Developing new approaches for monitoring and controlling the toxic cyanobacterium *Microcystis* through flow-cytometric analysis

A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

Ian James Chapman

Faculty of Science and Technology

Bournemouth University

September 2016

This page intentionally left blank

This page intentionally left blank

Abstract

Biological hazards principally those produced by microorganisms have been identified as a primary concern for drinking water, putting human health at great risk. One major threat to drinking water security is associated with cyanobacteria, where bloom-forming genera like *Microcystis* can cause anoxic environments, damage filtration systems and produce potent toxins. As future projections of climate change and anthropogenic nutrient loading continue to favour the growth of *Microcystis* it adds further stress to an already limited supply of clean drinking water, highlighting the need to develop new monitoring and controls of freshwater systems. Here, high through-put, real time protocols using a relatively low cost flow cytometer were developed for identifying, enumerating and analysing the single cell physiology of *Microcystis.* These methodologies were then adopted for monitoring environmental populations in drinking water supplies and assessed the potential of novel biological, chemical and biochemical controls.

Measurements based on light scatter and fluorescence emissions from uni-algal *Microcystis* culture lines and fresh isolates (derived from a novel technique) were used as calibration for a flow cytometric assay to monitor *Microcystis*-like cells in a local reservoir. The findings for the first time reported seasonal patterns of *Microcystis* in a British lowland reservoir, revealing increased local densities during late summer and early autumn with temperature being the most significant factor. To assess potential *Microcystis* controls the mortality rates in laboratory experiments were also sampled through flow cytometry incorporating molecular probes, which enabled the analysis of single-cell physiological states after exposure to particular stressors.

A grazing experiment was carried out which examined the trophic interactions of a ciliate protist, *Blepharisma americanum*, against a toxic and non-toxic strain of *Microcystis*. *B. americanum* died in the presence of toxic *Microcystis* at the same rate as a nutrient-starved control and recorded no grazing effects on cyanobacterium densities despite ingestion being observed. In contrast, non-toxic *Microcystis* populations were controlled when grazed by *B. americanum* with ciliate populations increasing, providing further insight into their ecological role within the microbial loop. The results also contradicted previous experimental organisms which were found to feed on toxic microcystin-rich cyanobacterial cells, contributing to the theory that the secondary metabolite may function as an anti-predatory molecule.

A cheap naturally degrading chemical agent (acetic acid) found to control terrestrial photoautotrophs was tested on a fresh isolate of *Microcystis*. Applications of acetic acid were trailed in parallel with of a well-known anti-cyanobacterial compound (hydrogen peroxide) resulting in the increased formation of reactive oxygen species (ROS), membrane permeability and consequently cell mortality. For the first time in cyanobacteria acetic acid was found to induce ROS and decrease densities. Although hydrogen peroxide had a more effective dose for dose control of *Microcystis* the concentrations were too high for UK operational limits, which have not been set for acetic acid. The results also highlighted that freshly isolated *Microcystis* require increased concentrations compared to established culture lines.

The production of a biochemical control derived through filtered bi-products from a nutrient depleted *Microcystis* culture demonstrated an auto-induced sub-lethal and lethal effect on low and exponential *Microcystis* densities. The cytotoxic effect was dose dependant and only induced cell mortality under light conditions, indicating a potential for anti-*Microcystis* compounds. Results also emphasised the importance of rigorous testing of any control measure through various cell densities / population life cycles and environmental parameters.

The flow cytometric monitoring protocols developed throughout this research can be used for accurate, real time measurements of cyanobacteria like *Microcystis* in aquatic systems. Enhanced by flow cytometry analysis all three biological, chemical and biochemical controls of *Microcystis* can potentially be integrated into water treatment management, thereby increasing drinking water security.

Table of contents

List of figures

List of tables

Publications and presentations

Publications

- Chapman, I.J., Esteban, G.F. and Franklin, D.J., 2016. Molecular probe optimization to determine cell mortality in a photosynthetic organism (*Microcystis aeruginosa*) using flow cytometry. *JoVE (Journal of Visualized Experiments)*, 107, e53036-e53036.
- Hartnell, D.M., Chapman, I.J., Esteban, G.F. and Franklin, D.J., 2016. Exploiting eco-physiological niche to facilitate the separation of the freshwater cyanobacteria *Microcystis* sp. and *Synechococcus* sp. *Journal of Microbiological Methods*, 122, 13-15.

Conference presentations

- Chapman, I.J., Esteban, G.F. and Franklin, D.J., 2014. Using flow cytometry to enumerate the cyanobacteria *Microcystis* in a lowland British reservoir and the relationship between cell abundance and environmental factors. *British Phycological Society 62nd Annual Meeting.* National University of Ireland, Galway, Ireland, 25th - 27th June 2014.
- Chapman, I.J., Esteban, G.F. and Franklin, D.J., 2015. Counting for water quality: using flow cytometry as a novel way to monitor our drinking supplies. *Bournemouth University Postgraduate Researcher Science and Technology 7th Annual Conference*. Bournemouth, U.K., 20th - 21st January 2015.
- Chapman, I.J., Esteban, G.F. and Franklin, D.J., 2015. Optimising the flow cytometric detection of the cyanobacteria *Microcystis* in a lowland British reservoir. *Association for the Sciences of Limnology & Oceanography, 2015 Aquatic Sciences Meeting, Aquatic Sciences: Global And Regional Perspectives - North Meets South*. Granada, Spain, 22nd - 27th February 2015.

Poster Presentations

- Chapman, I.J., Esteban, G.F. and Franklin, D.J., 2013. Flow cytometry: past, present and future applications in aquatic microbiology. *Bournemouth University Postgraduate Researcher Science and Technology 5th Annual Conference*. Bournemouth, U.K., 11th June 2013.
- Chapman, I.J., Esteban, G.F. and Franklin, D.J., 2015. Counting for water quality: using flow cytometry as a novel way to monitor our drinking supplies. *Wessex Conservation Forum "Biodiversity, Ecosystem Services and the Green Economy"*. Bournemouth University, U.K., 11th February 2015.
- Chapman, I.J., Esteban, G.F. and Franklin, D.J., 2015. Grazing of *Blepharisma americanum* on toxic and non-toxic *Microcystis aeruginosa* cells. *Federation of European Protistological Society, VII ECOP-ISOP Joint Meeting 2015*. Seville, Spain, 5th - 10th September 2015.

This page intentionally left blank

List of abbreviations and common terms

ADH	Aldehyde Dehydrogenase
ANOVA	Analysis of Variance
APC	Allophycocyanin
ATP	Adenosine Triphosphate
AU	Arbitrary Units
bEPS	Bound Extracellular Polysaccharide
BMAA	β-N-methylamino-L-alanine
$C_2H_4O_2$	Acetic acid
CAP	Catalase
CCAP	Culture Collection of Algae and Protozoa
CEFAS	Centre for Environment, Fisheries and Aquaculture Science
CET	Concentration / Exposure Time
CHABs	Cyanobacterial Harmful Algal Blooms
Chl	Chlorophyll
CM	Conditioned Media
CM-H_2DCFDA	5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester
CMFDA	5-chloromethylfluorescein diacetate
CV	Coefficient of Variation
DCDR	Drop-Coating Deposition Raman
DCF	Dichlorofluorescein
DCFH	2’7’-dichlorohydrofluorescin
DWI	Drinking Water Inspectorate
EA	Environmental Agency
EC	European Commission
EEA	European Economic Area
ELISA	Enzyme Linked Immunosorbent Assay
EPS	Extracellular Polysaccharides
ESI	Electrospray Ionization
EU	European Union
FAO	Food and Agriculture Organization
FCM	Flow Cytometry
FDC	Frequency of Dividing Cells
FL	Fluorescence (channel)
FSC	Forward Light Scatter
GSH	Glutathione
GST	Glutathione-S-transferase

GV	Gas vesicles
^{1}H NMR	Proton Nuclear Magnetic Resonance
H_2O_2	Hydrogen Peroxide
HPLC	High Performance Liquid Chromatography
LC	Liquid Chromatography
LDA	Laser Doppler Anemometry
LM	Linear Model
LPS	Lipopolysaccharides
MC	Microcystins
MC-LR	Microcystin + Leucine and Arginine
MeCN	Acetonitrile
MET	Meteorological
mycB	Microcystin synthetase cluster B
NSF/ANSI	National Sanitation Foundation / American National Standards Institute
Nod	Nodularin
NRCC	National Research Council Canada
NTU	Nephelometric Turbidity Units
OD	Optical Density
ODO	Optical Dissolved Oxygen
PAR	Photosynthetic Active Radiation
PBP	Phycobiliprotein
PC	Phycocyanin
PCC	Pasteur Culture Collection
PCD	Programmed Cell Death
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PEC	Phycoerythrocyanin
PMT	Photomultiplier Tubes
PPi	Protein Phosphatase inhibition
PUFAs	Polyunsaturated Fatty Acids
QC	Quality Control
RM	Repeated Measures
ROS	Reactive Oxygen Species
rRNA	Ribosomal Ribonucleic Acid
RubisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
SE	Standard Error
S-Layers	Surface Layers
SOD	Superoxide Dismutase

SPE	Solid-Phase Extraction
SPME	Solid Phase Microextraction
SRM	Selected Reaction Monitoring
SRP	Soluble Reactive Phosphorus
SSC	Side Light Scatter
TEM	Transmission electron microscopy
TOC	Total Organic Carbon
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labelling
UKTAG	United Kingdom Technical Advisory Group
UN	United Nations
UNESCO	United Nations Educational, Scientific and Cultural Organization
UNGA	United Nations General Assembly
UPLC	Ultra Performance Liquid Chromatography
UVD	Ultra Violet Detection
VIF	Variance Inflation Factor
WFD	Water Framework Directive

This page intentionally left blank

Acknowledgements

I would like, first and foremost, to express my deepest gratitude and appreciation to my supervisory team, Dr. Daniel Franklin and Professor Genoveva Esteban. Without their knowledge, guidance, expertise, dedication and extreme patience over the years none of this research would have seen the light of day. I am also fully indebted to my fellow PhD students, David Hartnell and Su Chern Foo, and undergraduate students, Eddie 'I do science' McCarthy and Amir Daly for their support with various parts of this thesis. In recognition of their contribution I would like to express my sincere gratitude to Ian Hayward and Natalie Jones of Bournemouth Water, as data collection at Longham reservoirs would not have been possible without their help, especially in adverse weather conditions. Thanks also go to Dr Andrew Turner from the Centre for Environment, Fisheries and Aquaculture Science for his toxicology analysis and assistance. My appreciation also extends to the Bournemouth University administration staff and laboratory technical team in particular Iain Green, Dean Burnard, Damian Evans, Paola Barbuto, Stephan Fordham and Mike Rowley. To my family, friends and Dorset House office colleagues for helping me survive all the stressful times during this research. In addition, I am very grateful to Bournemouth University for giving me the opportunity and funding to make this project possible.

Finally, a special thanks goes to my beautiful fiancé, Shannon, for her countless sacrifices made on my behalf. Words cannot express how grateful I am for your support throughout my PhD journey.

This page intentionally left blank

Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Code of Practice for Research Degrees and that it has not been submitted for any other academic award. Except where indicated by specific references in the text, the work is the candidate's own. Work done in collaboration with, or with the assistance of others, is indicated as such.

This page intentionally left blank

Chapter I: Introduction

1.1 Global freshwater resources and the significance of cyanobacteria

1.1.1 Freshwater resources

Access to clean freshwater is limited for most of the human population despite being recognised by the United Nations General Assembly as explicitly vital to the realisation of all human rights (Resolution 64/292, UNGA 2010). Currently water scarcity affects more than 40% of the human population and by 2025 projections estimate two thirds of the world's population will reside in regions of high water stress, with 1.8 billion in areas of absolute water scarcity (UNESCO 2006).

Population growth, urbanisation, migration and industrial demands through consumerism and technology have led to immense pressures on aquatic bodies, especially freshwater systems. Microbial hazards are the primary concern for drinking water quality both in developing and developed countries (WHO 2008). The greatest risks to public health from micro-organisms in aquatic sources are associated with human and animal waste alongside increased nutrient loading (WHO 2011). Therefore microbial threats underline the need to develop rapid, robust and accurate detection methods, so water management strategies can be implemented to control problematic organisms.

