Microcystin enhances the fitness of microcystin producing cyanobacteria at high light intensities by either preventing or retarding photoinhibition

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2013

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By

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Submitted in fulfilment/partial fulfilment of the requirements for the degree of *Philosophiae Doctor* to be awarded at the Nelson Mandela Metropolitan University

January 2013

Promoter: Prof T.G. Downing

To my late great-grandparents, Sydney and Kathleen Hart, who always believed in my potential for success. Your confidence in me has been a source of inspiration all my life. This is dedicated to you.

Table of Contents

Summary

Several genera of cyanobacteria produce microcystin, a monocyclic peptide, with a unique chemical structure. To date, there have been over a 100 different structural variants of microcystin which have been identified. Microcystin production is affected by numerous environmental factors. However, the primary modulating factor for intracellular microcystin quota is the intracellular N:C ratio. No clearly defined biological role has been described for microcystin. Proposed roles for microcystin include defence against plankton grazers, metal chelation, an infochemical and a protectant against oxidative stress. There is sufficient evidence to support a biological role for microcystin in photosynthesis: microcystin is predominantly located in the thylakoid membranes, the microcystin gene cluster is differentially expressed as a function of light and a growth advantage for the microcystin producer in saturating light intensities. The purpose of this study is to investigate a possible biological role for microcystin in preventing photoinhibition and thus explaining the growth advantage observed in toxin-producers over non-toxin-producers. The uptake of exogenous microcystin was observed in *Synechocystis* PCC 6803 which was internalized and located in the thylakoid membranes and caused the inhibition of photosynthesis. Microcystin variants and increasing concentrations of microcystin-LR had no effect on the fluidity of the thylakoid membranes. The exposure of thylakoid membranes from *Synechocystis* PCC 6803 to physiologically relevant concentrations of different microcystin variants resulted in the inhibition of photosystem II activity but not photosystem I activity. The inhibition of photosystem II was variant dependent and concentration dependent for microcystin-LR and microcystin-RR. Chlorophyll a fluorescence data showed that photosystem II inhibition was caused by the inhibition of the oxygen evolving complex. Furthermore, a completion study revealed that the microcystin-producing *Microcystis* PCC 7806 had a competitive advantage over the non-microcystin producing Δ*mcyA* mutant of *Microcystis* PCC 7806 at high light intensities. The data indicates that microcystin protects the toxin-producer by either retarding or preventing photoinhibition and thus identifying the first data supported function for microcystin in cyanobacteria.

Acknowledgements

The author would like to express his sincere appreciation to the following individuals:

Prof T.G. Downing, Department of Biochemistry and Microbiology, Nelson Mandela Metropolitan University, for his invaluable guidance and funding throughout this study

Prof S. Barnard and Dr J. Berner, North West University for their assistance and use of equipment

Dr J van Tonder, University of Pretoria for his assistance and use of equipment

W. Wilde, A. Erasmus, and S. Pambuka, Department of Biochemistry and Microbiology, Nelson Mandela Metropolitan University, for all their assistance and support

Prof V Oosthuizen, HOD, Department of Biochemistry and Microbiology, Nelson Mandela Metropolitan University, for all his encouragement and support throughout the course of this study

C. Sember, M. Esterhuizen-Londt, S. Downing (The CRG crew), Department of Biochemistry and Microbiology, Nelson Mandela Metropolitan University, for all their support and assistance in the lab

The staff, post-graduate and under-graduate students of the Department of Biochemistry and Microbiology, Nelson Mandela Metropolitan University, for all their encouragement, support and laughs throughout the course of this study

M. Scheckle and J. Long for editorial assistance

My family and friends for all their encouragement and support throughout the course of this study

My wife, Kara Phelan, for all her love, encouragement, support and tea throughout the course of this study

The National Research Foundation for their financial assistance throughout the course of this study

List of Abbreviations

3-PGA	Glycerate-3-phosphate
ADDA	2S,3ES,8S,9S-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-deca-4E,6E-dinioc acid
A_o	Primary electron acceptor
cyt	Cytochrome
DCPIP	Dichlorophenol-indophenol
DGDG	Digalactosyl diglycerides
DPH	1,6-diphenyl-1,3,5-hexatriene
E_m	Redox potential
F	Phenylalanine
HPLC-MS	High Pressure Liquid Chromatography-Mass Spectrometry
L	Leucine
MC	Microcystin
MCs	Microcystins
MGDG	Monogalactosyl diglycerides
M-PEA	Multi-functional Photosynthetic Efficiency Analyser
MT	Δ*mcyA* and Δ*mcyB*
OD	Optical Density
OEC	Oxygen Evolving Complex
PAR	Photosynthetically Active Radiation
PC	Plastocyanin
Pc	Phycocyanin
PCC	Pasteur Culture Collection
PE	Phycoerythrin
PEs	Phycoerythrins
PG	Phosphatidyl glycerol
Pheo	Pheophytin
PI_{ABS}	Performance Index Absorbance
POD	Peroxidase
PS I	Photosystem I
PS II	Photosystem II
Q_A	Quinone
Q_B	Plastoquinone
Q_i	Quinone-binding site
Q_o	Plastoquinol binding pocket
R	Arginine
RC	Reaction Centre
ROS	Reactive Oxygen Species
Rubisco	Ribulose -1,5- bisphosphate carboxylase/oxygenase
sGST	Soluble glutathione S-transferase
SQDG	Sulphoquinovosyl diglycerides
Tyr	Tyrosine
TyrZ	Tyrosine
W	Tryptophan
WT	*Microcystis* PCC 7806
Y_Z	Tyrosine

List of Figures

List of Tables

Chapter 1

1.1 Literature Review

1.1.1 Introduction

Cyanobacteria are Gram-negative, oxygenic photosynthetic prokaryotes that produce a wide range of biologically active metabolites (Carmichael 1997). Many of these metabolites are toxic, including the commonly occurring heptapeptide microcystin (MC) hepatotoxins. Numerous genera of cyanobacteria such as *Microcystis*, *Anabaena*, *Nostoc*, *Planktothrix*, *Oscillatoria*, *Hapalosiphon* and *Anabaenopsis* produce microcystins (MCs); however this is not true for all genera or taxa of cyanobacteria (Chorus and Bartram, 1999). Microcystin has been extensively researched and, given the complexity of the MC structure, the metabolic burden of MC production, and the degree of environmental modulation of MC cellular concentration and production rate, it is likely that the production of MC results in an ecological benefit to the producer (Orr and Jones, 1998). However, the exact physiological role or metabolic significance of MC remains unknown. Proposed roles for MCs include a defense mechanism against plankton grazers (Jang *et al*.,2003, Jang *et al*., 2004), potential intracellular functions, such as ferrous iron (Fe^{2+}) chelation (Humble *et al*., 1994, Utkilen and Gjølme, 1995), and putative extracellular functions relating to cyanobacterial interactions with phytoplankton, zooplankton and bacteria (Pearson *et al*., 2004). MC production is influenced by numerous environmental parameters. However, the most significant environmental parameter that affects microcystin production is medium nitrogen to phosphorus ratio, which corresponds to intracellular nitrogen to carbon ratio (Downing *et al*., 2005a; Downing *et al*., 2005b). Additionally two thirds of the total intracellular MC content is localized within the thylakoid area (Young *et al*., 2005, Shi *et al*., 1995) and a strong correlation between MC and growth rate and MC and performance index (PI_{ABS}) at saturating light intensities grown in excess nitrogen (Phelan, 2009) supports a possible role for microcystin in photosynthesis.

1.1.2 Cyanobacterial Photosynthesis

Cyanobacteria are capable of performing respiration and photosynthesis simultaneously on the same membrane system. The photosynthetic process in cyanobacteria is very similar to that of higher plants, with the main differences being that cyanobacteria use phycobilisomes for light harvesting, and there is a higher ratio of PS I to PS II. The photosynthetic apparatus of cyanobacteria is composed of five functional protein complexes, namely phycobilisomes, photosystem II (PS II), photosystem I (PS I), plastoquinone-plastocyanin oxidoreductase and ATP synthase (Reuter and Müller, 1993).

1.1.2.1 Photosynthetic apparatus

1.1.2.1.1 **Phycobilisomes**

The predominant function of the phycobilisomes is light harvesting and energy migration. These light harvesting antennae are sensitive to environmental factors such as light. Cyanobacteria can alter their phycobilisomes by changing the size or phycobiliprotein composition or rearrangement of the core to influence energy sharing in response to their environment (Reuter and Müller, 1993). There is a strong correlation between the number of PS II core particles and the number of phycobilisomes (Joshua *et al*., 2005). Phycobilisomes absorb light in the visible spectrum between 480 nm and 650 nm. The most common phycobilisome structures found in cyanobacteria are the hemidiscoidal phycobilisomes. These phycobilisomes consist of a tri-cylindrical core with six rods extending from the core. The phycobiliproteins in the phycobilisome are arranged so that energy can flow from the high-energy biliproteins to the low-energy biliproteins (figure 1.1). Phycoerythrins (PEs) and phycoerythrocyanin are examples of high-energy biliproteins that absorb light of the visible spectrum between 480 nm to 570 nm. Phycocyanin (Pc) absorbs light between 550 nm to 650 nm and is an intermediate-energy biliprotein, whereas allophycocyanins are the lowest energy phycobiliproteins which absorb light between 650 nm to 655 nm and transfer energy to PS II (MacColl, 1998).

1.1.2.1.2 Photosystem II

The PS II reaction centre (RC) is a large complex of pigment-bearing proteins that are embedded into the thylakoid membrane. This reaction centre is made-up of approximately 25 protein subunits and 13 redox cofactors (Hankamer *et al*., 1997). Electron transfer occurs in major proteins of the central core, D1 and D2. D1 and D2 form a protein heterodimeric complex which contains a Mn cluster, P680, pheophytin (Pheo), a quinone (Q_A) and a plastoquinone (Q_B) (figure 1.1). This complex contains all the co-factors involved in water oxidation and plastoquinone reduction (Goussias *et al*., 2002; Dwivedi and Bhardwaj, 1995). CP43 and CP47 are two symmetrically arranged proteins that surround the heterodimeric core and contain sites for chlorophylls, which play a role in light collection (Dwivedi and Bhardwaj, 1995). Cytochrome (cyt) b_{559} is another important protein that is associated with the core and contains a proto-haem and two small trans-membrane units. The exact function of cyt b559 is not known but it has been shown to play a role in the assembly of PS II RC and the Mn cluster and a possible protective function against oxidative damage (Goussias *et al*., 2002). In plants, three extrinsic polypeptides (33, 23 and 17 kDa) are found on the luminal surface of the RC. Their main functions are to maintain the levels of Ca^{2+} (23 kDa) and Cl^- (33, 23 and 17 kDa) and to protect the catalytic site of the RC from reductive attack. However, in cyanobacteria, these three polypeptides are absent and are replaced with other proteins, one of which is a cyt c_{550} that has no redox role (Nelson and Yocum, 2006). Another is Psb L, which is a small 4.4 kDa trans-membrane polypeptide that is needed for oxidation of tyrosine z (TyrZ) (Goussias *et al*., 2002).

1.1.2.1.3 Cytochrome b_6f

Cyt b_6f complex contains three subunits that are required for electron transport: cyt b_6 contains two b-haems with a redox potential of E_m = -84 mV and –158 mV, cyt *f* with a covalently bound c-type haem (E_m = 330mV) (Pierre *et al*., 1995) and the Riekse protein that has an $[Fe_2S_2]$ cluster with a redox potential of E_m = +290 mV (Nitschke *et al*., 1992). A high potential path is formed by the cyt *f* and Riekse protein and a low potential path is formed by the cyt b_6 on the opposite side of the membrane. The plastoquinol binding pocket (Q_o) is formed by subunit IV together with the Riekse protein and cyt b_6 on the lumen side of the membrane. On the opposite side of the membrane a quinone-binding site (Q_i) is predicted. The Cyt b_6f

complex is composed of another four small units and a chlorophyll *a* and a β-carotene molecule (Breyton, 2000). Cyt b_6f complex is involved with the transfer of electrons from the Q_B of PS II RC to either plastocyanin (PC) or cyt c_{553} (figure 1.1) and is a proton-pumping complex that generates a proton gradient which is used for ATP synthesis by the ATP-synthase (Campbell *et al*., 1998).

1.1.2.1.4 Plastocyanin

Plastocyanin (PC) is a small copper-binding protein made-up of 97 to 104 amino acids. This protein consists of an eight-stranded, antiparallel β-barrel with a copper atom. The copper atom forms liganded complexes with the side chains of cysteine, methionine and two histidines (Díaz-Quintana *et al*., 2003). The best known structure of plastocyanin is where the copper site is at one end of the molecule, most commonly known as the northern site. The surface at this end of the protein is referred to as the hydrophobic patch because it consists only of hydrophobic residues. On the eastern side of the plastocyanin molecule a negative patch is found. This patch consists of negatively charged side chains of glutamyl and aspartyl residues. The copper atom in the PC molecule is exclusively involved in electron transport and the negative and hydrophobic patch is thought to be involved in the interaction between PC and cyt b_6f complex and $P700^+$ of PS I RC (Díaz-Quintana *et al*., 2003). PC has three main functions: firstly to maintain the redox potential of the copper binding site so that it lies in-between cyt *f* and $P700^+$, secondly it must be able to interact with physiological reaction partners and finally allows electron transfer in and out of the copper centre (Nelson and Yocum, 2006).

1.1.2.1.5 Photosystem I

PS I in cyanobacteria is a large multi-subunit protein complex which consists of 12 proteins that is arranged in a trimeric form in cyanobacteria. The thermophilic cyanobacterium *Synechococcus elongates* has 96 chlorophyll *a* molecules, 22 carotenoids, three [4Fe4S] clusters and two phylloquinones associated with the 12 proteins of PS I (Jordan *et al*., 2001). PsaA and PsaB, are large membrane intrinsic subunits, which form a central heterodimer in the PS I trimer (figure 1.1). This central dimer contains all the components for the electron transport chain in PS I from P700 to F_X (Fromme *et al*., 2001). This includes six chlorophylls, two phylloquinones and the first of three [4Fe4S] clusters. The remaining two [4Fe4S] clusters are associated with the central positioned PsaC, which is located on the stromal side of the

thylakoid membrane (Jordan *et al*., 2001). The light harvesting complex consists of 90 chlorophyll *a* molecules and 22 carotenoids surrounding the inner core and two peripheral regions of PsaA and PsaB (Fromme *et al*., 2001). PsaF, PsaJ and PsaX are integral membrane proteins of PS I found on the distal side of the trimer axis where the N-terminus of PsaF is exposed to the luminal side of the membrane, whereas PsaL, PsaI and PsaM are intrinsic membrane proteins on the proximal side of the PS I trimer. These six proteins are associated and interact with the light harvesting complex of PS I (Jordan *et al*., 2001). PsaD and PsaE are found on the stromal side of PS I and form the stromal hump with PsaC (Fromme *et al*., 2001).

1.1.2.2 Electron flow in the cyanobacterial membranes

The RC chlorophyll of PS II is excited when energy is transferred from the phycobilisomes. This energy transfer is non-radioactive, directional and extremely efficient. An electron is raised to a higher energy level when light excites PE and PC rods in the phycobilisome. The neighbouring AP molecule of the phycobilisome core receives the energy from PE and PC via resonance energy transfer (MacColl, 1998).

The excited chlorophyll *a* molecule in turn excites P680 in the D1/D2 heterodimer to form P680* which is an exceptional donor (Remy *et al*., 2004). When P680* donates an electron to Pheo to form $Pheo^-$ a radical cation $P680^+$ is formed. This transfer is incredibly rapid as it only takes a few picoseconds. $Pheo^-$ is oxidised to Pheo when it transfers an electron to Q_A, a protein-bound plastoquinone, to form Q_A^-. Finally, Q_A^- transfers its electron to a more loosely bound plastoquinone Q_B, which forms Q_B^-. In order for the fully reduced form of Q_BH_2 to be formed (Masojidek *et al*., 2001), a double reduction and protonation of Q_B^- must occur. Once Q_BH_2 is fully reduced, it is released from the PS II binding site and exchanged for an oxidised plastoquinone (Breyton, 2000). $P680^+$ is returned to its ground state by accepting an electron from the oxidation of water (figure 1.1). The general pathway for electron transfer in the PS II RC can be summarised as follows:

$$H_2O \rightarrow [Mn_4CaCl] \rightarrow Y_z/Y_z^* \rightarrow P680/P680^+ \rightarrow Pheo/Pheo^- \rightarrow Q_A/Q_A^- \rightarrow Q_B/Q_B^-$$

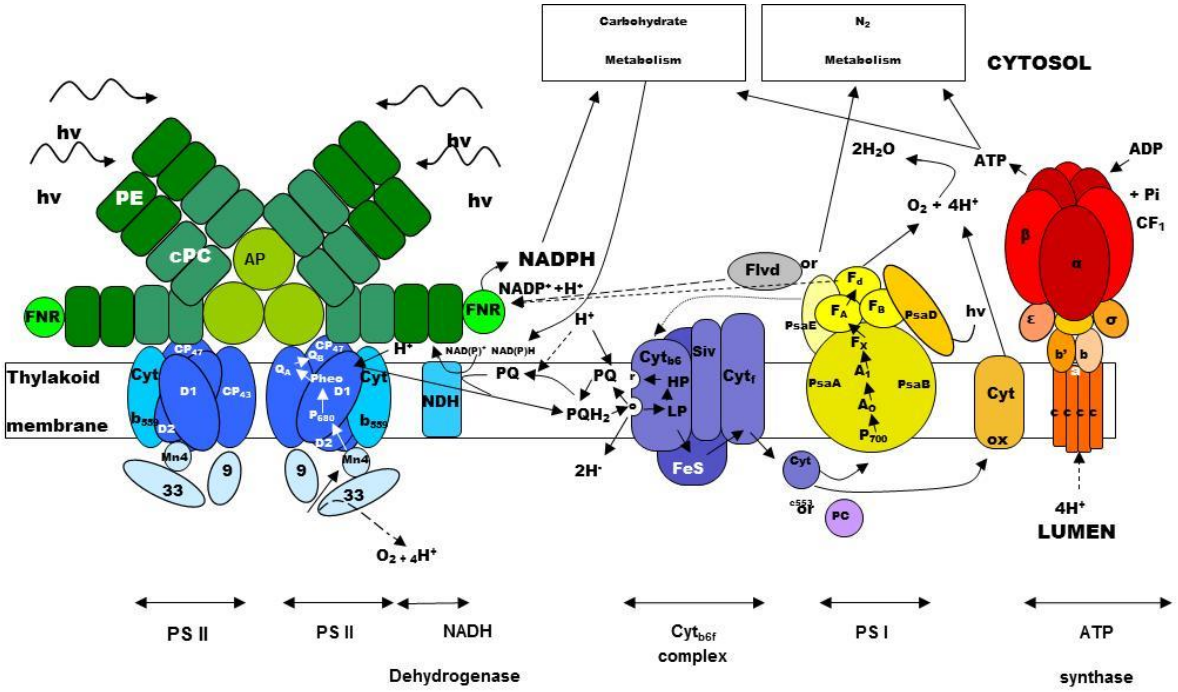

Figure 1.1: The transport of electrons in *Synechocystis* sp. strain PCC 6701 thylakoid membrane (see text for details).

Abbreviations: hv, photons of visible light; PE, phycoerythrin $\alpha_3\beta_3$ trimers; CPC, phycocyanin $\alpha_3\beta_3$ trimers; AP, allophycocyanin; Cyt b_{559}, cytochrome b_{559}; Mn4, manganese cluster of the O_2; P_{680}, dimeric chlorophyll center; Pheo, pheophytin; Q_A, quinine secondary electron acceptor; Q_B, plastoquinone; NDH, NAD(P)H dehydrogenase; cyt b_6, cytochrome containing high and low-potential heme centers; S_{IV}, subunit IV of cytochrome *bf* complex; Fe-S, iron-sulfur redox center; PC, plastocyanin; Cyt c_{553}, cytochrome c_{553}; PsaA and PsaB; chlorophyll *a* binding proteins (core of PS I); Fd, ferredoxin; FNR, ferredoxin/flavodoxin NADPH oxidoreductase; Cyt ox, cytochrome oxidase complex (modified from Campbell *et al*., 1998).

The redox potential (E_m) of the primary oxidant, $P680/P680^+$ has been estimated to be positive 1.12 V. This was based on the redox potentials of the intermediates that are summarised above for electron transport through the PS II RC: O_2/H_2O, + 0.93 V; Y_z^*/Y_z, + 0.97 V; Q_A^-/Q_A, -0.03 V and Q_B/Q_B^-, + 0.03 V (Nelson and Yocum, 2006).

The overall reaction initiated by light in PS II is shown by the following equation:

$$\mathbf{4\ P680 + 4H^+ + 2Q_B + 4\ photons \longrightarrow 4\ P680^+ + 2Q_BH_2}$$

The fully reduced Q_BH_2 binds to the plastoquinol binding pocket (Q_o) of the cyt b_6f complex. The plastoquinone molecule undergoes oxidation at the Q_o site. This leads to the expulsion of two protons into the lumen and the injection of two electrons. This turnover of the cyt b_6f complex is known as the Q-cycle (Breyton, 2000). The two

electrons which are released have two different potentials. The one electron is of a high-potential path and is used to reduce PC; the other is of a low-potential path (Breyton, 2000). PC then donates an electron to $P700^+$ which is a powerful oxidising agent (Mi *et al*., 1999). $P700^+$ is formed when light-induced charged separation oxidises P700 with a E_m of +0.43 V to form $P700^-$ and then an electron from $P700^-$ is transferred to the primary electron acceptor $A_{0,}$ which is a chlorophyll *a* monomer with an E_m = -1 V. A_1, a phylloquinone, (E_m = -0.8 V) is reduced by A_0 which in turn reduces F_X, an interpolypeptide which is an iron-sulfur protein (4Fe-4S). F_X has an E_m of -0.705 V. The electron from F_X is finally transferred to F_A and F_B with E_m's of -0.52 V and -0.58 V respectively (Nelson and Yocum, 2006). These two final electron acceptors are 4Fe-4S clusters (figure 1.1). The final electron carrier in the chain is a flavoprotein ferredoxin which is an $NADP^+$ oxidoreductase. This transfers electrons from the reduced ferredoxin to $NADP^+$ (Mi *et al*., 1999)

The overall reaction which occurs in PS I is represented by the following equation:

$$\mathbf{2Fd_{red} + 2H^+ + NADP^+ \longrightarrow 2Fd_{ox} + NADPH + H^+}$$

PS I and PS II are both required to move electrons from H_2O to $NADP^+$. A percentage of the light which is absorbed is conserved as NADPH. Simultaneously an electrochemical potential is being formed by protons being pumped across the thylakoid membrane. ATP synthase uses this proton gradient to drive the synthesis of ATP. Besides for ATP, NADPH is synthesised as well and is also an energetically-rich compound.

The overall reaction for non-cyclic photophosphorylation is summarised below:

$$\mathbf{2H_2O + 8\ photons + 2NADP^+ + 3ADP + 3\ Pi \longrightarrow O_2 + 2NADPH + 3ATP}$$

1.1.2.3 Oxygen-Evolution Complex

As stated above, $P680^+$ has to return to its ground state in order for it to capture another photon. In cyanobacteria this is achieved by an electron being donated to $P680^+$ by the splitting of two water molecules. This leads to the formation of four electrons, four protons and molecular oxygen. A single photon does not possess

sufficient energy to split the covalent bonds in water. Four photons are required for the photolytic cleavage of water. The four electrons that are released from splitting the two water molecules are not accepted immediately by $P680^+$. Instead, electrons are passed one by one by the OEC to $P680^+$. A Tyr residue of the OEC is responsible for passing the electron to $P680^+$ (Keren *et al*., 2004; Goussias *et al*., 2002).

The model for O_2 evolution is based on a set of oxidation states that is referred to as S-states. This model had five states designated from S_0 to S_4 of the enzyme and the number of the state represents the number of positive-charged equivalents. The light-driven formation of molecular oxygen is a linear reaction with a sequence of photocatalysed oxidations. The S_1 state is a highly stable state of the enzyme in the dark, therefore for oxygen to be released only three photochemical turnovers are needed. The S_2 and S_3 states are incredibly unstable and return to the S_1 state in darkness. Upon formation of the S_4 state, it reacts rapidly with water to form oxygen and returns the enzyme to its reduced form, S_0 (Kok *et al*., 1970; Barber *et al*., 2004). The S_0 state is the most stable state. In order to convert the S_0 state into the S_1 state a neutrally charged Tyr free radical, Tyr* is required (Styring and Rutherford, 1987). Tyr* is formed by Tyr donating an electron to $P680^+$ while a proton is lost. It is believed that Tyr* is oxidized through electron transfer equilibrium between the mediating co-factors (Goussias *et al*., 2002) (figure 1.2).

