
FISH, fluorescent *in situ* hybridization;
FOV, field of view; GF/C, glass fibre filter;
HPLC, high performance liquid chromatography;
LC, liquid chromatography;
mcy, microcystin synthetase gene cluster;
MC, microcystin(s);
min, minute(s)
MS, mass spectrometry;
MS/MS, tandem mass spectrometry;
m/z, mass-to-charge ratio;
n, sample size;
N, nitrogen;

NRPS, non-ribosomal peptide synthetase;
C_{18}, octadecyl carbon chain;
P, phosphorus;
PAR, photosynthetically active radiation;
PI, propidium iodide;
PCD, programmed cell death;
PKS, polyketide synthase;
QPCR, quantitative polymerase chain reaction;
×g, relative centrifugal force;
s, second(s);
v/v, volume per volume ratio.

CHAPTER 1: Introduction

1.1 Cyanobacteria and Bloom Formation

Cyanobacteria are photosynthetic prokaryotes that originated 3.5 billion years ago (Huisman et al., 2018); a long evolutionary history resulted in many adaptations and ecologically successful life strategies that allow members of this phylum to thrive in a wide range of environments, such as polar regions, oceans, lakes, rivers, desert soils, and geothermal hot springs (Omidi et al, 2018; Xiao et al., 2018). There are currently >270 cyanobacteria genera described, with >3,000 species found in planktonic and benthic habitats in marine, freshwater, as well as terrestrial systems (Komareck 2016; Huang et al., 2019). Cyanobacteria are commonly referred to as 'blue-green algae' because of their characteristic hue, caused by to the presence of the pigments phycocyanin (blue colored) and chlorophyll-a (green colored), which are often accompanied by the presence of other accessory pigments, such as carotenoids and phycoerythrin (Huisman et al., 2018).

Cyanobacterial dominance in aquatic systems can be explained by adaptations that allow them to efficiently exploit the resources available to them in the water column (Paerl and Paul, 2012). For example, some species are able to fix atmospheric nitrogen (N), and many genera possess high affinity uptake and intracellular storage capabilities for phosphorus (P). This supports cellular metabolism and growth, by allowing them to access these nutrients under limiting conditions (Pearl et al., 2011; Braun et al., 2018). Other cyanobacterial species can adjust their buoyancy via gas vesicles, an adaptation to stratified waters which they can exploit

to access photosynthetically active radiation (PAR) and CO_2 on the surface (Zurawell et al. 2005; Renaud et al., 2011). By accumulating polysaccharides and increasing cellular turgor pressure, cyanobacteria can then reduce their buoyancy or cause their gas vesicles to collapse (Zurawell et al. 2005).

Under optimal conditions, cyanobacteria can rapidly proliferate and form extensive blooms that can be seen even from space (Fig. 1.1). Blooms are defined as a rapid increase in algal density, with the most ancient occurrence reported by the Romans in 77 AD (Huang et al. 2019).

Figure 1.1 Satellite images of cyanobacterial blooms (from Huisman et al. 2018)

Anthropogenic activities may promote conditions that facilitate this cyanobacterial proliferation (Harke et al., 2016, Paerl et al., 2012). The dramatic increase in the availability of N and P resulting from agricultural activities (i.e., eutrophication), rising water temperatures, and the consequent stratification of the water column are the main factors driving bloom formation (Wood et al., 2011; O'Neil et al., 2012). In freshwater ecosystems, P is often the key limiting factor for cyanobacterial growth (Chaffin et al. 2018). Management procedures have therefore focused on reducing its input from agricultural activities (Lewis et al., 2008; Paerl et al., 2011). However, the resulting increase in the N:P ratio in the water column favors the

formation of blooms by genera that are not able to fix N (i.e., non-diazotrophic species) such as *Microcystis* and *Planktothrix*, which may also produce toxins (Huisman et al. 2018).

Bloom formation often occurs during late summer months, as most cyanobacteria species thrive at relatively high water temperatures (> 25°C; Paerl and Huisman, 2008). At this time of the year, more intense stratification of the water column occurs because the warm top layer does not mix with the cold bottom layer (Rabalais et al., 2017). Thermal stratification during warm months may be the main cause of bloom formation in Australia and Brazil, although high cyanobacterial biomass can also develop during periods of mixing (Dantas et al., 2011). Stratification of water bodies is particularly favorable for those species able to produce gas vesicles, which allow the cyanobacterium to adjust its buoyancy according to its needs (Smayda et al., 1997; O'Neil et al., 2012). As the bloom develops, the formation of scums on the water surface may lead to an increase in the temperature of the surrounding water, leading to a positive feedback process that maintains cyanobacterial dominance (Paerl et al., 2011; Stroom et al., 2016).

Cell aggregation and the formation of colonies has two major consequences: 1) decreased nutrient availability and light harvesting per cell, resulting in lower growth rates, and 2) enhanced buoyancy modulation (Yang et al., 2009). *Microcystis aeruginosa*, a species that forms large colonies in the natural environment, tends to disassociate into single cells if grown under laboratory conditions (Bolch and Blackburn, 1996). However, the same species aggregated when grown with a high concentration of zooplankton (Yang et al., 2009). The authors speculated that colony formation may be induced by the release of info-chemicals from the grazers, suggesting that this process is a defense mechanism adopted to reduce the risk of predation through an increased volume, as many grazers are extremely size selective.

Among the bloom-forming genera of cyanobacteria, *Dolichospermum* (formerly *Anabaena*), *Microcystis*, *Nodularia*, *Planktothrix*, *Cylindrospermopsis*, and *Trichodesmium* are the most commonly reported (Kaebernick et al., 2002). Cyanobacteria blooms often have deleterious effects on aquatic ecosystems (Janssen et al., 2019). For example, when a bloom senesces, bacterial respiration can deplete the water of its oxygen content, leading to mass deaths of the fish and aquatic invertebrates inhabiting the hypolimnion (Kardinaal et al., 2007; Rabalais et al., 2017). Blooms are especially associated with negative effects in terms of the aesthetic and recreational value of freshwater, through the production of foul odors and reduced water potability (Zurawell et al. 2005). The damage to drinking, agricultural, and recreational water resources has been estimated to cost the USA > $ 2.2 billion annually (Dodds et al. 2008). The main concern associated with bloom formation is that cyanobacteria can produce a variety of secondary metabolites that are toxic to plants and animals at naturally occurring concentrations (cyanotoxins; Fewer et al., 2007). Animal poisoning caused by the ingestion of cyanotoxins was first reported 142 years ago, when a *Nodularia spumigena* bloom formed in Lake Alexandrina (South Australia) and killed numerous sheep (Francis, G., 1878). In the past three decades, the frequency and distribution of harmful cyanobacterial blooms have increased dramatically (Neilan et al. 2013).

1.2 Cyanotoxins

Cyanotoxins are a diverse group of organic compounds, both in terms of their chemical structure and the nature of their toxicity to humans (Omidi et al. 2018). They are produced by a wide range of cyanobacteria, but *Microcystis*, *Planktothrix* and *Dolichospermum* (formerly *Anabaena*) are the most frequently reported toxin-producing genera in freshwater environments (Gagala et al., 2012). There are three main cyanotoxin chemical structures: cyclic

peptides (microcystins and nodularins), heterocyclic alkaloid compounds (anatoxins, anatoxin-a(S), cylindrospermopsins, saxitoxins, aplysiatoxins) and lipopolysaccharides (Kaebernick & Neilan, 2001; Stewart et al., 2006). Cyanotoxins are also classified into five groups, based on their effects on target organisms: (1) neurotoxins (anatoxins, anatoxin-a(S) and saxitoxins) affect the nervous system, disrupting the regular functioning of neuromuscular activities; (2) hepatotoxins (microcystins, nodularins, and cylindrospermopsins) are actively taken up by hepatocytes (i.e., liver cells) and can potentially lead to liver failure; (3) cytotoxins (cylindrospermopsins and alkaloids) inhibit protein synthesis, causing damage to the liver, kidneys, spleen, thymus, and heart; (4) dermatoxins (aplysiatoxins, debromoaplysiatoxins, lyngbyatoxins) affect the skin, causing dermatitis; (5) irritant toxins (endotoxins) are liposaccharides associated with the outer membrane of cyanobacteria, which elicit allergic reactions and can cause gastroenteritis and inflammation (Huisman et al., 2018).

1.2.1 Microcystins

Microcystins (MCs) are the most well-studied cyanotoxins, due to their widespread occurrence and high toxicity (Tillet et al., 2000). They form a large and structurally diverse group of cyclic heptapeptides produced non-ribosomally via a large enzyme complex comprised of peptide synthetases, polyketide synthases and additional tailoring enzymes. Non-ribosomal peptide synthetases (NRPSs) have a modular structure, with each module responsible for the activation, thiolation, modification, and condensation of one specific amino acid substrate (Christiansen et al. 2003). There are currently > 246 known variants of MCs reported (Huisman et al., 2018), which vary in peptide sequence, modifications, and toxicity (Rineheart et al., 1994; Sangolkar et al., 2006). One of the most toxic congeners, MC-LR, has recently been classified as potentially carcinogenic by the World Health Organization (WHO; Rogers et al., 2015).

Amino acid substitutions in the MC variants often includes non-proteinogenic amino acids (Christiansen et al. 2003). The existence of such a wide array of variants may be explained by the amino acid composition of the water column when blooms form (Moore et al., 1991). While *Microcystis* may produce different MC isoforms with considerable variation at the second and fourth positions and with MC-LR, MC-YR, and MC-RR as the predominant toxins, *Planktothrix* is often dominated by the desmethyl variant of MC-RR (dmMC-RR; Christiansen et al. 2003).

Due to differences in the number of N atoms in variable amino acids incorporated into MC, the availability of this nutrient in the water column may play an important role in controlling MC congener abundance (Puddick et al., 2016). Microcystins contain about 14% N by mass, yet the N content of *Microcystis* cells is approximately 7% N by dry mass (Chaffin et al. 2018). This stoichiometry suggests that N plays a vital role in cyanotoxin production. However, culturing experiments often show mixed results, sometimes finding no difference between the growth rates of potentially toxic and nontoxic isolates, grown in monocultures under optimal or suboptimal conditions (Chaffin et al. 2018). This suggests that factors other than nutrient form and availability may play a role in MC production.

The multi-enzyme complex responsible for MC biosynthesis is encoded by a relatively large gene cluster called MC synthetase (Nishizawa et al., 2000; Christiansen et al., 2003; Schatz et al., 2007). Phylogenetic studies of cyanobacteria capable of producing MCs have revealed a sporadic distribution of the MC synthetase cluster, caused by a partial or total loss of genes (Rantala et al., 2004; Christiansen et al., 2003). Microcystins are produced by several entirely different cyanobacteria, including unicellular, multicellular filamentous, heterocystous and non-heterocystous genera. Many strains can produce multiple MCs simultaneously, but usually

only one or two of these are dominant in any single strain (Christiansen et al. 2003). The presence of the toxin in distantly related groups seems to be consistent with the early origin of MC production in cyanobacteria (Rantala et al., 2004).

Microcystins are transported by anion transport proteins to hepatocytes (i.e. liver cells), where they inhibit the activity of the protein phosphatases 1 and 2A, resulting in excessive signaling and potentially leading to cellular disruption and tumor promotion (Neilan et al., 2013, Puddick et al., 2019). Several deaths caused by MC intoxication have been reported in birds, fish, and mammals, as well as humans (Wood et al. 2011; Elliot, 2012). In 1996 for example, 76 patients at a Brazilian hemodialysis center died of acute liver failure after being exposed to MCs in the water used for dialysis (Jochimsen et al., 1998). Concentrations as low as 50-70 mg kg^{-1} have been reported to cause mortality in humans (Neilan et al., 2013).

1.3 Microcystis

The *Microcystis* genus comprises unicellular cyanobacteria commonly encountered in eutrophic fresh waters, which frequently produce MCs and form colonies (Yoshida et al., 2008). The ability to form colonies and gas vesicles is crucial to the success of *Microcystis* in lake systems. These features enable fast vertical migration, offering them an advantage over other photosynthetic organisms (Kehr et al., 2006). While *M. aeruginosa*, *M. flos-aquae*, *M. ichtyoblable*, *M. novacekii*, and *M. wesenbergii* comprise both MC-producing and non-MC-producing strains, strains of *M. viridis* have only been shown to produce MCs to-date (Yoshida et al., 2008, Zilliges et al., 2008). Microcystins in this genus are produced continuously from the early logarithmic phase to the late stationary phase of a bloom (Lyck, S., 2004). Although MC production is generally constitutive, abiotic factors or growth can affect MC cellular content in *Microcystis* by a factor of 3-4 (Jacinavicius et al. 2019). Under P-limitation, non-toxic

Microcystis strains have a light quantum efficiency that is higher than that of their toxic counterparts. This suggests that blooms where toxic and non-toxic strains coexist will tend to be dominated by toxic *Microcystis* cells first, while non-toxic cells take over during the senescence stage, when nutrients are scarce (Wang et al. 2015).

1.4 Planktothrix

Planktothrix is one of the most prolific MC producing genera in temperate lakes (Catherine et al., 2008). Within the order Oscillatoriales, the genus *Planktothrix* is generally characterised by free-floating solitary trichomes distinguished in nine planktonic species, although a benthic strain was isolated in a freshwater biofilm in New Zealand (Wood et al., 2010) and more recently by Pancrace et al. (2017). The significance of *Planktothrix* blooms for public health became evident after an incident where soldiers suffered facial rashes, asthmatic symptoms and dry sporadic cough with vomiting after exposure to *Planktothrix agardhii* during canoe training in Hollingworth Lake, England (Codd et al., 1999). Individual *P. agardhii* strains may produce up to six different non-ribosomal peptides simultaneously (Janse et al., 2005). Both MC-producing and non-producing genotypes have been found for *P. agardhii* and *P. rubescens* (Janse et al., 2005).

The types of MC isoforms produced by *Planktothrix* often differ from that in *Microcystis,* and cellular production rates of the toxin have been found to generally be higher in filamentous strains from field studies (Christiansen et al., 2003). While most *mcy* genes present in *Planktothrix* have counterparts in the *mcy* clusters of *Microcystis*, the modules *mcy*F and *mcy*I are lacking in *Planktothrix* and *mcy*T is missing in *Microcystis*. While in *Microcystis* the *mcy* genes are organised in two operons transcribed in opposite directions, in *Planktothrix mcy* genes, except *mcy*T, are transcribed from the same DNA strand from a single operon.

Planktothrix agardhii is reported to produce more toxic MC variants during warm months, when recreational activities in lakes are most attractive (Tonk et al., 2005).

1.5 Monitoring for Microcystins

Monitoring of planktonic cyanobacteria in New Zealand waterbodies used for recreational activities generally follows the protocols and thresholds set out in the 'Interim New Zealand Guidelines for Cyanobacteria in Recreational Fresh Waters' (Ministry for the Environment and Ministry of Health, 2009). These biovolume thresholds were established using toxicity calculations based on the negative health effects of MCs, the likelihood of exposure to the toxins, and the expected MC cell quota (Puddick et al., 2019). It is difficult to predict the potential health risk posed by these toxins, because their concentration greatly varies within each bloom (Briand et al., 2012). Microcystin levels may increase dramatically in dense scums, such that estimates of 25,000 μg L^{-1} have been reported in lakes where the guideline limit for safe recreational water is 20 μg L^{-1} (Kardinaal et al., 2007). The presence of MCs in water bodies is regularly monitored using techniques that allow toxins to be detected and quantified in both natural and controlled environments, such as drinking water treatment plants, or clinical and pathological materials (Metcalf et al., 2009). Microcystin concentrations in environmental samples can be estimated using protein phosphatase inhibition assays, enzyme-linked immunosorbent assays (ELISAs) generally targeting the Adda portion of the MC structure, chemical derivatisation with gas chromatography-mass spectrometry analysis, and high-performance liquid chromatography (HPLC) in conjunction with either UV absorption or mass spectrometry (MS) detection (Metcalf et al., 2009; Rogers et al., 2015). Liquid chromatography-MS (LCMS) is a highly-specific analytical technique where complex mixtures

of compounds are separated by LC (generally using a reversed-phase C_{18} column) and analysed according to their mass-to-charge ratio (*m/z*) by MS (Guo et al., 2017).

All of these techniques are still in use, as they encompass a wide range of sensitivities, selectivities, and equipment costs, thus making it possible to choose analyses that meet specific requirements (Rogers et al., 2015; Guo et al., 2017). However, this wide range of measurement techniques also calls for the use of standardised procedures, to ensure that meaningful comparisons can be made between diverse datasets. Usually, the toxin biomass is concentrated by centrifugation or filtration through glass-fiber filters (GF/C), and then stored at a temperature ≤ -20 °C, or is lyophilized (<196 °C; Rogers et al., 2015). The extraction procedure usually involves the use of solvents (e.g., methanol or butanol), freeze/thaw cycles, sonication, or microwave-enhanced extraction (Rogers et al., 2015). One of the greatest difficulties encountered in co-culturing studies is that it is not possible to distinguish between toxic and non-toxic strains of the same species, on the basis of their morphology, thus excluding the use of traditional microscopy or flow cytometry for this purpose (Renaud et al., 2011). There is no apparent correlation between cell morphology and MC production (Yoshida et al., 2007), although it is sometimes possible to distinguish between cells, according to the type of photosynthetic pigments present (e.g., Kardinaal et al., 2007). However, in most cases molecular biology techniques are needed to determine the composition, and variation thereof, of strains within a bloom.

The genetic pathways for MC production have been characterised, and the genes involved in this process have been sequenced for many genera (Neilan et al., 2013). This proves particularly useful when adopting techniques such as quantitative PCR (or Q-PCR), which target specific genes within the MC synthase cluster (e.g. Kurmayer et al., 2003; Wood et al., 2011).

An alternative technique is fluorescent *in situ* hybridization (FISH), which localizes MC-encoding genes within a population, thus aiding a less-disruptive distinction between toxic and non-toxic strains within a bloom (Biegala et al., 2002; Zeller et al., 2016).