1.1.2 Microbial hazards and harmful algae in freshwater resources

The most common types of organisms that cause diseases in drinking water supplies are protozoans, helminths, viruses, bacteria and cyanobacteria. When dealing with such a range of organisms it becomes very challenging, as pathogenic species have such diverse behavioural, resistance and physiological responses to control techniques (Sih 2011; WHO 2011). Many microbes often require specific management approaches, as they can cause not only human health but also damage economies and local environments (e.g. toxic cyanobacteria).

Numerous phytoplankton species are prime agents of water quality deterioration, with cyanobacteria being a prominent harmful phylum. Cyanobacteria can form colonies or blooms which can clog filtration systems and reduce safe drinking water availability, thereby increasing costs and energy consumption for utilities companies (Figure 1.1). However, it is not just physical filtration that is the main concern, as several genera like

Microcystis can produce powerful toxins that threaten other aquatic organisms and human health (Chorus 2005).

Cyanobacteria that have the potential to produce harmful toxins can be found globally, including the Antarctic (Chorus and Bartram 1999; Hitzfeld *et al.* 2000). As such the World Health Organisation (WHO), many national governments and the scientific community has increased efforts into understanding the occurrence, causes and consequences of cyanobacterial harmful algal blooms (CHABs) found in freshwater sources (Chorus 2001). Consequently those efforts require appropriate monitoring programmes and control techniques in order to mitigate impacts on drinking water security.

Figure 1.1. A *Microcystis* sp. bloom in summer 2015, located at the south eastern point of Longham reservoir 1 where the *Microcystis* environmental monitoring research took place (Chapter 3).

1.1.3 The global significance of cyanobacteria

Cyanobacteria are photosynthetic prokaryotes with the ability to survive as unicellular, filamentous or colonial forms (Figure 1.2). They can dominate all aquatic ecosystems both in the water column and benthos, as well as in terrestrial settings through symbiotic relationships (Whitton 1992). Cyanobacteria are Earth's most ubiquitous photosynthetic organisms and the oldest known oxygen evolving phototrophic primary producers, with paleontological and geological evidence suggesting they existed as early as ~3.5 billion years ago (Schopf 2000; 2006). A conservative estimate of planktonic cyanobacteria in lakes alone is $3x10^{14}$ grams of carbon, which excludes polar, subarctic and benthic components (Garcia-Pichel and Belnap 2003).

Cyanobacteria have some common characteristics associated with many algae, such as a cell wall structure and the ability to perform oxygenic photosynthesis through a number of pigments (Sidler 1994; Chorus 2001). Cyanobacteria synthesise chlorophyll *a* (Chl) and additional light harvesting pigments, absorbing light from most regions of the photosynthetic active radiation (PAR) spectra.

Most species produce phycobilins (e.g. phycocyanin) which gives a characteristic colour in high densities, leading to the popular name of 'blue green algae' that cyanobacteria are often referred to (Whitton and Potts 2012). In some species, other intrinsic membrane chlorophyll-carotenoid-phycobiliprotein complexes are formed like phycoerythrin, producing a red colour in blooms as seen in *Oscillatoria* / *Planktothrix* (Komárek and Komárková 2004). Under optimal conditions high phytoplankton growth rates can occur, potentially forming blooms throughout the water column and / or in the benthos (Nielsen *et al.* 1996). Hydrological processes, biotic interactions and nutrient conditions strongly affect CHAB dynamics, which is exacerbated by seasonal and anthropogenic influences (Paerl 2008).

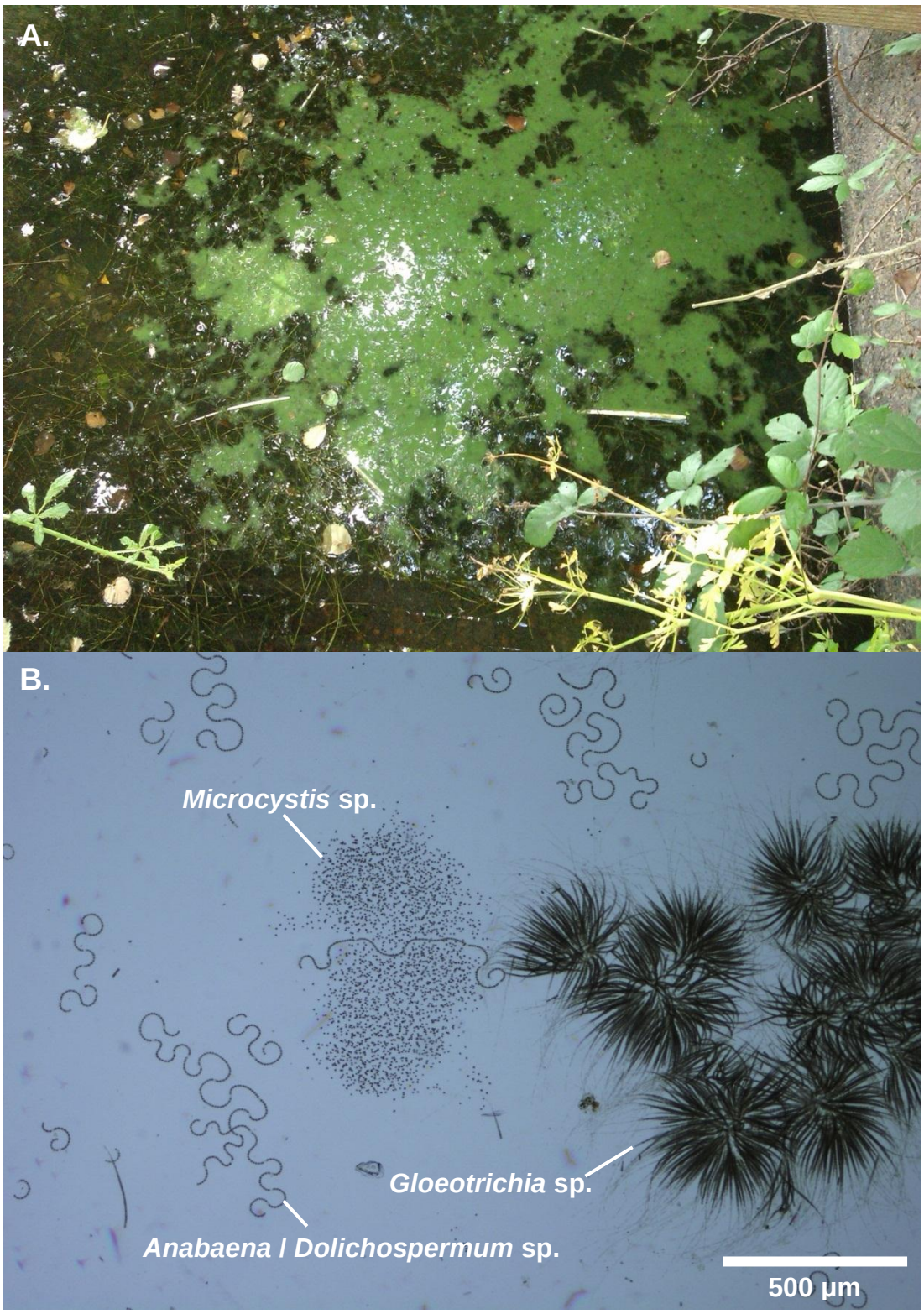


Figure 1.2. A) *Microcystis* bloom at Blashford lakes, Hampshire, U.K., where the original biomass for isolating *Microcystis* sp. CCAP 1450/17 was collected (Hartnell *et al.* 2016). B) A number of cyanobacteria species forming colonies and filaments were found amongst the *Microcystis* bloom (x40 magnification).

1.1.4 Cyanobacteria ecophysiology

Variation and succession in cyanobacterial populations can be explained by a number of environmental factors which include physical, chemical and biological pressures (Reynolds 1987; Schindler *et al.* 2008; Paerl and Huisman 2009). General trends report that temperate freshwater cyanobacterial densities increase through summer and early autumn, with lower populations in winter and early spring (Reynolds and Rogers 1976; Carvalho and Kirika 2003).

Seasonal spatio-temporal distribution also varies with species like *Microcystis*, recording a higher overwintering percentage population in the benthos compared to that of pelagic densities (Reynolds *et al.* 1981; Latour *et al.* 2004). The cyanobacterial community composition can also be very diverse, with one dominant species populating a water body to several species making up high percentages of a microbial ecosystem, as commonly seen in temperate European freshwater lakes (Chorus 2001).

In order to increase fitness / growth potential some cyanobacteria have evolved simple but effective survival adaptions. In species like *Microcystis* structures such as gas vesicles (GVs) can regulate buoyancy, optimising their light harvesting and nutrient acquisition in a water column (Walsby 1969; 1994). Cells in diazotrophic species (e.g. *Cylindrospermum*) can differentiate, forming heterocysts which can fix nitrogen when pools are limited (Van de Water and Simon 1981). When environmental conditions become too adverse cells can switch from a vegetative state to a resting state by forming akinetes (Adams and Duggan 1999). Akinetes found in *Anabaena* / *Dolichospermum* sp. remain viable even after exposure to extreme conditions such as UV radiation and long periods of desiccation (Olsson-Francis *et al.* 2009).

A number of secondary metabolites are also produced by cyanobacteria including hormones, antibiotics, allelochemicals, auto-inducers and toxins (Dagnino *et al.* 2006; Leão *et al.* 2012; Cohen *et al.* 2014). Certain cyanotoxins such as the microcystin class of toxins synthesised by *Microcystis* can be extremely harmful to a number of organisms, including humans. As drinking supplies can be severely impacted by *Microcystis* CHABs a robust detection method along with effective means of toxin quantification and the development CHAB controls are vital for human water security.

1.1.5 Toxic cyanobacteria and microcystins

Cyanotoxins in British water bodies include; anatoxins, β-N-methylamino-L-alanine (BMAA) cylindrospermopsins, lipopolysaccharides (LPS), microcystins (MC), nodularins and saxitoxins (Metcalf *et al.* 2008). The composition and quantity of cyanotoxins can vary between strains and cyanobacterial populations. The most frequently occurring and widespread cyanotoxins found in freshwater field samples are MCs, specifically microcystin-LR (MC-LR). *Microcystis* along with; *Anabaena* / *Dolichospermum*, *Anabaenopsis*, *Nostoc*, *Oscillatoria* / *Planktothrix* and the terrestrial *Hapalospihon* genus have all been documented producers of microcystin (Sivonen and Jones 1999). In addition to MCs *Microcystis* can also synthesise BMAA (Metcalf *et al.* 2008).

Since the first description of cyanobacterial livestock poisoning in the 19th century (Francis 1878) there have been a wide range of reports of intoxication in humans and animals (reviewed by Roegner *et al.* 2013). MCs are very potent cyanotoxins and considered to be very resistant to degradation due to their stable cyclic heptapeptide structure and to date consist of over 200 variants (Figure 1.3) (Codd *et al.* 2016).

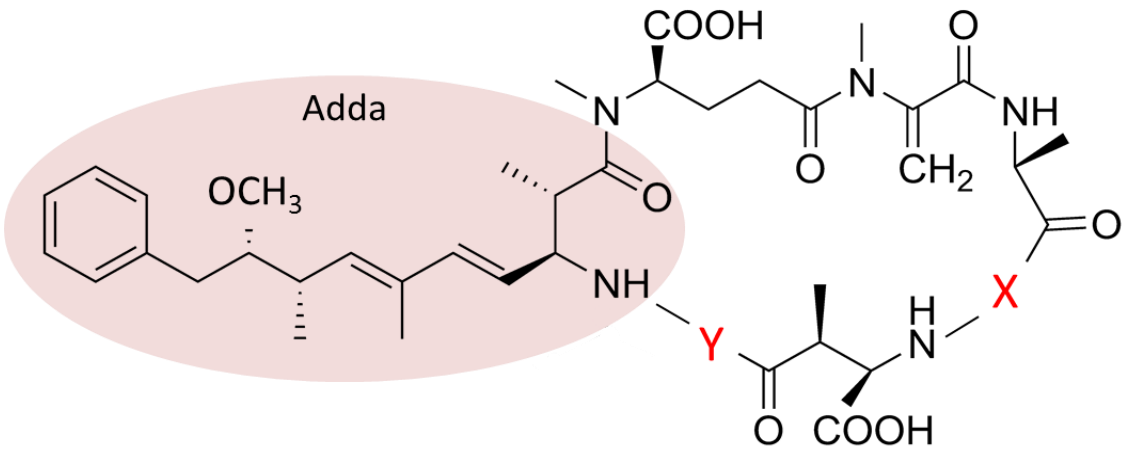


Figure 1.3. A microcystin molecule with a general structure cyclo(-d-Ala-L-X-D-erythro-β-methylAsp(iso-linkage)-L-Y-Adda-D-Glu(iso-linkage)-N-methyldehydro-Ala) where Adda is the novel β-amino acid, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4 (E), 6 (E)-dienoic acid and X and Y are sites of amino acid substitutions at positions 2 and 4 of the peptide ring. L-leucine and L-arginine would be the amino acid positions for MC-LR (adapted from Metcalf and Codd 2012).