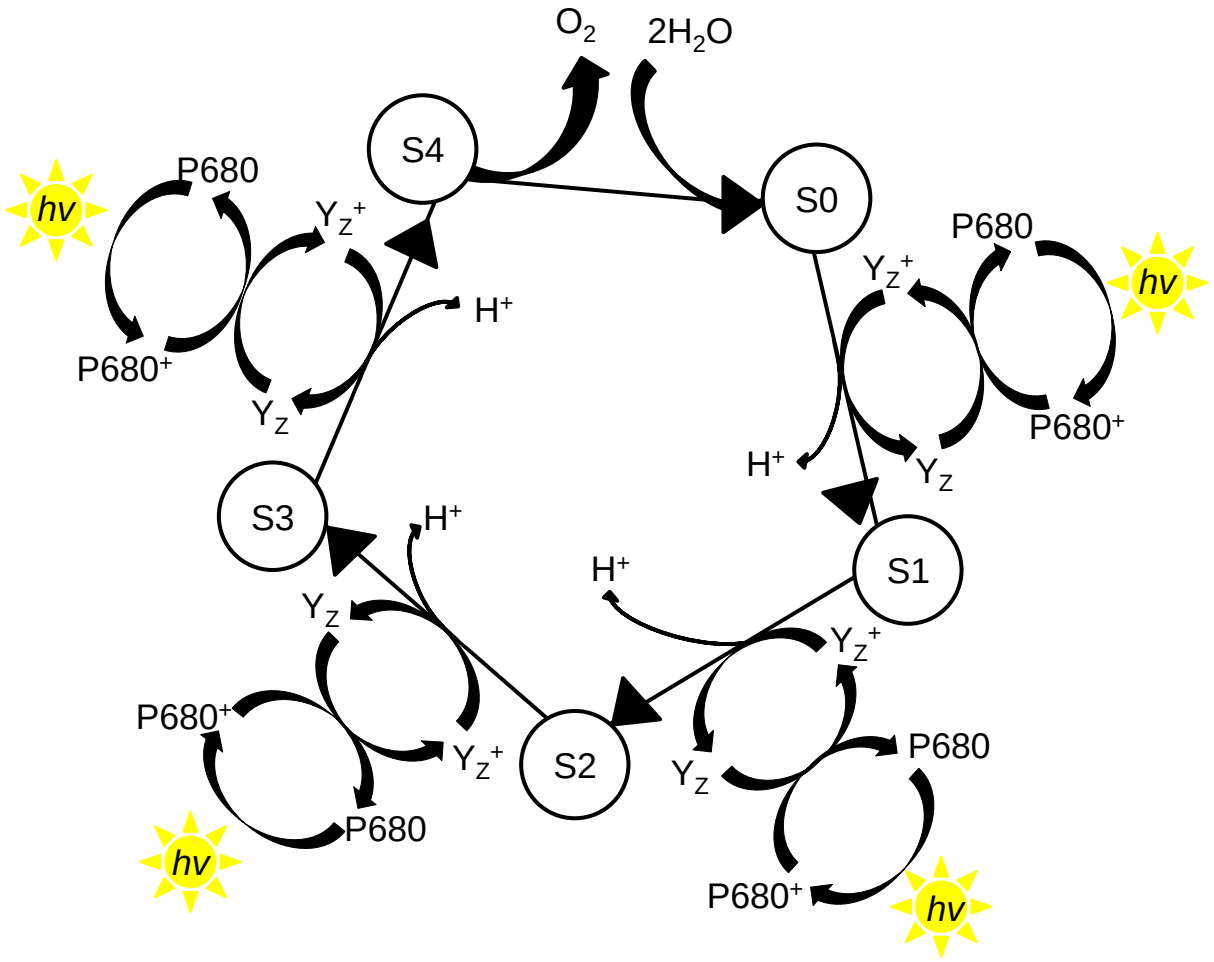

Figure 1.2: A model representing the five designated S-states required for O_2 evolution (modified from Kok *et al*., 1970; Barber, 2004).

Mn is the only redox active metal in the D1 protein and is involved with the O_2 evolution enzyme with removal of the Mn cluster resulting in the inhibition of the OEC. The assembly of a functional photochemical RC requires Mn Ca^{2+}, Cl^- and light. Light is required to produce the oxidant Tyr* which is necessary for oxidizing the Mn^{2+} ions to redox states of a higher potential. Ca^{2+} and Cl^- are needed for the S-state to advance past S_2 and Cl^- is required for the transition from the S_4 to the S_0 state as well. Ca^{2+} may play a role in stabilizing the ligand environment of the Mn cluster (figure 1.3) (Ananyev *et al*., 2001; Goussias *et al*., 2002; Umena *et al*., 2011).

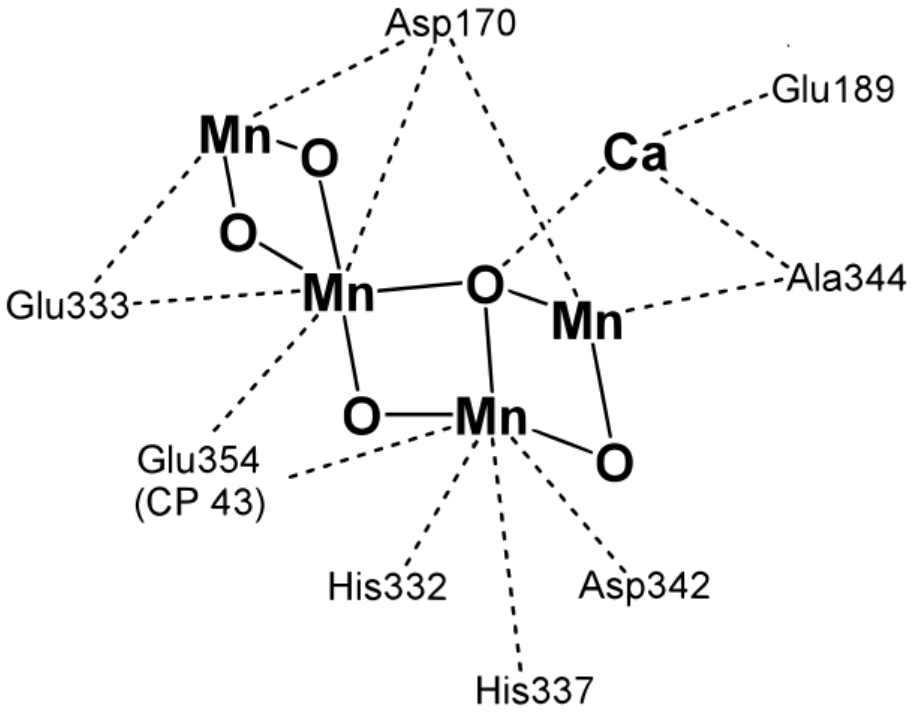

Figure 1.3: The suggested organization of the metalloenzyme core of the OEC (Umena *et al*., 2011)

1.1.3 Photoinhibition

Photoinhibition is the inhibition of the reaction center of photosystem II (PS II) when a photosynthetic organism is exposed to excessive photosynthetically active radiation (PAR) or UV radiation for a prolonged period of time. The OEC of PS II is sensitive to light therefore photoinhibition is inescapable for photosynthetic organisms because solar radiation is the driving force for photosynthesis. The extent of photoinhibition is dependent on the ability of the organism to efficiently repair PS II from the toxic effects of light (Murata *et al*., 2007).

During photoinhibition the functionality of the PS II components are sequentially lost. The OEC has been identified as the most sensitive component of PS II to solar radiation and therefore is the first to be effected by excessive light (Nishiyama *et al*., 2001). The loss of oxygen evolution under high light is associated with the release of a Mn ion to the lumen of the thylakoid membranes (Murata *et al*., 2007). Hakala *et al*. (2005) showed that the loss of oxygen evolution due to photoinhibition resulted in the

inability of isolated thylakoid membranes to reduce dichlorophenol-indophenol (DCPIP) in the presence of diphenylcarbazide (artificial electron donor). The loss of the movement of electrons in PS II is followed by the degradation of the D1 protein (part of the heterodimer core that forms the PS II reaction center).

1.1.3.1 Molecular mechanism of photoinhibition

The classical scheme for the molecular mechanism for photoinhibition was that PAR produces ROS by either charge recombination between the acceptor side and donor side of PS II or by excessive reduction of plastoquinone (Q_A). The excessive ROS produced can cause damage to the active centers of PS II and cleavage of specific fragments of the D 1 protein. However, results in recent studies in *Synechocystis* sp. PCC 6803 cannot be explained by the classical mechanism for photoinhibition (Allakhverdiev and Murata, 2004 and Nishiyama *et al*., 2004, Nishiyama *et al*., 2001). One of the most notable deviations from the classical mechanism is that ROS does not affect the proportionality between the initial rate of photodamage to PS II, calculated by kinetic analysis, in the presence of protein synthesis inhibitors and intensity of the incident light. Additionally, the action spectrum for photodamage was similar to the absorption spectra of compounds containing manganese and not the absorption spectra of chlorophyll and the carotenoids (Allakhverdiev and Murata, 2004 and Nishiyama *et al*., 2004). Furthermore, increasing the oxidative stress by either the addition of extracellular H_2O_2 or inactivation of H_2O_2-scavenging enzymes did not increase the rate of photodamage but inhibited the repair of PS II, which lead to the apparent photoinhibition (Nishiyama *et al*., 2001). The same result was observed by Nishiyama *et al*. (2004) when *Synechocystis* sp. PCC 6803 was illuminated in the presence of ethyl eosin, which causes an increase in the intracellular levels of $^1O_2$. These observations cannot be explained by the classical mechanism.

These observations have led to the development of a new scheme for the molecular mechanism of photoinhibition, which incorporates the previous and latest findings. The new molecular method states that photoinhibition occurs via a two-step process. The first step is a light driven destruction of the Mn cluster of the OEC (slow and the rate limiting step of photodamage) and the second step is the inactivation of the PS II reaction center by light that is absorbed by chlorophyll (rapid photodamage). The

extent by which photoinhibition occurs is compacted by ROS, which inhibits the repair of PS II. Studies in *Synechocystis* sp. have shown that repair of the reaction center, especially the synthesis of D 1 protein, is suppressed by high levels of intracellular H_2O_2 and $^1O_2$ (Murata *et al*., 2007).

MC may offer a protective role to the Mn cluster of the OEC by absorbing a proportion of the action spectrum that causes photodamage, thereby decreasing the rate or preventing photoinhibition.

1.1.3.2 Photoinhibition is enhanced by CO_2 limitation

Early studies have shown that the photoinhibition of PS II may be greatly enhanced in the suppression/absence of carbon fixation (Miller and Canvin, 1989). Melis (1999) observed that inhibition of carbon fixation in chloroplasts resulted in the rate of photodamage to be increased by the over reduction of Q_A. However, a recent study by Takahashi and Murata (2005) showed that interruption of the Calvin cycle by either the addition of exogenous glycoaldehyde (inhibitor of phosphoribulokinase) or by altering the gene of the large subunit of ribulose -1,5-bisphosphate carboxylase/oxygenase (Rubisco) via a missense mutation did not increase the rate of photodamage to the active center of PS II but the repair of PS II was inhibited.

The conversion of ribulose -1,5-bisphosphate and CO_2 to glycerate-3-phosphate (3-PGA) is catalyzed by Rubisco. NADPH and ATP are required for the conversion of 3-PGA to either glyceraldehyde-3-phosphate or dihydroxyacetone phosphate (Takahashi and Murata, 2006). Takahashi and Murata (2005) showed that the addition of exogenous 3-PGA completely stopped glycoaldehyde inhibition of D1 synthesis. This suggests that protein synthesis may be inhibited by the depletion of 3-PGA, caused by the suppression of carbon fixation. In the Calvin cycle the amount of NADPH used decreases as 3-PGA becomes depleted. This causes the amount of $NADP^+$ to decrease. $NADP^+$ is one of the most important electron acceptors in PS I. The rate of O_2 reduction increases as $NADP^+$ becomes depleted. This results in the formation of ROS. H_2O_2 production is increased when carbon fixation is suppressed. In cyanobacteria, the synthesis of D 1 protein is inhibited by H_2O_2.

1.1.4 Microcystin

Microcystin (MC) is the most common of all the hepatotoxins produced by cyanobacteria. The major producers of MC are *Microcystis, Anabaena, Nostoc, Planktothrix, Oscillatoria, Hapalosiphon* and *Anabaenopsis*.

1.1.4.1 Structure

MCs are non-ribosomal synthesized monocyclic peptides, with a general chemical formula of cyclo-D-alanine-L-X-D-erythro-β-methylaspartic acid –L-Z-ADDA-D-glutamate-N-methyldehydroalanine (Botes *et al*., 1985). There are over 100 structural variants of MC that have been identified (Meriluoto and Spoof, 2008). The main structural variations are in the L-amino acid residues which are labelled “X” and “Z” in the general formula. There are also many minor variations in the chemical structure, such as the lack of the methyl group on either methylaspartic acid or methyldehydroalanine, or both. Certain variants also have minor modifications in the ADDA chain of the molecule (Lawton *et al.,* 1994).

1.1.4.2 Environmental modulation

Intracellular MC quota is affected by a number of environmental factors and an examination of such factors may help to further clarify the role of MC.

Numerous environmental factors have been shown to influence the production and intracellular concentrations of MC. A significant amount of research has been conducted on the modulation of MC production and intracellular concentration has mainly focused on the effects of growth phase (Lee *et al*., 2000), light intensity (Lee *et al*., 2000, Long *et al*., 2001, Van der Westhuizen and Eloff 1985), temperature (Van der Westhuizen and Eloff 1985, Watanabe and Oishi,1985), growth rate (Lee *et al*., 2000, Long *et al*., 2001, Van der Westhuizen and Eloff 1985, Oh *et al*., 2000, Orr and Jones, 1998), uptake rates and availability of nitrogen and phosphorus (Lee *et al*., 2000; Long *et al*., 2001; Van der Westhuizen and Eloff 1985; Oh *et al*., 2000; Orr and Jones, 1998; Downing *et al*. 2005a) and rates of carbon fixation (Van der Westhuizen and Eloff 1985).

Jähnichen *et al*. (2007) showed that an increased $C_{i,i}$ deficiency by replacing Na-salts with K-salts in Z/4 medium enhanced intracellular MC quota. However, Downing *et al*., (2005b) showed that the intracellular N:C ratio is the primary modulator of cellular MC quota, with a higher MC content observed at ratios in excess of growth optima yielding for a given growth rate, indicating that MC production is enhanced under carbon stress.

1.1.4.3 Proposed functions for microcystin

The biological function of MC has been extensively investigated; however the exact physiological role or metabolic significance has not been elucidated. For many years it was thought that the biological role of MC was as a feeding deterrent because of the toxic effects that it had on various cyanobacterial grazers such as *Daphnia* (Jang *et al*., 2003). However, this proposed biological role for microcystin is problematic because feeding studies have shown that *Microcystis* strains can prevent grazing by Daphnia, whether microcystins are absent or present (Rohrlack *et al*., 1999; Rohrlack *et al*., 2001). Furthermore, phylogenetic analysis of cyanobacterial MC biosynthesis genes indicated that they co-evolved with house-keeping genes, which occurred well before the appearance of potential cyanobacterial grazers (Rantala *et al*., 2004).

Further studies showed that MC might have an extracellular function. The addition of exogenous MC to *Microcystis* and its response indicated that MC may be acting as an infochemical. Schatz *et al*. (2007) proposed that either stressed or dying *Microcystis* cells would release MC into the environment to signal to the rest of the population that stressful conditions are imminent. Additional evidence for an extracellular function for MCs was supported by the finding of a putative ABC-transporter protein encoded within the MC synthetase gene, which is predicted to be involved in both toxin biosynthesis and export (Pearson *et al*., 2004). Export of MC has not yet been shown; however an extracellular function of MC is highly improbable because only minute amounts of any MC are excreted into the external environment (Rohrlack and Hyenstrand, 2007). Berg *et al*. (1987) showed that the extracellular concentrations of MC ranged from non-detectable to about 10% of the

total MC quota. The upper range of extracellular MC concentrations can be explained by the lytic processes that occur in aged natural blooms and old laboratory cultures.

Studies have suggested that MC may have an intracellular function. Utkilen and Gjølme (1995) reported that there was a correlation between environmental iron and MC. Humble *et al*. (1994) showed that MC has the ability to chelate Fe^{2+}. This leads to the suggestion that MC may be an intracellular chelator which inactivates free Fe^{2+}, and therefore protects the cells from photo-oxidation. However, no further evidence has been found to support this intracellular function for MC. Furthermore, the effects of increased environmental iron and the vast array of cellular functions wherein iron plays a role suggest a coincidental correlation.

An increase in the accumulation of *mcy* mRNA was caused by high light intensities and iron limitation (Kaebernick *et al*., 2000; Sevilla *et al*., 2008). Strong illumination enhanced the transcription of the *mcy* genes and was accompanied by an equivalent increase in the amount of McyB. However, the intracellular quota of MC did not correspond to the enhanced transcription of the *mcy* genes. Kaebernick *et al*. (2000) noted that the MC quota decreased in conditions where there was enhanced transcription. This was observed with increasing light intensities, therefore the rate of carbon fixation would be increased. This results in a higher C:N ratio, which is unfavourable conditions for MC production (Downing *et al*., 2005b).

Recently, Zilliges *et al*. (2011) proposed a new role for MC as a protectant against oxidative stress by binding to redox sensitive cysteines via a covalent bond. They reported that a Δ*mcyB*$^{-}$ mutant of *Microcystis* PCC 7806 was more sensitive to continuous high light exposure at 300 µmol photons $m^{-2}.s^{-1}$ compared to the MC-producing *Microcystis* PCC 7806. The mutant became chlorotic after three to four days of continuous exposure to this high light intensity. No difference in growth was observed between these two strains when they were incubated at an optimal light intensity for growth of 30 µmol photons $m^{-2}.s^{-1}$. The recovery of *Microcystis* PCC 7806 was also superior to a Δ*mcyB*$^{-}$ mutant of *Microcystis* PCC 7806 from photo-bleaching after the culture was returned from the high light intensity for a week to a

light intensity of 30 µmol photons $m^{-2}.s^{-1}$. Oxidative stress in *Microcystis* PCC 7806 and the mutant of this strain was determined by chlorophyll *a* bleaching and a lipid peroxidation assay. However, reactive oxygen species (ROS) were not measured. The MC-producing strain may have been more resistant to oxidative stress because it was producing less ROS, due to a decrease in the water-splitting activity. Furthermore, Zilliges *et al*. (2011) showed that high light stimulates the binding of MC to proteins in MC-producing *Microcystis* PCC 7806 and the large sub-unit of Rubisco was less susceptible to protease degradation in the presence of MC.

1.1.4.4 Evidence for the involvement of microcystin in photosynthesis

A possible role for intracellular MC is either in the enhancement of photosynthesis or prevention of photoinhibition of photosynthesis. This is supported by the cellular location of MC, possible membrane viscosity modulation properties and increased MC production at reduced carbon fixation rates relative to nitrogen availability.

The cellular location of MC is predominantly in the thylakoid membranes and nucleoid areas (Young *et al*., 2005). The two possible transcription start sites contained in *mcyABC* and *mcyDEFGHIJ* are differentially expressed as a function of light, which suggests a role for MC in a light-dependent process (Kaebernick *et al*., 2000). Hesse *et al*. (2001) showed that a Δ*mcyB*$^{-}$ mutant of *Microcystis* PCC 7806 had substantial changes in pigments compared to the MC-producing *Microcystis* PCC 7806 and Jähnichen *et al*. (2007) reported that this mutant was unable to adapt to low intracellular inorganic carbon conditions by adapting its photosynthetic apparatus by an unknown mechanism. Phelan and Downing (2011) observed a strong positive correlation between microcystin and growth rate, with microcystin enhancing the growth of PCC 7806 under high light conditions.

The hypothesis for this study is that the production of MC enhances the fitness of the MC-producing cyanobacterium under high light by preventing/retarding photoinhibition. In order for MC to inhibit/decrease the rate of photoinhibition it must act directly on the photosynthetic apparatus or alter the fluidity of the thylakoid

membrane by the insertion of ADDA, or it must absorb potentially damaging light. Alternatively, microcystin stabilizes photosynthetic components damaged by photoinhibition. Regardless of the actual mechanism of how MC prevents/retards photoinhibition this provides a valid physiological and ecological function for MC in MC-producing cyanobacteria.

As per previous Chapter:

Studies have shown that exogenous microcystin have both beneficial and detrimental effects on non-microcystin-producing cyanobacteria (Singh *et al*., 2001; Sedmack and Kosi, 1998; Hu *et al*., 2004).

.

However:

There is insufficient evidence in literature which shows the uptake of exogenous microcystin by cyanobacteria.

Therefore:

Exogenous microcystin was fed to a non-microcystin producing, Synechocystis PCC 6803. The cells were fractionated into its different components to determine whether microcystin is taken up and if so, where the microcystin was located.

Chapter 2

2.1 The uptake of exogenous microcystin LR by a non-microcystin producing cyanobacteria

2.1.1 Abstract

The effect of exogenous microcystin on non-microcystin producing cyanobacteria has not yet been extensively studied. *Synechocystis* PCC 6803 was exposed to 10 $\mu g.L^{-1}$ of microcystin-LR for three days. Microcystin-LR was taken up by *Synechocystis* PCC 6803 and localized in the thylakoid membranes, which caused a decrease in photosystem II activity without any negative effects on the cell's survival.

2.1.2 Introduction

Cyanobacteria produce a wide range of biologically active metabolites many of which are toxic to higher organisms (Carmichael, 2001 and Duy *et al*. 2000). Microcystin (MC), a non-ribosomally synthesized peptide (Dittmann *et al*., 1997) is a potent inhibitor of eukaryotic protein phosphatases types 1 and 2A (MacKintosh *et al*., 1990). MC can form a covalent bond to a cysteine moiety of the catalytic domain of protein phosphatase1 (MacKintosh *et al*., 1995).

The effect and uptake of MC has been extensively studied in terrestrial plants (McElhiney *et al*., 2001; Gehringer *et al*., 2003; Chen *et al*., 2004 and Abe *et al*., 2006), and aquatic plants (Pflugmacher *et al*., 1998; Pflugmacher *et al*.,1999; Pflugmacher *et al*., 2001; Pflugmacher, 2002 and Pflugmacher, 2004; Mitrovic *et al*., 2005; Weiss *et al*., 2000; Yin *et al*., 2005 and Wiegand *et al*., 2002). The majority of these effects have been detrimental to eukaryotic photosynthetic organisms. These include growth inhibition (McElhiney *et al*., 2001; Gehringer *et al*., 2003; Chen *et al*., 2004 and Weiss *et al*., 2000), inhibition of photosynthesis (Abe *et al*., 1996; Pflugmacher *et al*., 2002; and Weiss *et al*., 2000), increases in glutathione peroxidase and glutathione–S-transferase (Gehringer *et al*., 2003), inhibition of superoxidase dismutase (Chen *et al*., 2004), increase in peroxidase activities (Chen

et al., 2004 and Mitrovic *et al*., 2005) and loss of chlorophyll content (Abe *et al*., 2006; Weiss *et al*., 2000 and Wiegand *et al*., 2002).

There have been limited studies on the effect of exogenous MC on cyanobacteria. The concentrations of exogenous MC that have been used in previous studies are much higher than would normally be found when a natural bloom collapses (Backer *et al*., 2008). Sedmack and Kosi (1998) reported that the addition of exogenous MC-RR resulted in the stimulation of growth to a non-toxic strain of *Microcystis aeruginosa* grown under low light conditions. Singh *et al*. (2001) and Hu *et al*. (2004) showed that exogenous MC has a detrimental effect on non-producing cyanobacteria, which included growth inhibition, chlorosis, cell lysis, inhibition of photosynthesis and change in pigment content.

These conflicting results are of little importance if the uptake of microcystin, and subsequent localization comparable with endogenous microcystin in the cell, cannot be certain. Here, the aim is to determine whether exogenous MC at relevant environmental concentrations is taken up by a non-MC-producing cyanobacteria, *Synechocystis* PCC 6803 and if there are any beneficial or detrimental effects to the organism.

2.1.3 Methods and Materials

2.1.3.1 Culture Conditions

A Mid-log phase culture of *Synechocystis* PCC6803 was collected by centrifugation (5000 *x g*; 10 min), washed twice in BG11 (Sigma) (pH 7.4) and resuspended in 5 L of fresh BG11. The resuspended culture was divided into 10 equal aliquots and each aliquot was placed into a 1000 ml erlenmeyer flask. A MC-LR stock solution in BG11 (1 $\mu g.\mu l^{-1}$) was added to half the flasks so that the final concentration of MC-LR was at 10 $\mu g.L^{-1}$ and the same volume of BG11 containing no toxin was added to the remaining flasks (control). The flasks were incubated at 20 °C (±0.5 °C) under continuous illumination of 100 µmol of photons $m^{-2}.s^{-1}$ for 72 hours (Triton Dayglo©).

2.1.3.2 Thylakoid membrane extraction

The cell walls and outer membranes, phycobiliproteins and thylakoid membranes were isolated from *Synechocystis* PCC 6803, according to Nishiyama *et al*. (1993) and Hagio *et al*. (2000), with the following modifications. Cells were pelleted by

centrifugation (4000 *x g*; 10 min) from 245 ml of culture. The pelleted cells were washed twice with 150 ml buffer A (50 mM HEPES-NaOH and 30 mM $CaCl_2$ at pH 7.5). The pellet was resuspended in 20 ml of buffer B (50 mM HEPES-NaOH, 30 mM $CaCl_2$, 0.8 M sorbitol, 1 M betaine and 6-aminohexanioc acid at pH 7.5). An equal volume of pre-chilled, acid-washed glass beads (150 – 212 µm) was added to the resuspended pellet. The mixture was vortexed at speed eight using an auto vortex mixer (Crown-scientific CSV90) for 60 seconds; following this, the mixture was placed on ice for 60 seconds. This was repeated five times. After the glass beads had settled to the bottom of the tube, the supernatant was removed and centrifuged at 1000 *x g* for five minutes to remove the unbroken cells. The supernatant was collected and centrifuged at 4500 *x g* for 10 minute to remove the outer membrane and cell wall (Omata and Murata, 1984). The pellet and supernatant were collected. The supernatant was centrifuged at 40 000 *x g* for 20 minutes. The supernatant containing the phycobiliproteins was collected and thylakoid membrane pellet was resuspended in buffer B.

2.1.3.3 Chlorophyll *a* extraction

One ml of either resuspended thylakoid pellet or whole cells was centrifuged at 13 000 *x g* for 10 minutes. The pellet was resuspended in one ml of 90 % methanol by vigorous vortexing with an auto vortex mixer (Crown-scientific CSV90) for 180 seconds. This was placed in the dark for two hours at 4 °C. The chlorophyll *a* concentration was determined by the method and formula used by Carvalho and Kirika (2005). The chlorophyll *a* concentration was used to normalize the PS II activity.