1.6 Ecological Role of Microcystins

The ecological role of MCs is still under debate and it is often difficult to compare results among a wide range of growth and toxicity assessment techniques (Kaebernick et al., 2000). However, it is conceivable that the function of the toxin may be related to factors that commonly enhance the production of peptides, such as culture age, temperature, light, nutrients, salinity, pH, and micronutrient concentrations. All of these affect the MC content of *M. aeruginosa*, *Anabaena flos-aquae* and *Planktothrix agardhii* (Dittman et al., 2001). Culturing experiments demonstrated that MC-producing strains have an advantage over their non-toxic counterparts under stressful conditions, such as low carbon concentrations, elevated temperatures, oxidative stress, or bright light conditions (Omidi et al., 2018). The effects of N and P on the production and toxicity of MC are highly variable. While P-limited conditions increased toxicity in *P. agardhii* (Sivonen, K., 1990), and *M*. *aeruginosa* (Oh et al.2000), higher levels of both N and P are usually found to promote the proliferation of toxic *Microcystis* strains over non-toxic ones (Vezie et al. 2002). For instance, high levels of N may promote the production of more toxic MC variants in *M. aeruginosa* (Oh et al. 2000).

Immuno-gold labeling techniques revealed that two-thirds of MCs were attached to the thylakoid membranes of producer cyanobacteria, leading to the hypothesis that MC is involved in photosynthesis (Shi et al., 1995; Young et al., 2005). Although very low light intensities decrease MC production, so do light intensities above the optimal range (Van der Westhuizen & Eloff, 1985), with no corresponding changes in growth rate (Kaebernick & Neilan, 2001;

Wiedner et al., 2003). The MC production rate generally depends on photon irradiance (Utkilen & Gjølme, 1992; Wiedner et al., 2003). Light quality (at the red end of the visible light spectrum), as well as high light intensity, were responsible for an increase in *mcy*B and *mcy*D transcripts in *M. aeruginosa* cells, suggesting that the MC synthetase gene cluster may require a certain irradiance threshold for initiation and up-regulation (Kaebernik et al., 2000). However, since numerous cellular processes are affected by light conditions, this is unlikely to be the only factor leading to these changes. For example, cell division is influenced by strong light, which may also have pronounced effects on transcription and/or toxin production (Wood et al., 2011). Under natural conditions, toxicity decreases with an increase in the depth of a bloom, suggesting there could be a negative relationship between toxin production and light penetration depth (Utkilen & Gjølme, 1992). In contrast to most general findings concerning *M. aeruginosa*, Sivonen et al. (1990) reported that in a *P. agardhii* strain, a low level of irradiance (12 and 24 µmol of photons m^{-2} s^{-1}) resulted in higher MC concentrations than a higher level of irradiance (50 and 95 µmol m^{-2} s^{-1}). However, the amino acid composition of MC produced by *P. agardhii* may vary substantially with light conditions, with more toxic variants being produced under higher irradiance (Tonk et al., 2005).

In addition to light, correlations between MC quota (toxin amount per cell) and a variety of other environmental factors such as water temperature, pH, and nutrient availability (mainly N and P) have been observed (Dai et al., 2016). Increasing temperature generally enhances toxicity, although temperatures exceeding 28 °C lead to a reduction in MC concentrations (Van der Westhuizen & Eloff, 1985). Colony size is also related to MC production, with larger colonies producing more than small ones (Jacinavicius et al., 2019). Iron deficiency triggered the production of toxins, suggesting that MC may contribute to the capture and incorporation

of iron in the cytosol (Sevilla et al.,2008). Nonetheless, these findings were only correlative, and further experimental verification is required.

Microcystin production may have evolved as a defense mechanism against grazing (Rohrlack et al., 1999). However, phylogenetic analyses revealed that the major MC-producing genus *Microcystis* likely evolved well before the arrival of metazoan cyanobacteria predators (Rantala et al., 2004). This suggests that, although we cannot be sure that the genus originally produced MCs, these toxins plausibly had a different function when they first appeared.

Autotrophic organisms often release chemicals and toxins that negatively affect their potential competitors, a process known as allelopathy (Li and Li 2012). Tillmann (2003) and Graneli and Hansen (2006) found that allelopathy, especially toxin production, intensifies under limiting conditions, such as low nutrient levels. Moreover, many bacterial species produce signal molecules, such as acylated homoserine lactones and oligo-peptides, to synchronize cellular activities in response to environmental changes, through a process referred to as "*quorum sensing*" (Borges et al., 2019).

Schatz et al. (2007) suggested that these toxins may have a potential role in intercellular communication, and acclimation to changing environmental conditions. They found that the presence of toxins outside the cells further stimulated toxin biosynthesis via enhanced expression of the *mcy* gene cluster. Specifically, there was a 12-fold increase in the expression of the *mcy*B module in a fresh culture containing *Microcystis* cells, after these were exposed to a 24 h treatment with a crude extract obtained from mechanically lysed *Microcystis* cells. However, these effects cannot be directly linked to MC because the crude extract contained other bioactive secondary metabolites known to be produced by some *Microcystis* strains, such as microviridins, microginin and aeruginosin, whose physiological role is also still poorly

understood (Huang et al., 2019). While the same study by Schatz et al., (2007) revealed an increase in the expression of the *mcy*B module also in cultures treated with just MC-LR, this was of negligible magnitude.

For MCs to play a role as infochemicals in intercellular communication, they must first be released from the cells to the extracellular environment. During growth and under different environmental conditions, MCs are released partly to their surrounding media as extracellular molecules (Wood et al., 2012). The effects of exogenous MCs on non-toxic cyanobacteria are variable and their role is still under debate. As an example, Sedmak and Kosi (1998) reported an increase in the growth rate of a non-toxic strain of *M. aeruginosa* when exposed to MC-RR under low lighting conditions. However, exogenous MC-RR also caused growth inhibition, chlorosis, cell lysis, photosynthesis inhibition, and a change in pigment color of non-MC-producing cyanobacteria, when these were exposed to intense lighting conditions (Singh et al., 2001; Hu et al., 2004; Downing et al., 2015). The non-MC producing *Synechococystis* PCC6803 took up exogenous MC-LR at environmentally relevant levels, and stored it in thylakoid membranes (Phelan et al. 2014), which is where it is stored by the majority of MC-producing cyanobacteria (Wang et al. 2015). However, the addition of MC-LR led to a significant decrease in the activity of photosystem II.

Since quorum sensing is a cell-density dependent mechanism, the potential release of MCs may require a certain cell density threshold to be initiated. Strains of *M. aeruginosa* with different initial cell densities showed that the final cell density was positively correlated with the initial cell numbers (Dunn & Manoylov, 2016), indicating that colonies proliferated in a cell density-dependent manner. In a mesocosm study, both *Microcystis* cell density and MC production increased by 14× over a period of 6 h (Wood et al., 2012), suggesting that MC

production may also increase in a cell-density-dependent fashion. While concentrations of extracellular MC did not increase, this could be due to the time required to initiate toxin export. As MC quotas escalated in Lake Rotorua (Kaikoura, New Zealand), there was a corresponding increase in extracellular MC concentrations (Wood et al., 2011). The significant and rapid up- and down-regulation of *mcy*E expression corresponding to variations in MC quota, extracellular MC levels and cell concentrations adds further evidence to support the hypothesis that these toxins may act as signaling compounds (Wood et al., 2011).

1.7 The Potential for Active Export of Microcystins

It is still not known whether MCs are exported to the extracellular environment by active transport, or whether the commonly observed increase in concentrations of extracellular MCs is a by-product of cell lysis. While little is known about the transport of organic substances through the membrane of cyanobacteria (Stebegg et al., 2019), the occurrence of protein transport through the cell membrane of cyanobacteria with surface layers in *Synechocystis* was reported (Agarwal et al., 2018), and some gram-negative bacteria can release outer membrane vesicles capable of long-distance transport (Bonnington et al. 2014). Some MCs have been detected in the cell wall and sheath area of *M. aeruginosa* cells, indicating the possible release of this toxin from intact cells (Shi et al., 1995). Moreover, although the release of MCs into the extracellular environment has long been attributed to the death and lysis of the blooms (Zurawell et al., 2005), there is evidence of a potential toxin export pathway. Genetic sequence analyses have shown a strong similarity between the *mcy*H module in the MC biosynthesis gene cluster and other ATP-binding cassette transporter genes, with homologs from plants, bacteria, nematodes and mammals involved in the transport of long-chain fatty acids structurally similar to the Adda sidechain of MCs (Pearson et al., 2004). However, inactivation

of the putative transporter protein encoded by *mcy*H in a mutant of *M. aeruginosa* led to complete blockage of MC production, preventing further functional characterisation of the *mcy*H module. This suggests that the putative MC export may be intrinsic to MC production.

A three-fold increase in the levels of MC-LR in *M. pinniformis* was detected during the light phase of a culturing experiment, for both light:dark and continuous light treatments, peaking at midday (Bittencourt-Oliveira et al. 2005). Levels of intracellular and extracellular MC also significantly oscillated under both continuous light and a light:dark cycle (12:12h), suggesting that the potential release of MC may be regulated by circadian rhythms and therefore that the toxins may play a key role in the central metabolism (Straub et al., 2011; Cordeiro-Araujo et al., 2013). Wiedner et al. (2003) found that extracellular levels of MC remained constant during the light:dark cycle, although the concentrations were 20 times higher at 40 µmol of photons m^{-2} s^{-1} than at 10 µmol of photons m^{-2} s^{-1}. However, none of these studies was able to determine whether the cells were still alive when the toxins were being released to the extracellular medium, or whether the increase in extracellular MCs was due to increased cell lysis at higher irradiances. This is indicative of the need to couple cell viability techniques with a close monitoring of variations in the levels of extracellular MCs. The presence of an active transport mechanism to secrete the toxins would be partially supported by an increase in concentrations of extracellular MC regardless of cell lysis.

1.8 Overview and Objectives of this Study

The principal aim of this study is to address the hypothesis that cell lysis explains the concentration of extracellular MCs commonly observed in two major genera of MC-producing cyanobacteria: *Microcystis* and *Planktothrix*. To address this hypothesis, cell lysis was

monitored during a culturing experiment using the nucleic acid stain SYTOX Green and was compared to the levels of extracellular MCs observed.

The SYTOX Green staining protocol was assessed and optimised for use on *Microcystis* and *Planktothrix* in Chapter 2, where the effects of different concentrations and incubation times on the fluorescence intensity of the dye were investigated and its accuracy was tested. This investigation was conducted to ensure the assay was reliable for detecting lysed cells during the culturing experiment described in Chapter 3.

In a culturing experiment conducted on *Microcystis* and *Planktothrix* (Chapter 3), the levels of cell lysis, as well as concentrations of extracellular MC, were monitored in an effort to understand whether the amount of toxins found in the medium could be explained by cell lysis. Evidence of the opposite case would provide further substantiation of a potential active export mechanism involved in the secretion of the toxins, therefore, supporting the role of MCs as signaling molecules.

In Chapter 4 the findings of these studies are discussed and future directions in this research area are provided.

CHAPTER 2: OPTIMISATION OF SYTOX GREEN STAINING METHOD WITH *MICROCYSTIS* AND *PLANKTOTHRIX*

2.1 INTRODUCTION

Toxic cyanobacteria blooms can negatively impact ecosystems, and the physiological state of the cells within a bloom can greatly alter its toxicity (Emerson et al., 2017). Monitoring for the presence and activity of cyanobacterial cells is therefore a pressing matter in environmental policy (Simis et al., 2012). Under stressful conditions or due to apoptosis, cells may sustain damage to their subcellular structures and physiological activities (Tashyreva et al. 2013). Cell lysis is a common and fundamental process in phytoplankton ecology, demanding the development of proper and fast methodologies to monitor cell viability (Peperzak et al., 2011).

Numerous colorimetric or staining assays have been developed to evaluate the cellular viability of micro-organisms. These techniques depend on one or both of two properties; the intactness of the cell membrane and the physiological state of the cell. Unlike dead cells, living, healthy and active cyanobacterial cells have intact plasma membranes and genomes, detectable metabolic activity, and significant content of pigments for effective photosynthetic performance (Zhu et al. 2013). A number of fluorescence-based assays that reflect various physiological functions are commercially available for detecting cell viability and activity, such as assessment of membrane integrity and potential, intracellular pH, respiration intensity, intracellular enzymatic activity etc. (Tashyreva et al. 2013). The fluorescent probe fluorescein diacetate (FDA) has commonly been applied to assess cell viability of microalgae (Steinberg et

al., 2011). The enzyme is cleaved by endogenous esterase activity following uptake into cells and results in the release of fluorescein, a compound that fluoresces bright green in metabolically active, viable cells. However, fluorescein rapidly leaks from cells, compromising the reliability of the results (Sato et al. 2004).

The loss of cell membrane integrity is one of the main features discriminating dead or severely injured cells from living cells, since it plays a key role in the operation of the whole cell and it is sensitive both to mechanical rupture and molecule driven alterations (Bischofberger et al. 2009). Assays intended for estimating membrane integrity are based on the passive exclusion of particular dyes (e.g., propidium iodide and SYTOX Green) by cells with structurally intact membranes (Tashyreva et al. 2015, Gallardo-Rodriguez et al. 2016). Ideal indicators of plasma membrane integrity concentrate only in cells with permeabilized membranes and exhibit marked fluorescence enhancement within these cells (Roth et al 1997). The efficacy of fluorescent compounds depends on their selectivity, brightness, excitation and emission maxima, and inherent biological toxicity (Roth et al. 1997). Because DNA and RNA provide large numbers of intracellular binding sites that promote marked fluorescence enhancement of many different stains, it is possible to obtain accurate information about the state of populations at a single-cell level (Roth et al., 1997). The application of fluorescent dyes makes the assessment of cell viability more accurate, rapid, and straightforward (Zhu et al. 2013). These dyes are often detected by fluorescent microscopy (Peperzak et al., 2011) but they can also be detected by solid state cytometry or flow cytometry (Berney et al. 2007). While the use of automated counting tools such as a flow cytometer significantly speeds up the process, fluorescence microscopy has the advantage of detecting low-biomass samples that might be more difficult to analyse with flow cytometry, where it may be difficult to distinguish them

from the background (Emerson et al., 2017). For microscopy, the sample is stained and applied to a microscope slide, and the counts are performed manually or using image-processing software (Emerson et al., 2017).

Dual fluorescence assays are commonly used to assess cell viability, usually by employing differently coloured nucleic acid stains to differentiate between viable and non-viable cells (Berney et al. 2007). One example is the LIVE/DEAD BacLight viability assay kit widely employed for bacteria, which uses two nucleic acid binding probes, SYTO® 9 and propidium iodide (Sato et al. 2004). SYTO 9 is a permanent intercalating green fluorescent dye capable of penetrating most plasma membranes and staining all cells containing nucleic acids, while propidium iodide is a red fluorescent dye that is normally membrane impermeable, and is thus excluded by the intact cell membranes of living cells. Non-viable cells will thus show fluoresce in the red end of the spectrum at 635 nm, with excitation at 490 nm (Johnson et al. 2015). However, this overlaps with the excitation and emission wavelengths of pigments such as chlorophyll (Darzynkiewicz et al., 1994; Sato et al. 2004), making this assay generally not suitable for microalgae. Nonetheless, Zhu et al. (2013) report the successful application of SYTO 9 and propidium iodide on two cyanobacterial genera (*Microcystis* and *Synechocystis*).

SYTOX dyes are another important family of nucleic acid stains commonly used as a marker for cell lysis (Thakur et al. 2015). These dyes exhibit >1,000-fold fluorescence enhancement upon DNA binding, have a high DNA binding affinity, show little base selectivity, and exist in different colours spanning the whole visible spectrum, including SYTOX® Blue and SYTOX® Green. These have been applied to various species of cyanobacteria and algae (Sato et al., 2004). For instance, SYTOX Blue was used as an alternative to the non-viable cell indicator propidium iodide in the filamentous cyanobacterium *Anabaena* (Johnson et al., 2016). However, the dye

also stained viable cells, possibly due to the passage of dye molecules through intercellular channels connecting cells within a filament (Mullineaux et al. 2008).

The SYTOX Green stain provides several advantages over the dyes described earlier due to its bright fluorescent signal, spectral properties, and relative non-toxicity to living cells (Tashyreva et al. 2013). Most importantly, SYTOX Green fluoresces bright green when excited with a 450-490 nm source (Sato et al. 2004), with optimal excitation wavelength at 504 nm and peak emission at 523 nm (Zetsche et al. 2011), which does not overlap with chlorophyll and phycobiliprotein fluorescence. This allows the simultaneous visualisation of intact and lysed cells in a simple dual fluorescence viability assay, where lysed cells fluoresce green (SYTOX Green) and intact cells fluoresce red (chlorophyll autofluorescence). Although there are reports of green autofluorescence by microalgae interfering with green-fluorescing stains, the SYTOX Green fluorescence is strong enough to outperform any green autofluorescence potentially emanating from microalgal cells (Tang et al. 2007). The dye has been successfully applied to bacteria, yeast cells, cyanobacteria, diatoms, dinoflagellates and green algae in both cultures and field samples for cell viability assessment (Veldhuis et al. 1997; Machado et al. 2012).

The manufacturer's recommended protocol for SYTOX Green is to use a concentration of 0.5 to 5 µM, incubating for > 5 minutes in the dark at room temperature, however, there is much variability in the parameters used by researchers for different cyanobacterial genera (e.g., Sato et al., 2004; Bouma-Gregson et al., 2017; Tashyreva et al., 2015). The optimal concentration and incubation time of SYTOX Green may vary significantly from sample to sample depending on interspecific differences in permeability, state of the cultures, and on culture conditions prior to the staining procedure (Tashyreva et al., 2013). For instance, SYTOX Green was

effective with two *Synechocystis* strains, *Planktothrix agardhii* and *Microcoleus autumnalis*, but not on *Anabaena* (Sato et al., 2004). Moreover, excessive dye concentrations and prolonged incubation periods resulted in heterogeneous staining of cell populations and overestimation of the proportion of lysed cells (Tashyreva et al. 2013), suggesting that different genera may require different staining procedures to ensure results reliability.

In the current study, I investigated the performance of the SYTOX Green stain for use with *Microcystis aeruginosa* and *Planktothrix sp*., to develop an optimised staining protocol. The optimal dye concentrations and incubation times were determined for each strain, the accuracy of the dual-fluorescence assay was assessed, and the half-life of the permeated cells was investigated for these two species. The optimised assay will later be applied to samples from the culturing experiment described in Chapter 3.