MCs are often referred to as being hepatotoxic, however, there are a number of mammal and fish studies reporting MC accumulation in multiple tissues and organs (brain, gonads, heart, kidney, liver and lung) resulting in physiological damage (Metcalf and Codd 2012). The acute and chronic pathology of MCs are not completely understood as they exhibit diverse system effects *in vitro*, including tumour promotion (Falconer 1991).

As yet no single removal strategy has been found to consistently remove MCs from dynamic drinking water sources, such as reservoirs (Roegner *et al.* 2013). Economically resource-rich countries have the ability to implement multistage treatments but regions without economic / legislative enforcement are at a disadvantage. Cyanotoxins, in particular MC contamination of drinking water sources represent a global challenge.

1.1.6 Cyanobacteria, a blooming nuisance

Many CHABs occur at the surface where irradiance is greater which can lead to accumulation of unpleasant odours, unpalatable tastes and increased toxicity (Chorus and Bartram 1999). Through rapid cell proliferation, CHABs may quickly deplete available nutrients, increase turbidity, reduce inorganic carbon supplies and induce a population 'crash' (Paerl *et al.* 2001). The following decay of biomass has the potential to create hypoxic or even anoxic environments, significantly altering the chemical, physical and biological characteristics of aquatic ecosystems, becoming a major concern for water quality managers. However, it is the principle effect on drinking water supplies by cyanotoxins, predominantly microcystins (MCs), that are of most concern (Codd *et al.* 1999; 2005).

Although the exact function of MCs in cyanobacteria are unknown the socio-economic implications are evident. Lake Taihu the third biggest lake in China fed by the Yangtze River between Shanghai and Nanjing provides drinking water for over 2 million people, irrigates millions of hectares of agricultural land and is China's most important fishery for carp, eels and crabs. In recent years the lake has seen a substantial rise in *Microcystis aeruginosa* (attributed to eutrophication), restricting areas of aquaculture, reducing tourism and increasing stress on drinking water supplies (Guo 2007; Qin *et al.* 2007).

On a global scale economic impacts from algal blooms range from tens of thousands to billions of dollars (depending on the scale and its usage) by primarily reducing aquaculture, tourism / recreation and health (EUR 27905, Sanseverino *et al.* 2016). Further understanding of *Microcystis* ecology, morphology and physiology would therefore benefit from the development of novel detection systems and control techniques, as well as contributed to the knowledge of their role in ecosystems.

Certain CHAB forming cyanobacterial species such as *Microcystis* are particularly prevalent in closed freshwater systems, like reservoirs (Figure 1.1 and Figure 1.2). As such, reservoirs will be a focal point of this study because they impact numerous stakeholders from water authorities to environmental, health and utilities agencies (Codd 2000). Despite *Microcystis* heavily influencing human water security, the factors regulating growth and death rates are mostly unknown. Further research is needed to better understand their ecology and impact on water supplies.

1.2 *Microcystis* ecophysiology

1.2.1 *Microcystis*

Microcystis are a genus of unicellular and colony-forming coccoid cyanobacteria, defined by spherical cells with colourless, stratified, mucilage. However, strains of species such as *aeruginosa* lack an individual sheath and have free floating smaller sub colonial characteristics (Figure 1.4). *Microcystis* colonies range from micro to macroscopic, differing in morphology during vegetative cycles. Cell diameter also varies through the cell cycle, with ranges of 2 - 8.5 µm measured in natural isolates of different species (Komárek and Komárková 2002). During cell proliferation *Microcystis* division occurs on three planes perpendicular to one another in succeeding generations, with the daughter cells being almost hemispherical directly after division (Šejnohová and Maršálek 2012).

Dominance by *Microcystis* in water sources is associated with higher temperatures, lower irradiance, high pH / low CO_2, wind mixing and N:P ratios (Whitton and Potts 2012), along with buoyancy control through GVs, a benthic storage strategy, resistance to some zooplankton grazers and higher requirements for trace metals than other phytoplankton (Šejnohová and Maršálek 2012).

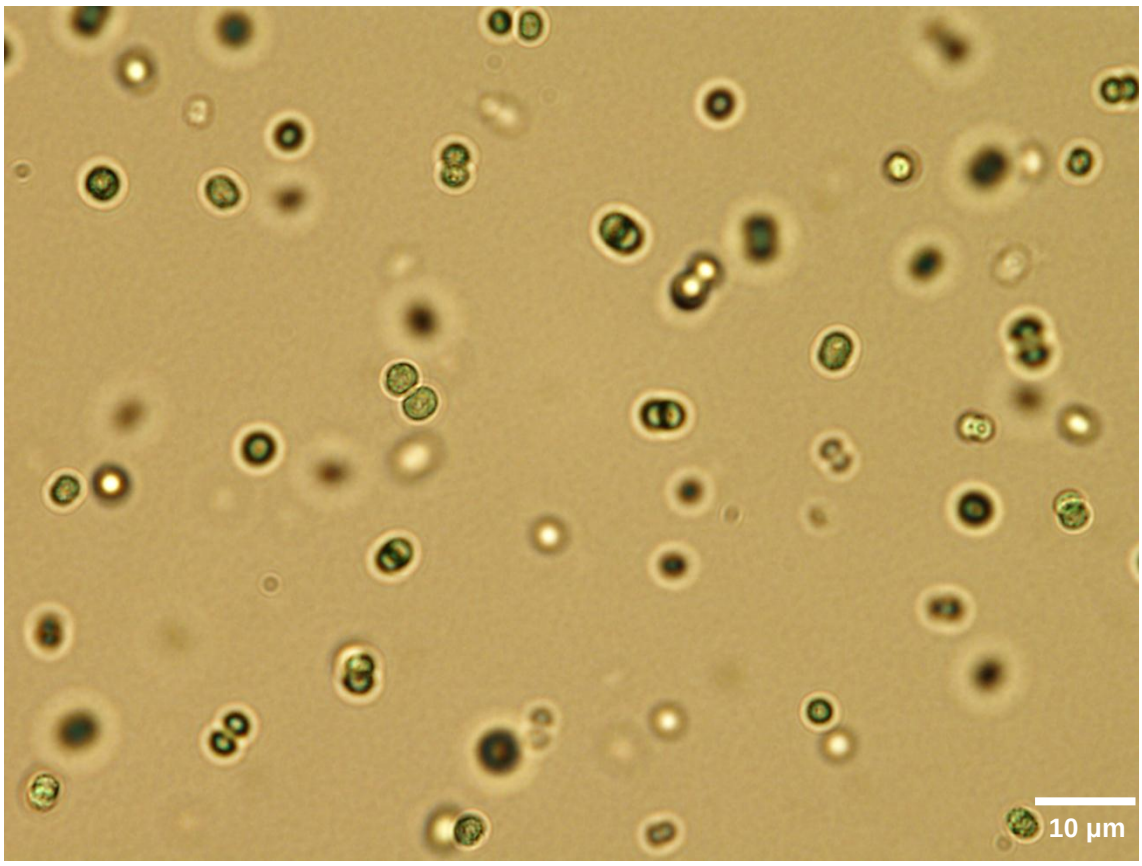


Figure 1.4. Image of *Microcystis aeruginosa* (PCC 7806) with clear differences in cell cycle stage (e.g. doublets / post division morphology) visible by light microscopy (x600 magnification).

Microcystis blooms have a global distribution and develop in slow-moving and standing waters ranging from small ponds to some of the largest lakes, which cause problems for open drinking water storage facilities like reservoirs. A typical annual cycle of *Microcystis* in temperate regions sees a planktonic bloom develop in the summer / autumn proceeded by a benthic stage in the winter / spring, where then the highest densities are found on the upper layers of sediments (Reynolds and Rogers 1976; Reynolds *et al.* 1981).

Microcystis colonies have been recorded as far down as 70cm in sediments with comparable morphology and physiology to planktonic biomasses. The deep benthic colonies also revealed distinct physiological heterogeneity with non-viable peripheral cells and viable central colonial cells (Latour *et al.* 2007). The sediment colonies reinvade the water column during spring with vertical migration through changes in buoyancy, achieved by modification of protein and carbohydrate content (Visser *et al.* 1997) and GV densities (Walsby 1972; Thomas and Walsby 1985). However, it is the higher growth rates of planktonic *Microcystis* that influence bloom formation, with the sediment phase used more as a sink than a source (Verspagen *et al.* 2005; Cires *et al.* 2013).

1.2.2 *Microcystis* morphology and taxonomic position

As with all taxonomic approaches for organisms modern classification of cyanobacteria requires a combination of techniques. Within the context of microbial molecular lineages (evolutionary), data must correlate with ecological relations and phenotypic variations (Komárek 2010). The systematic classification of cyanobacteria (Cyanoprokaryota, Cyanophyta) has seen significant changes throughout the past decade with the introduction of polyphasic taxonomy, where molecular and morphological analysis compliment biochemical, phenotypic, ecological and cellular ultrastructure data (Komárek 2012; Komárek *et al.* 2014; Komárek *et al.* 2016; Codd *et al.* 2016).

There has been a variety of names used to identify *Microcystis* in nature which has led to confusion. One of the greatest problems to date has been to transfer all modern molecular data to experimental research and establish the correct nomenclatural framework for cyanobacterial scientific practice. The application of molecular methods has enhanced cyanobacterium taxonomy, becoming a baseline in cyanobacterial systematics (Komárek 2012).

A *Microcystis* 'species' traditionally has been described according to its cell diameter, population density, colony formation (including intra-colonial organisation), mucilage structure, pigment content and life cycle (Komárek and Komárková 2002). The term 'morphospecies' has also been widely used based on the aforementioned criteria. There has to date been records of over 50 *Microcystis* morphospeices, 20 in temperate regions, with 11 found in European countries (Komárek and Komárková 2002). However, *Microcystis* colonies isolated from the wild have been shown to change form or disaggregate completely during laboratory culture (John Day, personal communication), which then poses the problem of re-identifying species and strains based solely on the 'morphospecies' concept (Otsuka *et al.* 2000).

Even molecular studies of *Microcystis* taxonomy has provided contradictory results, as reconstruction of phylogenetics through subunits of *Microcystis* ribosomes (16S rRNA) and deoxyribonucleic acid (DNA-DNA hybridisation) have revealed no differences amongst some morphospecies (Otsuka *et al.* 2001). In contrast, a phylogenetic study based on 16S rRNA from five strains of *M. smithii* isolated in Lake Dishui (China) were found to have intermixed with other morphospecies and could be placed at three different positions on the phylogenetic tree (Liu *et al.* 2011).

Whilst 16S rRNA can be used to delimit genera of cyanobacteria, the method becomes challenging when focusing on species. The variability from environmental adaptions combined with mutations further complicates criteria for defining a *Microcystis* species (Lidstrom and Konopka 2010). Subsequently the delimitation of *Microcystis* morphospecies into one uniform 'species' proposed by Otsuka (2001) may be a little premature, until the diversification process behind the 'morpho / ecotypes' can be explained (Komárek and Komárková 2002).

A review of European *Microcystis* from Šejnohová and Maršálek (2012) has indicated that there are at least three clusters within *Microcystis* that can be identified through both molecular markers and morphology, which have been defined as small (S), medium (M) and large (L) cell-size groups (Table 1.1). A recent systematic review of cyanobacterial genera has suggested a polyphasic approach to cyanobacterial identification gives more validity and characterisation to each genus (Komárek 2014; Komárek *et al.* 2016; Codd *et al.* 2016).

Table 1.1. *Microcystis* found in Europe with the debated morphospecies classification (adapted from Šejnohová and Maršálek 2012).