2.1.3.4 Photosystem II activity

An oxygraph containing a Hansatech oxygen electrode (Hansatech, King's Lynn, Norfolk, U.K) was used to measure the rates of oxygen evolution. The electrode was calibrated as per the manufactures instructions. Samples were dark-adapted for 15 minutes. Either one ml of the dark-adapted cell suspension or thylakoid membranes was added to the reaction chamber. The reaction chamber was illuminated with photosynthetic fluorescent tubes (Triton Dayglo©) under continuous illumination of 100 µmol of photons $m^{-2}.s^{-1}$. PS II activity was measured by the method of Stewart and Bendall (1980) by the addition of 2,6 –dimethyl-1,4-benzoquinone at a final concentration of 1 mM and $K_3Fe(CN)_6$ at a final concentration of 2 mM.

2.1.3.5 Microcystin extraction and quantification

MC-LR from the different fractions was extracted as in Phelan and Downing (2007) by sonication of pelleted cultures in 70 % ethanol in the dark for 24 hours and MC-LR was quantified by ELISA as per manufacturers' specifications (Abraxis Microcystins/Nodularins ELISA kit, 96T).

2.1.4 Results

Both cultures were uniformly dispersed in the medium. Microscopic examination revealed no morphological changes of the cells for either treatment. The three day exposure to exogenous MC-LR resulted in a significant decrease in PS II activity in both whole cell and thylakoid membrane measurements compared to the control (figure 2.1). Furthermore, there was no significant difference in PS II activity between the treated whole cell and treated thylakoid membrane measurements. There was no significant change in the PS II activity between the zero time control and day three of the control for both the whole cell and thylakoid measurements (figure 2.1).

No MC-LR was found in the phycobiliprotein fraction of the cell extract. However, MC-LR was found in the cell wall/outer membrane and thylakoid fraction (Table 1). This indicates that of the measured MC not remaining in the medium, almost half was in the thylakoid membrane and more than half in the cell debris or whole cell fractions.

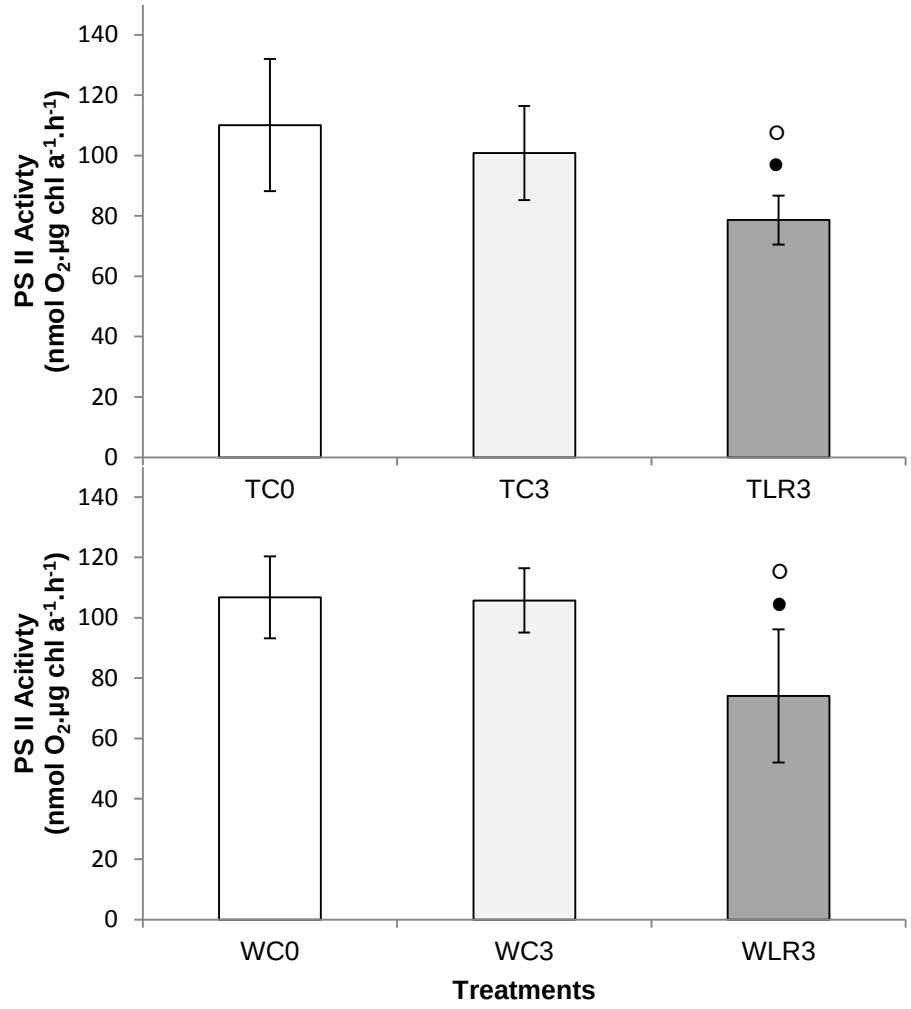

Figure 2.1: The effect of MC-LR (■) on PS II activity in *Synechocystis* PCC6803 (W) and thylakoid membranes extracted from *Synechocystis* PCC6803. Error bars are standard deviation; n = 5. ○ - indicates significant difference from Day 0 control (C0,(□)) ($p < 0.05$) and ● - indicates significant difference from Day control (C3,(■)) ($p < 0.05$)

Table 2.1: The distribution of MC-LR within the different cellular fractions of *Synechocystis* PCC 6803.

Medium and Cell fractions per litre of culture	[MC -LR] µg.L^{-1}			
	Day 0		Day 3	
	Avg	Std dev	Avg	Std dev
Medium	10	0	4.01	0.67
Cell Wall and Outer membrane	0	0	1.91	0.36
Phycobiliproteins	0	0	0	0.06
Thylakoid Membrane	0	0	1.52	0.14

2.1.5 Discussion

Singh *et al*. (2001) showed the uptake of semi-purified ^{14}C-labelled MC, from *Microcystis aeruginosa*, when added to actively growing cultures of *Anabaena sp.* BT1 and *Nostoc muscorum* C. Agardh. ^{14}C-labelled MC uptake was greater in the light compared to the dark and the uptake rate of the ^{14}C-labelled MC was greater in *Nostoc muscorum* C. Agardh compared to *Anabaena sp.* BT1. However, Singh *et al*. (2001) did not show where the ^{14}C-labelled MC was localized. The ^{14}C-labelled MC was added to whole cells and uptake of the toxin was measured with a scintillation counter. Unfortunately, this method does not provide any information about the location of MC and it is therefore possible that the MC may not have even been internalized but rather remained bound to the outside of the cell. It is also therefore not apparent if the MC which was possibly taken up was degraded or not. MCs are more susceptible to degradation by photolysis in the presence of pigments, which may have been present in the semi-purified MC extracts (Tsujl *et al*.; 1994). However in the data presented here, MC-LR was taken up and almost half of that taken up was located in the thylakoid membranes of the non-toxic *Synechocystis* PCC 6803. Young *et al* (2005) reported that in MC-producing cyanobacteria, 69 % of the total gold labelled MC was associated with the thylakoid membranes in PCC 7806 whereas in PCC 7820, 78 % of the total gold labelled MC was found in the thylakoid membranes. None of the MC-LR taken up by *Synechocystis* PCC 6803 was associated with the phycobiliproteins. In contrast, Jüttner and Lüthi (2008) showed binding of MC to the phycobiliproteins due either to Van der Waal's forces or electrostatic interactions between the positively charged domains of the proteins and the negatively charged MC. These interactions can be considered as non-specific and weak and the absence of MC in the phycobiliproteins could either be due to the

disruption of these forces and subsequent loss of the MC during the extraction procedure or the composition of the extraction phase (buffer B), which may have had an effect on the ethanol extraction of MC, whereas the thylakoid membranes and cell wall/outer membrane fragments were pelleted out and lacked buffer B.

The addition of exogenous MC-LR resulted in a significant decrease in PS II activity for both whole cell and thylakoid membrane measurements compared to the control. Singh *et al*. (2001) reported the complete inhibition of oxygen evolution in whole cells within 25 minutes in *Anabaena sp.* BT1 and *Nostoc muscorum* C. Agardh with the addition of 25000 $\mu g.L^{-1}$ of semi-purified MC-LR while 50000 $\mu g.L^{-1}$ of semi-purified MC-LR resulted in the complete inhibition of growth by cell lysis in both genera. However, the environmentally relevant concentration of MC-LR used in this study (10 $\mu g.L^{-1}$) (Backer *et al*., 2008) did not.

There was no significant difference between the PS II activities measured by oxygen evolution between the whole cell measurements and the thylakoid membrane measurements for both the control and the exposure to exogenous MC. This indicates that the inhibition of PS II was due to the uptake of exogenous MC-LR and subsequent localisation in the thylakoid membranes.

In conclusion, environmentally relevant concentrations of MC-LR are taken up by non-MC producing cyanobacteria. The uptake of the exogenous MC at environmentally relevant concentrations causes a decrease in PS II activity without any detrimental effects to the survival of the non-MC producing cyanobacteria as previously reported (Huiying *et al*., 2009; Singh *et al*., 2001 and Hu *et al*., 2004).

As per previous Chapter:

Exogenous microcystin was taken up by *Synechocystis* PCC 6803 and internalized into the thylakoid membranes which led to a decrease in photosynthetic activity.

However:

Cyanobacteria can alter the fluidity of their thylakoid membranes in response to environmental stress to alter their photosynthetic capacity.

Therefore:

The effect of microcystin on fluidity of thylakoid membranes from *Synechocystis* PCC 6803 was determined by measuring the degree of polarization of the fluorescent probe, DPH.

Chapter 3

3.1 Microcystin variants have no effect on the fluidity of thylakoid membranes from *Synechocystis* PCC 6803

3.1.1 Abstract

Cyanobacteria have been able to occupy a wide and diverse range of environmental niches because of their ecological diversity and their physiological responses to environmental stresses, including their ability to alter their membrane fluidity. Thylakoid membranes isolated from *Synechocystis* PCC 6803 were exposed to two artificial membrane fluidity modifiers and various variants of microcystin (MC). Alterations in the fluidity of the membrane were determined by the degree of polarization of the fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene (DPH). It was found that none of the MC variants had a significant effect on the fluidity of the thylakoid membranes, suggesting that variations previously observed in photosynthetic efficiency were not due to changes in the membrane fluidity

3.1.2 Introduction

Cyanobacteria are among a diverse group of organisms that are capable of performing both respiration and oxygenic photosynthesis simultaneously in the same membrane system. In the majority of cyanobacteria, photosynthetic electron transport occurs exclusively in the thylakoid membranes, whereas respiratory electron transport occurs in both the thylakoid and cytoplasmic membrane systems (Vermaas, 2001). This unique biochemistry of cyanobacteria has allowed them to adapt and survive some of the most extreme environments.

Cyanobacterial membrane lipids are usually esters of glycerol and fatty acids and can either be saturated or unsaturated. A few genera of filamentous cyanobacteria have been found to produce large amounts of polyunsaturated fatty acids in their lipids (Parker *et al*., 1967 and Kenyon *et al*., 1972).

The most abundant glycerolipids found in the thylakoid membranes are sulphoquinovosyl diglycerides (SQDG), monogalactosyl diglycerides (MGDG), digalactosyl diglycerides (DGDG) and phosphatidyl glycerol (PG) (Wada *et al*.; 1994), with PG being the only phospholipid found in the thylakoid membrane

(Hagio *et al*.; 2000). Besides these glycerolipids being the major component of the membrane-forming bilayers, they also act as hydrophobic ligands for membranous proteins (Singh *et al*., 2002 and Gombos *et al*., 1994). In cyanobacterial cells and chloroplasts of higher plants it has been suggested that these above mentioned glycerolipids play a pivotal role in electron transport through the thylakoid membranes (Singh *et al*., 2002). Pick *et al*. (1987) showed that SQDG is associated with ATP synthase complex and Murata *et al*. (1990) reported that MGDG is bound to the photosystem II (PSII) reaction centre.

In order to study the effect of SQDG, MGDG, DGDG and PG on photosynthesis, mutants were created which had various lipid components in their thylakoid membranes. A null mutant of *Synechococcus* sp. PCC 7942, unable to produce SQDG, was made by disrupting the *sqdB* gene. Studies on this mutant revealed that SQDG had no effect on photosynthesis and growth under optimal growth conditions for this culture (Güler *et al*., 1996). A DGDG deficient mutant of *Arabidopsis*, which had significantly less content of DGDG, had a lower photosynthetic activity and an altered structure of the thylakoid membranes compared to the wild type (Dörmann *et al*., 1995). Hagio *et al*. (2000) showed a drastic decrease in total photosynthetic activity in a *pgsA* mutant, which is incapable of producing PG, compared to the wild type. This decrease in photosynthetic activity was caused by a decrease in PS II activity, but PS I activity was not affected. Further studies have shown that PG may be involved in the dimerization of heterodimer proteins in the PS II complex. Studies so far have shown that DGDG and PG play important roles in photosynthesis and upkeep of the structure of the thylakoid membranes (Güler *et al*., 1996; Dörmann *et al*., 1995 and Hagio *et al*.; 2000).

The physical characteristics of the thylakoid membrane are not only determined by the type and content of glycolipids. The degree of unsaturation of the acyl residues of the glycolipids plays an important role as well (Murata and Wada, 1995). Cyanobacteria produce enzymes, called desaturases, which are intrinsic membrane proteins that catalyse the addition of double bonds into the hydrocarbon chains of fatty acids (Singh *et al*., 2002). These enzymes play a vital role in cold acclimation of cyanobacteria (Wada and Murata, 1990). Cyanobacteria desaturate their fatty acids in response to a decrease in ambient temperatures (causes the thylakoid membrane to become less fluid), which decreases the viscosity of the membrane at the lower

temperatures (Sato and Murata, 1982). Gombos *et al*. (1997) transformed *Synechococcus* sp. PCC 7942 with the *desA* gene for a Δ12 desaturase. This transformant had a higher tolerance for high light due to the increased unsaturation of the thylakoid membranes. This indicates that desaturation of the membranes can enhance the organism's ability to survive high light stress by enhancing the rate of the synthesis of the D1 protein *de novo*. Yamamoto *et al*., (1981) suggested that an increase in membrane fluidity improves the mobility of plastoquinone within the lipid phase. The plastoquinone pool is generally thought to be involed in electron transport between PS II and PS I, therefore resulting in more efficient photosynthetic activity. Fracheboud (2006) reported that a decrease in membrane viscosity enhance the water-splitting activity of photosynthesis.

Endogenous MC is predominantly located in the thylakoid membranes (Young *et al*., 2005). The interaction of MC within the thylakoid membranes is presumably due to the insertion of the ADDA residue (Orr and Jones, 1998). MC and surfactants seem to have similar structural properties due to their amphiphathic nature, where the carboxyl groups and the guanidine group of arginine of the MC molecule are hydrophilic and the ADDA residue is hydrophobic. Therefore, the insertion of ADDA moiety into the thylakoid membrane may cause the membrane to become less viscous because the degree of saturation of the ADDA moiety is less than some of the acyl residues of the glycerol lipids, hence affecting the membrane fluidity, which in turn affects photosynthesis.

The aim of this investigation is to investigate the effect of microcystin (MC) variants on the fluidity of cyanobacterial thylakoid membranes.

3.1.3 Methods and Materials

3.1.3.1 Culture condition

Mid-log phase culture of *Synechocystis* PCC 6803 were collected by centrifugation (4000 *x g*; 10 min), washed twice in BG11 (Sigma) (pH 7.4) and inoculated into 2000 ml Erlenmeyer flasks to a final volume of 1500 ml of BG11 (pH 7.4). Cultures were incubated at 25 °C (±0.5 °C) under continuous illumination of 20 µmol of photons $m^{-2}.s^{-1}$ (Triton Dayglo©).

3.1.3.2 Thylakoid extraction

Thylakoid membranes were extracted as per methods and materials of chapter two.

3.1.3.3 Chlorophyll *a* determination

The chlorophyll *a* extraction and concentration was determines as per methods and materials of chapter two.

3.1.3.4 Manipulation of the lipid phase order

Thylakoid membranes (10 $\mu g.ml^{-1}$ chlorophyll *a*) were incubated at room temperature for 60 min in the dark while being gently mixed on an orbital shaker with either 300 µM cholesterol to increases the rigidity of the thylakoid membrane, 50 mM benzyl alcohol to increase the fluidity of the membrane, MC-LR, MC-RR, MC-WR, and MC-LF. After incubation, the samples were centrifuged at 15 000 *x g* and the pellet was resuspended in a buffer containing 50 mM HEPES-NaOH, 30 mM $CaCl_2$, 0.8 M sorbitol, 1 M betaine and 6-aminohexanioc acid at pH 7.5. This was repeated twice.

3.1.3.5 Steady-state fluorescence polarization measurements

After the incubation period, the alterations to the viscosity of the thylakoid membranes were determined by measuring the degree of polarization (*P*) of the fluorescence emitted from the probe 1,6-diphenyl-1,3,5-hexatriene (DPH), according to Popova *et al*., (2007) and Velitchkova and Popova (2005), with the following modifications. DPH was added to a final concentration of 2.5 µM to the resuspended thylakoid membranes with a final chlorophyll *a* concentration of 5 $\mu g.ml^{-1}$. This mixture was incubated in the dark for 30 minutes. The different samples were measured using a Synergy 2 fluorimeter (Biotek) equipped with polarization filters. Samples were excited at 360 nm and the emitted fluorescence from each sample was measured at 450 nm. The slit widths were set at 10 nm.

3.1.4 Results

The viscosity of the thylakoid membrane is determined by the value of *P* of DPH. An increase in the value of *P* corresponds to a decrease in fluidity of the membrane. The incorporation of cholesterol into the thylakoid membranes resulted in a significant increase in the degree of polarization compared to the control, whereas

the incorporation of benzyl alcohol in the thylakoid membranes caused a significant decrease in the degree of polarization (figure 4.1).

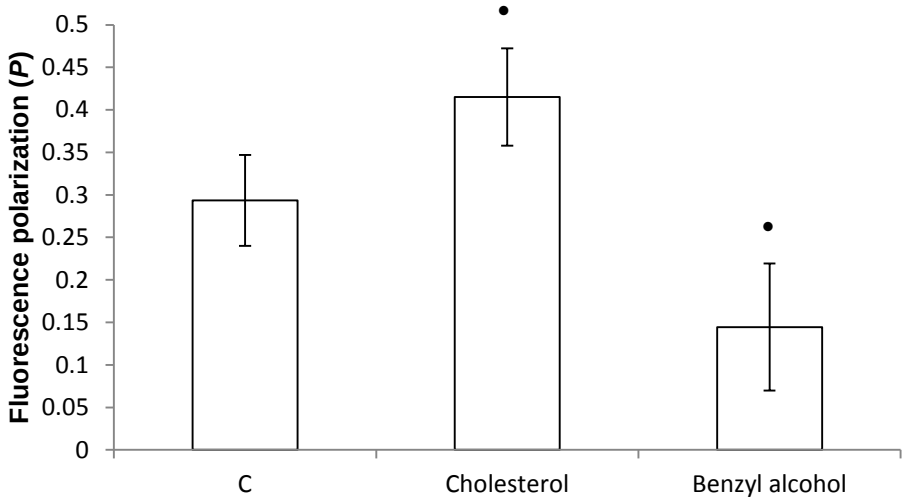

Figure 3.1: Fluidity of isolated thylakoid membranes from *Synechocystis* PCC 6803, which have been artificially modified with 300 µM cholesterol and 50 mM benzyl alcohol at room temperature for 60 minutes, was determined by the degree of polarization (P) of DPH. Error bars represent standard deviation, n= 6, $p< 0.05$

The addition of exogenous MC to the thylakoid membranes of *Synechocystis* PCC 6803 had no effect on the degree of polarization for all the variants (figure 3.2A) and concentrations (figure 3.2B) tested. This indicates that MCs have no effect on the fluidity of the thylakoid membranes.

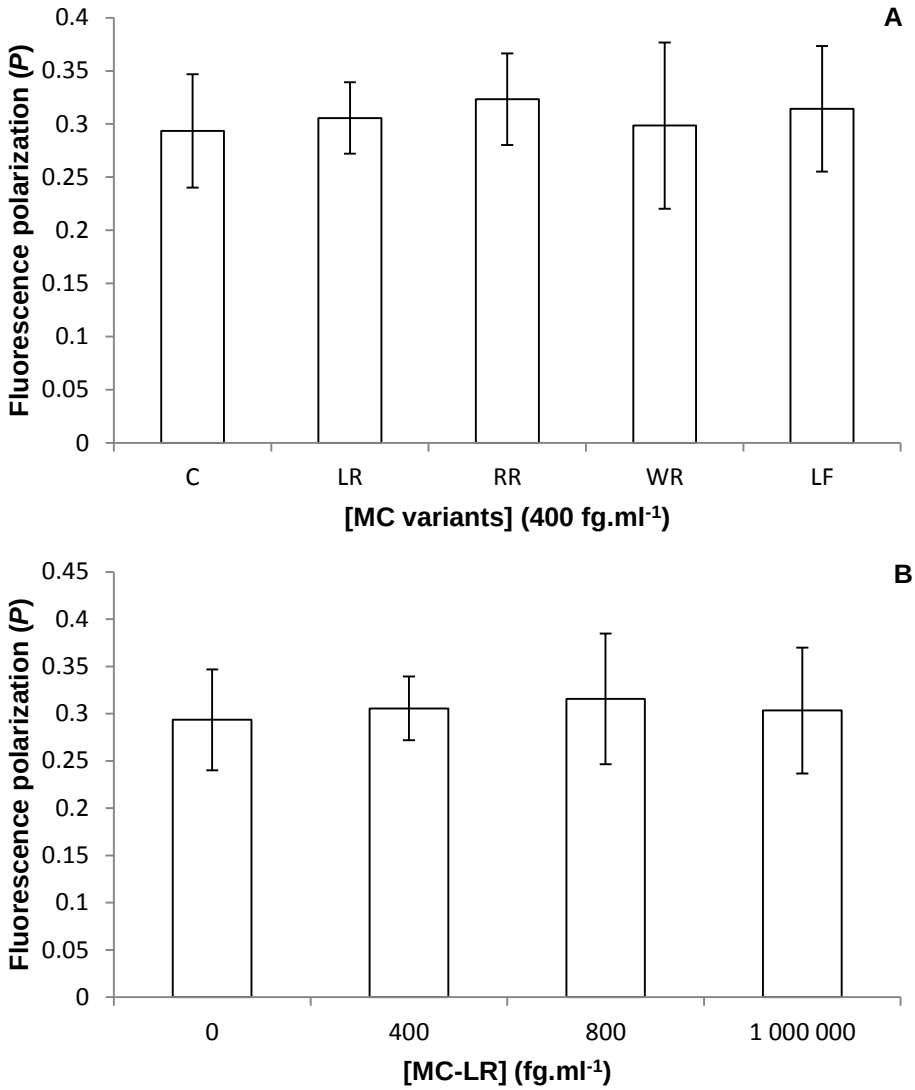

Figure 3.2: The effect of exogenous MC variants (A) and increasing concentrations of MC-LR (B) on the fluidity of isolated thylakoid membranes from *Synechocystis* PCC 6803 was determined by the degree of polarization (*P*) of DPH. Error bars represent standard deviation, n= 6, $p< 0.05$

3.1.5 Discussion

One of the responses by cyanobacteria to environmental stress is to alter the fluidity of the thylakoid membrane by changing the degree of saturation of their lipids present in the membrane. A decrease in membrane fluidity effects the electron transport between PS II and PS I. Thus, the observed decrease in photosynthetic activity observed in PS II (chapter 2) may be attributable to membrane fluidity alterations as a function of ADDA insertion into the thylakoid membrane. In this study, the incorporation of cholesterol into cyanobacterial thylakoid membranes' resulted in a decrease in the fluidity of the membrane. Popova *et al*. (2007),

Velitchkova and Popova (2005) and Velitchkova *et al*. (2001) observed the same trend in thylakoid membranes extracted from 14 day old pea leaves where the incorporation of cholesterol caused the membranes to become more rigid. The incorporation of cholesterol increases the rigidity of the membrane by limiting the motional freedom of the fatty acyl chains and reducing the degree of packing of the head groups. The incorporation of benzyl alcohol into the cyanobacterial thylakoid membranes lead to an increase in the fluidization of the membrane. Velitchkova and Popova (2005) and Velitchkova *et al*. (2001) reported a similar result when incorporating benzyl alcohol into thylakoid membranes from 14 day old pea leaves. The results obtained in this study for the incorporation of cholesterol and benzyl alcohol into the cyanobacterial thylakoid membranes indicated that the fluorescent probe, DPH, was working correctly and this method is suitable for testing the effect of MC on the fluidity of these membranes.

However, in this study, the addition of MC did not alter the membrane fluidity. This indicates that MC neither interacts with nor is incorporated into the lipid bilayer as suggested by (Shi *et al*., 1995; Young *et al*., 2005) and more likely interacts with a protein(s) involved in photosynthesis. Zilliges *et al*. (2011) showed that MC forms a covalent interaction with cysteine residues of proteins. The lack of alteration to the fluidity of the thylakoid membranes is further supported by the data in chapter two, where PS I activity was not affected by the addition of different MC variants at various physiologically relevant concentrations. If MC did alter the membrane fluidity, there would have been a significant decrease in PS I activity as membrane fluidity is very important for effective electron transport between PS II and PS I. The mobile electron carriers plastoquinone, which mediates electron transport from PS II to cytochrome b_6f ($Cytb_6f$), and plastocyanin, which transports electrons from $Cytb_6f$ to PS I, mediate the transport of electrons from PS II to PS I. The rigidity of the thylakoid membrane has a drastic effect on the mobility of the mobile electron carriers, which in turn has an effect on the activity of PS II and PS I (Gombos *et al*., 1988). Popova *et al*. (2007) reported that artificial rigidification of the thylakoid membranes causes more of a decrease in PS I-driven electron transport than PS II-driven electron transport. Yamamoto *et al*. (1981) and Busheva *et al*. (1998) noted the same trend that PS II-driven electron transport was less affected by an increase in membrane fluidity than PS I.