2.2 METHODS

2.2.1 Cyanobacteria Cultures and Materials

Microcystis aeruginosa CAWBG617 and *Planktothrix* sp. CAWBG59 were obtained from the Cawthron Institute Culture Collection of Microalgae (Cawthron Institute; Nelson, New Zealand) and grown in Erlenmeyer flasks in a climate chamber at 18 °C with a 12 h:12 h light/dark photoperiod and a photon-flux of 90 µmol photon m^{-2} s^{-1}. SYTOX Green was supplied by the manufacturer in a 250 µL tube at a concentration of 5 mM in dimethyl sulfoxide (DMSO). The stain was diluted to 1 mM using DMSO and, to preserve its integrity, was stored as individual stock solutions (500 µL) in amber glass vials at -20 °C.

2.2.2 Permeabilisation of Cyanobacteria

Samples of *Microcystis* and *Planktothrix* (10 mL) were centrifuged at 4,000 × g for 20 min in 15 mL falcon tubes. The cyanobacterial biomass was resuspended in 70% ethanol (5 mL) and allowed to stand at ambient temperature for 10 min. The cyanobacteria were subsequently pelleted by centrifugation at 4,000 × g for 10 min, washed twice with sterile MLA (Bolch & Blackburn, 1996), resuspend in MLA (5 mL), and analysed within several hours after the permeabilisation procedure.

2.2.3 Optimisation of SYTOX Green Dye Concentration

The SYTOX Green stock solution (1mM) was further diluted in 100% DMSO to obtain 0.1, 0.2, 0.4, 1, 2, 4 and 10 µM solutions of the dye. Subsamples of permeabilised cells (20 µL; see Section 2.2.2) were mixed with an equivalent volume of SYTOX Green and incubated in the dark at ambient temperature for 30 min. This yielded final SYTOX Green concentrations of 0.05-5 µM. Samples were microscopically analysed using the procedure described in Section 2.2.6.

2.2.4 Optimisation of SYTOX Green Incubation time

Ethanol-permeated cyanobacteria samples (20 µL; see Section 2.2.2) were mixed with an equivalent volume of SYTOX Green (2 µM; final concentration of 1 µM) and incubated in the dark at ambient temperature for 5, 10, 20, 30, 40 or 50 min. Samples were microscopically analysed using the procedure described in Section 2.2.6.

2.2.5 Testing the Accuracy of the SYTOX Green Dual-Fluorescence Cell Viability Assay

Samples with different fractions of lysed and intact cells were prepared to assess the accuracy of the SYTOX Green stain. For both *Microcystis* and *Planktothrix*, a subsample (15 mL) of each culture was transferred into a falcon tube and left untreated (intact cells), and another subsample (15 mL) was permeabilised according to the procedure described in Section 2.2.2 (lysed cells). From these, subsamples were mixed to obtain samples containing 0%, 20%, 40%, 60%, 80%, 100% lysed cell samples (20 µL). Samples were mixed with an equivalent volume of SYTOX Green (2 µM; final concentration of 1 µM) and incubated in the dark at ambient temperature for 30 min, then microscopically analysed using the procedure described in Section 2.2.6.

2.2.6 Fluorescence Microscopy Analysis

An aliquot of the stained sample (10 µL) was mounted under a coverslip on a microscope slide and sealed with nail polish. The sample was examined using an epifluorescence microscope (Olympus BX51; Tokyo, Japan) equipped with FITC (green channel) and Texas Red (red channel) fluorescence filters. The FITC filter (excitation 465-500 nm; emission 510-545 nm) was used to detect the fluorescence of the SYTOX Green stain, and the Texas Red filter (excitation 530-575 nm; emission 590-670 nm) was used to detect fluorescence emitted by the photosynthetic pigments present in the cells (i.e., chlorophyll-α and phycocyanin). The samples were analysed under 400× magnification for *Planktothrix* and 200× magnification for *Microcystis*. For each sample, ten fields of view (FOV) were observed, each composed of two fluorescence images per FOV acquired from the red and green channels using a digital camera attached to the

microscope (Olympus DP73; Tokyo, Japan). The two images were combined using CellSens software and subsequently processed on ImageJ where each photo was converted to an 8-bit black and white image, and the threshold values were adjusted for both the red and the green channels. The *Microcystis* cells were counted using the "analyse particles" function. Heterotrophic bacteria (< 1 µm) were found in the non-axenic *Microcystis* cultures and were only detected under the green channel. In order to avoid this background interference, particles smaller than 2×2 pixels were excluded from the analysis. The filamentous *Planktothrix* strain was analysed by selecting each filament and calculating their area.

Percentage of lysed cell was calculated as follows:

$$l = \left(\frac{a}{A}\right) \cdot 100\%$$

l = percentage of lysed cells in the sample

a = number of lysed cells counted

A = total number of cells counted

2.2.7 Half-life Evaluation of Permeabilised Cyanobacteria

The degradation rate of lysed cells after ethanol treatment was determined according to the time elapsed before the SYTOX Green stain would no longer yield a detectable fluorescent signal under the microscope. Cultures of *Microcystis aeruginosa* and *Planktothrix* sp. (grown as

described in Section 2.2.1) were permeabilised as described in Section 2.2.2. The cyanobacterial suspension was pelleted by centrifugation at 4,000 × g for 10 min, washed twice with sterile MLA (Bolch & Blackburn, 1996), resuspended in sterile MLA (50 mL), and incubated at the culturing conditions described in Section 2.2.1. The initial sampling was conducted immediately after the ethanol treatment, and additional samples were taken each day for 6 days, until the SYTOX Green fluorescent signal was no longer detected microscopically.

Each day ethanol-permeated cyanobacteria samples (20 µL) from each culture were incubated in the dark at ambient temperature with an equivalent volume of SYTOX Green (2 µM; final concentration of 1 µM) and microscopically analysed as described in Section 2.2.6.

2.2.8 Image Analysis

Analysis of concentration, incubation and half-life images was conducted in R using the EBImage package (Pau et al., 2010). Images were processed independently. Each image was composed of a red and a green channel, but only the green channel was analysed, as the ethanol permeabilisation process removed chlorophyll pigments and red autofluorescence was not detected. The intensity was measured as the log-transformed density of pixels that are identified as being cells. The SYTOX Green signal strength was measured by pixel density for *Planktothrix*, and by the number of objects detected for *Microcystis*. The 'makeBrush' function was used to detect the pixel density in each image, by dividing the image into two domains; pixels that are in cells, and pixels that are part of the background (of value 0). Labelled objects were pixel sets with the same unique integer value, and with the 'bwlabel' function all pixels for each connected set of foreground (non-zero) were set to a unique increasing integer. The minimum pixel value set to distinguish both *Microcystis* cells and *Planktothrix* filaments from the background was 0.05, in order to exclude heterotrophic bacterial contamination from

the analysis. If no cells were detected, no intensities were returned. The 'computeFeatures.basic' function was used to compute morphological features from the image and distinguish individual cell objects for *Microcystis.* The "agricolae" package (de Mediburu Felibe, 2019) was used to detect significant differences between groups. The "stringr" package (Wickham Hadley, 2019) was used to prepare the data for string-handling functions, which allowed all function and argument names to be consistent.

2.2.9 Statistical Analysis

All statistical analyses of the data were conducted in R software (R core team, 2018). Data was log-transformed and tested by one-way analysis of variance (ANOVA; PASW Statistics 18, SPSS Inc., Chicago, IL, USA) at a confidence level of 95%. Whenever the null hypothesis of the ANOVA was rejected, differences between groups were investigated with post hoc Tukey tests.

For the accuracy assessment, a linear regression model was used to assess the relationship between the known percentage of lysed cells and the detected percentage of lysed cells.

2.3 RESULTS

2.3.1 SYTOX Green concentration

There was no visible fluorescence of *Microcystis* cells using SYTOX Green at 0.05 µM, while the fluorescence intensity was visually similar between 0.5 and 5 µM (final concentration; Fig. 2.1).

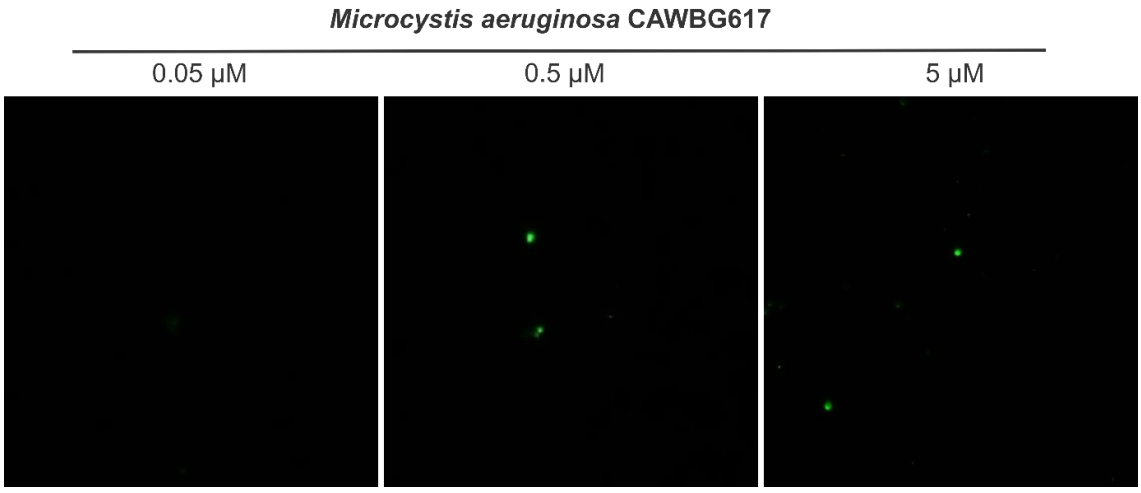


Figure 2.1 Images of lysed *Microcystis* cells detected under an epifluorescence microscope with a FITC filter at final concentrations of 0.05, 0.5 and 5 µM SYTOX Green stain.

The difference in fluorescence intensity of SYTOX Green between concentrations was statistically significant (one-way-ANOVA, $p = 0.02$). However, the Tukey's post hoc analysis revealed no significant differences between concentrations (Fig 2.2, $p > 0.05$).

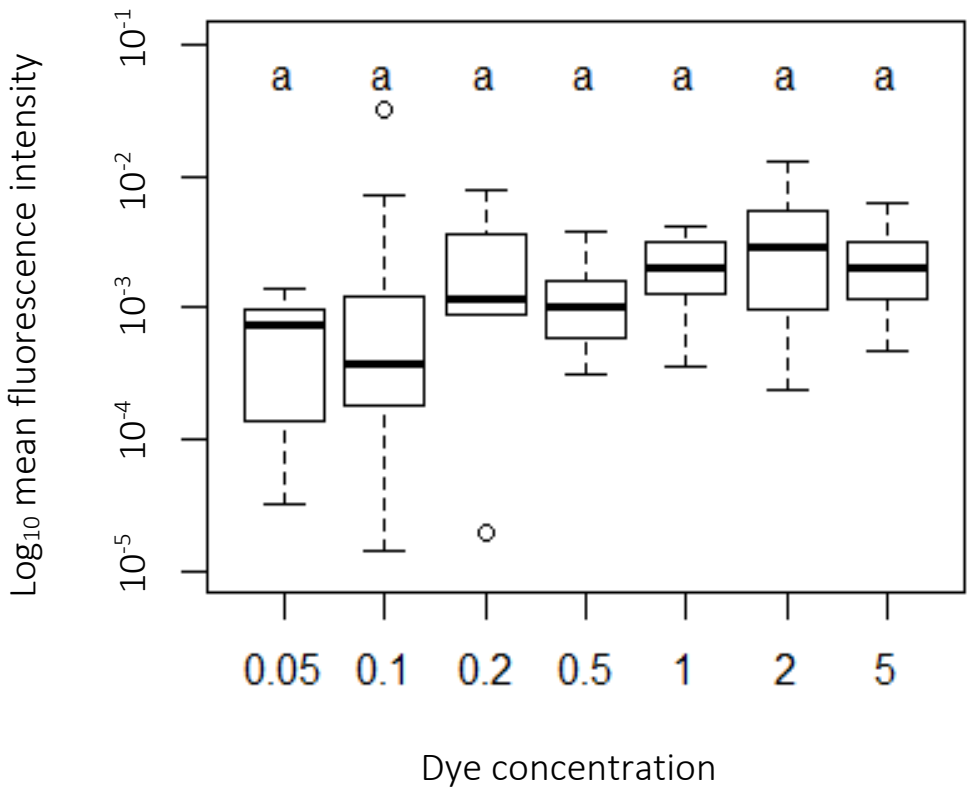


Figure 2.2 Fluorescence intensity of chemically lysed samples of *Microcystis* at final SYTOX Green stain concentrations of 0.05, 0.1, 0.2, 0.5, 1, 2, and 5 µM (note the log-scale on the y-axis).

No fluorescence signal was detected in the *Planktothrix* images at the 0.05 µM concentration. Compared to *Microcystis*, staining with a SYTOX Green concentration of 5 µM resulted in a much brighter fluorescence intensity, visibly different from the at 0.5 µM images (Fig. 2.3).

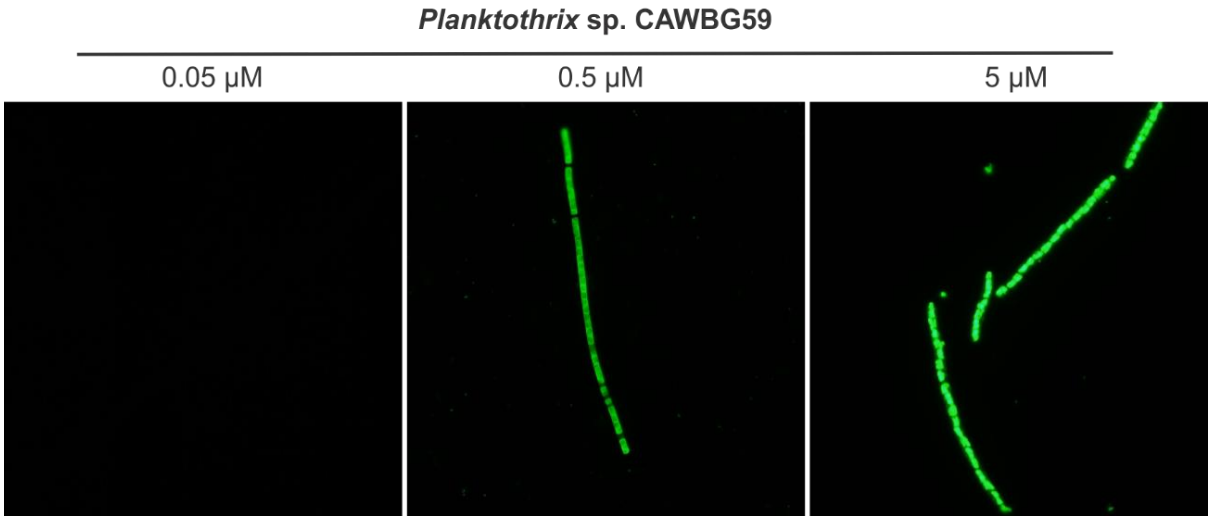


Figure 2.3 Images of lysed *Planktothrix* cells detected under an epifluorescence microscope with a FITC filter at concentrations of 0.05, 0.5 and 5 µM SYTOX Green stain.

Varying concentrations of SYTOX Green stain resulted in four significantly different groups of fluorescence intensities for the *Planktothrix* strain (Fig. 2.4; one-way ANOVA, $p < 0.001$). The intensity detected at 0.05 µM was significantly lower compared to all higher concentrations (post hoc Tukey test, $p < 0.01$) except for 0.1 µM. Samples stained with SYTOX Green at a 5 µM concentration yielded a fluorescence intensity significantly higher than all of the other concentrations ($p < 0.001$). The fluorescence intensity detected at 1 µM was slightly higher than that at 0.5 µM ($p = 0.04$) and was not different from that detected at 2 µM.

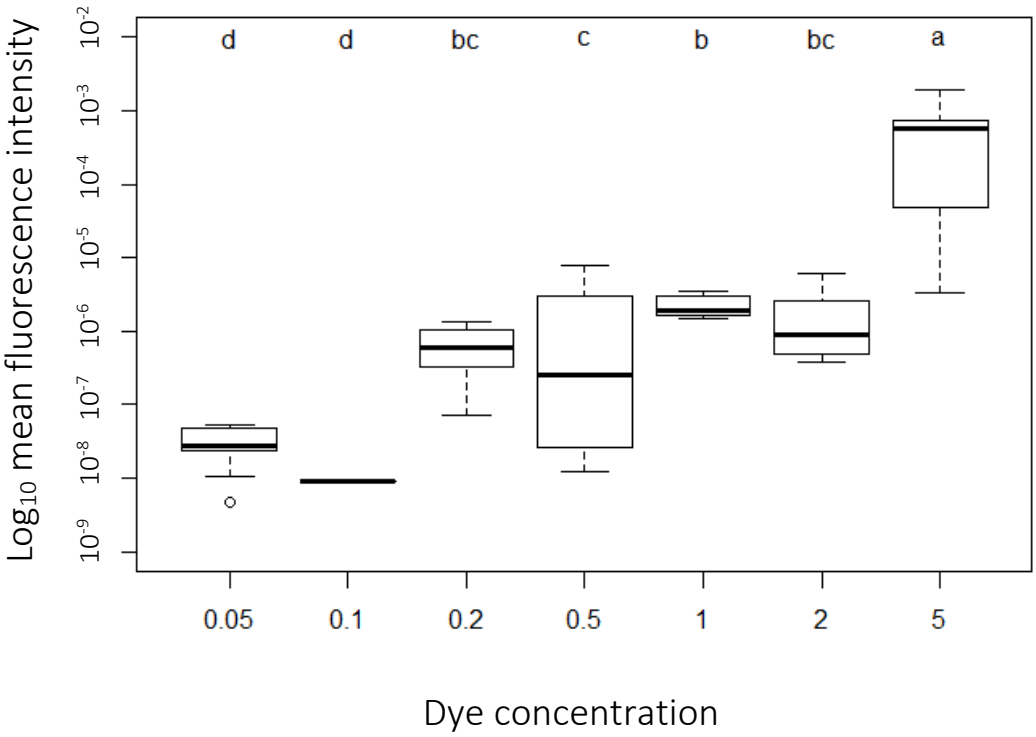


Figure 2.4 Fluorescence intensity of chemically lysed samples of *Planktothrix* at final SYTOX Green stain concentrations of 0.05, 0.1, 0.2, 0.5, 1, 2, and 5 µM (note the log-scale on the y-axis).