Cell Size cluster (µm)	Morpho-species	Cell diameter (µm)	Form of colony	Cell arrangement	Mucilage type
S (small)	*ichthyoblabe*	2 - 3.2	Irregular	Dense and regular	Indistinct
	flos-aquae	3.5 - 4.8	Spherical	Dense and regular	Diffuse, overlapping cells
M (medium)	*aeruginosa*	4 - 6.5	Irregular	Dense and regular	Diffuse, overlapping cells
	novacekii	2.4 - 6	Irregular	Dense, regular and lobbed	Diffuse, overlapping cells
	viridis	4 - 7.9	Irregular	Dense, regular and cubic	Diffuse, overlapping cells
L (large)	*wesenbergii*	4 - 8.5	Irregular	Dense, regular and lobbed	Diffuse, distant refractive margins

As the species concept in *Microcystis* is far from clear, a fresh isolate will provide a more logical experimental step when testing for control techniques (Lynch *et al.* 1991; Lakeman *et al.* 2009). Compared to decades old species / strains acquired from culture collections, establishing a fresh isolate would also be more representative of a locally adapted species.

Therefore, a part of this study will accordingly be dedicated to establishing a technique for isolating *in-situ Microcystis*, further facilitating the development of an environmental monitoring protocol. To successfully identify and detect specific a microbe requires a working knowledge of the target organism's morphological, biochemical, physiological and genetic characteristics, as well as their numerous ecological interactions.

1.3 *Microcystis* and cyanobacterial cell biology

1.3.1 Basic cell structure

Ultrastructure and morphological characteristics have been described in various *Microcystis* populations, both within laboratory and environment settings (Reynolds *et al.* 1981). As a prokaryote, the cytology of a cyanobacterium such as *Microcystis* is relatively simple. They have no nucleus or organelles (including chloroplasts, as seen in higher photoautotrophs) and use chlorophyll with an additional assortment of accessory pigments to carry out photosynthesis.

Cell surface structures are also an important cell features which are significantly affected by environmental factors. Mucilage or extracellular polysaccharides (EPS) play an essential role in the development of biofilms. However, the EPS is actually not a structural part of the cell wall and can be associated with other heterotrophic bacteria (Brunberg 1999). Proteinaceous filamentous structures such as fimbriae and pili do not exist amongst cyanobacteria, which have adapted to alternative methods of movement (e.g.GVs) and protection (debatably MCs).

Cyanobacterial cell walls are of a uniform gram-negative type containing a number of polymers and transporters with an outer membrane. The peptidoglycan cross linked architecture is very diverse amongst species and is primarily responsible for the cells strength. Usually gram negative prokaryotes have much thinner peptidoglycan layers (2 – 6 nm) compared to their gram positive counter parts (20 – 40 nm), however, cyanobacterial peptidoglycan layers are considerably thicker and have been measured over 700 nm (Hoiczyk and Hansel 2000).

One distinct difference between many cyanobacterial 'species' can be found in the cell wall structure, which differs in their crystalline monomolecular cell surface layers or S-layers (Reynolds *et al.* 1981). The S-layers are a well-defined supramolecular cell wall structure located outside the cell membrane and present in most prokaryotic organisms. This isoporous structure comprises of a single protein or glycoprotein species, exhibiting oblique square or hexagonal symmetry that appears during all stages of cell division and cell growth (Sleytr *et al.* 2014). The S-layers act as a molecular sieve, ion trap, inducer for cell adhesion and protective coating, in addition to increasing the affinity of micro-particles and keeping the rigidity and shape of the cell (Sleytr *et al.* 2014).

The chemical composition of purified cell walls from *Microcystis* sp. PCC 7806 has been isolated (Jürgens *et al.* 1989). The outer membrane contains lipopolysaccharide, fatty acid, carotenoids, two peptidoglycan-associated proteins and O-methyl sugars. The peptidoglycan is covalently linked to wall polysaccharides comprising of glucosamine, mannosamine, mannose, glucose and phosphate (Jürgens *et al.* 1989).

The structure of a cell wall may have a role in the ability of *Microcystis* to be grazed by other organisms. In addition the composition of a *Microcystis* cell wall and membrane will affect how any chemical will be taken up, be it beneficial (nutrients), antagonistic (chemical control) or passive (molecular probes). As the cellular architecture changes in cyanobacterial genera it will be essential to optimise any molecular probe designed to carry out in-depth physiological studies. Therefore research carried out here will also include optimisation of a number of molecular probes to increase our knowledge of how stressors from control techniques induce cellular physiological responses in *Microcystis*.

1.3.2 Intracellular structures

Gas vesicles

One of the most distinct features of *Microcystis* is the presence of gas vesicles (GVs) which can regulate buoyancy. GVs are proteinous, cylindrical ribbed structures filled with small gas molecules that are equal to the composition of the immediate environment such as; H_2, N_2, O_2, CO_2, CO, CH_4 and Ar (Walsby 1969). Movement in the water column by *Microcystis* is a result of GVs, carbohydrate reserve metabolism (from photosynthesis and respiration) and the physiochemical constitution of their environment.

Negative buoyancy is not only through the loss of GVs but also from a relative increase in dry matter, principally carbohydrate (Thomas and Walsby 1985). Such movement allows this genus of cyanobacteria to regulate its buoyancy in calmer waters (Reynolds and Rogers 1976). As a result this vertical or 'yoyo' model (Rabouille *et al.* 2003) can take advantage of nutrient and light levels in the water column, thereby augmenting photosynthesis in the upper layers (Brookes and Ganf 2001). The knowledge of *Microcystis* water column occupation will be integral for monitoring programmes, as population counts will vary in a spatio-temporal context. Currently routine monitoring programmes only take surface water samples for light microscopic analysis of microbes, which may not be reflective of water column densities.

Nutrient storage

There are a number of intracellular granules found in *Microcystis*, mainly functioning as a reserve substance. Glycogen granules associated with thylakoids are used as long term energy store of carbohydrate. Cyanophycin granules unique to cyanobacteria are nitrogen storing structures which can constitute up to 10% of a cells mass. Cyanophycin accumulates under light, sulphur or phosphorus stress (Allen 1984) and when nitrogen depletion occurs cyanophycin is broken down for nitrogen assimilation (Mackerras 1990). Polyphosphate granules (volutin) are used for storing phosphorus, with polyphosphate content being a function of initial orthophosphate (Jacobson and Halmann 1992). Lipid polymers are also formed from poly-beta-hydroxybutyric acid which is abundant in *Microcystis* and can be distinguished from polyphosphate granules by a 3 nm monolayer (Reynolds *et al.* 1981).

Structures assisting photosynthesis

Other specialised structures which aid cell survival include carboxysomes, siderophores and photosynthetic pigments found in the thylakoid. Carboxysomes inclusions are surrounded by a thin protein membrane consisting of RubisCO molecules (Ribulose-1,5-bisphosphate carboxylase/oxygenase). They appear to concentrate CO_2 in the cell, making it available to RubisCO which catalyses the photosynthetic fixation of CO_2 (Madigan 2012). Cyanobacteria are also capable of responding to low levels of iron by increasing their ability to scavenge from the environment. The activation of siderophores mediates a high affinity transport process which can easily bind to iron at levels as low as 1 nanogram per litre (Wilhelm 1995; Madigan 2012).

1.3.3 Photosynthetic pigments

Cyanobacteria such as *Microcystis* and rhodophytes contain water soluble chromoproteins known as phycobiliproteins (PBP), which act as the main light harvesting systems. PBPs assemble into aggregates called phycobilisomes that are bound to proteins in the thylakoid. PBPs have been placed into four main spectroscopic classes based on their visible absorption properties (Table 1.2).

Table 1.2. Phycobiliprotein main spectroscopic classes, adapted from Sidler (1994).

Phycobiliprotein	Wavelength of maximum absorption (λ max)
Phycoerythrocyanin (PEC)	575 nm
Phycoerythrins (PE)	565 - 575 nm
Phycocyanins (PC)	615 - 640 nm
Allophycocyanin (APC)	650 - 655 nm

Phycobilisomes have two main constructed elements i) a core substructure and ii) (hemidiscoidal) peripheral rods around that core substructure. Phycobilisomes are arranged such that molecules (e.g. allophycocyanin) are directly connected to the photosynthetic membrane. All cyanobacteria contain allophycocyanin and phycocyanin, with some species additionally containing phycoerythrin in varying ratios (Sidler 1994). Allophycocyanin is surrounded by phycocyanin, phycoerythrin or a mixture of both (Figure 1.5). *Microcystis* species only contain allophycocyanin and phycocyanin, although four strains from Thailand and China have recorded an absorption peak of 560 – 570 nm, indicating the presence of phycoerythrin (Otsuka *et al.* 1998).

In addition to phycobilisomes and Chl, carotenoids are found in many cyanobacteria species. Carotenoids are the largest class of naturally occurring light sensitive pigments in phototrophic organisms. Typically yellow, red, brown or green in colour they serve two major functions i) preventing toxic forms of oxygen through quenching reactive oxygen species (ROS) that cause photoxidation in PBPs, Chl and DNA (Jiang and Qiu 2005) and ii) as a part of the light harvesting antenna, absorbing photons and transferring energy to Chl (Hirschberg and Chamovitz 1994). Amongst the prokaryotes cyanobacteria only synthesise Chl *a*, whereas prochlorophytes produce both Chl *a* and *b*.

As a phylum cyanobacteria contain a unique composition of photosynthetic pigments which can be exploited by detection methods and control techniques alike. Light excitation and the resulting emission wavelengths can be analysed from native cyanobacterial autofluorescence and will be the basis of monitoring *Microcystis* throughout this research.

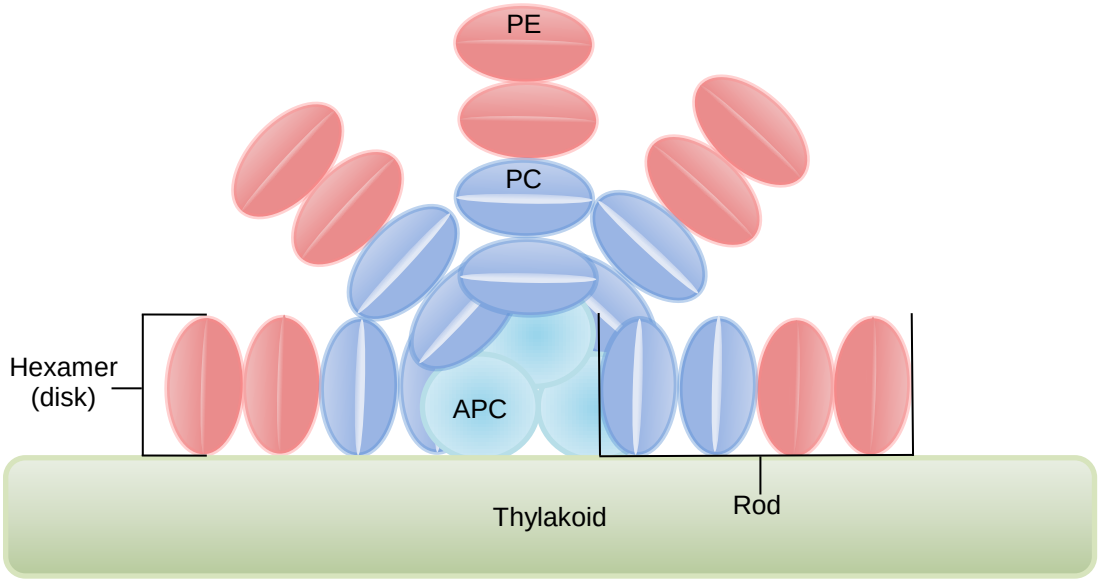


Figure 1.5. A generic phycobiliprotein (PBP) structure attached to the thylakoid for energy transfer to photosystem reaction centres. PBPs have a core structure (APC) and peripheral rods arranged in a hemidiscoidal fashion (PC and / or PE). Adapted from Sidler (1994).

1.4 Applied methods in cyanobacteria control

1.4.1 Controlling cyanobacteria

Policies surrounding the ecological and chemical status of drinking water have evolved over the past couple of decades, with major institutions like the European Union (EU) introducing international legislation to protect aquatic environments and increase water security. One initiative, the Water Framework Directive (WFD) has obligated all member states to achieve 'good' ecological status of both surface and ground waters (2000/60/EU). The requirements of such extensive environmental laws have made it challenging for managers and local authorities to gain and maintain these standards.