Furthermore, the lack of alteration to the viscosity of the thylakoid membranes by MC and the fact that MC has no significant effect on PS I activity (see chapter 4) suggests that the mobile electron carriers and $Cytb_6f$ are not the site of interaction for MC.

PS II activity (data from chapter two) indicates that PS II is the site of action for MC. Popova *et al*. (2007) reported that an increase in thylakoid membrane viscosity did not have a drastic effect on the photo-reducing ability of PS II. This provides further evidence that MC binds to a protein(s) that make up the PS II reaction centre because the addition of exogenous MC caused a significant decrease in PS II activity (chapter two) but did not alter membrane fluidity.

Therefore, MC may be involved in stabilizing the PS II reaction centre and enhancing the MC producer to tolerate high light. Phelan and Downing (2011) reported that *Microcystis* PCC 7806 (toxin producer) had a competitive growth advantage over a *mcyA*$^-$ knock out mutant of *Microcystis* PCC 7806 (non-toxin producer) at saturating light intensities. The *mcyA*$^-$ knock out mutant of *Microcystis* PCC 7806 become chlorotic and died after 72 hours. Furthermore, Zilliges *et al*. (2011) showed that high light stimulates the binding of MC to proteins and that the *mcyB*$^-$ mutant of *Microcystis* PCC 7806 (non-MC producer) was more sensitive to high light conditions compared to *Microcystis* PCC 7806 (MC producer).

These data therefore provide supporting evidence for a biological role of MC in photosynthesis specifically at the photosynthetic apparatus rather than by altering membrane fluidity.

As per previous Chapter:

The addition of microcystin to cyanobacterial thylakoid membranes had no effect on the membrane fluidity.

However

Approximately two thirds of the total intracellular microcystin is bound to the thylakoid membranes (Young et al., 2005; Shi et al., 1995). In chapter two, the exogenous microcystin was taken up and incorporated into the thylakoid membrane of the cyanobacterium. This caused a decrease in oxygen evolution.

Therefore:

Thylakoid membranes were extracted, purified and exposed to microcystin to determine whether the activity/activities of photosystem II, photosystem I or both were altered by the addition of microcystin.

Chapter 4

4.1 The effect of exogenous microcystin on PS II and PS I activity in thylakoid membranes of cyanobacteria

4.1.1 Abstract

Microcystin variants and increasing concentrations of MC-LR had no effect on the fluidity of the thylakoid membranes. However, the exact physiological role or metabolic significance of microcystin has not been determined. Approximately two thirds of the total intracellular microcystin content is localized within the thylakoid area and a strong correlation exists between both microcystin and growth rate, and microcystin and performance index (PI_{ABS}) at light intensities approaching saturation with excess nitrogen. These findings support a possible role for microcystin in photosynthesis prompting this investigation. Thylakoid membranes were isolated from *Synechocystis* sp. PCC 6803 by using a combination of lysozyme and glass beads to lyse the cells and ultracentrifugation to pellet the thylakoid membranes. The activity of photosystem II was determined by measuring oxygen evolution, and oxygen uptake was used to measure the activity of photosystem I. The addition of microcystin-LR, RR, and WR to the thylakoid membranes resulted in a decrease of photosystem II activity for all concentrations of microcystin used but had no significant effect on photosystem I, thus providing the first evidence that microcystin inhibits the activity of photosystem II at physiologically relevant concentrations.

4.1.2 Introduction

Over the past few decades, anthropogenic factors have led to an excessive release of nitrogen and phosphorus into the environment, which has resulted in eutrophication of man-made reservoirs and aquatic ecosystems (Carmichael, 2001). This has caused an increase in the incidence and intensity of cyanobacterial blooms resulting in several occurrences of human illness and animal poisoning (Falconer *et al*., 1999; Duy *et al*., 2000; Carmichael *et al*., 2001).

These cyanobacterial blooms have numerous negative impacts such as oxygen depletion during biomass decay, they are aesthetically non-pleasing, and they

produce unpleasant odours (Carmichael, 2001). However, the major concern with these blooms is that they are capable of producing a wide range of biologically active metabolites, some of which are toxic. Microcystins (MCs) are the most common of these toxic metabolites (Sivonen and Jones, 1999). They are produced by numerous genera of cyanobacteria such as *Microcystis Anabaena, Nostoc, Planktothrix, Oscillatoria, Hapalosiphon* and *Anabaenopsis* (Sivonen and Jones, 1999). However, microcystin (MC) production is not intrinsic to all genera or taxa of cyanobacteria (Chorus and Bartram, 1999).

MCs have an unusual β-amino acid, ADDA (2S,3ES,8S,9S-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-deca-4E,6E-dinioc acid) and belong to the family of monocyclic heptapeptides. The general formula for the MC structure is a cyclo-D-alanine-L-X-D-erythro-β-methylaspartic acid –L-Z-ADDA-D-glutamate-N-methyldehydroalanine (Botes *et al*., 1995). There are over 100 structural variants of MC that have been identified (Meriluoto and Spoof, 2008). The main structural variations are in the L-amino acid residues which are labelled “X” and “Z” in the general formula. The other minor variations occur at the other parts of the molecule (Spoof *et al*., 2003).

MC has been extensively researched and due to its unique and complex structure, the cost of MC production (Briand *et al*., 2012), the degree of environmental modulation of the MC cellular concentration and production rate, it has been suggested that the production of MC must be advantageous to the producer (Orr and Jones, 1998). However, the exact biological of MC has not yet been determined. Numerous roles for MCs have been proposed, these include a defence mechanism against plankton glazers (Jang *et al*., 2003; Jang *et al*., 2004), ferrous iron chelation (Humble *et al*, 1994); Utkilen and Gjølme, 1995) and a putative extra-cellular function relating to cyanobacterial interactions with phytoplankton, zooplankton and bacteria (Pearson *et al*., 2004).

In addition to the toxicity to mammals (Duy *et al*., 2000), MCs also negatively affects terrestrial and aquatic plants, microalgae and cyanobacteria .

Abe *et al*. (2006) exposed the leaves of *Phaseolus valgaris L*. by dipping, the leaves into a solution of MC-LR for 10 s. The final dose of MC-LR ranged between 15 and 1423 $\mu g.m^{-2}$. These single exposure treatments resulted in rapid inhibition of

photosynthesis, loss of chlorophyll content and leaf necrosis. Multiple treatments resulted in a greater degree of photoinhibition.

The effect of exogenous MC variants on aquatic plants has been well documented. *Chadophora sp*. and *Phragmites australis* were exposed to 0.5 µg.L-1 of exogenous MC LR for 24 h. This resulted in the inhibition of photosynthesis in both genera (Pflugmacher, 2002). The same was observed in *Vesicularia dubyana* when it was exposed to 50 $\mu g.L^{-1}$ of MC-LR for 24 hours (Weiss *et al*., 2000). *Lemna minor* was exposed to MC-RR concentrations ranging between 100 and 5000 $\mu g.L^{-1}$ for six days. The effect of this was chlorosis and inhibition of photosynthesis at the highest concentration of MC-RR and the inhibition of frond formation and growth at MC concentrations greater than 3000 $\mu g.L^{-1}$ (Weiss *et al*., 2000). *Ceratophyllum demersum* was exposed to pure MC-LR, MC-RR and a cyanobacterial extract containing MC at a concentration of 0.25 $\mu g.L^{-1}$, for 24 hours. The cyanobacterial extract had a significant effect on photosynthesis whereas the purified MC variants had a very weak to no effect on photosynthesis (Pietsch *et al*., 2001). However, Pflugmacher (2002) showed that there was inhibition of photosynthesis at a MC-LR concentration greater than 0.5 $\mu g.L^{-1}$ when the same strain was exposed for 24 hours.

When *Peridinium gatunense* Nygaard was exposed to 50 $\mu g.L^{-1}$ of MC-LR for 24 hours there was no significant inhibition of photosynthesis but there was an increase in the formation of reactive oxygen species (ROS) (Vardi *et al*., 2002). However, photosynthesis was not inhibited in the green alga *Scenedesmus armatus* when it was exposed to pure MC-LR and MC-RR at a concentration of 0.25 $\mu g.L^{-1}$ for one hour but POD (peroxidase) and sGST (soluble glutathione S-transferase) activities were increased. The cyanobacterial extract raised POD but inhibited sGST and photosynthesis (Pflugmacher, 2002).

The addition of semi-purified MC-LR, at a concentration of 25 000 $\mu g.L^{-1}$ and 50 000 $\mu g.L^{-1}$, to *Anabaena sp.* BT1 and *Nostoc muscorum* C. Agardh resulted in the inhibition of photosynthesis, nitrogenase activity, growth and CO_2 uptake. The degree of inhibition of nitrogenase activity, growth and CO_2 uptake was greater for both genera when exposed to the higher concentration of the toxin Singh *et al*., 2001).

Sedmack and Kosi (1998) exposed *Chroococcus minutus* (Kütz) Naeg. to 104 µg.L^{-1} and 519 µg.L^{-1} of exogenous MC RR at two different light intensities, a high intensity (40 µE.m^{-1}.s^{-1}) and a low intensity (4 µE.m^{-1}.s^{-1}). Growth inhibition was accompanied by the degradation of the cells at the higher light intensity. In contrast to this, the cultures grown under the lower light intensity and the lower concentration of MC-RR resulted in a stimulation of growth of cells which was then followed by growth inhibition after day 10 of exposure. However, when a non-toxic strain of *Microcystis aeruginosa* (Kütz) was exposed to 519 µg.L^{-1} of MC RR it resulted in significant stimulation of growth at the lower light intensity from day 10 when compared to the control. There was no significant stimulation in growth when cells were exposed to the lower concentration of MC-RR at the higher light intensity.

There have been very few investigations on the effect of exogenous MC variants on cyanobacterial cultures. Furthermore, the investigations that have been done are incredibly difficult to compare due to the fact that various cyanobacterial genera and strains were used, there were differences in experimental designs and a limited variety of MC variants have been tested and often at physiologically irrelevant concentrations. The aim of this study was to determine the effect of a range of exogenous MC variants, at different concentrations, on photosynthetic activity in thylakoid membranes of cyanobacterial cultures.

4.1.3 Methods and Materials

4.1.3.1 Culture conditions

Mid-log phase cultures of *Microcystis* PCC 7806 (Pasteur Culture Collection; France; 2011), a Δ*mcyA*$^{-}$ mutant of *Microcystis* PCC 7806 (B. Neilan, UNSW) and *Synechocystis* PCC 6803 were collected by centrifugation (4000 *x g*; 10 min), washed twice in BG11 (Sigma) (pH 7.4) and inoculated into 2000 ml Erlenmeyer flasks to a final volume of 1500 ml. Cultures were incubated at 20°C (±0.5 °C) under continuous illumination of 20 µmol of photons m^{-2}.s^{-1} (Triton Dayglo©). Thylakoid membranes extracted from *Synechocystis* strain PCC 6803 were used for the experiments to determine the effect of microcystin variants and concentrations of these variants on photosynthetic activity. *Synechocystis* strain PCC 6803 was

chosen for these investigations because it does not produce microcystin and it has a superior growth rate compared to the other two strains.

4.1.3.2 Thylakoid extraction

The thylakoid membranes were isolated from either *Microcystis* sp. PCC 7806, a Δ*mcyA* mutant of *Microcystis sp.* PCC 7806 or *Synechocystis* PCC 6803, according to Nishiyama *et al*. (1993) and Hagio *et al*. (2000), with the following modifications. Cells were pelleted by centrifugation (4000 *x g*; 10 min) from 1500 ml of culture with an optical density of one at 740 nm. The pelleted cells were washed twice with 150 ml buffer A (50 mM HEPES-NaOH and 30 mM $CaCl_2$ at pH 7.5). All the above procedures were performed at 22 °C. After washing, the pelleted cells were resuspended in 50 ml buffer A containing 0.8 M sorbitol, 1 M betaine, 6-aminohexanioc acid, 5 mM EDTA and 0.2 % (w/v) lysozyme. This mixture was incubated at 28 °C with continuous mixing for 90 minutes. The following steps were performed between 0 °C and 4 °C. The cell suspension containing lysozyme was centrifuged at 4500 *x g* and the resulting pellet was washed and resuspended in buffer B (50 mM HEPES-NaOH, 30 mM $CaCl_2$, 0.8 M sorbitol, 1 M betaine and 6-aminohexanioc acid at pH 7.5). This was repeated three times. After the final wash, the pellet was resuspended in 20 ml of buffer C. An equal volume of acid-washed glass beads (150 – 212 µm) was added to the resuspended pellet. The mixture was vortexed at speed eight using an auto vortex mixer (Crown-scientific CSV90) for 60 seconds; following this, the mixture was placed on ice for 90 seconds. This was repeated five times. After the glass beads had settled to the bottom of the tube, the supernatant was removed and centrifuged at 2000 *x g* for five minutes to remove the unbroken cells and cell debris. The supernatant was collected and centrifuged at 40 000 *x g* for 25 minutes. The supernatant was discarded and thylakoid membrane pellet was resuspended in buffer C. The extracted membranes were used to measure photosynthetic activities.

4.1.3.3 Chlorophyll *a* extraction

One ml of the resuspended thylakoid pellet was centrifuged at 10 000 *x g* for 10 minutes. The pellet was resuspended in one ml of 90 % methanol by vigorous vortexing with an auto vortex mixer (Crown-scientific CSV90) for 180 seconds. This

was placed in the dark for two hours at 4 °C. Samples were centrifuged at 10 000 *x g* for five minutes and the supernatant was measured spectrophotometrically at 665 nm and a turbidity correction at 740 nm. The following formula was used to determine the chlorophyll *a* concentration:

$[\text{Chl } a]\ (\mu g.L^{-1}) = (A_{665\ nm} - A_{740\ nm}) \times \{(13.9 \times v)/(V \times L)\}$

where: v is the volume of extract in ml; V is the volume of the original sample in L and L is the path length of the cuvettes used in cm (Carvalho and Kirika, (2005).

4.1.3.4 Photosynthetic activity

Photosynthetic activity was determined by measuring the rate of DCPIP reduction in the presence of cyanobacterial thylakoid membranes Anderson *et al*., 1994). Thylakoid membranes were diluted to 10 µg of chlorophyll *a* $.ml^{-1}$ with buffer B. Either MC-RR, MC-LR or MC-WR was added to each of the diluted thylakoid membranes at a concentration of 75, 125, 250, 375 or 500 $fg.ml^{-1}$. An equal volume of buffer B was added instead of MC for the control. Thylakoid membrane containing two µg of chlorophyll *a* (200 µl) was added to each well of a 96-well microtitre plate containing either the MC variants or the buffer B. The microtitre plate was placed on ice and incubated in the dark for 15 min. DCPIP was added to each well at a final concentration of four mM. The microtitre plate was covered with foil and the initial $A_{600\ nm}$ was determined using a microtitre plate reader. After taking the initial reading, the plate was immediately illuminated at 100 $\mu mol.m^{-2}.s^{-1}$ of photons for ten minutes. After illumination the samples were read again. The photosynthetic activity was determined as µmol DCPIP reduced. µg chlorophyll a^{-1}. min^{-1}.

4.1.3.5 Oxygen evolution

An oxygraph containing a Hansatech oxygen electrode (Hansatech, King's Lynn, Norfolk, U.K) was used to measure the rates of oxygen evolution or uptake. The electrode was calibrated with air-saturated distilled H_2O at 20 °C as per the manufactures instructions. Thylakoid membranes were diluted to a concentration of 10 µg of chlorophyll *a* $.ml^{-1}$. Either MC-RR, MC-LR or MC-WR was added to each of the diluted thylakoid membranes at a concentration of either 100, 200 or 400 $fg.ml^{-1}$. Buffer B was added instead of MC for the control. Samples were dark adapted for 15 minutes. One ml of the dark-adapted thylakoid membranes was added to the reaction chamber. The reaction chamber was illuminated with photosynthetic

fluorescent tubes (Triton Dayglo©) under continuous illumination of 100 µmol of photons $m^{-2}.s^{-1}$.

4.1.3.6 Photosystem II activity

The activity of photosystem II (PSII) was measured as O_2 evolution by the addition of 2,6 –dimethyl-1,4-benzoquinone at a final concentration of 1 mM as an electron acceptor, which was kept oxidized by $K_3Fe(CN)_6$ at a final concentration of 2 mM (Stewart and Bendall, 1980). Oxygen evolution was measured according to section 4.1.3.5.

4.1.3.7 Photosystem I activity

Photosystem I (PS I) activity was measured as O_2 uptake. The following reagents were added to the reaction chamber containing one ml of thylakoid membranes: 75 µM 2,6-dichlorophenol-indophenol, 2.5 mM sodium D-isoascorbate, 10 mM 3,4 – dichlorophenyl – 1,1-dimethylurea, 3 mM NH_4Cl and 30 µg of methyl viologen $.ml^{-1}$ (Stewart and Bendall, 1980). Oxygen evolution was measured according to section 4.1.3.5.

4.1.4 Results

MC-LR inhibits photosynthetic activity in *Microcystis* PCC 7806 and *Synechocystis* PCC 6803 (figure 4.1). The photosynthetic activity of *Synechocystis* is greater than that of the *Microcystis* strain PCC 7806 and a Δ*mcyA*$^-$ mutant of *Microcystis* strain PCC 7806. The control of the toxin producer was significantly lower than the mutant for the same strain (figure 4.1). The exogenous MC-LR caused a lesser degree of inhibition of photosynthetic activity in *Synechocystis* PCC 6803 compared to the other two strains. *Microcystis* strain PCC 7806 showed the greatest percentage of inhibition when compared to the Δ*mcyA*$^-$ mutant of *Microcystis* strain PCC 7806 (figure 4.1). This is most likely due to the endogenous production of MC-LR by *Microcystis* strain PCC 7806.

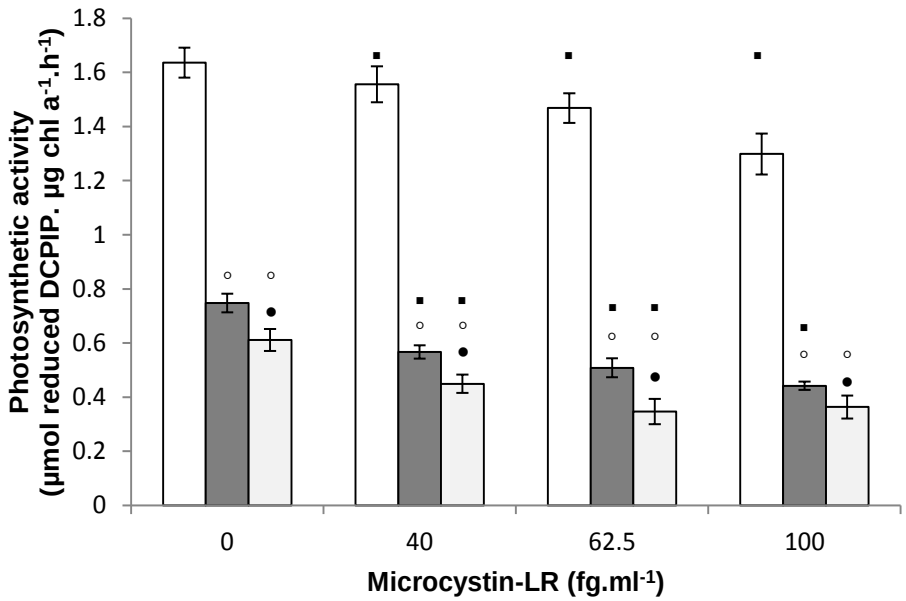

Figure 4.1: The effect of exogenous MC-LR on the photosynthetic activity of thylakoid membranes isolated from *Synechocystis* sp. PCC 6803 (□), *mcyB*$^-$ mutant of *Microcystis* sp. PCC 7806 (■) and *Microcystis* sp. PCC 7806 (■). Error bars are standard deviation; n = 10. ■ -indicates significant difference from previous concentration of MC ($p < 0.05$); ○ - indicates significant difference from *Synechocystis* sp. PCC 6803 ($p < 0.05$) and ● - indicates significant difference from *mcyB*$^-$ mutant of *Microcystis* sp. PCC 7806 ($p < 0.05$)

The addition of exogenous MC-LR, MC-RR, and MC-WR caused a significant decrease in PS II activity compared to the control, for all concentrations of exogenous microcystin variants tested (figure 4.2 and 4.3). There was a strong logarithmic relationship ($y = -44.35\ln(x) + 94.598$; $r^2 = 0.9959$) between PS II activity and the concentration of exogenous MC-LR (figure 3.3a).

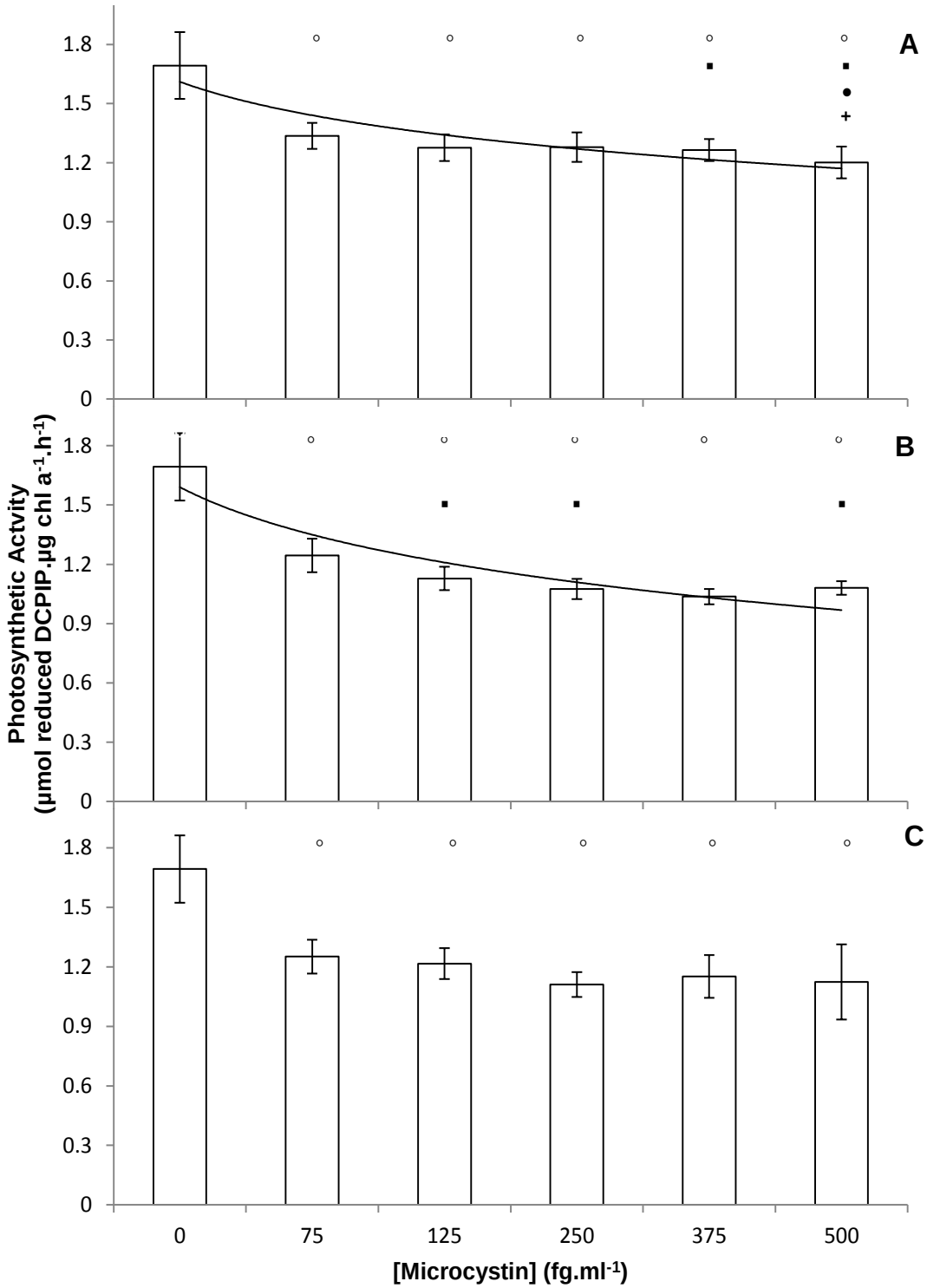

Figure 4.2: The effect of exogenous MC-LR (A), MC-RR (B) and MC-WR (C) on the photosynthetic activity of *Synechocystis* sp. PCC 6803. Error bars are standard deviation; n = 10. ○ - indicates significant difference from the control ($p < 0.05$); ■ -indicates significant difference from previous; ● - indicates significant difference from the 125 fg.ml^{-1} of MC-LR ($p < 0.05$) and + - indicates significant difference from the 250 fg.ml^{-1} of MC-LR ($p < 0.05$)

A similar logarithmic relationship was observed between PS II activity and different concentrations of exogenous MC-RR (y = -39ln(x) + 74.187; r^2 = 0.955) (figure 4.3b). A weaker logarithmic relationship was observed for photosynthetic activity and the concentration of exogenous MC-LR (y = -0.0764ln(x) + 1.6088; r^2 = 0.648) (figure 4.2a) when measured with the DCPIP reducing assay compared to PS II activity measured by oxygen evolution. A similar trend was observed for photosynthetic activity and the concentration of exogenous MC-RR (y = -0.347ln(x) + 1.5905; r^2 = 0.8641) (figure 4.2b). There was no relationship between PS II activity and exogenous MC-WR concentration (figure 4.2c and 4.3c).