A final concentration of 1 µM was chosen as the optimal staining concentration for using the SYTOX Green stain with both *Microcystis* and *Planktothrix*.

2.3.2 SYTOX Green Incubation Time

Longer incubation times did not visibly increase the fluorescence intensity of *Microcystis* cells under the microscope. Although one-way ANOVA initially revealed a significant difference in the fluorescence intensity of *Microcystis* lysed cells stained with SYTOX Green stain (1 µM final concentration) after 5, 10 ,20 ,30, 40, and 50 minutes ($p = 0.04$), the post hoc Tukey's analysis revealed no significant differences between time groups (Fig. 2.5-A). A significant increase in the fluorescence intensity of stained *Planktothrix* cells (Fig. 2.5-B) was detected between 5 and

10 minutes (post hoc Tukey test, $p = 0.01$) and between 5 and 40 minutes (post hoc Tukey test, $p = 0.001$), which matched observations under the microscope. Based on these results, an incubation time of 30 minutes was chosen to perform further tests with the dye for both strains.

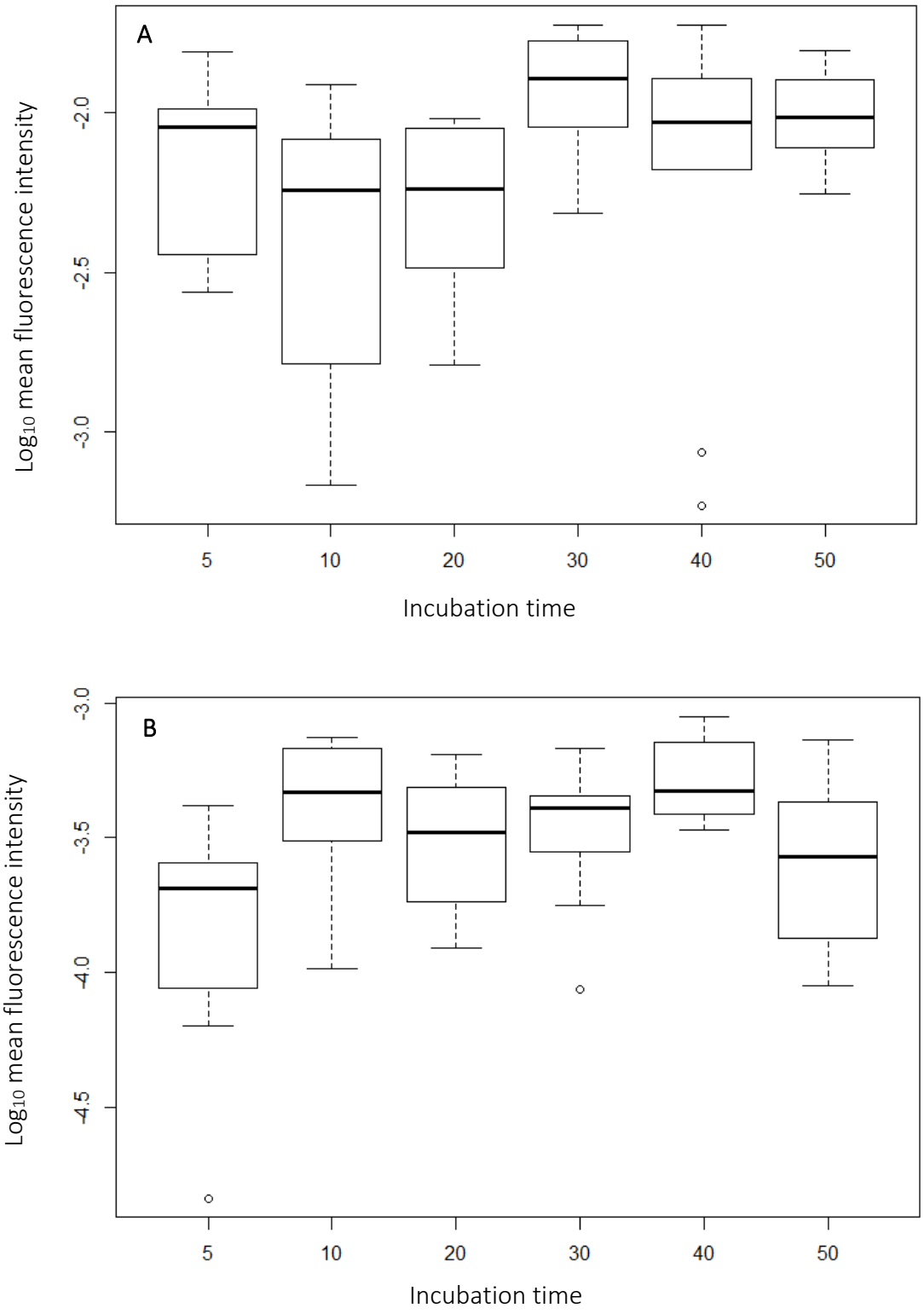


Figure 2.5 Mean fluorescence intensity of *Microcystis* (A) and *Planktothrix* (B) lysed cells after incubation in SYTOX Green stain for 5, 10, 20, 30, 40, and 50 minutes.

2.3.3 Accuracy of the SYTOX Green intact/lysed assay.

To test the reliability of the optimised SYTOX Green cell viability assay, different proportions of lysed and intact cells were mixed together and analysed under the fluorescence microscope. For both *Microcystis* and *Planktothrix* there was a strong linear relationship between the percentage of lysed cells measured and the known percentage of lysed cells in the intact/lysed cell mixtures (Fig 2.6-A and B). Both relationships were significant ($p < 0.001$ for *Microcystis* and *Planktothrix*), and the percentage of variance explained by the regression relationships were 97% for *Microcystis* ($R^2 = 0.97$) and 98% for *Planktothrix* ($R^2 = 0.98$).

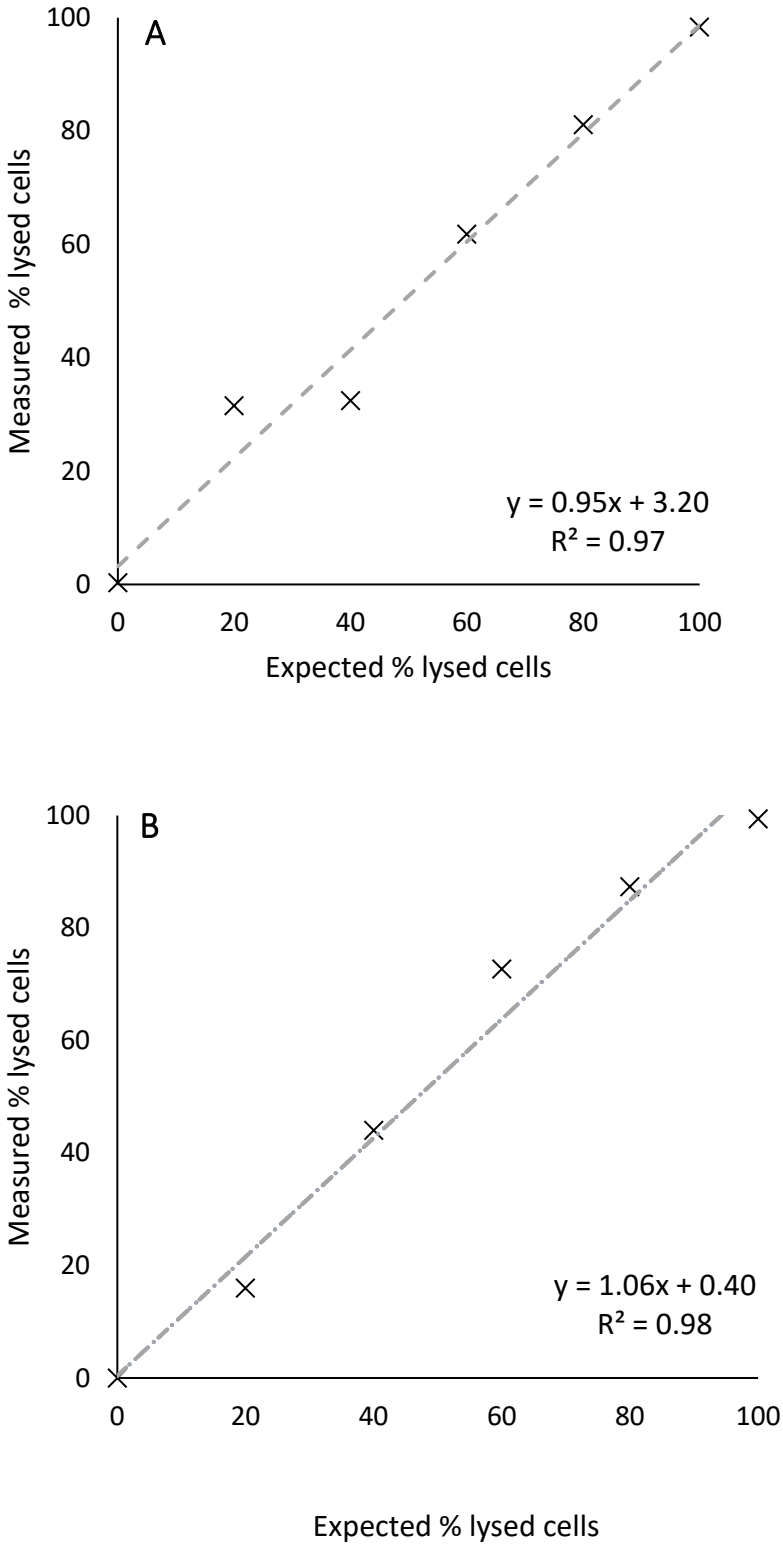


Figure 2.6 Regression analysis of fluorescently detected % lysed cells vs known % lysis for *Microcystis* (A) and *Planktothrix* (B).

2.3.4 Half-life Evaluation of Permeabilised Cyanobacteria

The half-life of *Microcystis* and *Planktothrix* cells lysed with ethanol was determined using the SYTOX Green viability assay. There was a strong and significant negative correlation between the age of ethanol-treated *Microcystis* cells and the percentage of cells detected over time (Fig. 2.7-A; linear regression, $R^2 = 0.96$, $p < 0.001$). There was also a strong and significant negative correlation between the age of ethanol-treated *Planktothrix* cells and the percentage of fluorescence intensity detected (Fig. 2.7-B; linear regression, $R^2 = 0.70$, $p < 0.001$). It took 1.5 days for SYTOX Green fluorescence intensity to decline by 50% in *Microcystis* cells (50% ÷ 33% per day decrease in fluorescence intensity), while it took 2.4 days in *Planktothrix* (50% ÷ 21% per day decrease in fluorescence intensity).

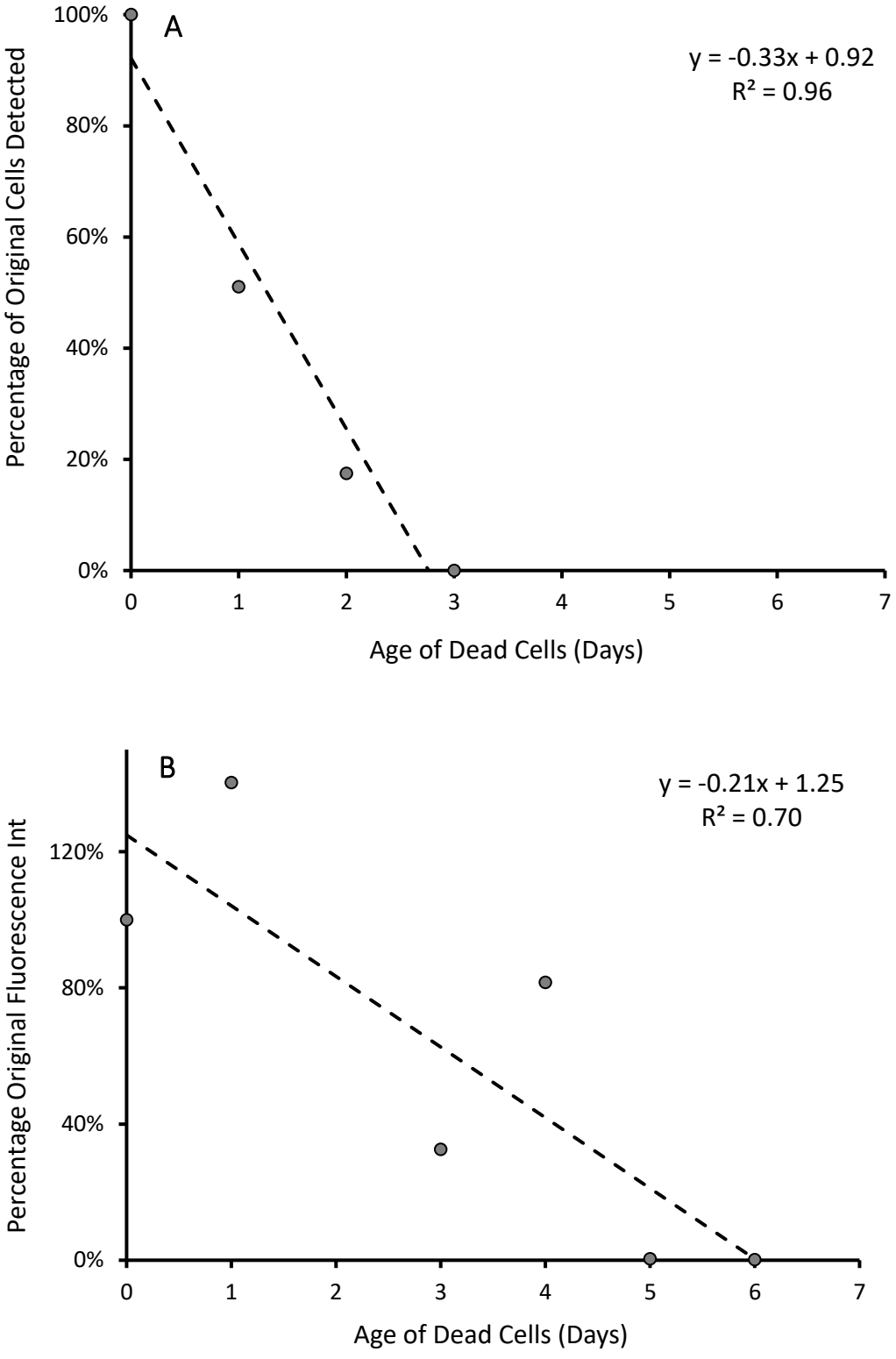


Figure 2.7 Age of Dead cells against percentage of original *Microcystis* cell objects detected (A) and age of dead cells against percentage of original fluorescence intensity detected in *Planktothrix* (B)

2.4 DISCUSSION

The use of fluorochromes for physiological assessment of bacteria provides accurate information about the state of individual cells in populations (Tashyreva et al., 2013). When the viability of the plasma membrane is compromised, compounds normally excluded from the cytoplasm because of size or charge are able to penetrate cells. During the present study, the performance of the nucleic acid stain SYTOX Green was investigated as a marker for cyanobacterial cell lysis. Because excessive dye concentrations and prolonged incubation periods have previously been reported to result in heterogeneous staining of *Microcoleus autumnalis* cell populations (Tashyreva et al. 2013), a preliminary assessment of the performance of SYTOX Green on different cell types was conducted to improve the accuracy of the staining method.

For both *Microcystis* and *Planktothrix* samples, the minimum staining concentration needed to microscopically detect SYTOX Green fluorescence was 0.5 µM, although statistical analysis revealed no significant difference in fluorescence intensity between concentrations for *Microcystis*. This concentration is slightly higher than that needed for *Microcoleus autumnalis* (0.2-0.3 µM; Tashyreva et al., 2013). In this study, a SYTOX Green concentration between 0.5 and 2 µM resulted in the even staining of nucleoids for both strains, with a bright green fluorescence intensity that matched in intensity the red fluorescence from the photosynthetic pigments. For *Planktothrix*, a concentration of 5 µM SYTOX Green yielded a much higher fluorescence intensity and resulted in higher background signal compared to the lower concentrations. Since the presence of nucleic acids from heterotrophic bacteria in the mucilage surrounding the filaments offers the dye more binding sites, concentrations higher than 2 µM should be avoided to reduce background noise in the images. Although a bigger

sample size may have improved the statistical significance for the differences between concentrations, a final SYTOX Green concentration of 1 µM was chosen for subsequent analyses, as it yielded robust fluorescent signals for both strains used in this study.

A minimum incubation time of 5 min, as suggested by the manufacturer, yielded sufficient fluorescence signal to detect ethanol-treated *Microcystis* and *Planktothrix* cells, and no statistical differences in fluorescence intensity between different incubation times were found for *Microcystis*. When observed under the microscope, cells incubated for 50 min did not yield a brighter fluorescent signal compared to shorter incubation times for either strain, suggesting that if needed, cell viability samples may be analysed up to 50 min after staining without altering the reliability of the results. An incubation time of 30 min was chosen to perform sampling in Chapter 3 to maintain a safe incubation time frame, because if *Microcoleus autumnalis* cell populations were left for longer than 60 min in SYTOX Green, the mean percentage of stained cells increased (Tashyreva et al., 2013).

When testing the accuracy of SYTOX Green for determining the proportion of lysed to intact cells, a strong and significant positive relationship was observed for both cyanobacterial genera studied (*Microcystis* and *Planktothrix*), and the slopes were close to 1, reinforcing the accuracy of the assay. This suggests that the aforementioned heterotrophic bacterial contamination did not significantly alter the proportion of lysed cells detected in the green channel. These results supported the applicability of the dual-fluorescence assay on *Microcystis* cells and *Planktothrix* filaments in the culturing experiment described in Chapter 3, using the SYTOX Green stain as a marker for lysed cells, and cell autofluorescence as a marker for intact cells. The staining method described in this study was specifically tested on laboratory samples. Important differences in the optimal staining procedure may be encountered in natural populations,

suggesting that further assessment of the SYTOX Green stain performance may be needed for environmental samples. For instance, *M. autumnalis* samples in the field required minimum dye concentrations 20-times higher than the laboratory samples to evenly label all cells (Tashyreva et al., 2013).