Nutrient loading has been perceived as the most influential factor that can be locally managed when dealing with microbial hazards, as it facilitates the growth of cyanobacterial populations (Downing *et al.* 2001; Schindler *et al.* 2008; Pearl 2009). Nutrient management is a viable long term strategy, however, the formation of CHABs presents a unique problem for routine water quality monitoring. As biological analysis is often only carried out every one or two weeks monitoring intervals are unlikely to detect hazardous cell densities that form rapidly (Falconer *et al.* 1999). Once a critical threshold has been reached large populations of cyanobacteria will need to be controlled quickly or negative socio-economic implications can occur.

Chemical, biochemical and biological measures have all been employed to reduce CHABs with varying results. As the pressure on our drinking water supplies intensifies more sustainable, fast-acting and less invasive control techniques are required, which subsequently will be addressed throughout this research.

Physical controls have also been developed, however, using mechanical approaches or extracting large amounts of water is energy intensive and requires a well maintained infrastructure. Economically disadvantaged or resource poor areas will often not be able to adopt physical approaches, so alternative methods need to be developed. As such this research will not investigate physical controls of cyanobacteria but a brief summary of recent techniques can be found in Appendix A1.

1.4.2 Developing novel approaches to cyanobacterial control

Chemical control

Chemical controls are commonly used to inhibit the growth of *Microcystis* and other nuisance cyanobacteria. Often potent, algalcides contain compounds like copper sulphate and potassium ions (Crance 1963; Parker *et al.* 1997). Although metal based chemicals have been effective in controlling or reducing densities, algalcides are not-species specific, resulting in the mortality of non-target organisms and facilitating oxygen depletion of water bodies (Cooke *et al.* 2005).

Alternate compounds that are natural, more specific to cyanobacteria, relatively cheap and have short residence times are greatly desired in water management practices. One popular natural chemical control method of *Microcystis* is to apply decomposing barley straw (Newman and Barrett 1993), however, the compound(s) directly associated with reducing growth rates have not been clearly identified. Although growth inhibition has been attributed to phenolics and / or carboxylic acids (Ridge and Pillinger 1996; Everall and Lees 1997) specifically identified decomposition products that can rapidly degrade and be produced cheap on an industrial scale have been neglected (e.g. acetic acid).

Biochemical control

It is not only synthetic chemicals that can affect cyanobacteria cells. Many prokaryotes have an intercellular signalling system, where excreted chemical signals regulate expression of specific genes in a density dependent manner. This biochemical process has been found in a number of prokaryotes and is also known as auto-induction or quorum sensing (Nealson *et al.* 1970; Fuqua *et al.* 1994). Cell free, filtered extracellular fluid (‘conditioned media’) from collapsing, nutrient-deplete *Synechococcus* cultures has been shown to have a toxic, light and density dependent inhibitory effect on conspecific cells (*Synechococcus*) (Cohen *et al.* 2014).

Microcystis has also seen growth rates reduced through the application of condition media which had a mixture of lethal and sub-lethal effects (Dagnino *et al.* 2006). However, Dagnino *et al.* (2006) did not investigate factors such as light conditions or concentration gradients like Cohen *et al.* (2014). Further examination of any *Microcystis* anti-microbial affects from conditioned media needs to be more comprehensive. Additional understanding of potential auto inducers also has to take into account any cellular physiological responses across a concentration gradient and incorporate the timings of application in batch / cell cycles.

Biological control

Although chemical treatment methods can result in rapid cyanobacterial mortality rates certain compounds may reside in the water column or sediments, causing long term damage to ecosystems or requiring additional treatment steps (Haughey *et al.* 2000; Nyström *et al.* 2002). Alternate methods such as natural controls (e.g. predators) can therefore be of great importance.

As *Microcystis* can be both toxic and non-toxic, the organisms that modify autotrophic biomass become extremely important. Zooplankton, especially ciliates play a significant role in aquatic functional ecology, where the grazing of cyanobacteria is poorly described and complex (Finlay and Esteban 1998). Grazing experiments of sustainable predators (e.g. encysting ciliates) will not only contribute to the knowledge of how efficient microbial consumers can be at controlling *Microcystis* blooms but also allows us to build a more comprehensive picture of trophic interactions in cyanobacterial ecology.

1.5 Aims and objectives

Experimental work into cyanobacterial cellular physiology has often relied on established culture lines, which have adapted to artificial conditions over many years or even decades. Stock culture lines have revealed a number of different phenotypic, metabolic and physiological characteristics compared to natural isolates and their use in functional ecology has subsequently been brought into question (Lynch *et al.* 1991; Lakeman *et al.* 2009). Therefore detection of *in-situ* cyanobacterial species for monitoring programmes and the testing of control methods will be more relevant to applied ecological problems if a recent environmental *Microcystis* species has been isolated.

Aim 1: To isolate a *Microcystis* species from a reservoir or lake for comparative study.

Objective 1: Local aquatic bodies were observed to find *Microcystis* populations, where a number of mechanical and physiological techniques were adopted (alone and in combination) to achieve a uni-algal culture.

Despite a wealth of knowledge concerning the factors that regulate growth rates the underlying processes involved in cell mortality is relatively limited (Franklin *et al.* 2012). Identification of drivers in cell mortality through the examination of physiological states will potentially provide fundamental insights into intracellular signalling, bloom development and ultimately population regulation (Franklin *et al.* 2006). Molecular probes combined with flow cytometry (FCM) can provide rapid physiological cell state analysis, however, commercial molecular probe kits often require optimisation for a specific organism otherwise results may be under or overestimated (Davey 2011).

Aim 2: To develop a valid replicable protocol indicating physiological states specific to *Microcystis* through FCM.

Objective 2: Molecular probes designed for cell membrane analysis, general hydrolytic enzyme activity and reactive oxygen species presence were optimised in *Microcystis* through varying concentrations and incubation times against positive controls.

Establishing a rapid and robust protocol for *Microcystis* seasonal monitoring would help protect drinking water sources and could be integrated into future water quality monitoring programmes. FCM is a high resolution semi-automated method of counting phytoplankton cells, which has been used to identify and enumerate cyanobacteria in temperate freshwater bodies. Although proven to be valuable FCM has only been used twice to analyse British seasonal patterns of *Microcystis* and that being in a lotic system (Read *et al.* 2014; Bowes *et al.* 2016 –*unpublished*).

Aim 3: To develop a rapid and accurate protocol for enumerating *Microcystis* cells in water supplies used for human consumption.

Objective 3: Batch cultures and fresh isolations of *Microcystis* were used to identify specific light scatter and fluorescence traits through FCM, enabling discriminative analysis of other organisms from environmental samples.

Aim 4: Monitor *Microcystis* seasonal patterns in a British lowland shallow reservoir and establish a relationship with environmental factors.

Objective 4: Using the protocol developed from aim 3 water samples and environmental parameters were recorded over a two years period determining the significant factors that affect *Microcystis* densities in a British lowland shallow reservoir.

Protozoan species, particularly ciliates are the largest and most complex of micro-organism which can significantly modify autotrophic biomass (Finlay and Esteban 1998). Being a ubiquitous grazer of primary producers the impacts of ciliates as a predator of cyanobacteria have been severely neglected. As *Microcystis* can produce toxins further investigation is required to understand ciliates ecological role in CHAB formation, where a potential control could exist through biomanipulation.

Aim 5: To assess the model ciliate *Blepharisma americanum* as a biological control of *Microcystis* and its potential ecological impact on toxic cyanobacterial bloom formation.

Objective 5: *Blepharisma americanum* was introduced to uni-algal cultures of toxic and non-toxic *Microcystis* at relatively low and high densities. Cell counts and intracellular toxin levels of *Microcystis* were recorded along with ciliate numbers, morphological dimensions and cyst formation to assess their specific trophic interactions.

Although very effective at killing aquatic microbes, algalcides often have a number of disadvantages such as non-specificity to organisms, short term operation, sediment accumulation and oxygen depletion (Cook *et al.* 2005). Identified from decomposing barley straw (Everall and Lees 1997) acetic / ethanoic acid ($C_2H_4O_2$) has been successful as a herbicide in macrophytes and as a potent medicinal anti-microbial agent (Spencer *et al.* 2003; Ryssel *et al.* 2009). To date acetic acid not been tested on cyanobacteria which could lead to a cheap, naturally degrading alternative for water treatment.

Aim 6: To evaluate the use of acetic acid as a potential chemical control in *Microcystis*.

Objective 6: Acetic acid was tested on a freshly isolated species of *Microcystis* through a number of concentrations in a short term experiment. *Microcystis* densities along with cell physiological states such as membrane injury and ROS generation were analysed through FCM. Acetic acid was also compared to a well-known oxidising agent and algalcide, hydrogen peroxide, to evaluate its potential as an anti-*Microcystis* chemical agent.

Although allelopathy is a well-established process between species the regulation of cell mortality in native cyanobacterial populations to date has not been well defined. It is only recently that any auto-inducing effects on cyanobacteria have been characterised (Sharif *et al.* 2008; Schatz *et al.* 2013; Cohen *et al.* 2014). The use of cell free, filtered extra-cellular media form a nutrient-depleted chlorotic (de-pigmented) population has shown promise as an anti-*Microcystis* biochemical agent but not extensively researched (Dagnino *et al.* 2006).

Aim 7: To evaluate *Microcystis* growth inhibition and mortality through exposure to auto-inducers from a cell free nutrient-deplete media.

Objective 7: *Microcystis* were grown from an exponential phase to a chlorotic density (typical of a crashed population). Filtered extracts from the nutrient-depleted sample was then added in a number concentrations to different batch cycle phases of viable *Microcystis* populations, under both light and dark conditions. Cell numbers, membrane integrity and hydrolytic enzyme activity were subsequently analysed to evaluate any auto-inhibitory effects.

Chapter II: Methodological development

"Water is a heritage which must be protected and defended"
European Union – Water Framework Directive (2000/60/EC)

2.1 Introduction

2.1.1 Developing new approaches for monitoring toxic cyanobacteria and *Microcystis* analysis

From a microbiological perspective the primary objective of drinking water management is to ensure the absence of pathogenic / nuisance organisms and to limit uncontrolled re-growth during water storage and distribution (Hammes *et al.* 2008). Preventive management is the preferred approach to ensure drinking water safety, where numerous stakeholders can be reliant on a limited aquatic source (WHO 2011). As microbes have been identified as a primary hazard in drinking water (WHO 2008) a proper understanding of their growth, survival and mortality factors are required.

As *Microcystis* can produce mass toxic blooms there is a need to develop rapid and accurate methodologies to assess a number biological parameters, both for detection in their natural environments and their response to particular stressors. To address the aims set out by this research this chapter has designed novel robust methods to detect and enumerate *Microcystis*, analyse their physiological state on a single-cell level, quantify intracellular toxin production (microcystins) and record morphological changes.

2.1.2 If you can't measure it, you can't control it. Rapid assessment of microbial hazards in water resources

Since algal species contain various photosynthetic pigments (Section 1.3.3) and can be morphologically distinct, a high through put analytical system taking advantages of these features would be extremely valuable. One such technology that can simultaneously analyse multiple characteristics of a particle or cell in real time is flow cytometry (FCM).

FCM is a powerful diagnostic tool which can use native autofluorescence and light scatter to provide information for marine and freshwater phytoplankton community structures (Gasol and Giorgio 2000; Read *et al.* 2014), total cell counts (Marie *et al.* 2005), single-cell physiological states (Mikula *et al.* 2012; Chapman *et al.* 2016) and physically sort out heterogeneous samples to provide monocultures (Perelman *et al.* 2004). As FCM is becoming increasingly established for microbial investigations, here the core analytic method for identifying, enumerating, assessing physiological states and morphological characteristics of *Microcystis* were recorded through FCM.

2.1.3 Principles of flow cytometry

FCM is made up of three main systems: fluidics, optics and electronics, with five main operating units: a flow cell / chamber, light source(s) (laser), optical filters, signal detectors and a data processor (Figure 2.1). When a sample (cell suspension) is run through FCM the internal sheath fluid hydrodynamically focus cells into a stream to intercept the laser(s) evenly. The optical system, consisting of one or more lasers, interrogates each cell to produce light scatter and fluorescence signals.

A detector directly in front of and to the side of a laser beam will record forward and side light scatter, measuring cell size and internal complexity respectively. Additional fluorescence detectors will also record colour signals emitted from each cell. These detectors are referred to as photomultiplier tubes (PMTs), which have fluorescent filters so each detects a specific wavelength. PMTs convert photon energy into electronic signals where a computer can process the data collected from every cell (Shapiro 1994). Any successful FCM microbiological investigation then needs to be an established robust protocol which should contain at least three types of controls, i) Instrumental control (FCM setup), ii) Specificity (both precise and accurate gating) and iii) Biological comparison (Maecker and Trotter 2006).