MC-WR was the most potent inhibitor of PS II activity at the following concentrations; 100 and 200 fg.ml^{-1}. MC-RR inhibited PS II activity to a greater extent compared to MC-WR, but to a lesser extent than MC-LR at the above-mentioned concentrations. MC-WR was a more potent inhibitor of PS II activity compared to MC-RR at a concentration of 400 fg.ml^{-1}, but the difference in inhibition between these two toxins was not significant. MC-LR, at this concentration, was the least potent inhibitor.

PS I activity was not inhibited by any of the MC variants, for all concentrations that were tested (figure 3.4).

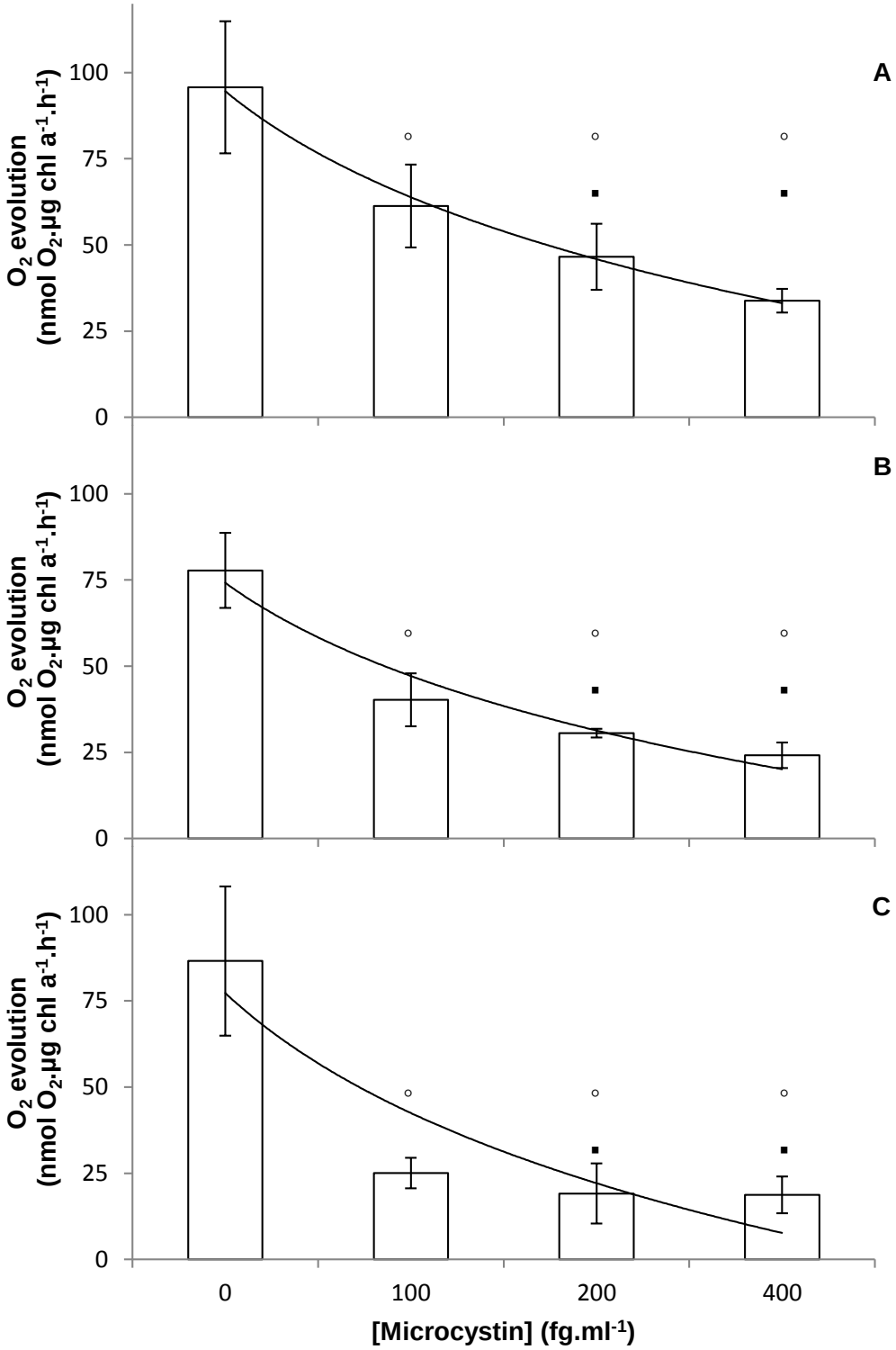

Figure 4.3: The effect of MC-LR (A), MC-RR (B) and MC-WR (C) on PS II activity of thylakoid membranes isolated from *Synechocystis* sp. PCC 6803. Error bars are standard deviation; n =5. ● - significant from control ($p < 0.05$) and □ - significant from previous ($p < 0.05$)

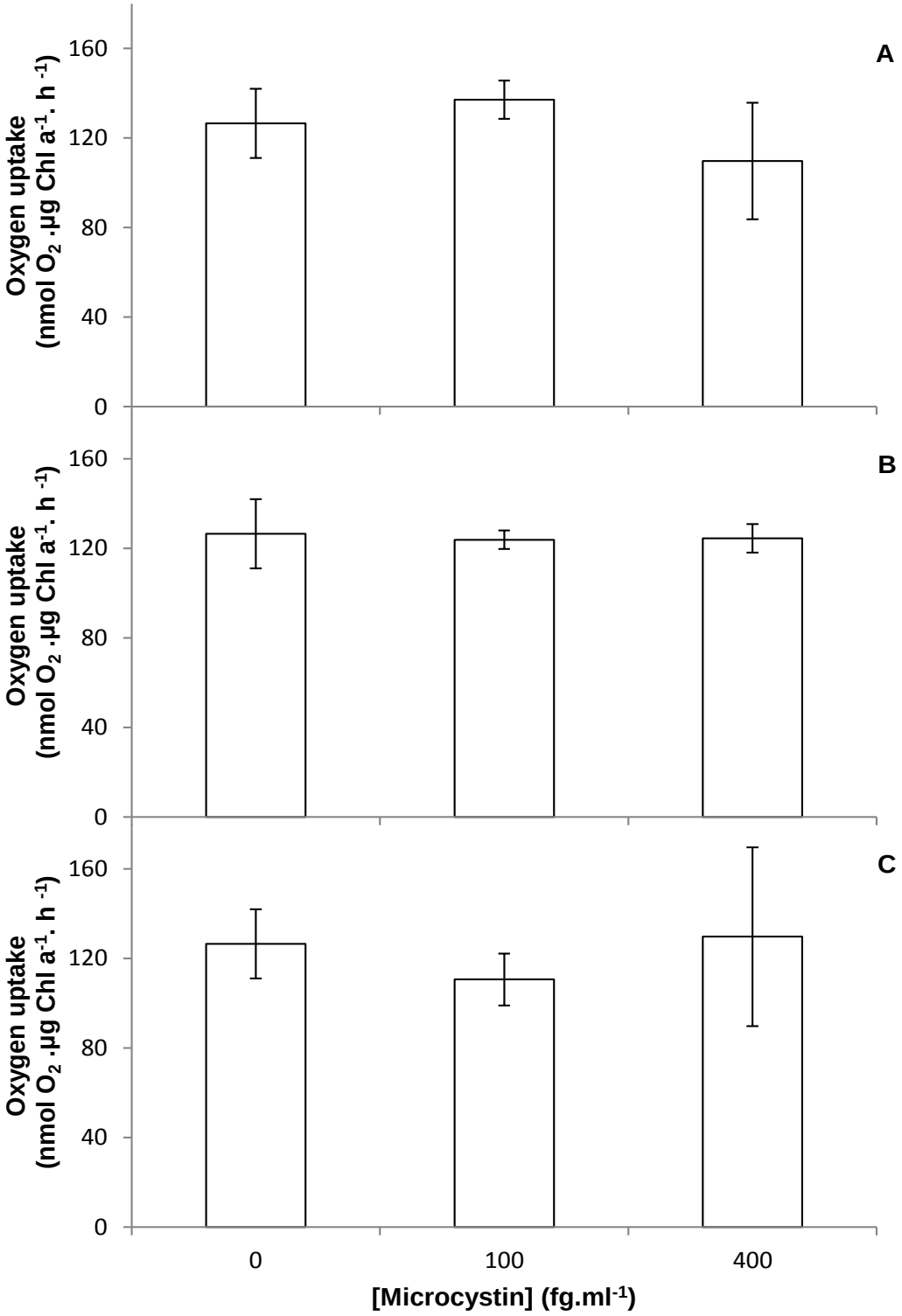

Figure 4.4: The effect of MC-LR (A), MC-RR (B) and MC-WR (C) on PS I activity of thylakoid membranes from *Synechocystis* sp. PCC 6803. Error bars are standard deviation; n =5

4.1.5 Discussion

It has been noted that exogenous MC inhibits photosynthesis in cyanobacteria [Hu *et al*., 2004; Singh *et al*., 2001). However, the concentrations of exogenous MCs that have been used in previous studies have not been physiologically relevant. Singh *et al*. (2001) exposed the following genera of cyanobacteria, *Anabaena sp.* BT1 and *Nostoc muscorum* C. Agardh, to either 25 $\mu g.ml^{-1}$ or 50 $\mu g.ml^{-1}$ of semi-purified MC-LR. This resulted in complete inhibition of oxygen evolution in both strains within 25 minutes. Hu *et al*. (2004) noted a similar trend that MC-RR concentrations greater than 100 $ng.ml^{-1}$ inhibited PS II activity significantly on day four of exposure, however, from day six PS II activities had recovered to its original position.

The data showed that MC-LR, RR and WR inhibited photosynthesis in thylakoid membranes of *Synechocystis* strain PCC 6803 for concentrations that are substantially lower than those previously tested, and typical of intracellular concentrations of MC [Downing *et al*., 2005a; Downing *et al*., 2005b; Lyck, 2004; Okello and Kurmayer, 2011; Phelan and Downing, 2007; Phelan and Downing, 2011; Wiedner *et al*., 2003) as determined by converting the data into a MC quota per cell. This was done by determining the average chlorophyll *a* quota per cell which was determined to be approximately 500 $fg.cell^{-1}$ for the cultures that were used for this study. This is consistent with what was reported by Lyck (2004) who observed that the chlorophyll *a* quota per cell ranged between 184 and 1799 $fg.cell^{-1}$, of which three quarters of the data ranged between 184 and 800 $fg.cell^{-1}$. Therefore, the MC quota per cell can be extrapolated as 0.005 $fg.cell^{-1}$, 0.01 $fg.cell^{-1}$ and 0.02 $fg.cell^{-1}$ for each treatment of each variant that was tested. These extrapolated MC content per cell are lower than the MC content determined from previous studies. Wiedner *et al*. (2003) reported MC quotas ranging from 40 to 80 $fg.cell^{-1}$ for *Microcystis* PCC 7806 grown in light conditions from 10 to 100 $\mu mol.m^{-2}.s^{-1}$. Long *et al*. (2001) observed a variation in MC quotas ranging from 0.053 to 0.116 $fmol.cell^{-1}$ under nitrogen limiting conditions for *Microcystis* strain MASH 01-A19. Okello and Kurmayer (2011) reported that there was a greater than 100 fold difference in MC quota between Lake George (Kahendero) and Lake Saka. Lake George consistently showed the lowest MC quota ranging from 0.03 to 1.24 $fg.cell^{-1}$ for a *Microcystis* sp. whereas a *Microcystis* sp. from Lake Sake consistently showed a higher MC quota (14 to 144 $fg.cell^{-1}$). Downing *et al*. (2005b) showed that intracellular MC quotas

ranged between 0.01 to 1 fg.cell^{-1} for *Microcystis aeruginosa* at various N:C ratios. The data showed that MC inhibits PS II activity of thylakoid membranes from *Synechocystis* strain PCC 6803 at physiologically relevant concentrations.

The only difference between the MC variants that were used for this study was the hydrophobicity of the L-amino acids at position two and four of the MC molecule. Therefore, the differences in inhibition of PS II activity were most probably caused by the hydrophobicity of the L-amino acids. MC-WR, the most hydrophobic variant, caused the greatest inhibition of PS II activity (figure 4.3C). This is probably due to the higher surface activity on the thylakoid membranes. Vesterkvist and Meriluoto (2003) reported that MC-LW, the more hydrophobic variant, had the highest surface activity on an egg phosphatidylcholine-cholesterol monolayer, whereas the hydrophilic variant had a lower surface activity. The hydrophobicity of the MC variants may either alter the membrane fluidity or enhance binding to the thylakoid membranes, hence altering the electron transport through the thylakoid membranes.

The data from this study indicated that MC affects PS II while having no significant effects on PS I activity for any of the MC treatments (figure 4.3 and 4.4). Furthermore, this experiment indicated that the MC producer *Microcystis* PCC 7806 had a significantly lower photosynthetic activity compared to the Δ*mcyA*$^{-}$ mutant of *Microcystis* PCC 7806 (figure 4.1). PS II is very susceptible to excessive light (Murata *et al*., 2007). There are several possible explanations for the decrease in the rate of electron transport through PS II by MC. MC may protect or stabilise PS II from photoinhibition or photodamage by either acting as a light protectant by absorbing the excess excitation energy of the action spectrum that causes PS II damage or binding to the oxygen-evolution complex and decreasing the amount of reactive oxygen species generated.

MC inhibits PS II activity at physiologically relevant concentrations and that the degree of inhibition is affected by the hydrophobicity of the variable L-amino acids. Furthermore, this data provides further support that the biological function of MC may be involved in either regulating or protecting the photosynthetic apparatus. This may provide a competitive advantage for MC producers in unfavourable

environmental conditions that lead to photoinhibition in other aquatic photosynthetic organisms.

As per previous Chapter:

Microcystin reduced photosystem II activity at physiologically relevant concentrations. The degree of inhibition of photosystem II was concentration-dependent and variant-dependent. The activity of photosystem I was not affected by microcystin.

However

It is not yet known which component of the photosystem II reaction centre that is inhibited by microcystin.

Therefore:

Chlorophyll *a* fluorescence was used to determine the action site of microcystin in photosystem II.

Chapter 5

5.1 Use of chlorophyll *a* fluorescence to identify the effects of microcystin variants on energy fluxes and components of the photosystem II of *Synechocystis* PCC 6803

5.1.1 Abstract

Cyanobacterial blooms have increased over the past few decades and have been mainly linked to anthropogenic eutrophication of water bodies. These blooms sometimes produce toxic metabolites, one of which is microcystin. The biological function of microcystin has been extensively researched. However, no definitive function has been described. Microcystin has been observed to inhibit PSII in higher plants and cyanobacteria. Chlorophyll *a* fluorescence is a valuable tool in studying photosynthesis and provides a non-invasive technique for real-time information about the photosynthetic status of an organism. *Synechocystis* PCC 6803 was exposed to different concentrations of a number of microcystin variants. After exposure to microcystin, a Multi-functional Photosynthetic Efficiency Analyser (M-PEA) fluorimeter was used to measure the chlorophyll *a* fluorescence of the different treatments. The JIP-test and the change in variable fluorescence were used to examine the photosynthetic status of the cyanobacterium. The results showed that microcystin variants LR, RR and LF affected the photosynthetic efficiency and energy flow through photosystem II suggesting a mode of action for microcystin at the photosynthetic apparatus level. This represents the first data-supported potential biological function for microcystin in cyanobacteria; protection against photoinhibition.

5.1.2 Introduction

The photosynthetic apparatus of cyanobacteria is composed of five functional protein complexes; phycobilisomes, photosystem II (PS II), photosystem I (PS I), cytochrome b6f and ATP synthase (Reuter and Müller, 1993)

MCs are non-ribosomally synthesized (Dittmann *et al*., 1997) mono-cyclic heptapeptides. These heptapeptides have a unique chemical structure which includes the β-amino acid, 2S,3ES,8S,9S-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-deca-4E,6E-dienoic acid (ADDA). The general formula for MC is cyclo-D-alanine-L-X-D-erythro-β-methylaspartic acid –L-Z-ADDA-D-glutamate-N-methyldehydroalanine (Botes *et al*., 1985). Currently, over 100 different structural variants have been identified (Meriluoto and Spoof, 2008). The majority of these variations occur at the variable L-amino acid residues which are designated as "X" and "Z" in the general formula. For example, MC-LR has leucine and arginine, MC-RR has two arginine residues, MC-LF has leucine and phenylalanine and MC-WR contains tryptophan and arginine. Other variations include minor alterations to other parts of the molecule, such as the loss or the addition of methyl groups (Spoof *et al*., 2003).

MC is not produced by all genera of cyanobacteria (Chorus and Bartram, 1999). *Microcystis*, *Anabaena*, *Nostoc*, *Oscillatoria* and *Planktothrix* are the most common genera that have been found to produce MCs (Sivonen and Jones, 1999). MC toxicity has been extensively studied. In mammals (including natural human intoxication), fishes, amphibians and invertebrates MCs are potent hepatotoxins (Carmichael, 2001; Dao *et al*., 2010; Deblois *et al*., 2008; Magalhaes *et al*., 2003). MCs have numerous harmful effects on photosynthetic organisms such as aquatic plants, algae and terrestrial plants. These include: rapid inhibition of photosynthesis, decrease in chlorophyll content, leaf necrosis, and inhibition of growth (Abe *et al*., 2006; Pflugmacher, 2002; Wiegand *et al*., 2002; MacKintosh *et al*., 1990). However, the effect of MCs on cyanobacteria has not been adequately studied. The studies that have been conducted (Sedmack and Kosi, 1998; Pflugmacher *et al*., 1999; Singh *et al*. 2001; Hu *et al*., 2004) have not shown any consistency in the results obtained due to the use of different genera of cyanobacteria, variation in experimental design, and non-relevant MC concentrations.

Singh *et al*. (2001) reported that when *Anabaena sp.* BT1 and *Nostoc muscorum* C. Agardh were exposed to semi-purified MC-LR, at a concentration of 25 000 $\mu g.L^{-1}$ and 50 000 $\mu g.L^{-1}$, photosynthesis was inhibited and nitrogenase activity, growth and CO_2 uptake decreased. This was more evident for the higher concentration for the semi-purified MC-LR.

Sedmack and Kosi (1998) exposed *Chroococcus minutus* (Kütz) Naeg. to 104 $\mu g.L^{-1}$ and 519 $\mu g.L^{-1}$ MC-RR at a light intensity of 4 $\mu E.m^{-1}.s^{-1}$ which resulted in the stimulation of growth at day eight of incubation for both concentrations that were used. After day eight a decrease in growth rate was observed for both concentrations at the same light intensity. However, an increase in *C. minutus* biomass relative to the control was observed on day eight at 40 $\mu E.m^{-1}.s^{-1}$ of continuous illumination for the lower concentration of MC-RR and also on day six for the higher concentration. On day 12 of exposure to MC-RR, reduced biomass relative to the control was observed for both concentrations of MC-RR (Sedmack and Kosi, 1998).

However, when a non-toxic strain of *Microcystis aeruginosa* (Kütz) was exposed to 519 $\mu g.L^{-1}$ of MC RR it resulted in significant increase in biomass relative to the control at the lower light intensity from day 10 and from day 12 when exposed to 104 $\mu g.L^{-1}$ of MC-RR. The addition of 519 $\mu g.L^{-1}$ of MC-RR resulted in an increase in cell numbers relative to the control from day 10 in the non-toxic strain of *Microcystis aeruginosa* (Kütz) when grown under a light intensity of 40 $\mu E.m^{-1}.s^{-1}$ and from day 12 when non-toxic strain of *Microcystis aeruginosa* (Kütz) was exposed to 104 $\mu g.L^{-1}$ of MC-RR at the same light intensity (Sedmack and Kosi, 1998).

The current research on the effect of exogenous MC on cyanobacteria is limited and one of the major shortcomings of these published works is that experiments have been done at physiologically irrelevant concentrations of MC. Furthermore, it is difficult to compare prior studies because of the use of different methods and different cyanobacterial genera, and a limited variety of MC variants.

Chlorophyll *a* fluorescence was used by Perron *et al*. (2012) to examine the effect of exogenous MC variants at different concentration on green algae. They noted that after a 15 minute exposure to various MC variants, the photosynthetic efficiency in *Chlorella vulgaris* CPCC111 was negatively affected. MC-LF at a concentration of 1 $\mu g.ml^{-1}$ decrease the photosynthetic efficiency the most, followed by MC-YR, MC-LR and MC-RR at that concentration. In addition, a concentration dependent relationship was observed for MC-LF and MC-RR with increasing MC concentrations decreasing the photosynthetic efficiency. Chlorophyll *a* fluorescence data from

Deblois and Juneau (2010) revealed a logarithmic relationship between MC concentration and electron transport rates.

Chlorophyll *a* fluorescence is a useful tool that offers non-invasive, quick, real-time information about the photosynthetic status of a photosynthetic organism. Unlike higher photosynthetic organisms, cyanobacteria have accessory pigments called phycobiliproteins which function in light-harvesting and energy migration to photosystem II (PSII) (Bogorad, 1975). The phycobiliproteins have an effect in the interpretation of the fluorescent signals with fluorescent intensity at 50 µs (F_0) being affected by the phycobiliprotein content (Campbell *et al*., 1995). Campbell (1996) reported that the F_0 value increased with an increase in the phycocyanin content. A mutant of *Synechococcus* sp. PCC 7942 lacking phycocyanin had a much lower F_0 compared to the wild type.

The maximal photochemical efficiency (F_v/F_m [$(F_m - F_0)/F_m$]) in higher plants is approximately 0.8. Values less than this indicate inhibition of PSII. In cyanobacteria the average F_v/F_m ratio for the cyanobacterium *Synechococcus* sp. PCC 7942 is between 0.4 and 0.6. However, a mutant of this strain, unable to produce phycocyanin has a F_v/F_m ratio of approximately 0.75 when cultured in the same conditions (Campbell *et al*., 1998). Campbell *et al*. (1995) and Clarke *et al*. (1995) showed that if the phycobiliprotein content remained constant, the variations in the F_v/F_m ratio correlated with oxygen evolution. This means that as long as the phycobiliprotein quota in the cyanobacterium remains constant, the parameters from the JIP-test and the fluorescent induction curves can be compared. This can easily be achieved by comparing aliquots of an identical culture.

The aim of this investigation was to determine the component of PSII that was inhibited by MC variants at different concentrations in the non-MC producing cyanobacterium, *Synechocystis* PCC 6803, by chlorophyll *a* fluorescence, so as to further elucidate the metabolic significance or ecological benefit of MC production.

5.1.3 Methods and Materials

5.1.3.1 Culture conditions

Synechocystis PCC 6803 was chosen for this study because it does not have differing pigment quota like *Microcystis* PCC 7806 and the Δ*mcyA* mutant of *Microcystis* PCC 7806 which will affect the interpretation of fluorescent data. *Synechocystis* PCC 6803 was grown to on OD_{740} of 0.7 in BG11 (Sigma) at pH 7.4. Cultures were incubated at 20 °C (± 0.5 °C) under continuous illumination of 20 µmol of photons $m^{-2}.s^{-1}$ (Triton Dayglo©). The cells were pelleted by centrifugation (5000 *x g*). The pellet was washed and resuspended twice in freshly made BG11 at pH 7.4. The resuspended culture was divided into 45 equal volumes of 20 ml and placed into 50 ml Falcon tubes. The Falcon tubes containing 20 ml of culture were divided into nine groups of five. The first group was the control and the remaining eight groups contained two different final concentrations (2.5 $\mu g.l^{-1}$ or 10 $\mu g.l^{-1}$) each of the following MC variants: MC-LR, MC-RR, MC-WR and MC-LF (Alexis Biochemicals). Cultures were incubated at 25 °C (±0.5 °C) for 16 hours under continuous illumination of 60 µmol of photons $m^{-2}.s^{-1}$. After the incubation period, 10 ml aliquots of each sample were dark-adapted for 1 hour and then filtered through a glass fibre membrane (Whatman).

5.1.3.2 Measurement of fluorescence

A Multi-functional Photosynthetic Efficiency Analyser fluorimeter (M-PEA, Hansatech Instrument Ltd. Kingslynn, UK) was used to monitor chlorophyll *a* fluorescence of dark-adapted cultures in pentuplicate for two places on each filter where the cultures were collected per treatment. Fluorescence induction curves of each sample were initiated by a homogenous red light (peak 650 nm) on a 12.56 mm^2 area of glass fibre membrane for one second.

The JIP-test formulae (Table 5.1) were used to analyse the OJIP transients (Strasser *et al*., 1995). The JIP-test formulae translate the chlorophyll *a* fluorescence induction curves into phenomenological and biophysical parameters that quantify the flow of energy through PSII (Strasser *et al*., 2004). The M-PEA plus software was used to solve the equations of the JIP-test. The parameters that refer to the onset of fluorescence induction are: (i) the specific energy fluxes per reaction centre for absorption (ABS/RC), trapping (TR_0/RC), electron transport (ET_0/RC) and dissipation

at the level of the antenna chlorophylls (DI_0/RC); (ii) the flux ratios or yields for maximum quantum yield of primary photochemistry ($\varphi_{Po} \equiv TR_0/ABS$), probability (at t = 0) that a trapped exciton moves an electron into the electron transport chain from Q_A^- ($\psi_o \equiv ET_0/TR_o$), quantum yield of electron transport (at t = 0) ($\varphi_{Eo} \equiv ET_0/ABS$), quantum yield (at t = 0) of energy dissipation (φ_{Do}); (iii) the phenomenological energy fluxes per excited cross sections (CS) for absorption flux per CS (ABS/CS), trapped energy flux per CS (TR_0/CS), electron transport flux per CS (ET_0/CS), dissipated energy flux per CS (DI_0/CS), fraction of functional PSII reaction centres per excited cross section (RC/CS) (Strasser *et al*., 1995; Strasser *et al*., 2004; Strasser *et al*., 2000).