As DNA degradation occurs following the loss of cell membrane integrity, the number of dead cells determined by SYTOX Green staining may gradually decrease with time. However, a delay in the degradation of the cells may occur after membrane integrity is lost, depending on environmental conditions and species (Lebaron et al., 1998). In a culturing experiment, if the dye binds to nucleic acids of lysed cells from a previous sampling time, cell lysis counts may be overestimated. This indicates that values for the concentration of lysed cells detected using SYTOX Green may need to be adjusted according to the half-life of the lysed *Microcystis* and *Planktothrix* cells. In this study, there was a strong negative relationship between the fluorescence intensity of SYTOX Green stain and the days elapsed since cultures were ethanol-treated, as also noted by Lebaron et al. (1998). The stain had 50% reduced efficacy after 1.5 days for *Microcystis* and after 2.4 days for *Planktothrix*. This suggests that studies with frequent sampling intervals may need to account for this SYTOX Green artifact to improve the accuracy of lysed cell counts. To the best of my knowledge, the half-life of the SYTOX Green stain has not been previously taken into account when using this dye for cyanobacterial cell viability assessment.

2.5 CONCLUSIONS

The efficacy and accuracy of SYTOX Green stain to measure membrane integrity in *Microcystis* and *Planktothrix* cultures was assessed. Staining with final concentrations between 0.5 and 2 µM yielded sufficient fluorescence intensity to detect permeated cells, and analysing the

samples between 5-50 minutes after staining provided reliable results. For both *Microcystis* and *Planktothrix*, the dye accurately measured lysed cells. The half-life of lysed cells was 1.5 days for *Microcystis* and 2.4 days for *Planktothrix*. This suggests that when sampling at frequent intervals, accounting for this SYTOX Green artefact would improve the accuracy of the lysed cell counts. Overall, the nucleic acid stain SYTOX Green provided an effective tool for detecting cell lysis in *Microcystis* and *Planktothrix* samples using fluorescence microscopy, supporting its use in the culturing experiment described in Chapter 3.

CHAPTER 3: INVESTIGATING THE POTENTIAL ACTIVE EXPORT OF MICROCYSTINS IN CYANOBACTERIA

3.1 INTRODUCTION

Cyanobacteria are photosynthetic prokaryotic organisms that originated 3.5 billion years ago (Omidi et al., 2017). Under favourable conditions, some cyanobacteria may proliferate to form extensive blooms, and toxin-producing species are often responsible for the poisoning of birds, fish, and mammals (including humans; Salmaso et al., 2015, Kubickova et al., 2019). The most commonly reported cyanotoxins are microcystins (MCs), which pose health risks especially to consumers that may use MC-contaminated waters as a habitat and for drinking water (Paerl et al., 2011; Buratti et al., 2017). Many morphologically different cyanobacteria can produce MCs, including; unicellular, filamentous, heterocystous and non-heterocystous genera. *Microcystis*, *Planktothrix*, *Oscillatoria* and *Dolichospermum* are among the most notable MC-producers (Huisman et al., 2018). To-date, only three cyanobacteria genera have been confirmed to be MC producers in New Zealand: *Microcystis*, *Planktothrix* and *Nostoc* (Puddick et al., 2019). These toxins are generally quantified in water samples and cyanobacteria extracts using liquid chromatography-mass spectrometry (LC-MS) and Adda-specific enzyme-linked immunosorbent assays (ELISA).

The various hypotheses concerning the physiological basis for MC production include; defence against grazers (Lürling, 2003), gene regulation (Dittmann et al., 2001); allelopathic interactions (Sukenik et al., 2002), intra-specific regulation (Schatz et al., 2007), and

siderophoric scavenging of metals such as iron (Martin-Luna et al., 2006; Saito et al., 2008; refer to Section 1.6 for more information). Although extracellular concentrations of MC have long been attributed to cell lysis (Rapala et al., 1997, Zurawell et al., 2005), most of these ecological functions, and those investigated for secondary metabolites in other bacteria and fungi, require the export of the compound from cells, under certain environmental conditions or growth stages (Dittman et al., 2001). The genetic sequence of the *mcy*H gene (part of the MC synthetase operon) is similar to that of the ATP-binding cassette (ABC) genes involved in the transport of long-chain fatty acids, structurally similar to the Adda sidechain of MC. Whilst no cyanotoxin transport system has been demonstrated in cyanobacteria, MCs have been detected in the cell wall and sheath area of intact *M. aeruginosa* cells, suggesting the possible release of this toxin from intact cells (Shi et al., 1995).

Autotrophic organisms often release chemicals and toxins that negatively affect their potential competitors, generally in response to environmental changes (Li and Li, 2012). The role of MC as a signalling molecule, in a process known as 'quorum sensing', has received increasing attention (Borges et al., 2019). For MC to play such a role, they must first be released from the cells to the extracellular environment. Fluctuations observed in the concentrations of intra- and extra-cellular MC support the hypothesis of an active release of toxins to the extracellular environment (Cordeiro-Araújo et al., 2013). The presence of toxins outside of the cell may serve as an indication of cell lysis within cyanobacteria populations, a function characteristic of intercellular signalling molecules (Schatz et al., 2007). It has also been observed that during growth, MCs are excreted partly to the surrounding media under a range of different environmental conditions (Wood et al., 2012). The significant and rapid up- and down-regulation of *mcy*E gene expression corresponding to variations in MC quota, extracellular MC

levels, and cell concentrations in Lake Rotorua (New Zealand) adds further evidence to support the hypothesis that MCs may act as signalling compounds (Wood et al., 2011).

Little is known about the transport of organic substances through the cyanobacterial membrane (Stebegg et al., 2019). However, there is evidence of protein transport through the cell membrane in cyanobacteria, e.g. *Synechocystis* (Agarwal et al., 2018). Moreover, all gram-negative bacteria release outer membrane vesicles, which are capable of long-distance transport (Bonnington and Kuehn, 2014). Microcystin production may be regulated via circadian photosynthetic rhythms and plausibly participates in cyanobacterial central metabolism (Straub et al., 2011). For example, a three-fold increase in the levels of MC-LR in *M. pinniformis* was observed during the illuminated phase of light:dark treatment, and under continuous lighting in a culturing experiment, reaching a peak during the middle of the day (Bittencourt-Oliveira et al., 2005). Moreover, the levels of both intra- and extra-cellular MC oscillated significantly under both continuous light, and during a light:dark cycle (12:12h), in a different culturing experiment using *M. aeruginosa,* thus suggesting that circadian rhythms may also regulate the release of MC to the extracellular environment (Cordeiro-Araujo et al., 2013). However, this study (like others) lacked a means to distinguish between extracellular MCs that have been released via cell lysis, and those released via active export.

Whilst several studies have attempted to understand whether MC export in cyanobacteria occurs actively or passively through cell lysis, to the best of my knowledge, the role of cell lysis as a major contributor to the observed extracellular MC has not been demonstrated. During the present study, I investigated the hypothesis that cell lysis explains the concentration of extracellular MC observed in cyanobacterial cultures. A culturing experiment was conducted using two major MC-producing species, *M. aeruginosa* and *Planktothrix* sp., where cell lysis

was monitored in the cultures using the SYTOX Green dual-fluorescence cell viability assay and levels of intracellular and extracellular toxins were determined by LC-MS.

3.2 METHODS

3.2.1 Culture Preparation

Autoclaved glass Erlenmeyer flasks containing sterile MLA medium (400 mL, Bolch & Blackburn, 1996) were inoculated with *M. aeruginosa* CAWBG617 (single-celled) or *Planktothrix* sp. CAWBG59 (filamentous). The cultures were obtained from the Cawthron Institute Culture Collection of Microalgae (Cawthron Institute; Nelson, New Zealand). These strains were chosen as common MC-producing cyanobacteria representing two morphology types (unicellular and filamentous) with different patterns of toxin production.

Before the beginning of the toxin export experiment, the MLA medium was exchanged twice to remove toxins that had already entered the extracellular environment, whilst at the same time encouraging healthy cell growth. For *Planktothrix* CAWBG59, the culture was passed through plankton netting (20-µm mesh size) and the residue was diluted with a fresh sterile MLA medium (400 mL) in a new autoclaved Erlenmeyer flask. Since the single-celled *Microcystis* CAWBG617 was not retained by the plankton netting, the culture was centrifuged (3,200 × g, 15 min, 18 °C) prior to re-suspending the cell pellet in fresh sterile MLA medium (400 mL) and transferring to a new flask. Three replicate cultures per strain were grown in a climate chamber at 18 °C with a 12 h:12 h light:dark photoperiod and a photon-flux of 90 µmol photon m^{-2} s^{-1}.

3.2.2 Experimental Design and Sampling

Each culture was grown for 57 days, with sampling occurring on days 0, 3, 6, 9, 13, 16, 23, 37, 44, 51, 57. On each sampling day, a sample for cell enumeration (7.5 mL) was preserved with Lugol's iodine (20 µL) and stored at ambient temperature in the dark and a sample for MC analysis (5 mL) was loaded onto a glass fibre filter (Whatman GF/C, 25 mm diameter) using a syringe. The first 0.8 mL of filtrate was collected in a glass autosampler vial (extracellular MC sample). The GF/C filter was placed in a microcentrifuge tube (cellular MC sample) and both MC samples were stored in the freezer (-20 °C) until their extraction and analysis. A sample for the dual fluorescence intact/lysed assay (1 mL for *Planktothrix* and 20 µL for *Microcystis*) was also collected on each sampling day and analysed immediately.

3.2.3 Dual Fluorescence Intact/Lysed Assay

The intact/lysed assay samples were assessed with the dual-fluorescence technique described in Chapter 2, using cyanobacterial autofluorescence from photosynthetic pigments and the nucleic acid stain SYTOX® Green. Aliquots of *Planktothrix* (1 mL) were centrifuged at 14,100 × *g* (18 ˚C) for 30 min in microcentrifuge tubes, 980 µL of supernatant was removed, and the pellet (20 µL) was resuspended and transferred to a new microcentrifuge tube. *Microcystis* was able to be assessed without pre-concentration by centrifugation. The cell samples were mixed with an equivalent volume of 2 µM SYTOX Green (20 µL; final dye concentration of 1 µM) and incubated in the dark at room temperature for 30 min. An aliquot of the stained sample (10 µL) was mounted onto a microscope slide, sealed with nail polish and examined under an epifluorescent microscope (Olympus BX51, Tokyo, Japan) equipped with FITC and Texas Red fluorescence filters.

The FITC filter (excitation 465-500 nm; emission 510-545 nm, green channel) was used to detect the SYTOX Green fluorescence and the Texas Red filter (excitation 530-575 nm; emission 590-670 nm, red channel) was used to detect the autofluorescence emitted by the photosynthetic pigments present in the cells (i.e., chlorophyll-α and phycocyanin). The samples were analysed under 400× magnification for *Planktothrix* and 200× magnification for *Microcystis*. For each sample, ten fields of view (FOV) were observed, with each of these comprising two fluorescence images per FOV, acquired in the red and green channels, using a digital camera attached to the microscope (Olympus DP73, Tokyo, Japan).

The images were combined using CellSens software and subsequently processed with ImageJ, where each photo was converted to an 8-bit black and white image, and the threshold values were adjusted for both the red and the green channels. The *Microcystis* cells were counted using the "analyse particles" function. Heterotrophic bacteria (< 1 µm) were found in the non-axenic *Microcystis* cultures and were detected in the green channel only. In order to avoid this background interference, particles smaller than 2×2 pixels were excluded from the analysis. The filamentous *Planktothrix* strain was analysed by selecting each of the filaments and then calculating their surface area.

The percentage of lysed cells was calculated as follows:

$$l = \left(\frac{a}{A}\right) \cdot 100\ \%$$

where:

l = percentage of lysed cells in the sample

a = number of lysed cells counted

A = total number of cells counted

3.2.4 Cell Enumeration

An aliquot of each cell count sample (0.5-2 mL) was settled in 12-well plates (Corning Constar) with milli-Q water, so that each well contained a total volume of 4 mL. The volume to be settled and the dilution factor were adjusted in order to have between 20 and 60 cells per FOV for the *Microcystis*, and between 5 and 20 filaments per FOV for the *Planktothrix*. Cell enumeration was performed with an inverted microscope (Olympus CKX41) using 10 FOVs per sample. *Microcystis* samples were assessed at 800× magnification and cell counts were obtained using a manual differential counter. Cell concentrations for *Microcystis* samples were determined as follows:

$$N = \frac{S/b \cdot n \cdot f}{V}$$

where:

N = cell concentration in the culture (cells mL^{-1})

n = average of number of cells counted in 10 FOVs

S = total surface area of the bottom of the settling chamber (mm^2)

b = FOV area (mm^2)

f = dilution factor

V = volume of sample settled (mL)

The *Planktothrix* cell count samples were mechanically ground prior to microscopic observations, using a mortar and pestle to disperse amalgamations into individual filaments. Cell enumeration was carried out by measuring the length of all filaments in ten FOVs, using

the polyline tool on CellSens with an inverted microscope (Olympus CKX41) at 400× magnification. The total length of filaments in ten FOVs was divided by ten to yield an average filament length per FOV. The number of cells per FOV was calculated by dividing the average filament length per FOV by the average cell length. The proportion of the well surface observed in one FOV, any sample dilutions and the volume settled were then used to determine the cell concentration using the formula below. To determine the average *Planktothrix* CAWBG59 cell length, a light microscope (Olympus BX51) attached to a digital microscope camera (Olympus DP70) was used to measure the length of 50 *Planktothrix* cells (randomly chosen from at least 10 different filaments) at 1,000× magnification.

Cell concentrations for *Planktothrix* were calculated as follows:

$$N = \frac{\frac{S}{b} \cdot \frac{P}{p} \cdot f}{V}$$

where:

N = cell concentration in the culture (cells mL^{-1})

P = total length of *Planktothrix* filaments per FOV (µm)

p = average *Planktothrix* cell length (µm)

S = total surface area of the bottom of the settling chamber (mm^2)

b = FOV area (mm^2)

f = dilution factor

V = volume of settled sample (mL)

The concentration of lysed cells was calculated as follows:

$$L = N \cdot (\frac{l}{100})$$

where:

L = Concentration of lysed cells (lysed cells mL^{-1})

N = Cell concentration (cells mL^{-1})

l = Percentage of lysed cells in the sample

3.2.5 Microcystin Analysis

The cellular MC samples were extracted by adding 80% methanol + 0.1% formic acid (v/v; 1 mL) to each microcentrifuge tube containing the GF/C filter loaded with a sample. The tubes were sonicated in a bath sonicator (60 min, 53 KHz, 100% power) filled with water and ice. The toxin extracts were clarified by centrifugation (12,000 × *g*; 5 min, 18 °C) and the extract supernatant (0.8 mL) was transferred into a glass autosampler vial and stored in the freezer (-20 °C) until analysis. The extracellular samples did not require any further preparation. Microcystin concentrations were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The compounds were separated in an Acquity I-Class ultra-performance liquid chromatography system (Waters Co.), using a C_{18} column (Waters Acquity BEH-C_{18}, 1.7-µm, 50 × 2.1 mm) maintained at 40 °C in a column oven. The sample components were eluted using a flow rate of 0.4 mL min^{-1} and a gradient of 10% acetonitrile (mobile phase A) to 90% acetonitrile (mobile phase B), each containing 100 mM formic acid and 4 mM ammonia as described in Table 3.1.

Table 3.1 Liquid chromatography gradient for microcystin quantitation by LC-MS.

Time (min)	Flow (mL/min)	% A[a]	% B[b]
Initial	0.4	95	5
0.2	0.4	95	5
0.6	0.4	65	35
1.8	0.4	50	50
2.5	0.4	35	65
2.6	0.4	0	100
2.7	0.6	0	100
3.3	0.6	0	100
3.5	0.6	95	5
4.0	0.4	95	5
4.1	0.4	95	5

[a] Mobile Phase A (90:10 water/acetonitrile + 0.1% formic acid). [b] Mobile Phase B (10:90 water/acetonitrile + 0.1% formic acid).

The sample components were analysed on a Xevo-TQS mass spectrometer (Waters Co.) operated in positive-ion electrospray ionization mode (source temperature 150 °C; capillary voltage 1.5 kV; N desolvation gas 1,000 L h^{-1} at 500 °C; cone gas 150 L h^{-1}). Multiple reaction monitoring (MRM) channels that assessed the *m/z* 135 fragment ion from the protonated molecular cations were used to quantify each toxin ($[M + 2H]^{2+}$ for MC-RR and associated

variants; $[M + H]^+$ for the other MCs). The MRM conditions for each MC congener that was assessed are summarised in Table 2.

Table 3.2. Multiple reaction monitoring conditions for liquid chromatography.

Microcystin (MC)	Parent ion (*m/z*)	Daughter ion (*m/z*)	Cone Voltage (V)	Collision Energy (eV)
didesmethylMC-RR	505.70	135.1	40	27
desmethylMC-RR	512.70	135.1	40	27
MC-RR	519.70	135.1	40	27
MC-AR / MC-RA	953.60	135.1	40	65
didesmethylMC-LR/MC-RAba	967.70	135.1	40	65
desmethylMC-LR	981.65	135.1	40	65
MC-LR	995.70	135.1	40	65
MC-FR	1029.70	135.1	40	65
MC-YR	1045.70	135.1	40	70
MC-WR	1068.75	135.1	40	65

Primary standards of MC-RR, MC-YR and MC-LR (DHI Lab Products) were used to produce external calibration curves (2-100 ng mL^{-1}) and to quantify the MC congeners observed in the samples. The MC-YR and MC-RR congeners were calibrated using the MC-YR and MC-RR calibration curves, respectively. The MC-LR calibration curve was used to quantify the other congeners. To ensure that MC concentrations were within the linear response of the method, samples lying outside the standard curve (> 100 ng mL^{-1}) were diluted (either 1/20 or 1/50) in 50% MeOH and re-analysed.