Instrumental controls involve adjusting FCM settings, such as entering a threshold or trigger so only data points with specific parameters are acquired. Any data points above or below a threshold will not be recorded, thereby increasing the resolution (data peaks) of FCM outputs. Fluidic speeds around the FCM system can also be changed resulting in more or less cells passing the laser intercept per second. Gating controls are used to distinguish between specific signals, such as molecular probe positive uptake for identifying physiological states. Biological controls are also significant as

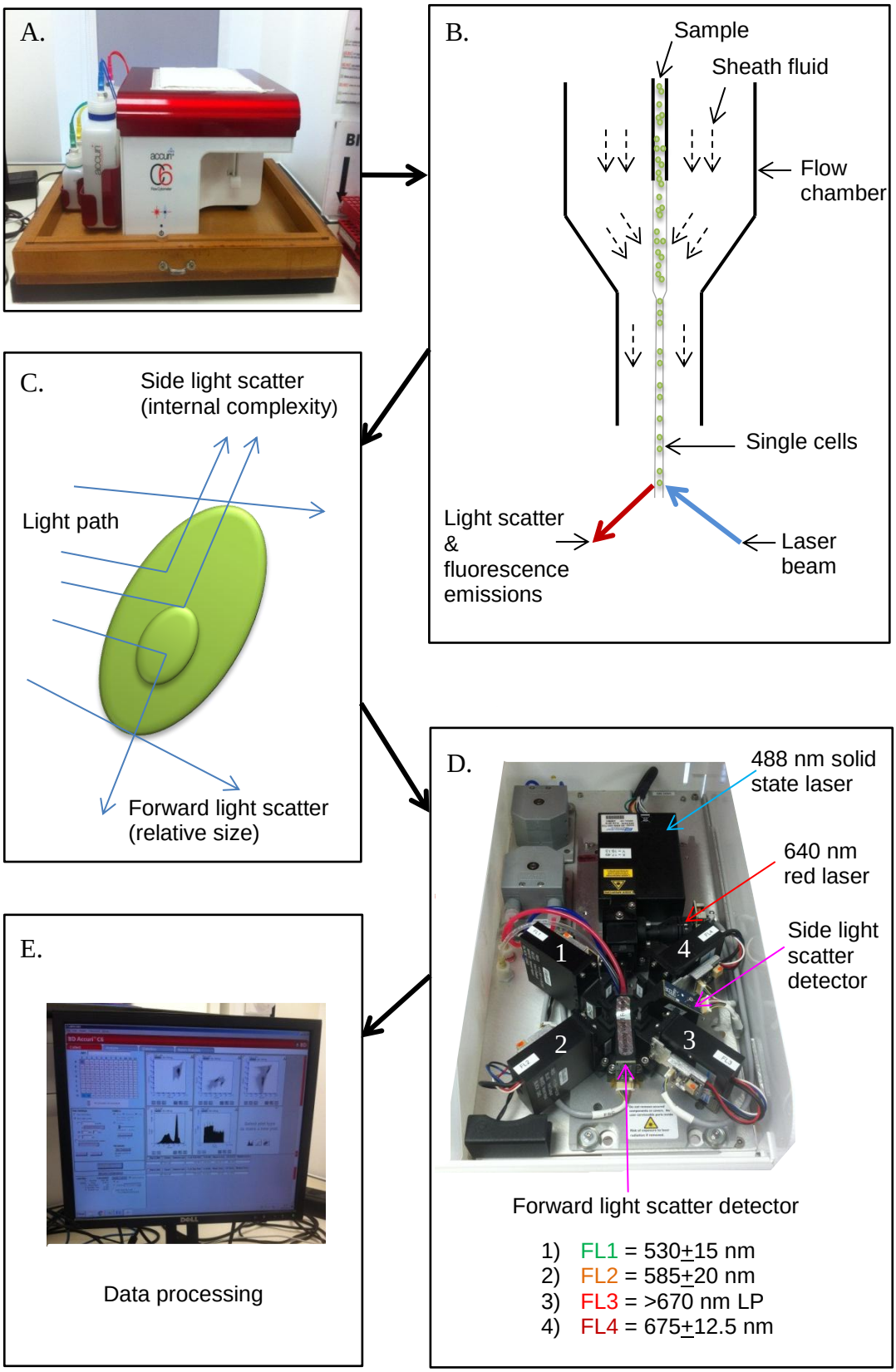


Figure 2.1. An overview of flow cytometry (A) The FCM used in this research (BD Accuri C6, BD, USA) with technical details found in the appendices (B1). (B) Schematic of the flow cell with hydrodynamic focusing of samples. (C) Light scatter paths from a cell. (D) Accuri C6 detectors for recording light scatter and fluorescence emissions at specific wavelengths. (E) All data collected from each cell is then processed by computer.

they provide relevant comparisons, such as a cyanobacterium standard which can be analysed under different conditions (Maecker and Trotter 2006).

2.2 Instrument controls

2.2.1 Thresholds and triggers for *Microcystis* detection with the Accuri C6 flow cytometer

Introduction

As a threshold is lowered more events (data points) are detected from sample debris and background electronic noise, which can dominate and reduce the resolution of target organism detection. An ideal value for a threshold should be placed somewhere between the cells of interest and background noise. The Accuri C6 (BD Bioscience, USA) instrument specifications used in this research can be found in the appendices (B1), where the manufacturer recommends a forward light scatter threshold of 80,000 AU (arbitrary units) with pulse height (FSC-H). However, for each sample analysed optimisation must take place to obtain the highest resolution of data for that target organism.

Methods

To find the appropriate threshold FSC-H was set to 10 AU (lowest possible) and a sample containing a uni-algal *Microcystis aeruginosa* (PCC 7806) population was run for 1 minute on various settings; slow, medium, fast and custom (Table 2.1). The threshold was then increased up to 100, 1000, 10,000 and 80,000 AU to establish how background noise can affect *Microcystis* cell detection, where a subsequent threshold was chosen for running the proceeding experiments. This was also repeated with side light scatter (SSC-H) and all fluorescence detection channels (FL1: 533±15 nm; FL2: 585±20 nm; FL3: >670 nm; FL4: 675±12.5 nm).

Results

Two populations were seen at thresholds up to 10,000 AU (Figure 2.2) regardless of the fluidic speed and core size. When threshold levels were raised above 10,000 AU one population (R1) decreased in terms of total events (percentage) per acquisition and one population (R2) increased. At 80,000 AU on all settings the R1 population completely disappeared and >99% of the data was recorded in R2 (Figure 2.2 B). To confirm that R1 was background noise and R2 were the cells of interest (*M. aeruginosa*), a sample containing 0.2 µm filtered ultrapure water (≥ 18 MΩ, Milli-Q, Millipore, USA) was put through the FCM on all settings. This time only one population was seen (R1) recording events only up to a threshold of 80,000 AU (Figure 2.2 C). This again was recorded on all other detectors (SSC-H, FL1,2,3 and 4-H) up to 80,000 AU.

Table 2.1. Fluidic speeds and core sizes used to test *Microcystis aeruginosa* (PCC7806) cell counts over various acquisition times.

Setting	Fluidic speed (µL/min)	Core size (µm)
Slow	14	10
Medium	35	16
Fast	66	22
Custom	100	15

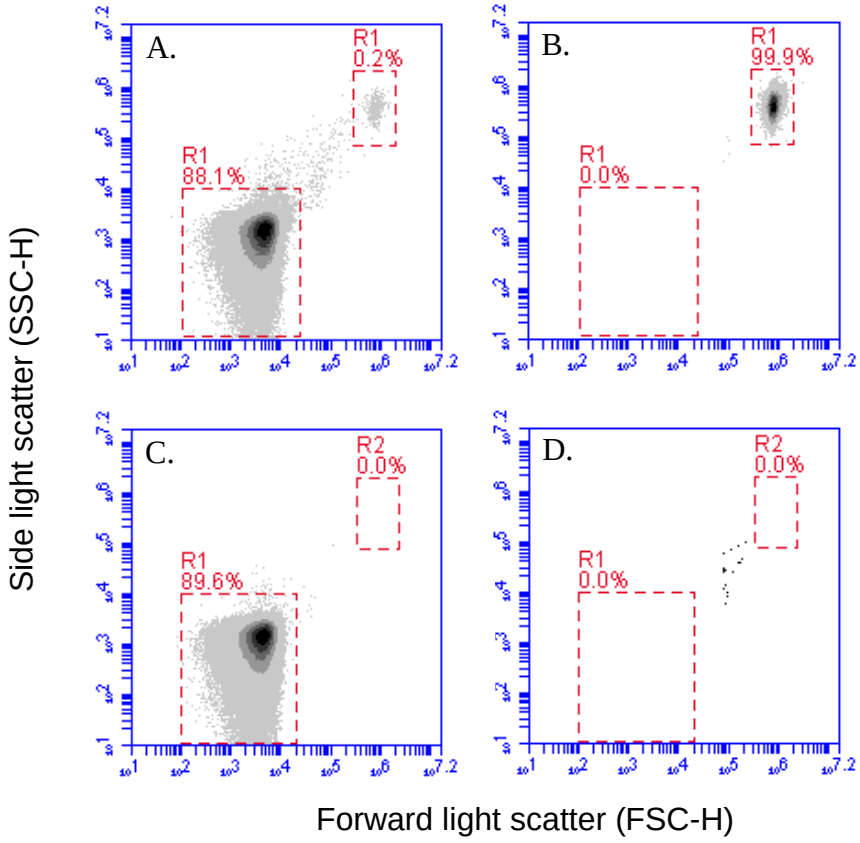


Figure 2.2. The reduction of background noise (R1) tested with samples from a *Microcystis aeruginosa* (PCC 7806) population (R2). Thresholds are increased from A) 10 AU to B) 80,000 AU. Confirmation of background noise in R1 gated areas was carried out with samples containing only filtered 0.2 µm H_2O (and no *M. aeruginosa* cells) using again thresholds from C) 10 AU to D) 80,000 AU.

Discussion

In many FCM models only one signal is used as a threshold / trigger and is sufficient as long as characteristics of other light scatter or fluorescence detectors are not effected (Shapiro 1994). Using the data collected from FSC-H threshold optimisation, SSC-H and all fluorescence detection channels produced a similar background noise region to R1, however, they were eliminated at the same time when the FSC-H threshold was raised to 80,000 AU. As such, the threshold was set to 80,000 FSC-H for all *Microcystis* analysis unless otherwise stated.

During threshold optimisation a slight shift in all parameters (light scatter and fluorescence emissions) was observed when going from the pre-set; slow, medium and fast settings to custom (Figure 2.3). As the acquired data shifted slightly between settings there is the potential that cell counts could do the same. Therefore, cell counts would need to be analysed between different flow speeds, core sizes and sample run times.

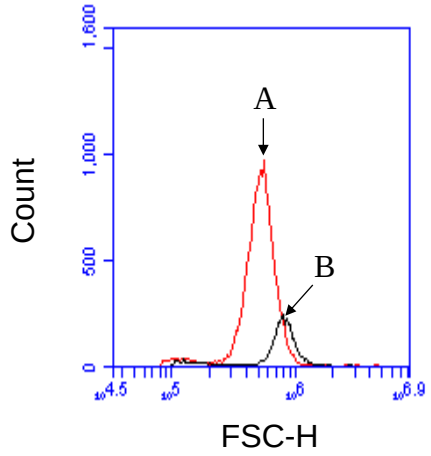


Figure 2.3. Changing settings caused a shift in histogram FCM outputs for all parameters. The above example shows a shift in mean peak FSC-H wavelengths (log scale) from different densities of *Microcystis aeruginosa* between settings A) Custom = 539,512.43 (AU) and B) Slow = 825,935.12 (AU).

2.2.2 Instrumental controls (fluidic speeds and core sizes) incorporating gating and biological controls for *Microcystis* detection and enumeration

Introduction

Settings to measure *Microcystis* cell numbers between different FCM instruments have ranged significantly in studies, with fluidic speeds from 14 µL/min (Chapman *et al.* 2016) up to 600 µL/min (Zhou *et al.* 2012) being published. As a mean histogram peak shift was observed with FSC-H between different settings (Figure 2.3) there was a need to identify any potential differences in overall cell counts between them and over time.