As described by Strasser *et al*. (2004), the performance index ($PI_{(ABS)}$) is a multi-parametric expression of absorption of light (ABS), trapping of excitation energy (TR) and conversion of excitation energy to electron transport, which contribute to the initial stage of photosynthetic activity of the reaction centre (RC) complex:

$$\mathbf{PI_{(ABS)} = (\gamma/(1-\gamma) \times (\varphi_{Po}/(1-\varphi_{Po}) \times (\psi_o/(1-\psi_o)}$$

where γ is the fraction of reaction centre chlorophyll (Chl_{RC}) per total chlorophyll ($Chl_{RC\ +\ antenna}$), therefore $\gamma/(1-\gamma) \equiv Chl_{RC}/Chl_{antenna} \equiv RC/ABS$, $\varphi_{Po}/(1-\varphi_{Po})$ is the contribution of the light reactions for primary photochemistry derived by the JIP-test and $[\varphi_{Po}/(1-\varphi_{Po})] \equiv TR_0/DI_0 \equiv k_p/k_N \equiv F_V/F_0$, $\psi_o/(1-\psi_o)$ is the contribution of the dark reactions as derived by the JIP-test in that the following equivalences are valid $[\psi_o/(1-\psi_o)] \equiv ET_0/(TR_0 - ET_0) \equiv (F_M - F_{2\ ms})/(F_{2\ ms} - F_{50\ \mu s})$.

Table 5.1: The JIP- test formulae using data obtained from the O-J-I-P chlorophyll *a* fluorescence transient (Strasser *et al*., 2004).

Extracted and technical fluorescence parameters
$F_0 = F_{50\ \mu s}$, fluorescence intensity at 50 µs
$F_{100\ \mu s}$ = fluorescence intensity at 100 µs
$F_{300\ \mu s}$ = fluorescence intensity at 300 µs
F_J = fluorescence intensity at 2 ms
F_I = fluorescence intensity at 30 ms
F_M= maximal fluorescence intensity
t_{F_M} = time to reach F_M
V_J = relative variable fluorescence at the J-step = ($F_{2\ ms}$- F_0)/ (F_M – $F_{2\ ms}$)
Quantum efficiencies
$\varphi_{Po} = TR_0/ABS = [1- (F_0/F_M)] = F_V/F_M$
$\psi_o = ET_0/TR_o = 1 - V_J$
$\varphi_{Eo} = ET_0/ABS = [1- (F_0/F_M)]\ \psi_o$
Specific fluxes
$ABS/RC = M_0(1/ V_J)(1/\varphi_{Po})$
$TR_0/RC = M_0(1/ V_J)$
$ET_0/RC = M_0(1/ V_J)(\ \psi_o)$
$DI_0/RC = (ABS/RC) – (TR_0/RC)$
Phenomenological fluxes
$ABS/CS = ABS/CS_{Chl}$ = Chl/CS or $ABS/CS_0 = F_0$ or $ABS/CS_M = F_M$
$TR_0/CS = \varphi_{Po}(ABS/CS)$
$ET_0/CS = (\varphi_{Po})(\psi_o)(ABS/CS)$
$DI_0/CS = (ABS/CS) – (TR_0/CS)$
Density of reaction centers
$RC/CS = \varphi_{Po}(V_J/ M_0)(\ ABS/CS)$
Performance index
$\mathbf{PI_{(ABS)} = (\gamma/(1- \gamma)(\varphi_{Po}/(1- \varphi_{Po})(\psi_o/(1- \psi_o)}$

Further examination of the chlorophyll *a* fluorescent transients was done by calculating and plotting the difference in V of the treatment normalized to the control between F_0 and F_m. These plots have distinct bands where the treatments are either above or below the reference line. The ΔK, ΔJ and ΔI-bands appear approximately at 0.2 ms, 2ms and 30 ms respectively. The uncoupling of the oxygen evolution complex is linked to the ΔK-bands. The accumulation of Q_a^- is associated with the ΔJ-bands, whereas ΔI bands show that the final reduction of the end acceptors is inhibited (Cosgrove and Borowitzka, 2010).

5.1.3.3 Statistics

Statistical analysis was performed using Statistica8 (StatSoft Inc., Tulsa, OK, USA). Students T-test was used to determine significance between treatments. Data were considered to be significantly different when *p* values < 0.05.

5.1.4 Results

Excitation of the dark-adapted *Synechocystis* PCC 6803 with a saturated homogenous red light induced a typical chlorophyll *a* fluorescence transient (Kautsky) curve (figure 5.1). The Kautsky curve for the control (no MC) was higher than the Kautsky curves when the different concentrations and variants of MC were added. However, there were no clear trends in these differences relating to the concentrations used or the different variants.

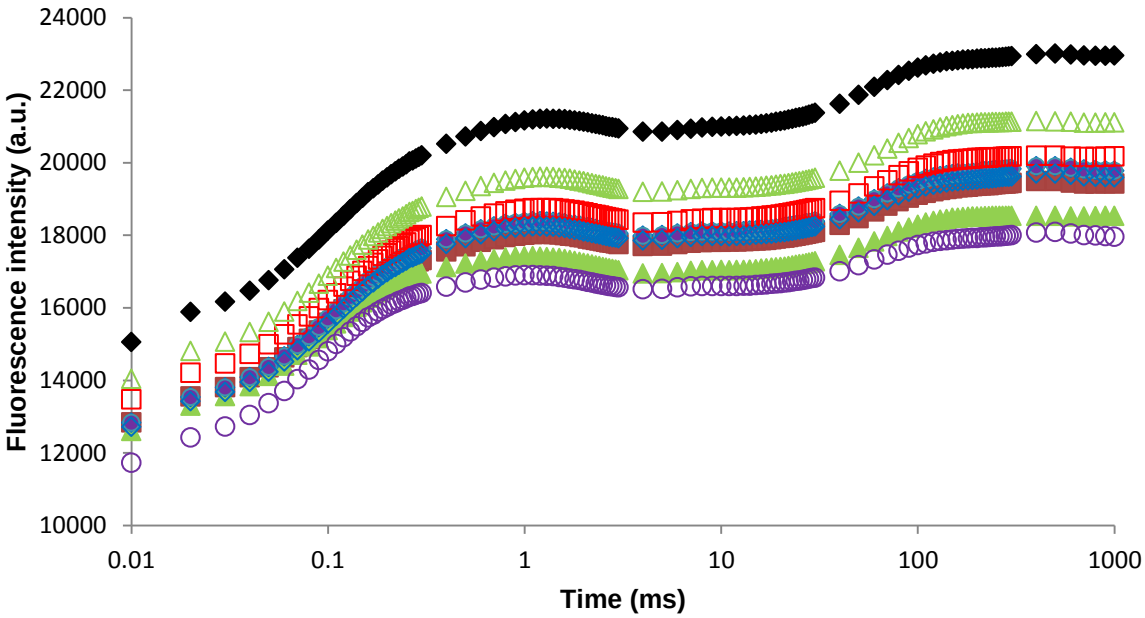

Figure 5.1: Chlorophyll *a* fluorescence transients exhibited upon illumination of dark adapted *Synechocystis* PCC 6803 by saturating red light (650 nm), plotted on a logarithmic scale. The effects of a 16 hour exposure to 0 $\mu g.L^{-1}$ (♦); 2.5 $\mu g.L^{-1}$ of MC-LR (■), MC-RR (▲), MC-WR (♦) or MC-LF (●) and 10 $\mu g.L^{-1}$ of MC-LR (□), MC-RR (△), MC-WR (◊) or MC-LF (○) were recorded from filtered *Synechocystis* PCC 6803. n = 10

To further examine whether there were any differences between the concentrations and the variants of MC used, the MC-dependent changes in the fluorescence

kinetics were investigated further by calculating and plotting the difference in relative variable fluorescence by normalizing between F_0 and F_m ($\Delta V = V_{treatment} - V_{control}$) (figure 5.2). A positive ΔK-band (± 0.2 ms) was observed for all the concentrations and variants of MC that were added exogenously to *Synechocystis* PCC 6803 (figure 5.2). The positive shift in the ΔK-band was the largest for MC-RR at a concentration of 2.5 µg.L^{-1} followed by MC-WR, MC-LF and MC-LR. When the concentration of MC was increased to 10 µg.L^{-1} the relationship between the different variants used in this study and the positive shift in the ΔK-band was altered. The greatest positive shift in the ΔK-band was observed for MC-LF followed by MC-WR, MC-RR and MC-LR.

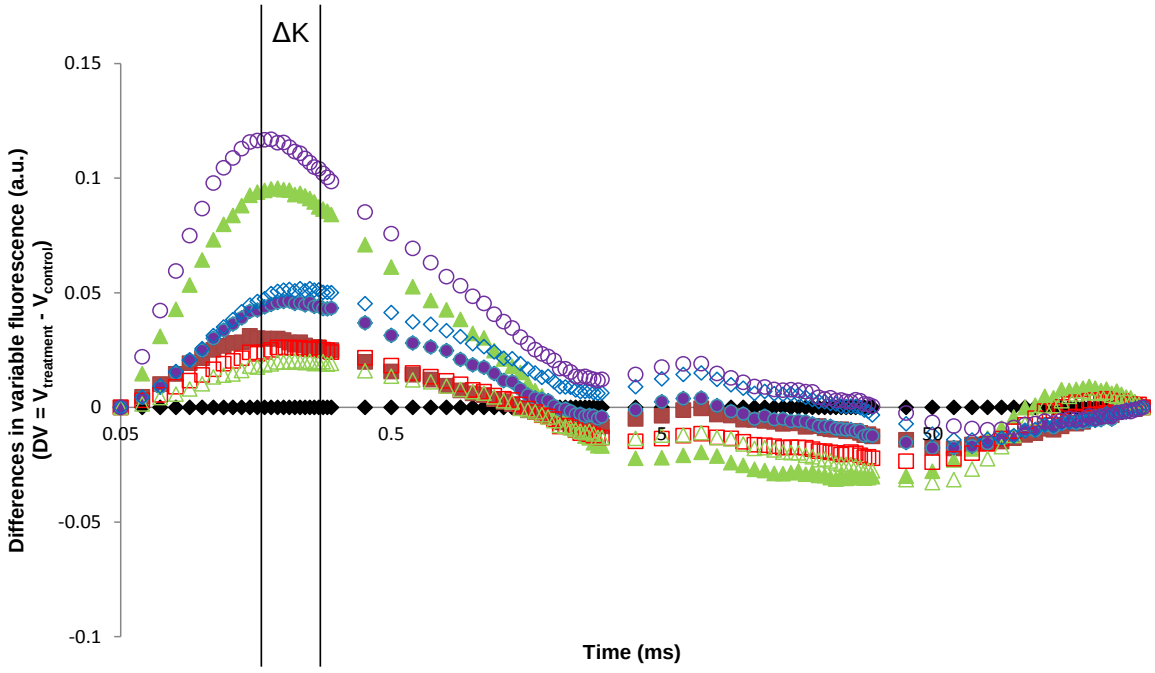

Figure 5.2: Difference in variable fluorescence of the double normalization at the OP phase, V_{OP} = (Ft – F0)/(FP – F0), of the V_{OP} of the control subtracted from the treatment. The effects of a 16 hour exposure to 0 µg.L^{-1} (♦); 2.5 µg.L^{-1} of MC-LR (■), MC-RR (▲), MC-WR (♦) or MC-LF (●) and 10 µg.L^{-1} of MC-LR (□), MC-RR (△), MC-WR (◊) or MC-LF (○) were recorded from filtered *Synechocystis* PCC 6803. n = 10

The concentration-dependent differences between the hydrophobic and hydrophilic variants of MC were more evident in the positive shifts of the ΔK-bands (figure 5.2).The JIP-test formulae were applied to determine specific differences in the photosynthesis of *Synechocystis* PCC 6803 for the different concentrations and variants of MC that were used.

MC-RR at a concentration of 2.5 $\mu g.L^{-1}$ caused a significant increase in the ABS/RC compared to the control whereas MC-LR, MC-WR and MC-LF were higher than the control but not significantly so (figure 5.3A). When the exogenous MC concentration of each variant was increased to 10 $\mu g.L^{-1}$ (figure 5.3B), a significant concentration-dependant relationship was observed between MC-RR and ABS/RC and MC-LF and ABS/RC, whereas this relationship was not observed for MC-LR and MC-WR. As the concentration of MC-LF increased, the ABS/RC increased as well. The opposite was observed for MC-RR and ABS/RC.

The addition 2.5 $\mu g.L^{-1}$ of MC-LF to *Synechocystis* PCC 6803 had no effect on the DIo/RC compared to the control. MC-RR, MC-LR and MC-WR significantly increased the DIo/RC, of these, MC-RR enhanced the DIo/RC the most followed by MC-LR and then MC-WR. MC-RR increased the DIo/RC the greatest, followed by MC-LR and then MC-LF. The same trends were observed for DIo/RC as in the ABS/RC for an increase in the concentration of exogenous MC for the variants used in this study (figure 5.3A and figure 5.3B).

No significant changes were observed (figure 5.3A) in the TRo/RC when 2.5 $\mu g.L^{-1}$ of MC-LR, MC-WR and MC-LF was added compared to the control. However, there was a significant increase when MC-RR was added at the same concentration (figure 5.3). MC-RR caused the greatest amount of TRo/RC compared to the other variants. No concentration-dependant relationship was observed in the TRo/RC when the concentrations of MC-LR, MC-RR and MC-WR were increased to 10 $\mu g.L^{-1}$. An increase in concentration of MC-LF caused a significant increase in the TRo/RC (figure 5.3B).

MC-RR, MC-LR and MC-WR enhanced ETo/RC compared to the control, whereas no real effect was observed when the culture was exposed to 2.5 $\mu g.L^{-1}$ of MC-LF (figure 5.3A). No significant differences were observed between the different variants used in this study. A significant concentration-dependant relationship was observed between MC-WR and ETo/RC and MC-LF and ETo/RC, whereas this relationship was not noted for MC-LR and MC-WR. As the concentration of MC-LF increased, the ETo/RC increased as well. The opposite was observed for MC-WR and ETo/RC (figure 5.3B).

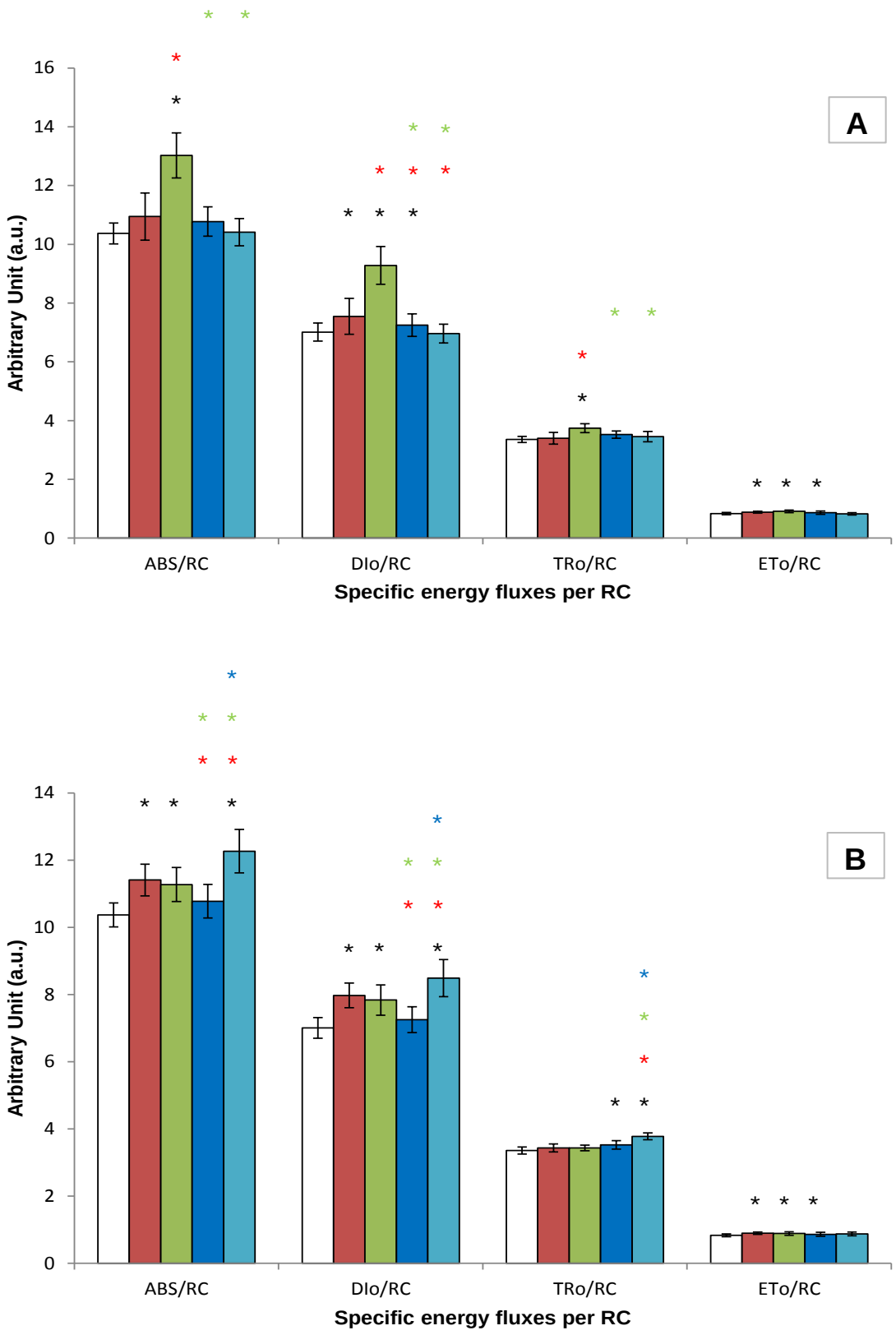

Figure 5.3:The effects of a 16 hour exposure to either 2.5 µg.L^{-1} (A) or 10 µg.L^{-1} (B) of MC-LR (■), MC-RR (■), MC-WR (■) or MC-LF (■) on the specific fluxes of *Synechocystis* PCC 6803 calculated by the JIP test formulae relative to the control (□). The specific fluxes are ABS/RC, absorption; TR0/RC, trapping; ET0/RC, electron transport and DI_0/RC, dissipation at the level of the antenna chlorophylls. n = 10, error bars are standard deviation. * - indicates significant difference from the control ($p < 0.05$); * -indicates significant difference from MC-LR ($p < 0.05$); * -indicates significant difference from MC-RR ($p < 0.05$; * -indicates significant difference from MC-WR ($p < 0.05$)

The addition of exogenous MC had both positive and negative effects on the quantum efficiencies. $\Phi(P_0)$ was significantly enhanced by the addition of 2.5 $\mu g.L^{-1}$ MC-WR compared to the control whereas the opposite was seen for MC-LR. No differences were seen between the control and the MC-WR and MC-LF in comparison to the control. MC-RR at this concentration decreased $\Phi(P_0)$ the most followed by MC-LR and then MC-WR and MC-LF. No significant differences were observed between MC-WR and MC-LF at this concentration (figure 5.4A. When the concentration of MC was increased to 10 $\mu g.L^{-1}$ (figure 5.4B) there was a significant decrease in $\Phi(P_0)$ when *Synechocystis* PCC 6803 was exposed to MC-LR, MC-RR and MC-LF compared to the control. No significant difference was observed when MC-WR was exposed to the culture. A concentration-dependant relationship between $\Phi(P_0)$ and MC-LR and MC-WR was not observed for when the concentration of these variants was increased. However, an increase in MC-RR concentration resulted in an increase in $\Phi(P_0)$ and an increase in the concentration of MC-LF caused a decrease in $\Phi(P_0)$.

The addition of 2.5 $\mu g.L^{-1}$ (figure 5.4A) of MC-WR enhanced $\Psi(E_0)$ significantly compared to the control, whereas the other variants had no significant effect on $\Psi(E_0)$. The relationship between $\Psi(E_0)$ and the MC variants was altered when the concentration of each variant was increased to 10$\mu g.L^{-1}$(figure 5.4B) The significant relationship between MC-WR and the control was lost. As the concentration of MC-WR increased, the $\Psi(E_0)$ decreased. MC-LR at this higher concentration enhanced $\Psi(E_0)$ but MC-LF inhibited $\Psi(E_0)$ significantly. No concentration-dependant relationship was observed for these variants. As the concentration of MC-RR increased the $\Psi(E_0)$ increased as well.

No significant changes were observed in $\Phi(E_0)$ with the addition of 2.5 $\mu g.L^{-1}$of MC-LR and MC-LF compared to the control, however $\Phi(E_0)$ was significantly increased compared to the control with the addition of MC-WR and significantly decreased with the addition of MC-RR at the same concentration (figure 5.4A). As the concentration of MC increased (figure 5.4B) the significant relationship between $\Phi(E_0)$ and MC-RR and MC-WR respectively was lost. MC-LF inhibited $\Phi(E_0)$ whereas the other variants of MC had no effect on this parameter. An increase in MC-WR and MC-LF caused a significant decrease in $\Phi(E_0)$ in contrast to MC-RR where an increase in concentration significantly enhanced the $\Phi(E_0)$.

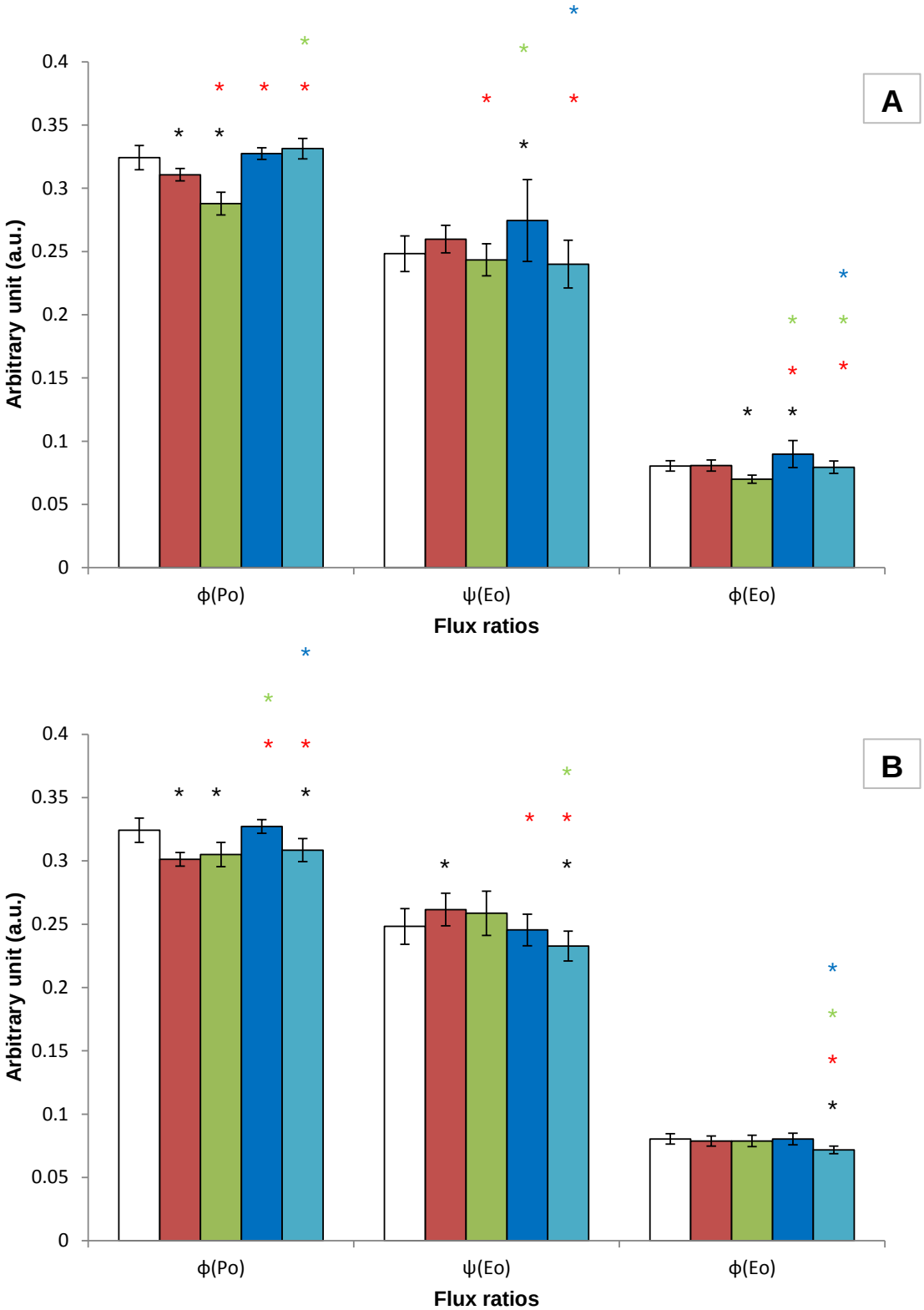

Figure 5.4: The effects of a 16 hour exposure to either 2.5 µg.L^{-1} (A) or 10 µg.L^{-1} (B) of MC-LR (■), MC-RR (■), MC-WR (■) or MC-LF (■)on the flux yields of *Synechocystis* PCC 6803, calculated by the JIP test formulae relative to the control (□). The flux yields are $\varphi_{Po} \equiv TR_0/ABS$, maximum quantum yield of primary photochemistry; $\psi_o \equiv ET_0/TR_o$, probability (at t = 0) that a trapped exciton moves an electron into the electron transport chain Q_A^-; $\varphi_{Eo} \equiv ET_0/ABS$, quantum yield of electron transport (at t = 0) and φ_{Do}, quantum yield (at t = 0) of energy dissipation. n = 10, error bars are standard deviation. n = 10, error bars are standard deviation. * - indicates significant difference from the control ($p < 0.05$); * -indicates significant difference from MC-LR ($p < 0.05$); * -indicates significant difference from MC-RR ($p < 0.05$); * -indicates significant difference from MC-WR ($p < 0.05$)

No significant difference was observed for the $PI_{(ABS)}$ with the addition of the different MC variants at a concentration of 2.5 $\mu g.L^{-1}$ compared to the control. There were variances in the $PI_{(ABS)}$ value when the different variants of MC were compared to each other (figure 5.5). When the concentration of MC was increased, a similar trend was observed for the lower concentration of MC variants except MC-LF which was significantly greater than the control.