The MC quota is the amount of MC in each individual cell. This was calculated for both strains by dividing the cellular MC concentration by the concentration of cells in the sample:

$$MC_q = \frac{CM}{C}$$

where:

MC_q = MC cell quota (ng $cell^{-1}$)

CM = Cellular MC concentration (ng mL^{-1})

C = Cell concentration (cells mL^{-1})

At times, the MC quota was converted to pg $cell^{-1}$ by multiplying by 1,000.

3.2.6 Prediction of Extracellular MC Concentration Using Lysed Cell Concentration

The lysed cells' contribution of MCs to the extracellular environment was calculated as follows:

$$MC_l = MC_q \times L$$

where:

MC_l = concentration of extracellular MC from lysed cells (ng mL^{-1})

MC_q = MC quota (ng $cell^{-1}$)

L = Concentration of lysed cells in the culture (lysed cells mL^{-1})

An adjustment was made for the concentration of lysed cells, using the half-lives of lysed cells determined for *Microcystis* and *Planktothrix* described in Chapter 2. The number of lysed cells that remained detected in the cultures was calculated as follows:

$$E = L \times (0.5^{\frac{TL}{HL}})$$

where:

E = Excess number of lysed cells detected

L = Concentration of lysed cells in the culture (lysed cells mL^{-1})

TL = Time lapse between sampling days

HL = SYTOX Green stain half-life (1.5 days for *Microcystis* CAWBG617 and 2.4 days for *Planktothrix* CAWBG59; see Section 2.2.7)

The surplus number of lysed cells was then subtracted from the original lysed cell concentration, to obtain a more accurate estimation of the concentration of lysed cells in the sample.

3.2.7 Statistical Analyses

All statistical analyses of the data were performed with R software (R core team, 2018). The influence of sampling time on the proportion of lysed cells, cell concentrations, and extracellular MC concentration were tested by one-way analysis of variance (ANOVA; PASW Statistics 18, SPSS Inc., Chicago, IL, USA), at a confidence level of 95%. Whenever the null hypothesis of the ANOVA was rejected, differences between groups were investigated with post hoc Tukey tests. Most of the data did not pass the normality and variance homogeneity Levene test and, therefore, was log-transformed prior to statistical analysis. A linear regression model was used to assess the relationship between the predicted extracellular MC contributed by the lysed cells and the observed extracellular MC concentration. The data in all graphs are represented as mean values ± standard error.

3.3 RESULTS

Cultures of *Microcystis* aeruginosa and *Planktothrix* sp. were monitored over 57 days. During this time, samples for cell enumeration, toxin concentration and an intact/lysed cell assay were collected and analysed. The SYTOX Green nucleic acid stain successfully stained the lysed cells from both cultures, emitting a bright green fluorescence that could easily be differentiated from the red fluorescence of chlorophyll-rich, healthy cells (Fig. 3.1).

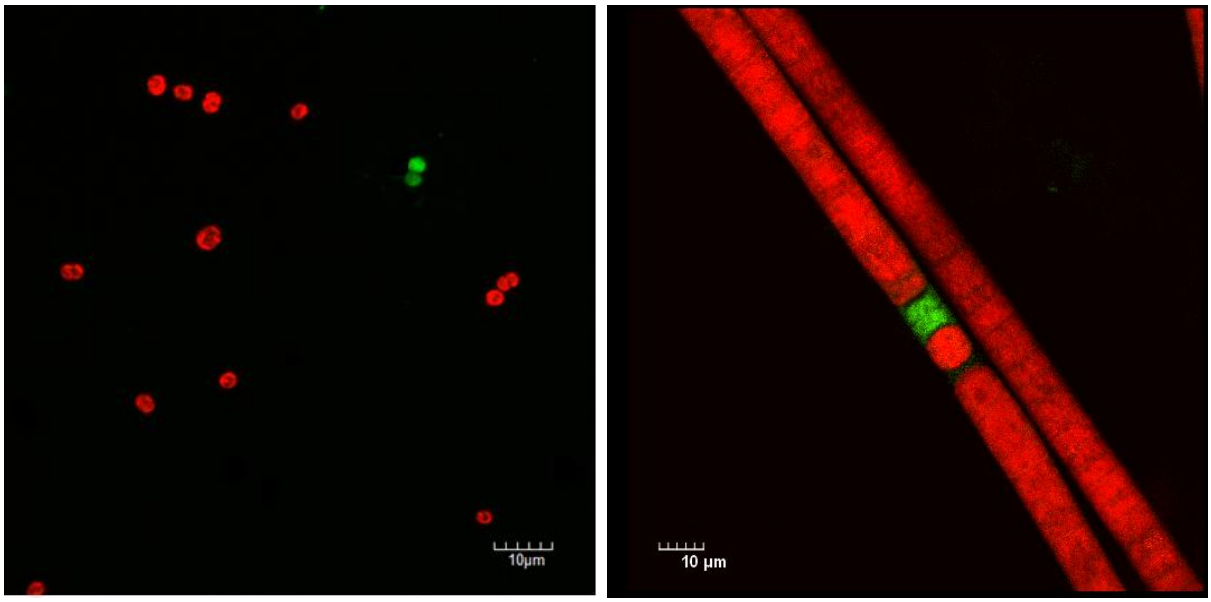


Figure 3.1 Confocal microscopy images of *Microcystis* (left) and *Planktothrix* (right) samples stained using SYTOX Green stain. Red fluorescence indicates cells with intact cell membranes; green fluorescence shows SYTOX Green permeated cells; Scale bar = 10µm.

3.3.1 *Microcystis* Culturing Experiment

Culture density and MC quota increased throughout the experiment (one-way ANOVA, $p < 0.001$). The *Microcystis* culture concentrations increased by over 3 times from 7×10^6 cells mL^{-1} to 25×10^6 cells mL^{-1} (Fig. 3.2). During this time, the MC quota increased by 2.3-times,

from 0.03 pg cell^{-1} at the start of the experiment to 0.07 pg cell^{-1} on the final sampling day ($p = 0.008$). The MC quota peaked at 0.11 pg cell^{-1} on day 51 (Fig. 3.2).

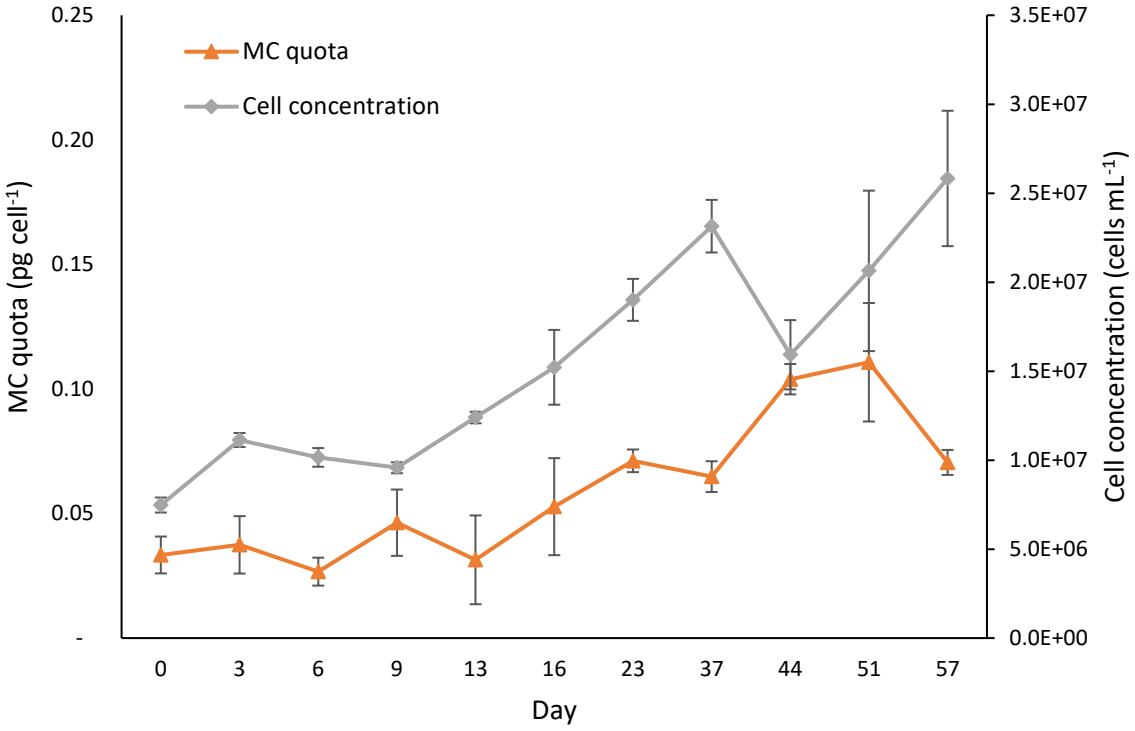


Figure 3.2 Time series of cell concentration and microcystin (MC) quota in the *Microcystis* culture over the 57 day sampling period. Data correspond to mean ± one standard error, n= 3.

There was a significant increase in the proportion of lysed cells occurred during the experiment (one-way ANOVA, $p = 0.002$). The percentage of lysed cells increased from 4% on the first day to 17% on the final day (Fig. 3.3). A post hoc Tukey test revealed that the proportion of lysed cells were significantly different between days 6 and 51 ($p = 0.03$), and days 13 and 51 ($p = 0.03$). The concentration of extracellular MCs also increased significantly during the experiment ($p < 0.001$), reaching a concentration of 196 ng mL^{-1} on the final sampling day

(Fig. 3.3-A). The first significant increase in extracellular MC since the beginning of the experiment (day 0) occurred on day 9 ($p < 0.001$). The next significant increase occurred between days 9 and 23 ($p = 0.015$), then between days 23 and 37 ($p= 0.004$), and the last significant increase occurred between days 37 and 51 ($p = 0.002$). At many of the sampling points there was a very low variability in extracellular MC between cultures (i.e., days 0, 3, 9, 13, 16, 23). There was a strong and significant correlation between the concentration of lysed cells in the *Microcystis* cultures and the concentration of extracellular MC ($R^2 = 0.52$, $p < 0.001$; Fig. 3.3-B).

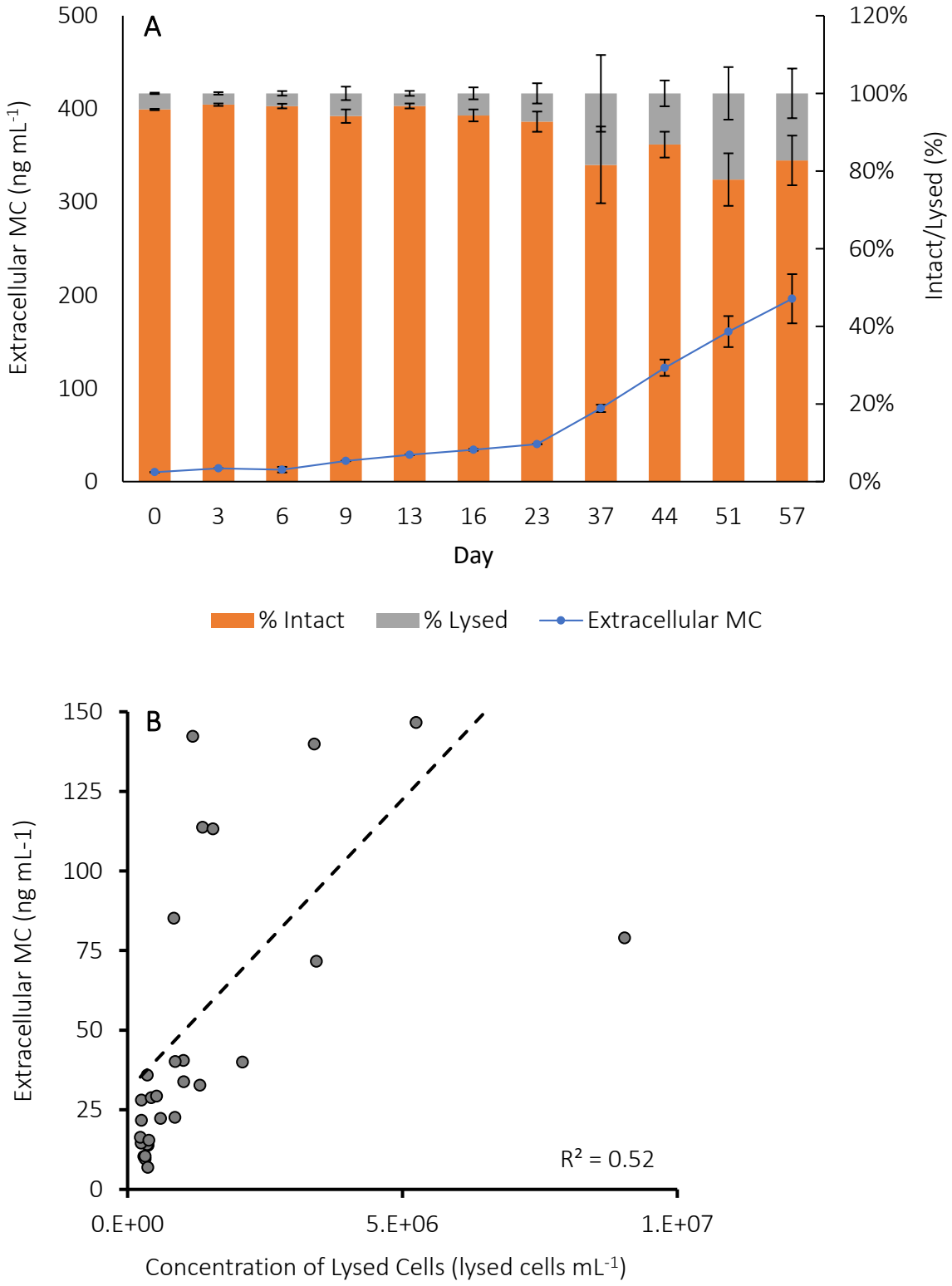


Figure 3.3 Time series of the concentration of extracellular microcystins (MCs) and the proportion of lysed and intact cells in the *Microcystis* cultures (A) and concentration of extracellular MC as a function of the concentration of lysed *Microcystis* cells (B).

To further investigate the significant relationship between cell lysis and extracellular MC, a model was developed to predict the concentration of extracellular MC in the culture medium from the *Microcystis* cell lysis data (see Section 3.2.6 for more details). When the predicted extracellular MC was compared to the extracellular MC measured in the cultures, a strong and significant relationship was observed (R^2= 0.55, $p < 0.001$, Fig. 3.4-A). However, the gradient of the linear regression showed that the concentration of extracellular MC was overestimated by a factor of 2.6. When the model was adjusted to compensate for excess signals from lysed cells that had not degraded between sampling points (using the half-life for *Microcystis* cells determined in Section 2.2.7), the strength and accuracy of the model was not substantially improved (R^2= 0.50, $p < 0.001$; gradient = 2.48 vs. 2.59; Fig. 3.4-B).

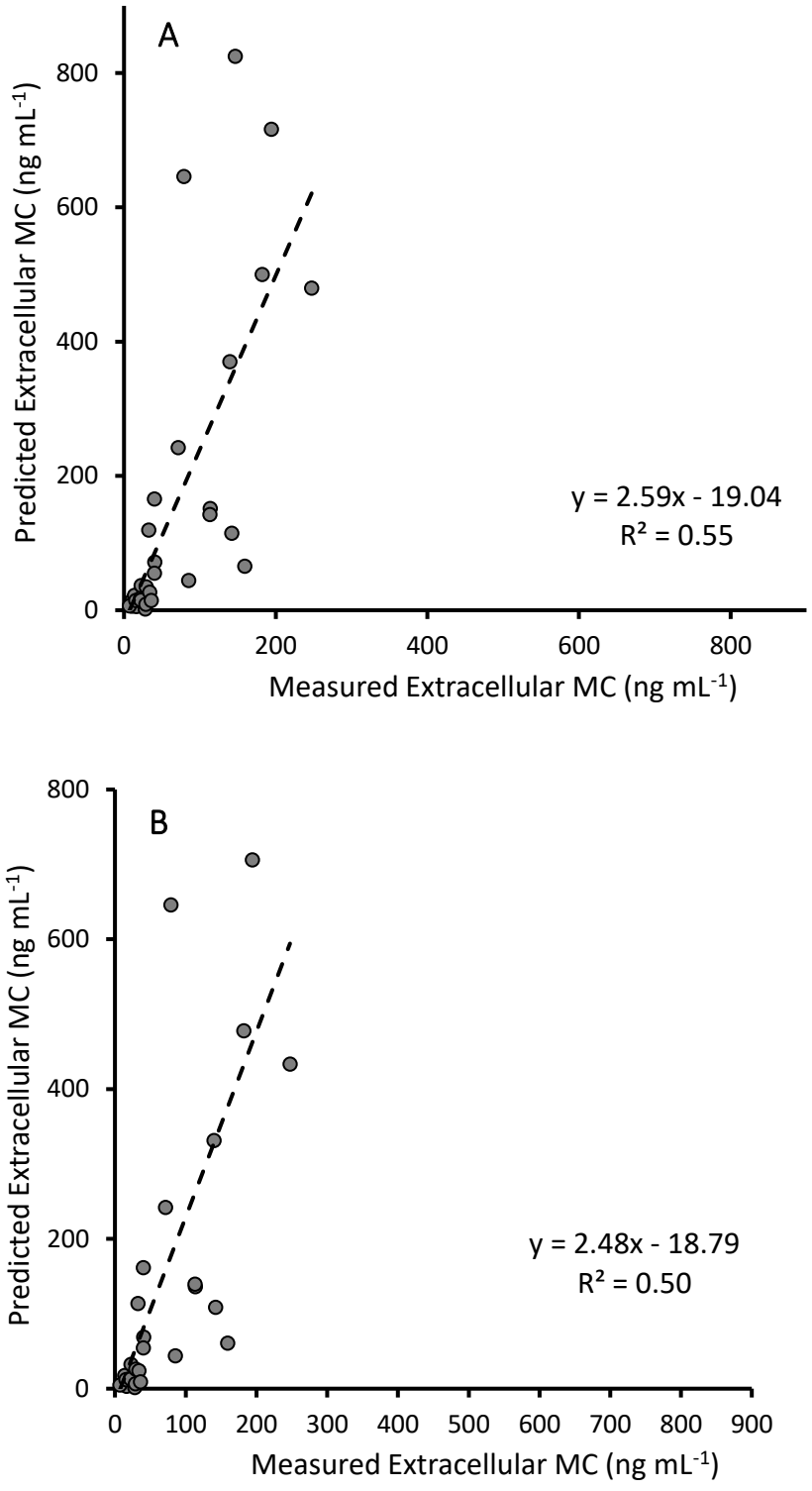


Figure 3.4 Predictive model for the contribution to extracellular microcystins (MCs) from lysed *Microcystis* cells: without adjustment for excess lysed cells (A); with adjustment for excess lysed cells (B).