Methods

A uni-algal population of *M. aeruginosa* (PCC 7806) was subsequently enumerated on four different settings (Table 2.1) against a number of acquisition times (10s, 30s, 60s, 120s and 300s). As populations of phytoplankton are discriminated using a combination of parameters, here *M. aeruginosa* cells were distinguished by gating the highest histogram peak found in the far red channel, representing cells with non-degraded phycocyanin fluorescence (FL4-H: excitation 640 nm: emissions 675±12.5 nm) into a FSC-H histogram plot representing cell size. The FSC-H peak was then gated in a density plot of both FSC-H and SSC-H determining the final count (Figure 2.4).

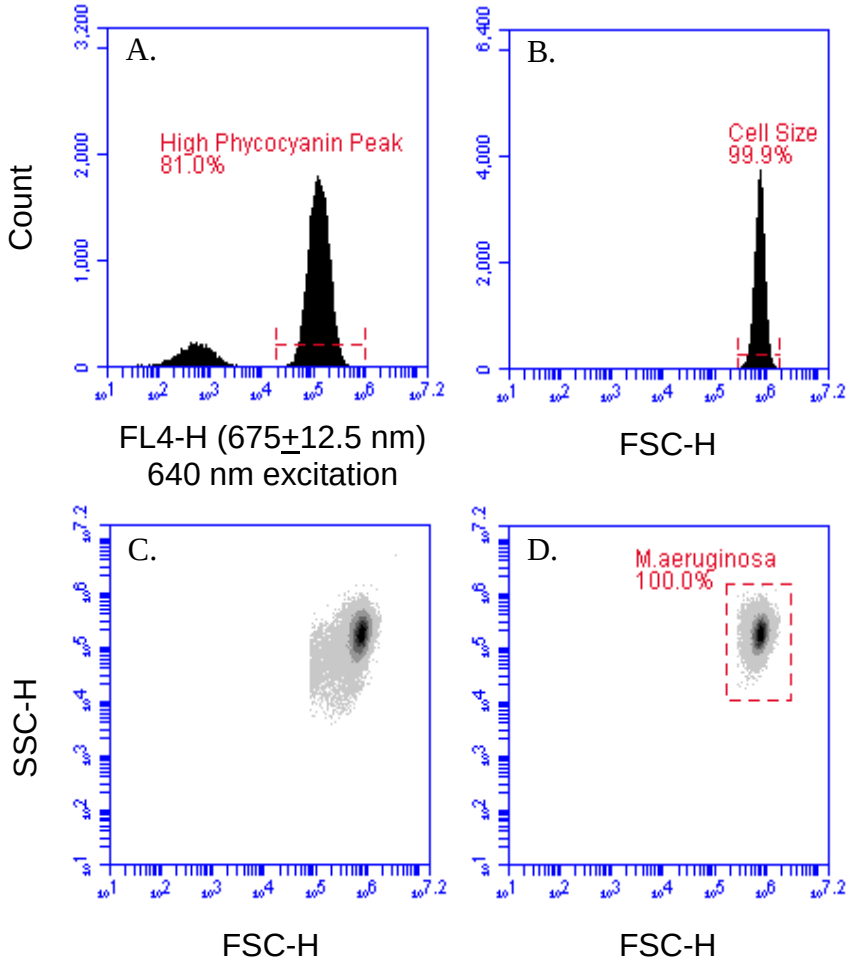


Figure 2.4. Gating from A) *Microcystis aeruginosa* (PCC 7806) highest phycocyanin peak into B) cell size (FSC-H) to refine data from C) an original FSC-H / SSC-H density to record D) final cell count.

In addition the percentage coefficient of variation (CV) was measured to identify precision of counts, where %CV looks at the spread of flow data in histogram peaks. %CV is calculated automatically through the FCM analytical software using the standard deviation of the peak, divided by the mean channel number of the peak, then multiplied by 100. A channel number is a data point that has been converted from light signals produced by cells to an electronic signal through PMTs. To record any significant difference in *Microcystis* cell densities (Figure 2.4 D), cell counts and %CV (FSC-H) were tested as a response to different settings and sample run times through a two-way repeated measures (RM) ANOVA (SPSS 20.0, SPSS Inc., Chicago, IL, USA).

Results

A two-way RM ANOVA reported there was a significant difference in cell counts and %CV between the different settings and time ($p < 0.001$) (Table 2.2). For cell counts a *post hoc* test revealed two homogeneous subsets i) slow and medium, with a combined mean of 36,0879±5,379 cells/mL across all settings and times and ii) fast and custom (combined mean 31,1882±3,507 cells/mL). When calculated through individual settings a very low standard error was recorded (S.E. ≤ 2.8%) (Figure 2.5). A *post hoc* analysis of %CV reported that the custom setting had a higher FSC-H peak data spread at 24.88±0.07% compared to the other three settings grouped (slow, medium and fast) at 21.78±0.05%. Both cell counts and %CV also reported that anything above 60s was not significantly different ($p > 0.05$).

Table 2.2. Statistical information for optimisation of *Microcystis aeruginosa* (PCC 7806) cell counts (cells/mL) and FSC-H peak coefficient of variation (%CV) between settings and various sample run times. Homogeneous subgroups were defined and indicated by similar symbols (a and b) per column.

FCM settings	Cell count (cells/mL) Tukey's post hoc		FSC-H coefficient of variation (%CV) Holm-Šídák post hoc	
Slow	a		a	
Medium	a		a	
Fast	b		a	
Custom	b		b	
Two way RM ANOVA	*F*	*p*	*F*	*p*
Setting effect ($_{3,40}$)	61.62	< 0.001	673.20	< 0.001
Time effect ($_{4,40}$)	23.32	< 0.001	5.75	< 0.001
Setting x time ($_{12,40}$)	3.14	< 0.01	3.41	< 0.01

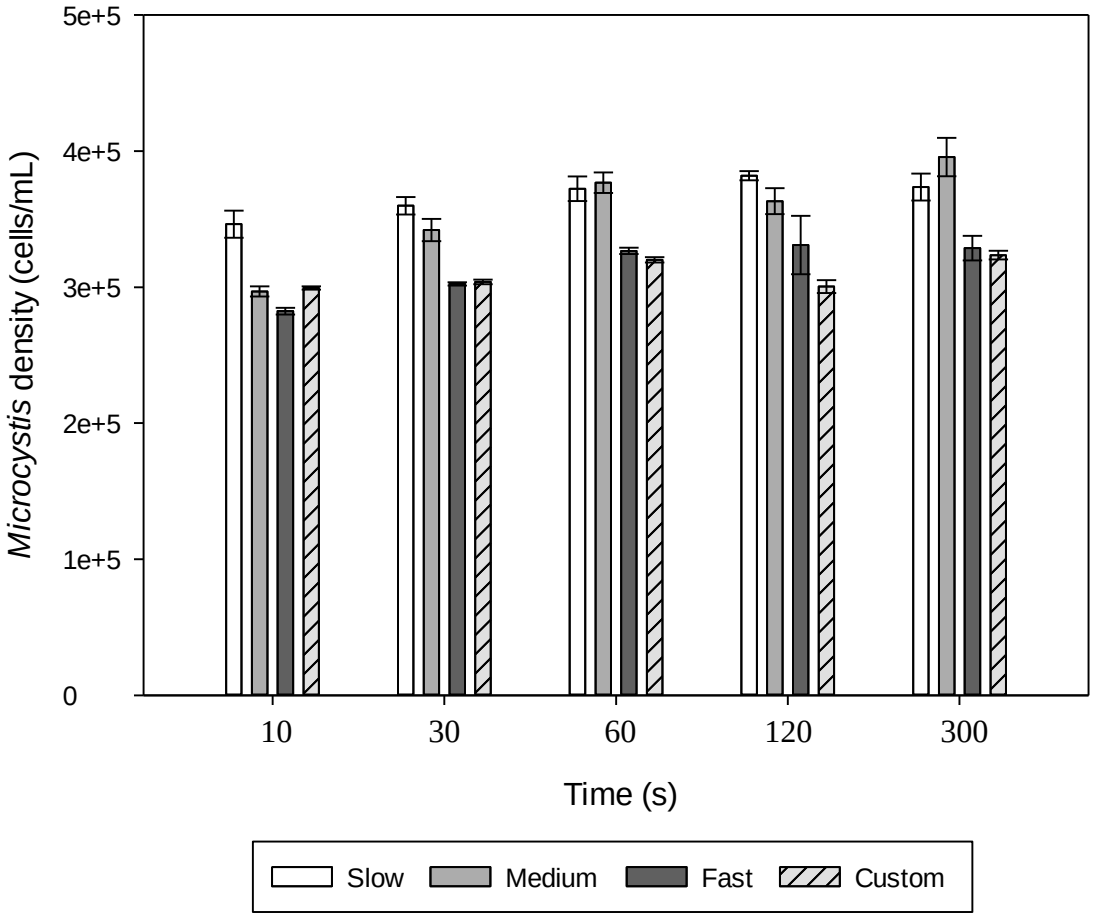


Figure 2.5. *Microcystis aeruginosa* (PCC 7806) FCM cell counts acquired from different settings and time periods, ranging from 10 to 300s (includes S.E. bars where *n* = 10).

Discussion

The lower settings (slow and medium) provided a slightly different cell count compared to the higher settings (fast and custom). The lower settings had a decreased fluidic speed and core size width which creates a greater resolution of data, as cells would intercept the laser in a more uniform fashion (Shapiro 1994; BD Bioscience 2000). Through higher speeds and core sizes there is a potential to analyse two or more cells as one data event. Although the higher flow rates recorded a slightly less resolved data output settings such as fast or custom will increase the sample volume analysed. Larger volumes analysed will give a more representative view of an environmental algal community or allow the detection of cells residing in very low densities.

As the cell cycle in *M. aeruginosa* is not synchronised within a population different cell sizes will be encountered, recording relatively high %CV of FSC-H across all settings compared to non-biological standards (e.g. sizing or fluorescence beads used for

calibration). In addition the daughter cells are almost hemispherical immediately after division (doublets) which will give wider %CV values for FSC-H histogram peaks (Figure 1.4).

For laboratory culture experiments a slow flow setting with a sample run time of two minutes was subsequently chosen and for environmental samples a custom flow setting with a longer acquisition period of five minutes. To simplify the counting protocol in laboratory experiments only one fluorescence channel was selected for counting. Although both FL3 (>670nm: excitation 488nm) and FL4 (675±12.5nm: excitation 640nm) are suitable detection channels, FL4 was chosen.

The gating parameters through FL4 provide information on allophycocyanins which include phycocyanin (PC), an accessory pigment that is commonly found in cyanobacteria and rhodophytes (Sidler 1994). It has also been published that in *Microcystis* the absorption maxima of Chl *a* disappears 12 hours before phycocyanin when exposed to chemicals with low pH levels (Arii *et al.* 2015). Chl *a* signals could therefore potentially under-represent *Microcystis* cells counts when conducting laboratory investigation with low pH levels, such as the chemical control experiment in this research involving acetic acid.

As the custom settings recorded a shift in FSC-H mean peak wavelengths it was also necessary to identify changes that would result from calibration curves for cell size calculations. This was required as two different settings were used for laboratory culture experiments and environmental detection / monitoring.

2.3 Cell size and biovolume calculations

Introduction

Cell size and biovolume are important cell characteristics. FCM not only enumerates cells but can also give information on their respective diameters and subsequent biovolumes. Through FSC and SSC recordings it is possible to calculate cell diameters from appropriate calibrations (Hoffman 2009). SSC has been shown to be a very good proxy for cell sizing in mammalian cell lines (Tzur *et al.* 2011), however, some cyanobacteria including *Microcystis* contain gas vacuoles, which can collapse and regenerate causing shifts in side light scatter outputs (Walsby 1972; Lee *et al.* 2000).

FSC is the most commonly used parameter for measuring cell diameter, as the diffracted light from the incident laser beam is detected in a forward direction relative to the cells size. The resulting electronic signal or pulse from FSC is then measured as either a pulse; height, area, or width.

Pulse height is the maximum value of a pulse when the cell is focused in the centre of the light source and pulse area records the total amount of light emitted by a cell. The pulse width is the time taken for a cell to pass through a laser beam and proportional to cell surface area or size. As pulse width is more representative of a cells size, FSC-W will be used to analysis *Microcystis* and define size (diameter).