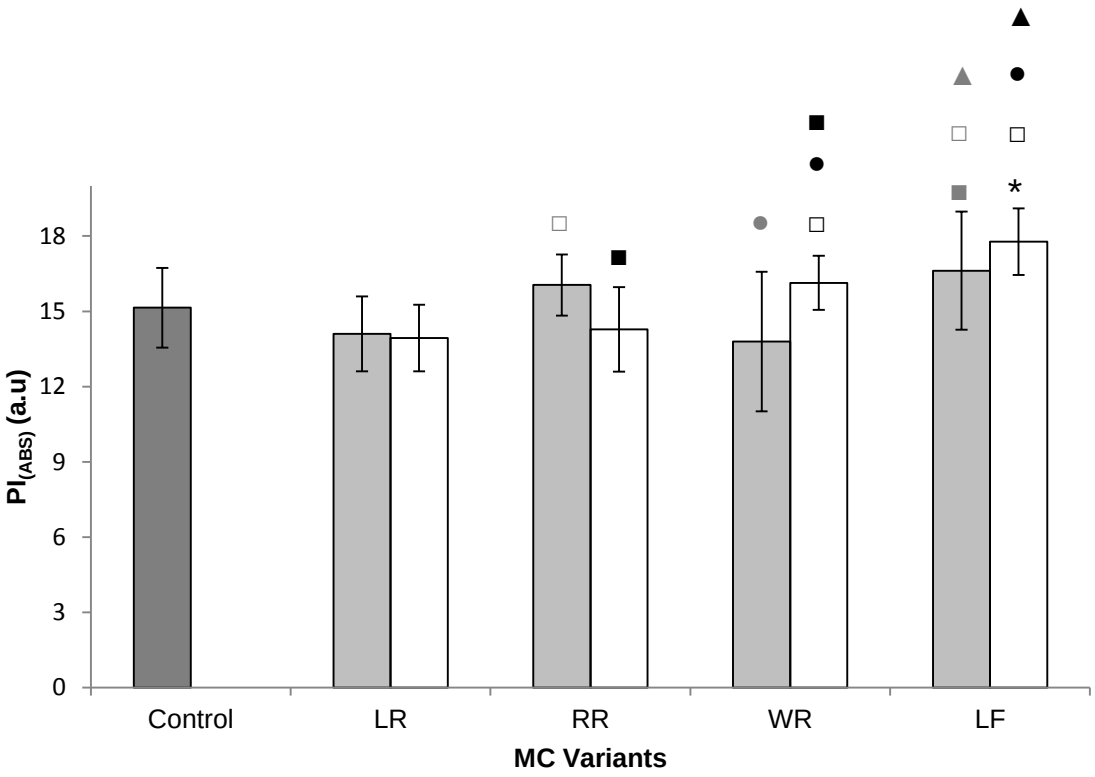

Figure 5.5: The effect of a 16 hour exposure to either 2.5 $\mu g.L^{-1}$ (■) or 10 $\mu g.L^{-1}$ (□) of MC-LR, MC-RR, MC-WR or MC-LF on the $PI_{(ABS)}$ of *Synechocystis* PCC 6803, calculated by the JIP test formulae relative to the control (■). n = 10, error bars are standard deviation. * - indicates significant difference from the control ($p < 0.05$); □-indicates significant difference from 2.5 $\mu g.L^{-1}$ of MC-LR ($p < 0.05$); ●-indicates significant difference from 2.5 $\mu g.L^{-1}$ of MC-RR ($p < 0.05$); ▲-indicates significant difference from 2.5 $\mu g.L^{-1}$ of MC-WR ($p < 0.05$); □-indicates significant difference from 10 $\mu g.L^{-1}$ of MC-LR ($p < 0.05$); ●-indicates significant difference from 10 $\mu g.L^{-1}$ of MC-RR ($p < 0.05$); ▲-indicates significant difference from 10 $\mu g.L^{-1}$ of MC-WR ($p < 0.05$); ■ -indicates significant difference from previous concentration of MC ($p < 0.05$)

The $PI_{(ABS)}$ was broken-up into its three parameters (fig 5.6): absorption of light, trapping of excitation energy and conversion of excitation energy to electron transport and further analysed in order to determine whether there were changes within these parameters of $PI_{(ABS)}$. A concentration dependent relationship was observed for RC/ABS with MC-RR and MC-LF. The RC/ABS was significantly higher

than the control for both concentrations of MC-LR and MC-RR and the highest concentration of MC-LF used in this study (figure 5.6A). The TR_0/DI_0 was significantly lower than the control for both MC-LR and MC-RR and the highest concentration of MC-LF used in this study; however the lower concentration of MC-LF was significantly greater than the TR_0/DI_0 of the control. A concentration dependent relationship was seen between TR_0/DI_0 and MC-LR, MC-RR and MC-LF (figure 5.6B). There was no significant difference in $ET_0/(TR_0-ET_0)$ between the control and all the MC variants that were tested in this study (figure 5.6C).

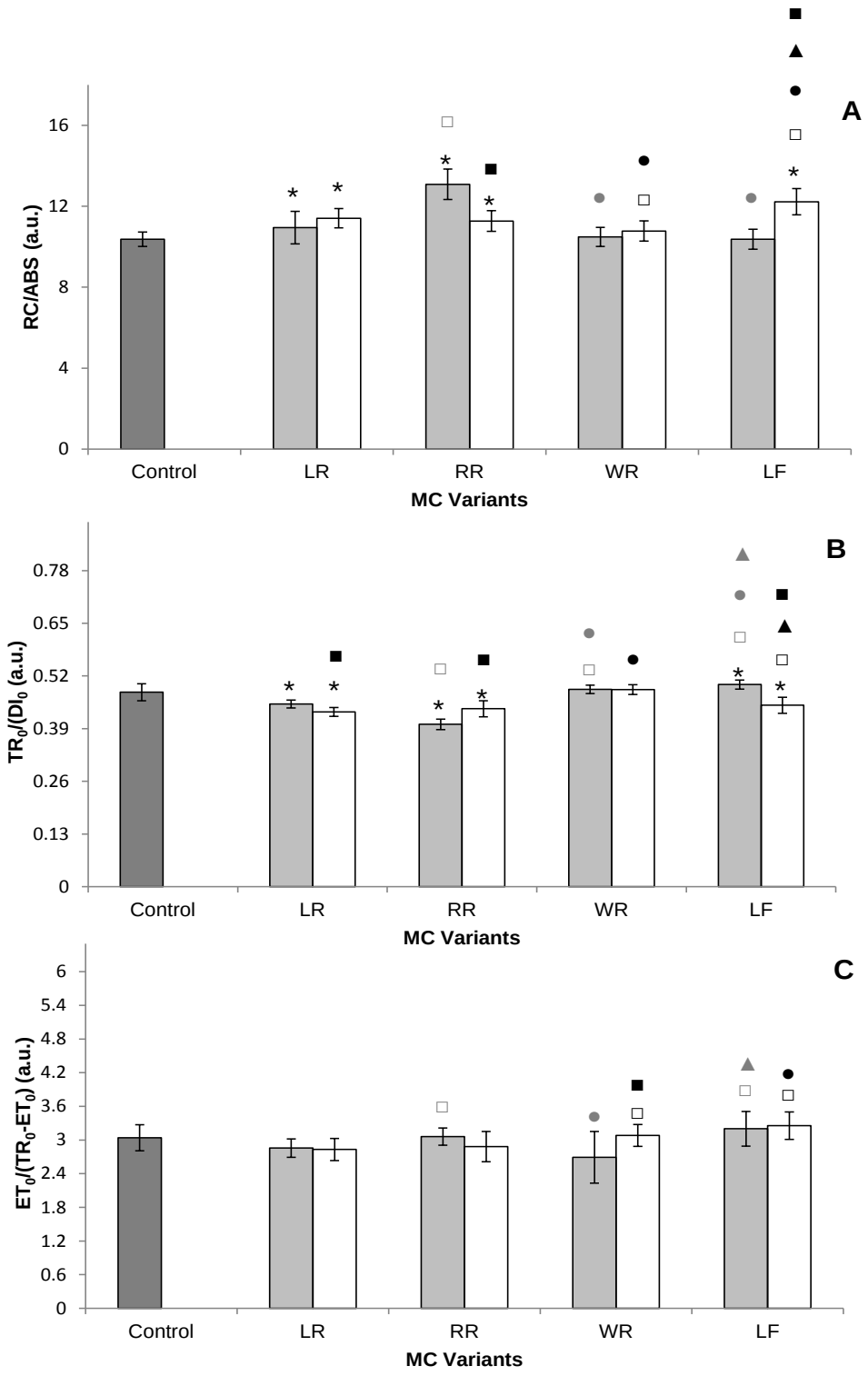

Figure 5.6: The effect of a 16 hour exposure to either 2.5 µg.L^{-1} (■) or 10 µg.L^{-1} (□) of MC-LR, MC-RR, MC-WR or MC-LF on the three parameters that make-up the PI_{ABS} of *Synechocystis* PCC 6803, calculated by the JIP test formulae relative to the control (■). n = 10, error bars are standard deviation. n = 10, error bars are standard deviation. * -indicates significant difference from the control ($p < 0.05$); □-indicates significance difference from 2.5 µg.L^{-1} of MC-LR ($p < 0.05$); ●-indicates significant difference from 2.5 µg.L^{-1} of MC-RR ($p < 0.05$); ▲-indicates significant difference from 2.5 µg.L^{-1} of MC-WR ($p < 0.05$); □-indicates significant difference from 10 µg.L^{-1} of MC-LR ($p < 0.05$); ●-indicates significant difference from 10 µg.L^{-1} of MC-RR ($p < 0.05$); ▲-indicates significant difference from 10 µg.L^{-1} of MC-WR ($p < 0.05$); ■ -indicates significant difference from previous concentration of MC ($p < 0.05$)

5.1.5 Discussion

The results of this study showed that the effect of exogenous MC on photosynthesis, especially PS II photochemistry reactions, was variant-dependent and concentration-dependent for some of the variants that were tested. A positive ΔK-band was obtained after subtracting the double normalisation of the O-P phase of the control from the treatment which indicates the uncoupling of the OEC (Strasser *et al*., 2004; Strasser *et al*., 2007). MC-RR at a concentration of 2.5 $\mu g.L^{-1}$, caused the greatest degree of inhibition of the OEC but no relationship was observed between the ΔK-band and the hydrophobicity of the MC variants which were tested. However, when the concentration of each MC variant was increased to 10 $\mu g.L^{-1}$, the two hydrophobic variants, MC-WR and MC-LF caused a greater degree in uncoupling of the OEC compared to the two hydrophilic variants, MC-LR and MC-RR. Although, there was an apparent reverse relationship between the shift in the ΔK-band and concentration of the hydrophobic and hydrophilic MC variants, there were insufficient variants used in this study to make any definite conclusions about this relationship. That inhibition of O_2 evolution was occurring was further supported by the significant inhibition of TR_0/DI_0 (figure 5.6). Inhibition of TR_0/DI_0 indicates that there is a decrease in the reduction of the plastoquinone Q_A to Q_A^- (Strasser *et al*., 2004). The degree of reduction of Q_A was variant-dependant, suggesting that the 2 variable L-amino acids of MC may affect either the intermolecular forces, intramolecular forces or both. Non-specific interactions have been shown between MC and proteins (Vela *et al*., 2008; Jüttner and Lüthi, 2008). However, Zilliges *et al*. (2011) showed that there was a specific covalent interaction between the N-methyldehydroalanine position of MC and cysteines of proteins. In order for a covalent bond to be established, non-covalent interactions need to initially occur. Thus, the variable L-amino acids of MC may play a role in the establishment of a covalent bond between cysteine and N-methyldehydroalanine by enhancing the non-specific reactions. Secondly, the hydrophobicity of these L-amino acids may alter the uptake of the toxin by the cyanobacterium, hence, the variation in the JIP-test parameters with the different variants in MC. Chlorophyll *a* fluorescence was attempted on thylakoid membranes, however whole cells were used instead because the F_V/F_M values were below the threshold value for cyanobacteria and therefore could not be analysed and

compared with statistical relevance. The low F_V/F_M values were probable due to the the removal of the light harvesting pigments from the active reaction centres.

There was a significant increase in ABS/RC and DI_0/RC compared to the control as the MC concentrations was increased. A similar trend was observed by Perron *et al*. (2012) when they exposed various genera of green algae to different concentrations and variants of MC. An increase in these specific energy fluxes per reaction centre indicates that the organism is experiencing stress to its photosynthetic apparatus (Redillas *et al*., 2011). Thus an increase in this parameter either indicates that the size of the antenna has increased or the reaction centres have been inactivated (Strasser *et al*., 2004). The increase in ABS/RC is most likely attributed to the inactivation of the reaction centres due to the lack of distinct positive L-bands (Redillas *et al*., 2011). However, the lack of significant difference between the control and the TR_0/RC (maximum reduction rate of Q_A) for the hydrophilic MC variants and the significant increase in the TR_0/RC for the hydrophobic variants suggests that the active reaction centres have maintained a high conversion efficiency of excitation energy (Strasser and Strasser, 1995). This suggests that MC may be involved with the down-regulation of PS II reaction centres and that the excitation energy is transferred to PS I, thus protecting the PS II apparatus from excessive excitation energy (Zhang *et al*., 2009).

A typical polyphasic OJIP rise was observed for the cyanobacterium *Synechocystis* PCC 6803 for all treatments of MC and the control, indicating that photosynthetic apparatus of the cyanobacterium is active. In cyanobacteria, the difference between the J-level and the P-level is not as pronounced as in plants and in some cases the J-level may be be higher than the P-level (Strasser *et al*., 1995) because cyanobacteria have a higher ratio of PS I to PS II (Campbell *et al*., 1998). Furthermore, no significant differences were noted in the PI_{ABS} for all MC variants and concentrations tested. The PI_{ABS} is an equation that describes the potential for redox reactions and Gibbs free energy to move through biochemical systems. In terms of photosynthesis, the PI_{ABS} indicates the vitality of the photosynthetic organism.

Schatz *et al*. (2007) reported that the release of MC into the environment by cell lysis may act as an infochemical which signals to the cyanobacterial population that the

population is experiencing stressful conditions. However, this is an unlikely function for MC because export of the toxin has not yet been shown, even though the finding of a putative ABC-transporter protein encoded within the MC synthetase gene is predicted to be involved in both toxin biosynthesis and export (Pearson *et al*., 2004). Furthermore, examination of acyl homoserine lactone (297.39 Da), a common infochemical in bacteria that is involved in quorum sensing (Waters and Bassler, (2005) it is found that acyl homoserine lactone is approximately one third of the size of MC which ranges between 900 and 1100 Da depending on the variant. Additionally, the complexity of the MC structure, the metabolic demand of MC production and environmental modulation of MC indicates that MC is not likely to be involved in communication between cyanobacterial cells.

There is sufficient evidence to support a biological function of MC in cyanobacterial photosynthesis: two thirds of the total internal MC quota is found in the thylakoid membranes (Young *et al*., 2005); there is a correlation between MC and growth rate (Lee *et al*., 2000; Long *et al*., 2001); growth rate advantage for MC producers under high light (Phelan and Downing, 2011); the *in vivo* binding of MC to proteins involved in photosynthesis (Zilliges *et al*., 2011); and an environmental modulation of MC production by light intensity (Lee *et al*., 2000; Long *et al*., 2001), uptake rates and availability of phosphorus and nitrogen (Lee *et al*., 2000; Long *et al*., 2001) and increased MC production at reduced carbon fixation rates relative to nitrogen availability (Downing *et al*., 2005b).

Recently Zilliges *et al*. (2011) proposed a new role for MC in MC-producing cyanobacteria as a protectant against oxidative stress. *Microcystis* PCC 7806 was less sensitive to high light exposure of approximately 300 µmol photons $m^{-2}.s^{-1}$ compared to a mutant of this strain unable to produce MC. The mutant became photo-bleached after three to four days of continuous exposure to this high light intensity. It was also noted that the wild-type was able to recover more efficiently to continuous exposure of high light when it was moved back into lower light intensities. Oxidative stress in *Microcystis* PCC 7806 and the mutant of this strain was determined by chlorophyll *a* bleaching and a lipid peroxidation assay. However, reactive oxygen species (ROS) were not measured. Data presented here revealed that the addition of exogenous MC inhibited the OEC in whole cells and in chapter two it was shown that MC inhibited O_2 evolution in PS II in thylakoid membranes. A

decrease in water-splitting activity decreases O_2 evolution and consequently the amount of ROS being produced. Therefore, the cyanobacterium would experience less oxidative stress causing retardation of photoinhibition and other oxidative stress-related changes. Hence, the protective function against oxidative stress shown by Zilliges *et al*. (2011) may not be due to the covalent modification of oxidative stress proteins by MC, but by the decrease in the amount of ROS by either inhibition or inactivation of the OEC.

Previous studies have shown that MC production enhances the fitness of the producer under limiting and stressful environmental conditions (Phelan and Downing, 2011; Zilliges *et al*., 2011). The results from this study provide further evidence for the involvement of MC in photosynthesis and show that MC may perform a protective function in MC-producing cyanobacteria at saturating light intensities by inhibiting O_2 evolution, thus decreasing the amount of ROS produced which would decrease the oxidative stress that the cyanobacterium experiences and inactivation of the PS II apparatus which may provide sufficient time for repair before permanent damage is done or MC may stabilize the OEC with the consequential reduction in the water activity.

As per previous Chapter:

Microcystin causes inhibition of the OEC and may act as a protectant against photoinhibition.

However:

Microcystin-producing cyanobacteria have a growth advantage over non-microcystin-producing cyanobacteria at high light intensities (Phelan and Downing, 2011; Zilliges et al., 2011).

Therefore:

A competition study was performed at a high light intensity to determine whether production of microcystin is beneficial to the toxin producer under these conditions.

Chapter 6

6.1 Competition between the microcystin-producing Microcystis PCC 7806 and the non-microcystin-producing ΔmcyA mutant of Microcystis PCC 7806 under a high light intensity

6.1.1 Abstract

Cyanobacterial populations consist of a mixture of microcystin-producing and non-microcystin-producing cells. The proportion of toxic to non-toxic cyanobacteria varies depending on environmental conditions. Competition studies were performed to determine whether microcystin production enhances the organism's ability to withstand high light intensities compared to a non-microcystin producer. *Microcystis* PCC 7806 and a Δ*mcyA* mutant of *Microcystis* PCC 7806 were inoculated into separate dialysis cassettes and then both cassettes were placed into large beakers containing BG11 and incubated at a high light intensity. Results showed that the Microcystis PCC 7806 out-competed the Δ*mcyA* mutant of *Microcystis* PCC 7806 with the mutant becoming chlorotic after 72 hours where *Microcystis* PCC 7806 did not. Addition of exogenous microcystin to the mutant culture prior to light exposure gave the mutant wild-type growth characteristics until the microcystin was diluted by growth of the culture. These data suggest that microcystin production has a physiological benefit to the producer in light intensities which cause photoinhibition.

6.1.2 Introduction

Not all cyanobacterial blooms produce microcystin (MC); some blooms contain only non-toxic strains whereas others contain a mixture of both toxic and non-toxic strains which can produce numerous MC variants (Kaebernick and Neilan, 2001; Kardinaal and Visser, 2005).

A major problem for all photosynthetic organisms is that excessive exposure to high light intensities causes inactivation of photosystem II (PS II). Therefore, an organism's ability to withstand strong light for long periods of time gives that

organism a competitive advantage over other photosynthetic organisms. Phelan and Downing (2011) described a competitive growth advantage for the MC-producing *Microcystis* PCC 7806 over the non-MC-producing Δ*mcyA* mutant of *Microcystis* PCC 7806 grown under batch culture conditions at continuous light intensities of 37 µmol of photons.m^{-2}.s^{-1}. Zilliges *et al*. (2011) reported the same observation when the cultures were incubated under a high light intensity of 700 µmol of photons.m^{-2}.s^{-1}.

Previous studies have shown that cyanobacterial populations consist of both MC-producing and non-MC-producing genera and strains. Several papers have indicated that there is a negative relationship between the proportion of cells that can produce MC and the abundance of cyanobacterial cells (Sabart *et al*., 2010; Briand *et al*., 2008a; Briand *et al*., 2009; Manganelli *et al*., 2010).

Competition studies have been performed in controlled conditions in order to gain a better understanding in the relationship between toxic and non-toxic cells within a population. To date, two freshwater cyanobacteria, *Microcystis aeruginosa* (toxic and non-toxic strains) and *Planktothrix agardhii* (toxic and non-toxic strains), have been used for co-culture experiments (Kardinaal *et al*., 2007; Briand *et al*., 2008b). These competition studies have shown that the non-MC-producing strains out-compete the wild type in conditions which are optimal for growth, whereas for environmental conditions which are unfavourable for growth, the MC-producing strains out-compete the non-MC-producing strains (Kardinaal) *et al*., 2007; Briand *et al*., 2008b). However, a major concern with these two investigations is that these two strains were of different genotypes and therefore the results obtained from the competition studies may have been influenced by other metabolic functions. In an attempt to alleviate competition bias, Briand *et al*. (2012) used *Microcystis* PCC 7806 (WT) and a Δ*mcyA* mutant of *Microcystis* PCC 7806 (MT), which are genetically identical in every aspect except for their ability to produce MC. Briand *et al*. (2012) noted that the MT had a higher growth rate than the WT grown under optimal light and varying nitrogen concentrations in co-culture experiments. However, they did not examine the effects of growth-limiting conditions on these two cultures. They used a combination of two incident light intensities of 39 µmol of photons.m^{-2}.s^{-1} and 5 µmol of photons.m^{-2}.s^{-1} and $NaNO_3$ at the following concentrations 0.036 mM and 9 mM for their co-culture experiments.

The data from the previous chapters indicates that MC inhibits photosystem II (PS II) by the uncoupling the oxygen evolving complex (OEC) without detrimental effects to the cyanobacterium. The uncoupling of the OEC leads to a decrease in O_2 evolution, which would decrease the amounts of reactive oxygen species (ROS) produced. MC may offer an protectant role to the producer in saturating light conditions by retarding photoinhibition. The aim of this investigation was to confirm whether there is a competitive advantage in MC production under high light intensities by performing co-culture experiments with the WT and MT strains, and also to further clarify the ecological benefit of MC production.

6.1.3 Methods and Materials

6.1.3.1 Culture conditions and preparations

Microcystis PCC 7806 and a Δ*mcyA* mutant of *Microcystis* PCC 7806 (B. Neilan, UNSW) were pre-cultured in BG11 at pH 7.4 (Sigma) grown under continuous illumination of 15 µmol of photons.m^{-2}.s^{-1} at 20 °C (±0.5 °C). Both cultures were collected by centrifugation (5000 *x g*; 10 min), washed twice and resuspended in fresh BG11 at pH 7.4.

6.1.3.2 Competition experiment

60 ml of each culture was inoculated into separate 70 ml Slide-A-Lyzer® G2 Dialysis Cassettes (Thermo Scientific) as per manufacturer's instructions. The dialysis cassettes were used because they allow mixing and equal exposure to medium compinents but no loss of the cells. Two dialysis cassettes were placed into a 3 L beaker containing 2.5 L of sterile BG11. Cultures were then grown under continuous illumination of 1000 µmol of photons.m^{-2}.s^{-1} for 120 hours. This was performed in triplicate.

6.1.3.3 Effect of Exogenous MC on a Δ*mcyA* mutant of *Microcystis* PCC 7806

The Δ*mcyA* mutant of *Microcystis* PCC 7806 was divided into 8 equal aliquots of 10 ml that were placed into eight sterile 20 ml McCartney bottles. A MC-LR stock solution in BG11 (10 ng.μl^{-1}) was added to four of the McCartney bottles so that the final concentration of MC-LR was at 10 µg.L^{-1} and the same volume of BG11 containing no toxin was added to the remaining four McCartney bottles (control). Cultures were exposed to the MC for 24 hours. Cultures were then incubated as

described above, under the same conditions and period as for the competition experiment.

The changes in population dynamics for both experiments were measured by $OD_{750\ nm}$, every 24 hours using a PowerWave HT Microplate Spectrophotometer (BioTek).

6.1.4 Results

Initially, the Δ*mcyA* mutant of *Microcystis* PCC 7806 had a significantly higher growth rate compared to *Microcystis* PCC 7806. However, from 24 hours the OD 750 nm of Δ*mcyA* mutant began to decrease, whereas the MC-producer *Microcystis* PCC 7806 continued to increase. The Δ*mcyA* mutant strain declined in numbers after 48 hours and after 72 hours it became chlorotic, while *Microcystis* PCC 7806 remained viable and continued to grow. *Microcystis* PCC 7806 began to clump and settle to the bottom of the dialysis cassette towards the end of the exposure. The Δ*mcyA* mutant was completely photo-bleached at this stage (figure 6.1).