3.3.2 *Planktothrix* Culturing Experiment

Planktothrix cultures also significantly increased in density (one-way ANOVA, $p < 0.001$) and MC quota (one-way ANOVA, $p < 0.001$) throughout the experiment (Fig. 3.5). The cultures grew from 1×10^3 cells mL^{-1} to 28×10^3 cells mL^{-1} on the final sampling day, and MC quotas increased by 6-times, from 0.02 pg $cell^{-1}$ on day 0, to 0.12 pg $cell^{-1}$ on the last sampling day, peaking at 0.17 pg $cell^{-1}$ on day 51 (Fig. 3.5). There was a 7.8-fold increase in MC quota between day 13 and day 23 (from 0.001 pg $cell^{-1}$ to 0.088 pg $cell^{-1}$; $p = 0.025$), and a 2.5-fold increase between day 44 and 51 (from 0.07 pg $cell^{-1}$ to 0.17 pg $cell^{-1}$; $p = 0.021$).

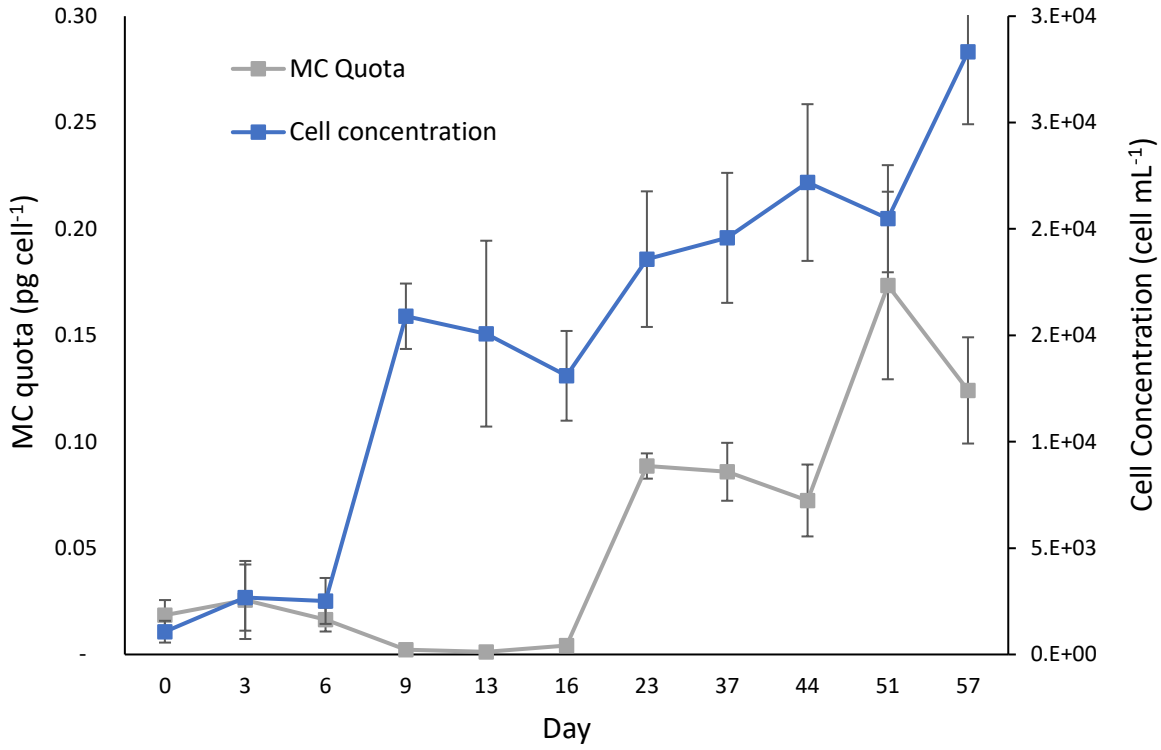


Figure 3.5 Time series of cell concentration and microcystin (MC) quota in the *Planktothrix* cultures over the sampling period. The datapoints correspond to the mean ± one standard error, n= 3.

There was a significant increase in the proportion of lysed cells in the *Planktothrix* cultures during the experiment (Fig. 3.6-A; one-way ANOVA, $p < 0.001$). While almost no cell lysis was

detected at the beginning of the experiment, by the final sampling day (day 57), an average of 37% of the *Planktothrix* cells had been lysed. Although a post hoc Tukey test revealed a significant drop in the proportion of intact cells between days 13 and 16 ($p = 0.002$), this proportion remained relatively constant until another statistically significant increase in cell lysis occurred between day 44 and day 57 ($p < 0.001$). During the experiment, there was a significant increase in the extracellular MC concentrations (one-way ANOVA, $p < 0.001$), which rose from 2.2 ng mL^{-1} on day 0 to 103.3 ng mL^{-1} on day 57 (Fig. 3.6-A). The highest extracellular MC concentration of 113 ng mL^{-1} was detected on day 44, but the concentration did not drop significantly thereafter (post hoc Tukey test, $p = 0.999$). As with the *Microcystis* cultures, at many of the sampling points the variations in extracellular MC between cultures (i.e., on days 0, 3, 6, 9, 16, 23, 37) were very low. There was a weak but significant relationship between the concentration of lysed *Planktothrix* cells and the extracellular MC concentration, with only 10% of the variance explained by the linear regression ($R^2 = 0.10$, $p = 0.006$; Fig. 3.6-B). The cell lysis model previously described for the *Microcystis* culturing experiment was also applied to the *Planktothrix* data. Its predictive accuracy for the *Planktothrix* data was weak and had a low slope value ($R^2 = 0.15$), but it resulted in a statistically significant (linear regression, $p < 0.001$; Fig. 3.7-A). As for the case of the *Microcystis* data, adjusting for excess lysed cells did not improve the model's strength (linear regression, $R^2 = 0.14$, $p < 0.001$; Fig. 3.7-B).

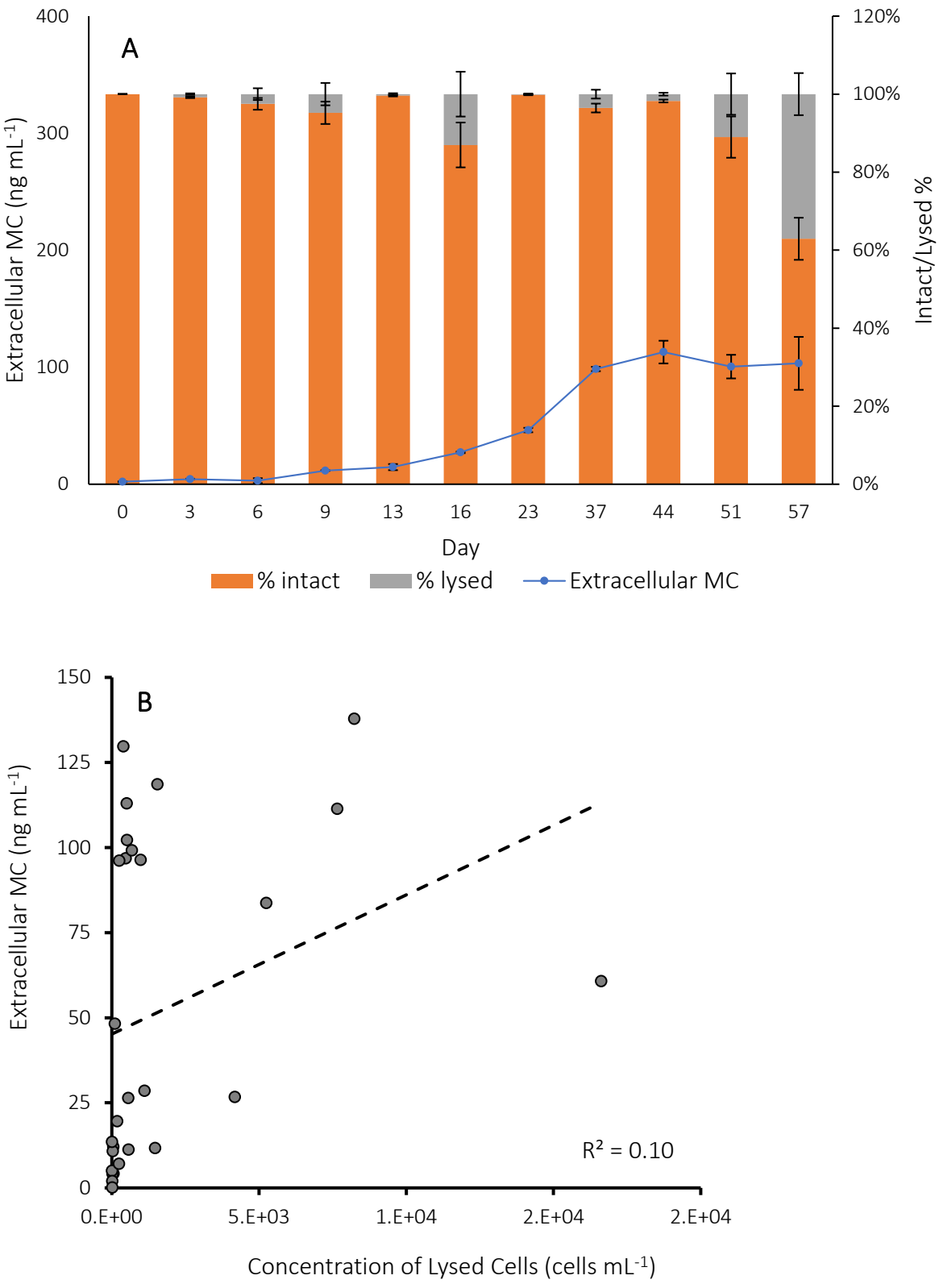
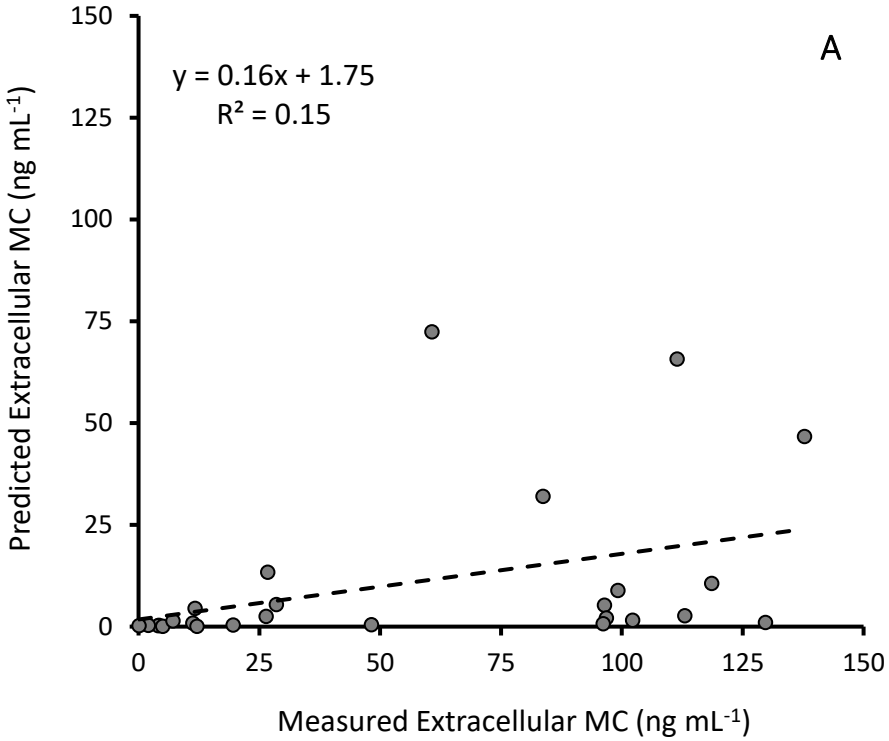


Figure 3.6 Time series of the concentration of extracellular microcystins (MC) and the proportion of lysed and intact cells in *Planktothrix* cultures (A), and concentration of extracellular MC as a function of the concentration of lysed *Planktothrix* cells (B).



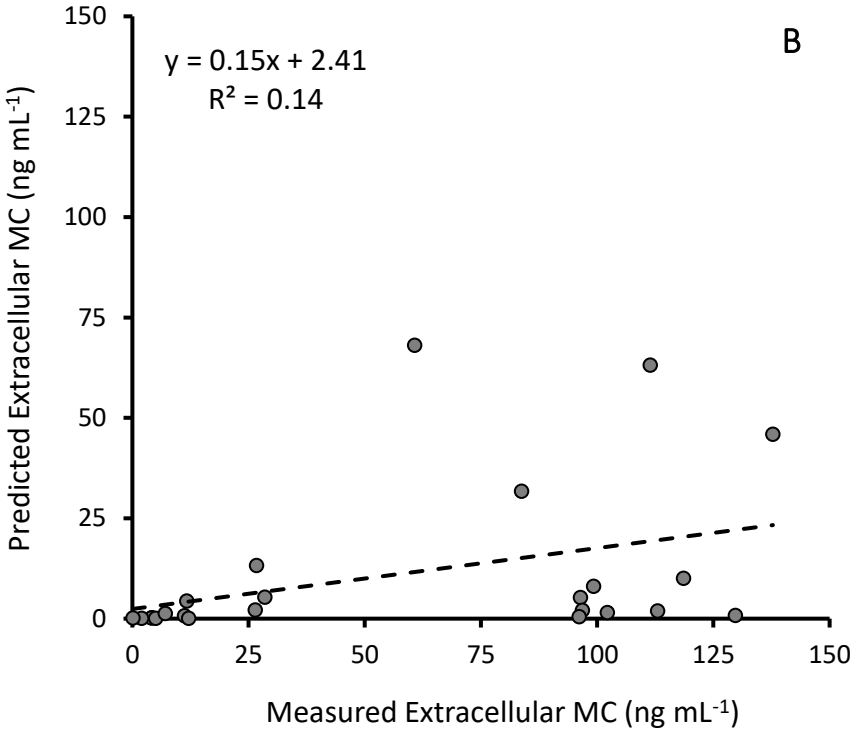


Figure 3.7 Predicted contribution of lysed cell MC to the extracellular environment, without adjustment for excess lysed cells (A), and with adjustment (B) for the *Planktothrix* cultures.

3.4 DISCUSSION

Microcystis and *Planktothrix* are common genera of bloom-forming freshwater cyanobacteria that are frequently associated with the production of the cyanotoxin MC. Although the physiological role of MC is still debated, there is growing evidence of its role as a signalling molecule (Huang et al., 2019). However, in order to perform such functions, it is implied that the toxin must be released into the extracellular environment. Whilst cell lysis provides a simple mechanism for this, this export strategy is lacking in control, when compared to active export. The presence of an ABC transporter protein in the *mcy* gene cluster (Pearson et al., 2004), as well as abnormal oscillations in extracellular toxins reported in previous studies (Wiedner et al., 2003; Cordeiro-Araujo et al., 2013), have provided some support for the theory proposing that MC are actively exported by cyanobacteria.

In a previous culturing experiment, the levels of extracellular aeruginosin, another secondary metabolite produced by *Microcystis*, remained constant while the intracellular aeruginosin quota fluctuated, suggesting that aeruginosins were likely not being actively exported out of the cell (Rogers, S., 2015). On the other hand, the same experiment showed a disproportional increase in extracellular MC, when compared to the extracellular aeruginosins observed on the same days, suggesting the potential for the active export of the former peptide. In further support of this hypothesis, the increase in extracellular MC also coincided with an increase in intracellular MC. While an attempt was made to monitor cell lysis during Rogers' experiment, the cell viability assay used for these measurements had a poor sensitivity and could not be used to assess cell viability. The dual-fluorescence assay developed in the present study could readily distinguish between intact and lysed cells, allowing the monitoring of cell lysis throughout the experiment.

During the present experiment, increases in extracellular MC were coupled with increased cell lysis for both species. There was a strong positive relationship between the concentration of lysed *Microcystis* cells and the concentration of extracellular MC, supporting the hypothesis that the concentration of extracellular MC observed in the cultures is explained by cell lysis. There was also a strong relationship between the measured extracellular MC concentrations and those predicted by the model for this strain. However, this model overestimated the concentration by a factor of 2.6 with respect to that measured by LC-MS/MS, possibly because protein-bound or degraded MCs were not detected by the analysis.

There was a weak but significant relationship between the observed quantities of cell lysis and the concentration of extracellular MC for *Planktothrix*. When the concentration of extracellular MC was predicted using the model with the *Planktothrix* data, the slope value obtained was low and there was a weak relationship between the measured extracellular MC concentration and the predicted concentration of lysed cells, and these results are considered unreliable.

Performing cell enumerations on the New Zealand strain of *Planktothrix* was problematic, in comparison to *Microcystis*. The cultures were viscous, with cells being visibly lost on pipette tips, grinder, and mortar during the sampling procedure and during the preparation of the 12-well plates for cell counts. As noted in Chapter 2, the organic sheath secreted by *Planktothrix* may have prevented the SYTOX Green stain from evenly permeating the lysed cells. Various difficulties have been encountered in the past with the application of dyes belonging to the SYTOX family to other filamentous cyanobacterial strains (Johnson et al., 2016, Sato et al., 2004), attributed to the presence of pores connecting the cells within a filament. Together, these factors may explain the different responses observed between the two types of cyanobacteria.

The levels of extracellular MC in *Microcystis* and *Planktothrix* were generally 3-10% of the cellular MC values. The extracellular MC concentration increased significantly during the experiment for both strains ($p < 0.001$). Whereas the MC concentrations continued to increase throughout the experiment with *Microcystis*, extracellular MC in *Planktothrix* peaked on day 44 and plateaued thereafter. The extracellular concentration of MC detected on days 37 for *Microcystis* was 6.5 times higher than that reported in a study of another *Microcystis* strain, at the same growth stage and grown under similar culturing conditions (Wei et al., 2016). In general, the external fraction of MC may contribute between as little as 5% and as much as 40% to the cellular fraction in axenic cultures (Orr and Jones, 1998; Rapala et al., 1997; Wiedner et al., 2003; Lyck et al., 2004; Jähnichen et al., 2011). It is also possible that the MC concentrations detected in the present study were underestimated, due to their degradation by heterotrophic bacteria or photobleaching (Kaebernick et al., 2000), or to the binding of MCs to proteins and peptides of other decaying cells (Wei et al., 2016). Further analysis of the kinetics of these reactions and processes should allow the model to be refined, leading to an improvement in its accuracy.

In the present study, the MC quotas increased despite the use of a constant light intensity throughout the experiment, following a pattern similar to that observed with extracellular MC concentrations. Lyck, S. (2004) found that MC quotas were the same at all stages of cell growth, whereas Long et al. (2001) observed a minimum of production at the lowest growth rate, and the highest production at maximum growth rate. However, high MC quota is not necessarily the consequence of a high rate of toxin synthesis, nor is a low MC quota necessarily the result of a low rate of MC synthesis. The lowest reported global MC quota for *Microcystis* strains is 0.006 pg cell^{-1} and the highest is 5.9 pg cell^{-1}, however, the New Zealand MC quotas are

generally higher than in other parts of the world (Puddick et al., 2019). In the present study, MC quotas doubled for *Microcystis* (0.03 pg cell^{-1} to 0.07 pg cell^{-1}), reaching levels comparable with the lowest median value observed in New Zealand (Puddick et al., 2019), whereas they increased by a factor of 8.5 in the case of *Planktothrix* (0.02 pg cell^{-1} to 0.17 pg cell^{-1}).

An increase in the transcription of two MC biosynthesis genes (*mcy*B, *mcy*D), which was not coupled with an increase in intracellular levels of MC (Kaebernick et al., 2000), nor with a rapid increase in extracellular MC (Liu et al., 2017), was also observed in *M. aeruginosa* cultures under high illumination intensities. This suggests that the toxins may be constitutively produced under low and medium light intensities, but could be released to the extracellular environment only when a certain higher threshold of light intensity is reached, showing that the putative MC export may be enhanced by the higher rates of cell lysis induced by higher levels of irradiance. Monitoring the same variables investigated in the present study under high irradiances may lead to an improved understanding as to whether the increase in extracellular MCs observed under such conditions may be the result of increased cell lysis, and whether this occurs after a certain light intensity threshold is passed.

A possible SYTOX Green artefact comes from the lysed cells that may remain detectable by the dye after several days. This was addressed in Chapter 2, where the half-lives of decaying cells were determined for *Microcystis* and *Planktothrix*. However, even after adjusting the model for this, the relationship between measured and predicted extracellular MC did not significantly improve, and this was probably caused by the interval between sampling days, which allowed most of the damaged cells to degrade. However, this adjustment to the model could prove to be advantageous in experiments where the sampling is carried out more frequently.

Overall, the findings of the present study indicate that cell lysis is potentially a major contributor of MC to the extracellular environment. However, investigations encompassing a wider range of MC producers, and further refinements of the methods should be undertaken to elucidate the mechanism behind the release of these toxins from cells. An improved understanding of this process would have important implications for the assessment of toxin concentrations in the water column, thus providing a useful tool for the prediction of MC bioaccumulation and its ecological impact on aquatic organisms (White et al., 2005). Progress in this area should contribute to the development of management strategies, which could be used to mitigate the toxicity of producing genera and allow the periods of greatest health risk during bloom events to be anticipated.

3.5 Conclusions

In order to test the hypothesis that the concentration of extracellular MC could be explained by cell lysis, a cell viability assay was coupled with analytical measurements of MC concentrations in two genera of bloom-forming cyanobacteria (*Microcystis* and *Planktothrix*). There was a strong and significant relationship between extracellular MC and cell lysis for *Microcystis*, whereas in the case of *Planktothrix* the relationship was weak, although surprisingly still significant. The model developed to predict the amount of extracellular toxins contributed by cell lysis showed that cell lysis over-estimated the concentration of extracellular MC. The same model again yielded weak but significant results for *Planktothrix*, suggesting that the conclusions for this strain should be treated cautiously. These results suggest that cell lysis is a substantial contributor to the development of extracellular MC in laboratory-grown *Microcystis* cultures. However, the data was not sufficiently accurate to conclude that the active export of MC did not also contribute to the observed quota of extracellular MC. A future

study combining this staining technique with other physiological markers, while monitoring extracellular concentrations of MC as well as MC quota, may further elucidate the pathway followed by MC once they have been produced.

CHAPTER 4: Discussion and Future Directions

While the biosynthesis pathway and toxic effects of the hepatotoxin microcystin (MC) are well understood, the physiological function of these metabolites remains a subject of debate (Huisman et al., 2018). The presence of an active export mechanism involved in regulating the release of toxins to the extracellular environment would partially support the role of these toxins as signalling molecules. Whilst increases in MC outside of cyanobacterial cells have long been attributed to cell lysis, studies suggest the potential for an active export mechanism involved in the release of the toxins: evidence of a putative ABC transporter gene in the *mcy* gene cluster (Pearson et al., 2004); increases in the translation of MC synthesis genes not matched by an increase in levels of intracellular MC (Kaebernick et al., 2000); localisation of MCs in the cell wall and sheath of intact cells (Shi et al., 1995); and abnormal oscillations in intra- and extracellular MCs (Cordeiro-Araujo et al., 2013). However, these studies have not excluded cell lysis as the primary contributor of MCs to the extracellular environment. In the first part of the current study, a dual-fluorescence assay was optimised for use on two common MC producers, *Microcystis* and *Planktothrix*. This staining procedure was then successfully employed in a culturing experiment, in an effort to assess whether the concentration of extracellular MC would be explained by the amount of cell lysis occurring in the cultures. As levels of cell lysis steadily increased in batch culture experiments, so did concentrations of extracellular MCs and the relationship between the two was strong and significant. This supports the hypothesis that cell lysis is a major contributor of MCs to the extracellular environment for *Microcystis.*

The dual-fluorescence assay described in Chapter 2 readily discriminated between lysed cells and intact cells for both *Microcystis* and *Planktothrix*. This was essential to the development of a model that predicts the contribution of extracellular MC from lysed cells. The assay worked best on *Microcystis*, but background fluorescence interference was detected when applied to *Planktothrix*. This background signal was due to the presence of a mucilaginous sheath containing heterotrophic bacteria. However, this problem was easily overcome by adjusting the threshold for image analysis so that heterotrophic bacteria could be excluded.

The optimal fluorescence parameters for these two cyanobacteria apply to laboratory-grown cultures, and SYTOX Green staining concentration and incubation times may need to be considerably different for field samples, as previously observed for *Microcoleus autumnalis* (Tashyreva et al., 2013). Moreover, while *Microcystis* is often found as single cells in laboratory conditions, it commonly forms colonies in the natural environment and secretes higher levels of mucilage (Yang et al., 2009), potentially leading to further complications with field samples. Problems were encountered when measuring cell concentrations for the *Planktothrix* strain because filaments were visibly lost in the preparation of samples for cell enumeration. Not surprisingly, when the lysis model was applied to the data collected for this strain, it did not yield a strong relationship between the observed levels of extracellular MCs and the levels contributed by lysed cells.

Determining whether naturally lysed cells stain similarly to ethanol-treated cells when incubated with the SYTOX Green dye would also further validate this assay and consequently the prediction model for the concentration of extracellular toxins. Since protein-bound or degraded MCs may not be detected by the LC-MS/MS analysis, refining MC sampling

procedures and determining whether any MC degradation occurs during sampling timeframes under the culturing conditions used may also improve the model.

The results described for *Microcystis* in Chapter 3 suggest that lysed cells contribute all of the MC detected in the extracellular environment, and do not support the MC active export hypothesis. Nonetheless, the role of these toxins as signaling molecules is still plausible, since for MCs to play such a role, they must first be released to the extracellular environment. In this scheme, it is possible that the release of MCs is associated with programmed cell death (PCD; Ding et al., 2012). Certainly, some MCs are released to the surrounding media during growth and under different environmental conditions (Wood et al., 2011). The occurrence of apoptosis-like death in both toxic and non-toxic *Microcystis* strains has also been demonstrated under various environmental conditions, with a significant release of MCs into the environment (Hu et al., 2019). Moreover, MC production and PCD are associated: they are assumed to confer a relative physiological advantage to producers; the occurrence of PCD naturally accompanies the release of MC; both processes have an ancient evolutionary root; there exists a strong association between PCD and upregulation of MC production; and metacascapases (i.e., genes linked with apoptosis induction) are found in both *Microcystis* and *Nodularia* (Hu et al., 2019).

In *Planktothrix,* MC release may be caused by necrosis, a more passive form of PCD (Ding et al., 2012). Necridia, or 'sacrificial' cells, are generally used for reproductive purposes by members of the order Oscillatoriales. In the dual fluorescence assay of the current study, SYTOX Green-stained *Planktothrix* cells were often located at either end of the filament, likely where the parent filament would have broken. Since cyanobacteria do not possess mitochondria, and PCD death is a light-dependent process, the photosynthetic apparatus of

cyanobacteria may have full control over cell death regulation (Ding et al., 2012). Programmed cell death and the subsequent release of toxins may be a mechanism adopted by cyanobacteria to cope with changing conditions, such as drastic fluctuations in light intensity and temperature (Schatz et al. 2007), and the advantages may include enhanced colony formation (Sedmak et al., 2005) and protection against photo-oxidation stresses such as high light or carbon deficiency (Latifi et al., 2009; Foyer and Shigeoka, 2011; Janichen et al., 2008). Under such conditions, MC biosynthesis becomes advantageous despite its high energy demand and a form of controlled death could confer an advantage to the remaining cells (Ding et al., 2012). In light of this, results from the current study do not necessarily exclude the possibility of a controlled release of MCs to the extracellular environment.

Bacteria must often coordinate physiological processes based on limited information about the external environment, and in many cases this is aided by the secretion and detection of small diffusible molecules, in a process called 'quorum sensing' (Cornforth et al., 2014). Quorum sensing systems are well known in gram-negative bacteria, as they can aid the coordination of important biological functions such as motility, aggregation, plasmid conjugal transfer, symbiosis and biofilm maintenance (Romero et al., 2011). This process is usually mediated by the accumulation of signaling molecules under higher cell densities, and it often controls the expression of more complex secondary metabolites. For instance, MC appears to be a proximal cue for the induction of genes responsible for the production of other secondary metabolites (Briand et al., 2016). Moreover, the presence of toxins outside the cells further stimulate its biosynthesis via enhanced expression of the *mcy* gene cluster (Schatz et al., 2007). Interestingly, recent studies with the gram-negative bacterium *Pseudomonas aeruginos*a provided evidence that, by using only multiple and interconnected signals with distinct half-

lives, the cells could infer both their social (density) and physical (mass-transfer) environments and simultaneously match gene expression to both (Cornforth et al., 2014; Moghaddam et al., 2014).

Cyanobacteria often need to coordinate movements within the colony and within the water column, for instance to access photosynthetically active radiation and nutrients, or to avoid high light intensities. Molecules involved in quorum sensing systems often guide the performance of such tasks, and the presence of such systems has been demonstrated to some extent for *Anabaena* (Romero et al., 2011), *Gloeothece* (Shariff et al., 2008) and *Trichodesmium* (Van Mooy et al., 2012). The association of MC production with colony formation (Sedmak et al., 2005), and protection against photo-oxidation stresses such as high light intensity or carbon deficiency (Latifi et al., 2009) suggests MC may play a part in quorum sensing systems and the extracellular movement of the toxins may trigger responses in the remaining cells. For instance, it was reported that extracellular MC-LR observed under high irradiances in *Microcystis* cultures is not actively transported into cells, but it is rather perceived and transduced by a receptor-mediated signaling cascade, providing some evidence for the involvement of MC in some intercellular communication process (Makower et al., 2015). One main argument against the allelopathic effects of MC is that this process will be effective only if toxin producing strains are sufficiently abundant and produce enough toxin to suppress toxin-sensitive strains (Kardinaal et al., 2007). Natural MC concentrations, as commonly found in *Microcystis* blooms, are generally too low to have allelopathic effects on other photoautotrophic organisms (Babica et al., 2006). Despite high *Microcystis* densities observed in the cultures, the extracellular fraction of MC detected in this experiment may still have been

too low to have any potential allelopathic effects, especially considering that it was between 3 and 10% of the total MC produced.

Although there is some evidence that MC provides an advantage over competing phytoplankton such as chlorophytes and diatoms (Pflugmacher, 2002; Wang et al., 2015), and offers defense against grazing (Rohrlack et al., 1999; Jang et al., 2008), other studies have found no correlation between toxin production and presence of *Microcystis* ecological competitors (Banerji al., 2019) or between direct exposure to grazers and MC gene expression (Harke et al., 2017). Since phylogenetic analyses revealed an early origin of MCs that predates the origin of cyanobacterial predators, it is possible that the release of MCs to the extracellular environment may have co-opted different purposes over the course of cyanobacterial evolution, such as anti-predator defense and allelopathy. It is also conceivable that MC toxicity to other organisms is purely fortuitous, and that the loss of MC-biosynthesis genes by non-toxic strains is explained by the presence of similar, structurally related but non-toxic compounds that may serve a similar physiological function, possibly at lower energetic costs.

Since in this study cell lysis explained the extracellular concentration of MC, and considering the high amount of carbon and nitrogen devoted to toxin biosynthesis, MC may also play a primarily intracellular role, conferring a direct adaptive advantage to the producer species (Ceballos-Laita et al., 2017). The relationship between MC production and cell division rates suggests an important role of MC in cellular metabolism of toxigenic strains (Orr and Jones, 1997). The role of MC in photosynthesis is partly supported by the finding that two-thirds of the intracellular MCs are located around the thylakoids of immuno-gold labelled *Microcystis* cells (Shi et al., 1995). However, MCs may also play a role in: binding to proteins under high light and oxidative stress conditions (Zilliges et al., 2011); maintaining the photosynthetic

machinery (Wang et al., 2018); sequestration or storage of P and trace metals such as iron (Sevilla et al., 2008; Shi et al., 2013; Harke and Gobler, 2013); removal of oxidative by-products (Latifi et al., 2009; Foyer and Shigeoka, 2011); and binding of the global nitrogen regulator NtcA protein in the bidirectional *mcy* promoter region, which is important in the carbon-nitrogen metabolism (Makower et al., 2014).

4.1 Future Directions

Broadening the applicability of the dual-fluorescence method to different MC-producing genera (e.g., *Dolichospermum, Anabaena*, *Nodularia*) could prove a useful tool to further determine the fate of MCs after they are produced, thus further elucidating the potential role of these toxins. For instance, the *mcy* gene cluster encoding for MCs between producing genera, and the *nda* gene cluster encoding for nodularins (hepatotoxins structurally similar to MC), share similar multienzyme components, but the position of the tailoring genes and of the putative ATP binding cassette transporter gene differ (Dittmann et al., 2005). The finding that nodularins produced by *Nodularia spumigena* form pores in lipid bilayers (Spassova et al., 1995) suggests that these toxins may also act as transmembrane transporters. A comparison between extracellular nodularins and MCs could therefore provide valuable information about the role of these toxins. However, while SYTOX Green stain has been successfully applied to *Planktothrix*, *Dolichospermum* and *Microcoleus* (Sato et al., 2004; Tashyreva et al., 2013), applying such staining techniques on other filamentous strains is often problematic. For instance, SYTOX Blue did not reliably stain *Anabaena* cells, potentially due to the leakage of the dye through intercellular pores (Johnson et al., 2015). Moreover, mucilage production and heterotrophic bacterial contamination may substantially complicate the application of SYTOX Green to *Nodularia*. Strategies to overcome these problems may encompass chemically

removing the mucilage sheath or growing the cultures with antibiotics, respectively. Considering that many MC-producers are filamentous, optimising the dye for these strains may prove an important development.

The use of 'dead cell' stains (including SYTOX Green) does not allow for the identification of intermediate states of cell injury that can be followed by cell recovery rather than death (Berney et al. 2007), which may partly explain why the model developed in this study overestimated the concentrations of extracellular MC contributed by lysed cells. The SYTOX Green stain has been successfully applied, in combination with other physiological markers, to detect different stages of cell injury on *Microcoleus autumnalis* (Tashyreva et al., 2013). The authors could classify cyanobacterial populations into four categories: (i) active and intact; (ii) injured but active; (iii) metabolically inactive but intact; (iv) inactive and injured, or dead. By combining the proposed set of physiological markers to monitor respiratory rate, membrane integrity, the presence and integrity of a nucleoid, and photosynthetic pigments, it should be possible to understand the physiological state of MC-producing cells, while at the same time following the intra- and extra-cellular movements of MC.

The ability to produce MC is not intrinsic to all taxa or strains. However, it is assumed that MC production provides an ecological advantage to the producer strains, considering its high energetic cost (Jahnichen et al. 2008). The increase in MC biosynthesis and higher concentrations of extracellular MC detected under high irradiances (Utkilen and Gjølme, 1992), suggest that light intensity alterations could provide an excellent tool for further investigations of the fate of MCs after they are produced. Because numerous cellular processes are also controlled by light intensity, factors such as cell division should also be closely monitored as this also has an impact on toxin transcription/production. Moreover, using a set of

physiological markers to monitor respiratory rate, membrane integrity, the presence and integrity of a nucleoid, and photosynthetic pigments may provide a better insight into other processes possibly involved in the release of the toxins and whether an active export mechanism may exist.

Climate change is likely to exacerbate the dominance of harmful cyanobacteria in aquatic ecosystems globally, increasing the need for a better understanding of toxin production and dynamics in the water column (Carey et al. 2012; Paerl and Paul 2012). Determining whether MCs or other cyanobacterial secondary metabolites are released in a controlled fashion by producer cells may shed light over the highly debated physiological role of the toxins. This could in turn assist monitoring and management procedures when toxic blooms form, and it may help predicting when levels of highest toxicity are reached.

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