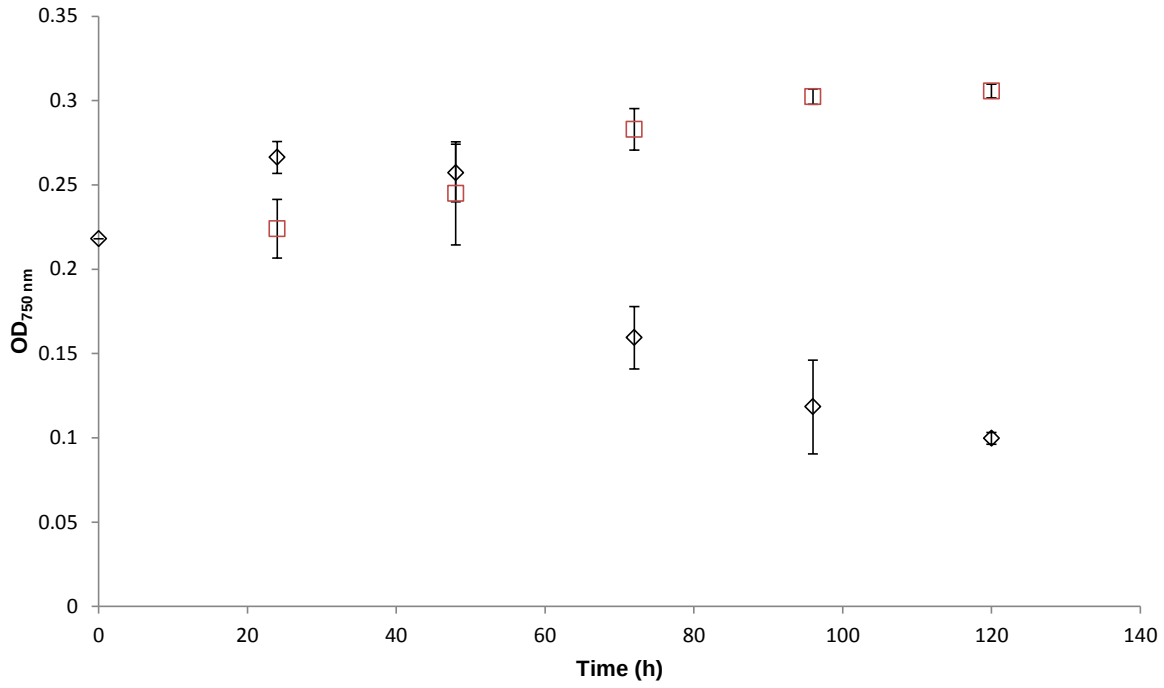

Figure 6.1: Growth curves of the WT (□) and the MT (◊) strains in competition experiment under a light intensity of 1000 µmol of photons.m^{-2}.s^{-1}. Error bars represent standard deviation, n= 3

The addition of exogenous MC-LR to Δ*mcyA* mutant of *Microcystis* PCC 7806 prevented the mutant from becoming chlorotic (figure 6.2) for a 120 hrs. Furthermore the growth of the mutant was not as dramatically impacted in the batch culture experiments compared to the competition experiments.

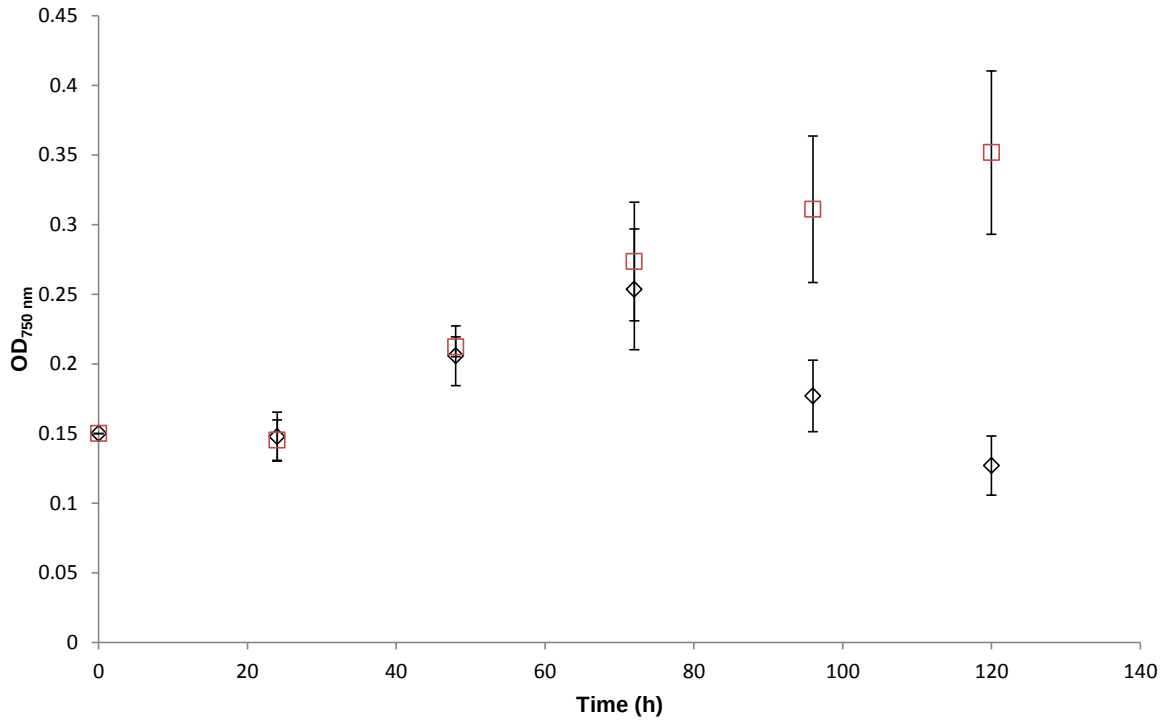

Figure 6.2: Growth curves of the Δ*mcyA* mutant incubated with 10 µg.L^{-1} of exogenous MC-LR (□) and the MT (◊) without the addition of exogenous MC-LR in batch culture under a light intensity of 1000 µmol of photons.m^{-2}.s^{-1}. Error bars represent standard deviation, n= 4

6.1.5 Discussion

In this study, the data showed that a MC-producer has a competitive advantage over a non-producer of MC in high light conditions and that the production of MC protects the cyanobacterium from photoinhibition and chlorosis due to excessive light (figure 6.1). Furthermore, the addition of exogenous MC to Δ*mcyA* mutant of *Microcystis* PCC 7806 prevented the chlorosis observed in the competition experiment and the control (figure 6.2). This is consistent with what was reported by Phelan and Downing (2011), who reported that *Microcystis* PCC 7806 had a competitive growth advantage in high light intensities compared to the Δ*mcyA* mutant of *Microcystis* PCC 7806. Furthermore, Zilliges *et al*. (2011) reported the

same growth advantage for *Microcystis* PCC 7806 over Δ*mcyA* mutant of *Microcystis* PCC 7806.

Competition studies done by Kardinaal *et al*. (2007) showed that a non-toxic strain of *Microcystis* CYA43 out-competed the toxic strain of *Microcystis* CYA140 for light. This is inconsistent with the data presented here. A much higher light intensity of 1000 µmol of photons.m^{-2}.s^{-1} was used in contrast to the study conducted by Kardinaal *et al*. (2007) where the highest light intensity was 25 µmol of photons.m^{-2}.s^{-1}. Presumably at low light intensities the metabolic burden of microcystin production reduces growth rate as there is no corresponding advantage of high light protection.

A possible reason for the non-MC producer out-competing the MC producer is that at lower light intensities there are lower carbon-fixation rates and this results in a high intracellular N:C ratio, which favours MC production (Downing *et al*., 2005b). However, Briand *et al*. (2012) reported in a recent publication that Kardinaal *et al*. (2007) did not show competition for light because as the light intensity penetrating through the culture vessel was not significantly different at the end of the experiment, there was no light limitation at the end of their experiments. A likely explanation for the non-MC producer out-competing the MC-producing strain uses its available resources to synthesise MC, whereas the non-MC-producing strain does not have the burden of MC production and therefore its resources are invested in growth. This was confirmed in a study, by Briand *et al*. (2012), where, under optimal light conditions, irrespective of nitrogen concentration the Δ*mcyA* mutant of *Microcystis* PCC 7806 had a significantly higher growth rate compared to the WT strain in co-culture conditions whereas in monoculture conditions there was no significant difference in growth rates. Furthermore the intracellular MC quota was higher in the co-culture conditions than the monoculture conditions, explaining the different growth rates.

Another competition study between a MC and a non-MC producing *Planktothrix agardhii* showed that the MC producer out-competed the non-MC producer in conditions which limited cell growth by increasing the light intensity and temperature, whereas the opposite trend was observed in optimal conditions for growth. This indicates that the production of MC is beneficial in growth-limiting conditions

(Briand *et al*., 2008b) because MC seems to prevent light-induced damage of PS II by the uncoupling of the OEC (chapters 2 and 5). Dziallas and Grossart (2011) noted that growth-limiting conditions (by increasing oxygen radicals and water temperature) selected for a MC producing *Microcystis* PCC 7806 over a non MC-producing strain.

In conclusion, the data from this study shows that there is an ecological benefit in producing MC. The production of MC enhances the ability of the producer to out-compete the non-MC producer at high light intensities by preventing light-induced damage to the photosynthetic apparatus.

Chapter 7

7.1 Final Discussion and Conclusions

The uptake of exogenous microcystin (MC) by cyanobacteria was confirmed using *Synechocystis* PCC 6803, a non-MC-producing cyanobacterium, as a model. Furthermore, once the toxin had been taken up it was internalised and located in the thylakoid membranes, which resulted in a decrease in the photosynthetic activity. This indicated that *Synechocystis* PCC 6803 responses to exogenous MC could be used to evaluate the effects of endogenous MC in MC-producing cyanobacteria because the MC was both taken up and localised where endogenous MC was typically found (Young *et al*., 2005; Shi *et al*., 1995). The *ΔmcyA* mutant of *Microcystis* PCC 7806 had a higher photosynthetic activity, determined by the reduction of DCPIP by light-driven reactions of photosynthesis, compared to *Microcystis* PCC 7806, suggesting that the rate of electron transport is less in the MC-producer compared to the non-MC-producing strain.

The fluidity of the thylakoid membranes plays an important role in regulating the light reactions of photosynthesis (Yamamoto *et al*., 1981). Popova et al., (2007) showed that the artificial modification to the thylakoid membranes of pea plants effected both photosystem II (PS II) and photosystem I (PS I) activity. However, increasing concentrations of MC-LR did not alter the fluidity of the thylakoid membranes. This suggests that there is an alternative binding site in the thylakoid membranes and that there is also a different mechanism of modifying photosynthesis.

The exposure of exogenous MC variants to purified thylakoid membranes resulted in a decrease in the activity of PS II, but PS I activity was not affected. There was a concentration-dependent relationship between the inhibition of PS II and all the variants used. Singh et al., (2001) reported that oxygen evolution was drastically inhibited in two genera of cyanobacteria, *Anabaena sp.* BT1 and *Nostoc muscorum* C. Agardh. However, Singh et al., (2001) used concentrations of MC that are physiologically irrelevant, 25000 and 50000 $\mu g.L^{-1}$, and in addition to this the MC extract that they used was only semi-purified, therefore the possible decrease in

oxygen evolution could not only be attributed to the MC. This study therefore showed the first evidence that MC inhibits PS II activity of cyanobacteria at physiologically relevant MC concentrations.

Since MC is taken up by the cyanobacterium, *Synechocystis* PCC 6803, and internalized into the thylakoid membranes. Chlorophyll *a* fluorescence was used to determine the action site of PS II inhibition by exposing *Synechocystis* PCC 6803 to exogenous MC at two different physiologically relevant concentrations. Analysis of the difference in the variable fluorescence of the double normalization at the OP phase revealed a ΔK-band, which indicates that MC leads to the uncoupling of the OEC. This was further supported by a decrease in the reduction of quinone, Q_A to Q_A^- showing that the water splitting activity of the oxygen evolution complex (OEC) was been inhibited by MC. The specific fluxes calculated by the JIP-test formula indicated that individual reaction centres were being deactivated but the maximum reduction rate of Q_A per RC was not inhibited. This combination of results of the specific fluxes suggests that the PS II is being down regulated to protect it from excessive excitation energy (Zhang et al., 2009). A similar trend in the specific fluxes was noted by Perron et al. (2012) when they exposed green algae to exogenous MC variants. The data from the chlorophyll *a* fluorescence study indicates that MC may protect the PS II apparatus from photoinhibition by inhibiting the water-splitting activity of the OEC.

In the previous chapters, the data suggests a biological role for MC by protecting the PS II apparatus by either preventing or decreasing the rate of photoinhibition. A competition study was performed to confirm whether the production of MC gives a completive advantage to the MC-producer over the non-MC-producer in high light conditions. The data from this study showed that the production of MC enhanced the ability of the MC-producer to survive in saturating light intensity. Phelan and Downing (2011) and Zilliges et al. (2011) reported a growth advantage for the MC-producing *Microcystis* PCC 7806 at higher light intensities over the non-MC-producing *ΔmcyA* mutant of *Microcystis* PCC 7806. Briand et al., (2008) reported the same competitive growth advantage for a toxin-producing *Planktothrix agardhii* strain over a non-toxin-producing strain of *Planktothrix agardhii* in co-culture experiments. However, different metabolic activities due to strain differences may have contributed to this

growth advantage as well, whereas *Microcystis* PCC 7806 and *ΔmcyA* mutant of *Microcystis* PCC 7806 are genetically identical except for their ability to produce MC. Briand et al. (2012) reported a competitive growth advantage for *ΔmcyA* mutant of *Microcystis* PCC 7806 over *Microcystis* PCC 7806 in co-culture experiments, this was however at optimal light conditions for growth but they did not examine higher light intensities which are not optimal for growth. Furthermore, the addition of exogenous MC-LR to the *ΔmcyA* mutant of *Microcystis* PCC 7806 restored the organism's ability to survive high light intensities whereas the control with no exogenous MC-LR, become chlorotic.

Schatz et al. (2007) proposed a biological role for MC as an infochemical. This was further supported by the identification of a putative ABC-transporter protein encoded within the MC synthetase genes which has been identified (Pearson et al., 2004). However, this is an unlikely biological role for MC because the export of MC has never been demonstrated. Furthermore, quorum sensing molecules generally cause an effect and are generally not the effector (Waters and Bassler, 2005), whereas the data in chapters two, four and five show that MC is directly responsible for the effects observed.

Zilliges et al. (2011) reported a putative novel function for MC as a protectant against oxidative stress by binding to redox sensitive cysteines, therefore stabilizing enzymes which are involved in oxidative stress. They determined this by exposing *Microcystis* PCC 7806 and a *ΔmcyA* mutant of *Microcystis* PCC 7806 to a high light intensity of 700 µmol of photons.$m^{-2}.s^{-1}$ and H_2O_2 to induce oxidative stress. The *ΔmcyA* mutant of *Microcystis* PCC 7806 becomes chlorotic after three to four days of these treatments whereas *Microcystis* PCC 7806 did not. Lipid peroxidation and chlorophyll *a* bleaching was used to determine whether the organism is experiencing oxidative stress. However, they did not measure the reactive oxygen species (ROS). A possible explanation for the above mentioned result is that the MC reduced the water splitting activity of the OEC (chapter 5). The concentration of ROS decreases when the water splitting activity is reduced thus causing less oxidative stress on the organism. MC as a protectant against oxidative stress is highly unlikely because the rate of photoinhibition is not increased by the addition of H_2O_2 or by the inactivation of oxidizing stress enzymes. However, these conditions inhibit the synthesis of the

D1 protein (Nishiyama *et al*., 2001). In addition, the degradation of the Mn centre of the OEC is the first step in photo-degradation, thus indicating a biological role for MC in stabilizing the OEC and preventing/retarding photoinhibition.

7.1.1 Possible Mechanisms of the inhibition of the oxygen evolving complex

Studies have shown that the UV spectrum is more effective at causing photodamage to PS II than the visible spectrum (Jones and Kok, 1966), thus under the same light intensity, the UV spectrum is the most destructive to PS II and results in rapid photoinhibition of photosynthesis (Takahashi et al., 2010). MC may prevent photoinhibition by absorbing the excess energy from the UV light. However, this is unlikely because when the thylakoid membranes were incubated in light containing both the UV and visible spectrum there were no significant differences in the photosynthetic activity of the thylakoid membranes which were exposed to different MC variants and the control (see appendix A; figure 9.1).

The more likely mechanism of MC protecting/stabilising the OEC is that it binds directly to the OEC by binding to the Ca^{2+} which is necessary for the advancement of the S_3 and S_4 state and thus reducing the efficiency of the Mn complex, stabilizes the existing confirmation while altering the flexibility of catalytic site and thus reducing the activity of the OEC or a conformational change in the OEC.

Orr and Jones (1998) indicated that MC may have ionophroic properties towards calcium, magnesium, manganese and iron (II). Calcium and manganese are vitally important for the functioning of the water-splitting activity of the OEC. If the Mn is removed from the OEC, oxygen evolution is completely inhibited. Calcium plays a role in the stabilisation of the ligand in the Mn cluster and is required for the S-state to advance past S_2. MC may either be interacting with the Ca^{2+} or Mn by preventing the loss of these two metals from the OEC and thus stabilising the OEC.

7.1.2 Possible explanation of variant-dependant relationship

In chapters three and five, there was a variant-dependant relationship between PS II activity and the uncoupling of the OEC. In chapter five, there was an apparent reverse relationship between the uncoupling of the OEC and the concentration of the hydrophobic and hydrophilic variants. As the concentration of MC variants with the hydrophobic amino acids increased, the uncoupling of the OEC increased, whereas

the reverse trend was observed for MC variants with the hydrophilic L-amino acids. Insufficient variants were used in this study and therefore no statistically significant conclusions can be drawn about this reverse relationship between the uncoupling of the OEC and different hydrophobicities of the L-amino acids.

A possible explanation for the MC variant changes in the inhibition of OEC is the environmental modulation of MC. Downing *et al*. (2005b) reported that the primary modulation factor of MC is the intracellular N:C ratio. Therefore, a decrease in carbon-fixation will result in an increase in the intracellular MC quota. In chapter five, the data shows that the uncoupling of the OEC decreases as the concentration of the hydrophilic MC variant increases, whereas the opposite trend is observed for the hydrophobic MC variant. Hong-Ling (2001) reported that there was a negative correlation between MC-RR and nitrate concentration, whereas MC-YR correlated with total nitrogen, organic nitrogen and nitrates. This may explain the apparent relationship between MC variants and the ΔK-band. A cyanobacterium which produces multiple variants of different hydrophobicities may modulate the intracellular concentrations of the individual variants in response to light and intracellular nitrogen. In conditions that do not favour MC production (low N:C ratio), hydrophilic variants may be produced because they cause a greater degree of inhibition of the OEC at a lower concentration compared to the hydrophobic variants. However, the environmental conditions which favour toxin production, especially an increased nitrogen concentration, will favour the production of the hydrophobic variants at higher concentration. Tonk et al. (2005) reported that there was a change in the intracellular quota of MC-deLR increased and MC-deRR decreased with an increase in light intensity, but the total intracellular MC quota was not significantly different. This further supports the idea that cyanobacteria capable of producing multiple variants alter their MC variants in response to environmental conditions, and in light of the data in chapter five, modulates the inhibition of the OEC complex.

Another possible explanation for the observed variant difference in OEC inhibition is that the two variable L-amino acids may alter either the intermolecular forces, intramolecular forces or both. Vela *et al*. (2008) and Jüttner and Lüthi (2008) reported that non-specific interactions are evident between MC and proteins. However, a covalent bond between N-methyldehydroalanine of MC and the cysteines of proteins was formed (Zilliges *et al*., 2011). Non-covalent interactions are

initially required for the formation of a covalent bond. It is likely that the variable L-amino acids of MC play a role in the establishment of the covalent bond which exists between cysteine and N-methyldehydroalanine by increasing the non-specific interactions. Secondly, the hydrophobic nature of these L-amino acids may aid in strengthening the binding/interactions between MC and to the hydrophobic region of PS II.

In conclusion, this study provides the first evidence for a possible physiological and ecological function for MC in MC-producing cyanobacteria. Physiologically, MC is linked to the inhibition of the water splitting activity of the OEC and thus may be a potential protectant against photoinhibition. The evidence to support this is the positive shift in the ΔK-band and a decrease in the reduction of Q_A to MC relative to the control. Furthermore, the specific fluxes data indicate that MC prevents PS II against photoinhibition. A possible physiological role for MC is that the production of MC enhances the ability of the producer to tolerate higher light intensities while being less susceptible to photodamage, thus favouring the growth of the toxin-producer over the non-toxin-producer in light intensities which are unfavourable growth and will result in photoinhibition,

7.2 Future Work

7.2.1 Mechanism of how microcystin prevents photoinhibition

Synechocystis PCC 6803 will be grown in 5 l flasks containing BG11. *Synechocystis* PCC 6803 will be grown up to a final volume of 4 l and a final OD_{740} of one. PS II will be isolated from the above mentioned culture by differential centrifugation using a sucrose gradient. The purified PS II particle will be exposed to different MC variants at different concentrations for an hour in the dark. Samples will be analysed by NMR spectroscopy to determine if MC alters the confirmation of the OEC.

The PS II particle of *Microcystis* PCC 7806 and a *ΔmcyA* mutant of *Microcystis* PCC 7806 will extracted by differential centrifugation using a sucrose gradient and analysed by NMR spectroscopy to determine whether there are any confirmation changes in the OEC of the non-MC-producing mutant and MC-producing strain.

7.2.2 Physiological significance of producing multiple microcystin variants

A mid-log phase culture of Microcystis PCC 7820, which produces four MC variants: MC-LR, MC-LY, MC-LW and MC-LF, will be inoculated in a chemostat. Numerous steady states will be established by varying the intensity of illumination and nitrate concentration in order to determine the effect of the MC variant composition on PS II activity. The following parameters will be measured: intracellular microcystin quota by high pressure liquid chromatography and mass spectrometry (HPLC-MS), oxygen evolution, chlorophyll *a* and chlorophyll *a* florescence.

7.2.3 Ecological significance of producing multiple microcystin variants

Competition experiments will be run between two toxic strains *M. aeruginosa* PCC 7820 and PCC 7806 using different intensities of light at a constant nitrate concentration to determine whether there is a benefit in producing multiple variants of different hydrophobicities. Cultures will be placed in separate dialysis cassettes and placed into a beaker containing BG11. The dialysis cassettes will be used because it allows sufficient mixing of the nutrients without mixing of the cells. The change in population dynamics will be monitored by means of cell counts by flow cytometry and the intracellular microcystin quota will also be determined by (HPLC-MS).

Chapter 8

8.1 References

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Chapter 9

9.1 Appendix A

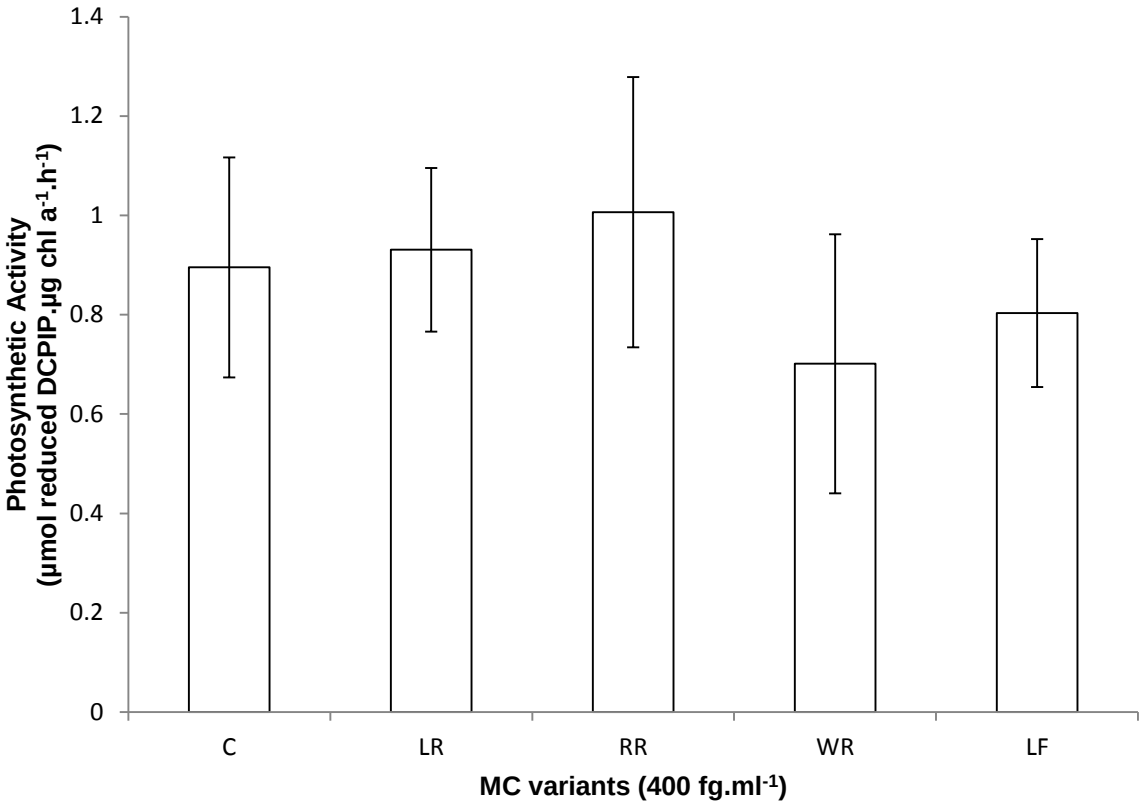

Figure 9.1: The photosynthetic activity of thylakoid membranes which were exposed to light containing both the visible and UV spectrum with the addition of four MC variants and buffer for the control. See Methods and Materials section (chapter four) for detailed description of culture conditions, thylakoid extraction and photosynthetic activity measured by the reduction of DCPIP. Error bars are standard deviation; n = 10