Methods

To analysis and obtain an accurate calibration curve for cell size in both slow and custom FCM settings (Table 2.1) flow cytometric grade polystyrene sizing beads ranging from 2.0 - 14.5 µm (Spherotech, US) were sampled with a run time of 2 min. Mixed samples of *M. aeruginosa* (PCC 7806) were also added to a number of single bead samples to confirm the diameters through both FCM (Figure 2.6) and light microscopy (Figure 2.7).

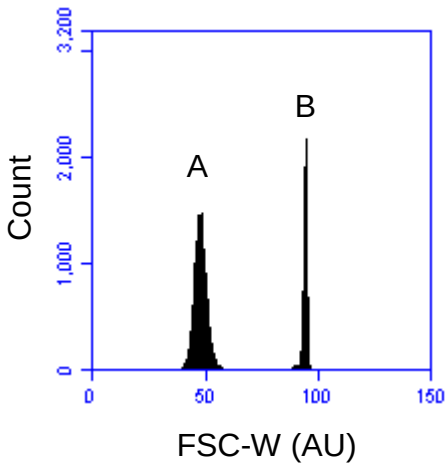


Figure 2.6. FCM output of two separate sized groups using FSC-W: A) PCC 7806 (-mycB) *Microcystis aeruginosa* (mean cell diameter 3.77 µm) and B) 14.5 µm flow cytometry grade polystyrene sizing beads, both on slow FCM settings (linear scale).

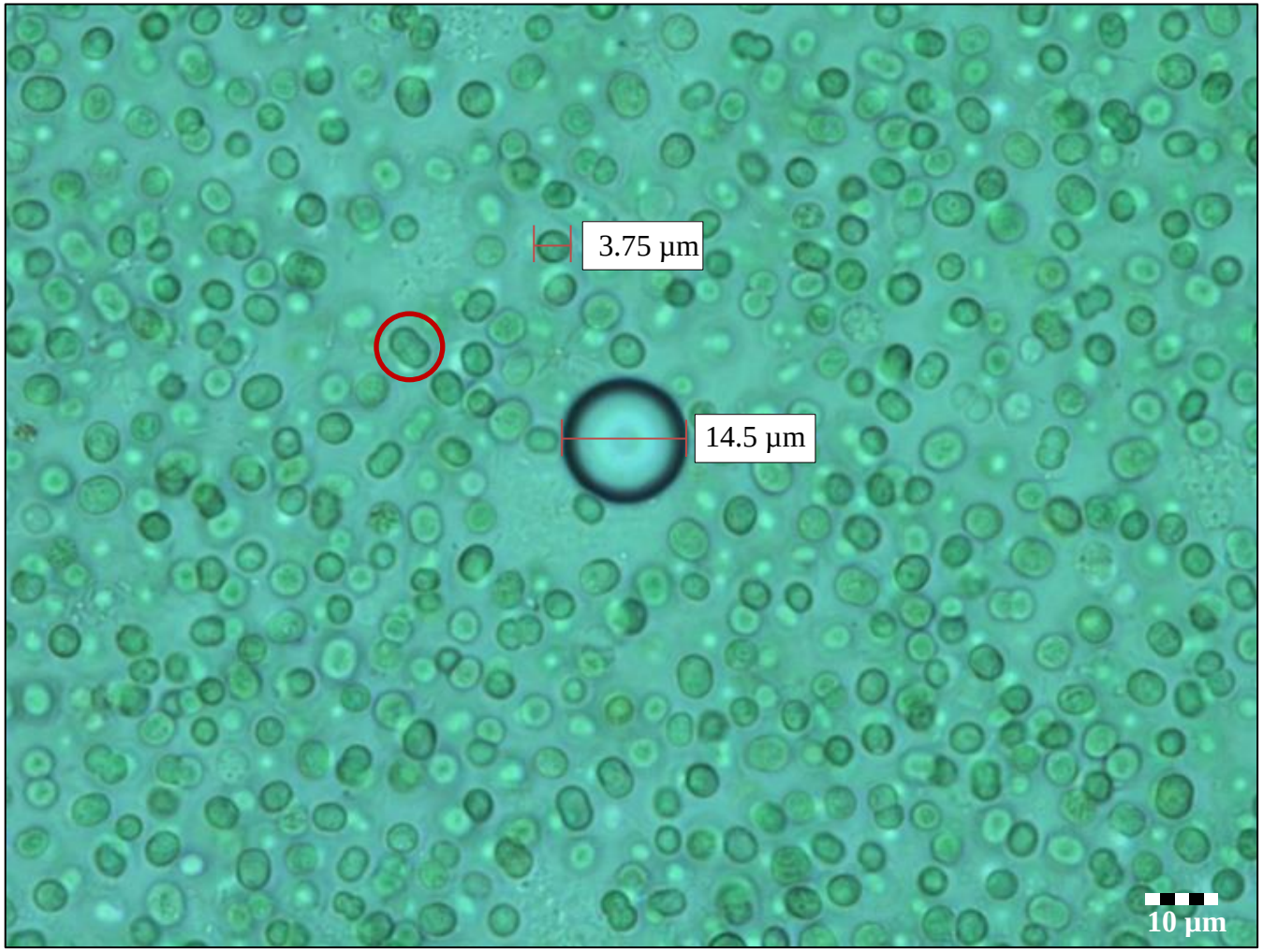


Figure 2.7. Light microscope image of pelleted PCC 7806 *Microcystis aeruginosa* (-mycB) and 14.5 µm sizing beads (x600 magnification), with semi-automated software cell measurements included. The red ring identifies *M. aeruginosa* reproduction, revealing a doublet formation.

Results

Both calibration curves had R^2 values with trend lines which fitted the data (slow = 0.998 and custom = 0.987) (Figure 2.8). However, it seemed by changing the fluidic speed and core size FSC-W outputs were affected. FSC-W arbitrary wavelength values (AU) decreased approximately 50% when speed and core size increased (Appendix B2).

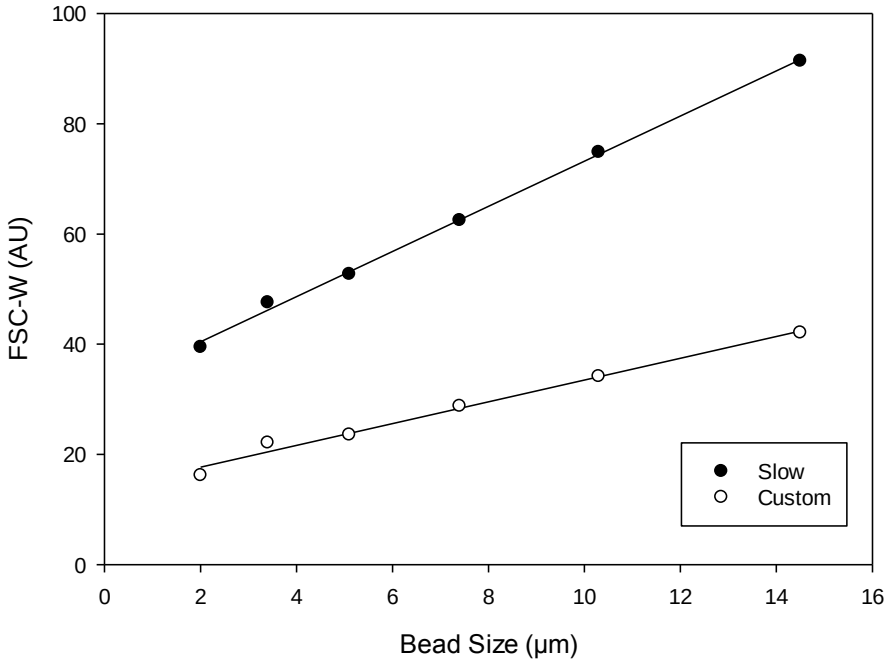


Figure 2.8. Two six-point calibration curves from sizing beads (2.0 - 14.5 µm) using settings: Slow (●) y = 4.099x + 32.228, R^2= 0.998 and Custom (o) y = 1.977x + 13.715, R^2 = 0.987 (n=10). The standard error bars are too small to be visualised.

Discussion

The shift of FSC-W mean peaks from slow to custom (14 – 100 µL/min) was due to Laser Doppler Anemometry (LDA) (Durst *et al.* 1981). When light is reflected from a moving object the frequency of scattered light is shifted proportional to the speed of the moving object. Here the LDA saw a shift in FSC-W between slow and custom because the fluidic speed increased, causing the recorded light scatter frequency to shift.

The calibration curves also did not intercept with 0 on the x axis as the limit for size detection of any particle or cell using the Accuri C6 is 0.5 µm. In addition the threshold was set at FSC-H 80,000 to avoid background noise and cell debris. Each calibration curve can be used depending on the type of FCM settings applied (e.g. slow for laboratory culture experiments or custom for environmental monitoring).

Microcystis cell biovolumes were subsequently calculated based on the volume of a sphere: $V = 4/3\ \pi\ r^3$. However, as cyanobacteria can form large blooms FCM data may be inaccurate because of aggregated cell analysis. A pre-sample colony fragmentation technique prior to sample analysis may be required for some samples. Slight caution to the exact size measurements must also be noted, as the presence of a cell in the laser beam changes the composition of the medium for which that light sources travels through.

The wavelength of laser illumination, collection angle of light scatter, the refractive index of the particle and flow medium (sheath fluid) will all affect the FSC outputs. However, this is often attributed to small cells / particles that are smaller than the illumination wavelength (Nolan 2015). As *Microcystis* are larger than 0.5 µm (Accuri C6 detection limit) and the wavelength for FSC is determined by a 488 nm laser the resulting calibration curve is acceptable to measure cell diameter.

2.4 Isolation of *Microcystis*

Experimental work into the ecology and physiology of important organisms like *Microcystis* often relies on long-established culture strains, which may have been maintained under optimal conditions for years or decades. The physiological representativeness of these laboratory strains can consequently be questioned (Lynch *et al.* 1991; Lakeman *et al.* 2009), providing impetus to develop simple protocols to isolate fresh cultures from natural populations. A number of isolation techniques were adopted to culture a freshly isolated *Microcystis* species, with success coming after replication of ecological characteristics (temperature and nutrient levels) and centrifugal washing. The new culture line has since been deposited into the Culture Collection of Algae and Protozoa (CCAP, Oban, UK) with the accession number 1450/17 and the resulting work published (Hartnell *et al.* 2016) (Appendix B3).

2.5 *Microcystis* colony fragmentation and FCM analysis

Microcystis can form large irregular shaped colonies, which is problematic for optimal FCM cell analysis and it may be necessary to use physical or chemical separation techniques to achieve accurate cell counts. Pre-treatment with potassium hydroxide, enzymes, vortexing, boiling and ultrasound have all been used to separate *Microcystis* colonies, with ultra sound exposure more commonly applied when establishing cell counts for *in-situ Microcystis* (Reynolds 1973; Reynolds and Jaworski 1978; Kurmayer *et al.* 2003; Joung *et al.* 2006; Karatan and Watnick, 2009).

Samples of an isolated *Microcystis* sp. that formed colonies found at Longham reservoirs (Dorset, UK) (Hartnell *et al.* 2016) were subjected to high and low impact separation techniques (ultrasound and vortexing) to assess colonial fragmentation and cell conditions. The low impact separation technique of vortexing had no significant ($p > 0.05$) impact on fragmenting colonies over any time periods of application (0, 20, 40, 50 and 60 s).

Only sonication for 50 seconds in an ultrasound bath (38kHz±10%, 75W, KC2, Kerry, USA) resulted in sufficient colony fragmentation. A lower ultrasound application time would result in colonies being too large to accurately count densities (Figure 2.9) and at 60s cells would lyse. A more in depth analysis of this experiment can be found in the appendices (B4). However, during ultrasound exposure the side light scatter FCM mean peak wavelengths (SSC-H) significantly shifted from the resulting collapse of gas vesicles (GV).

This SSC-H shift will need to be taken into consideration when detecting *in-situ Microcystis*, as GVs will naturally collapse through a diurnal cycle due to osmotic pressures in the water column and carbohydrate ratios (Walsby 1972; Thomas and Walsby 1985; Visser *et al.* 1997). There may also be an absence of GVs as found in a non-gas vacuolated mutant stock culture lineage CCAP 1450/14 generated from UV light exposure. Additionally mechanical equipment used for the extraction or treatment of drinking water may generate acoustic parameters to induce *Microcystis* GV collapse. Shifts in SSC-H from *Microcystis* would therefore be essential to understand when monitoring through FCM from open drinking water storages facilities like reservoirs.

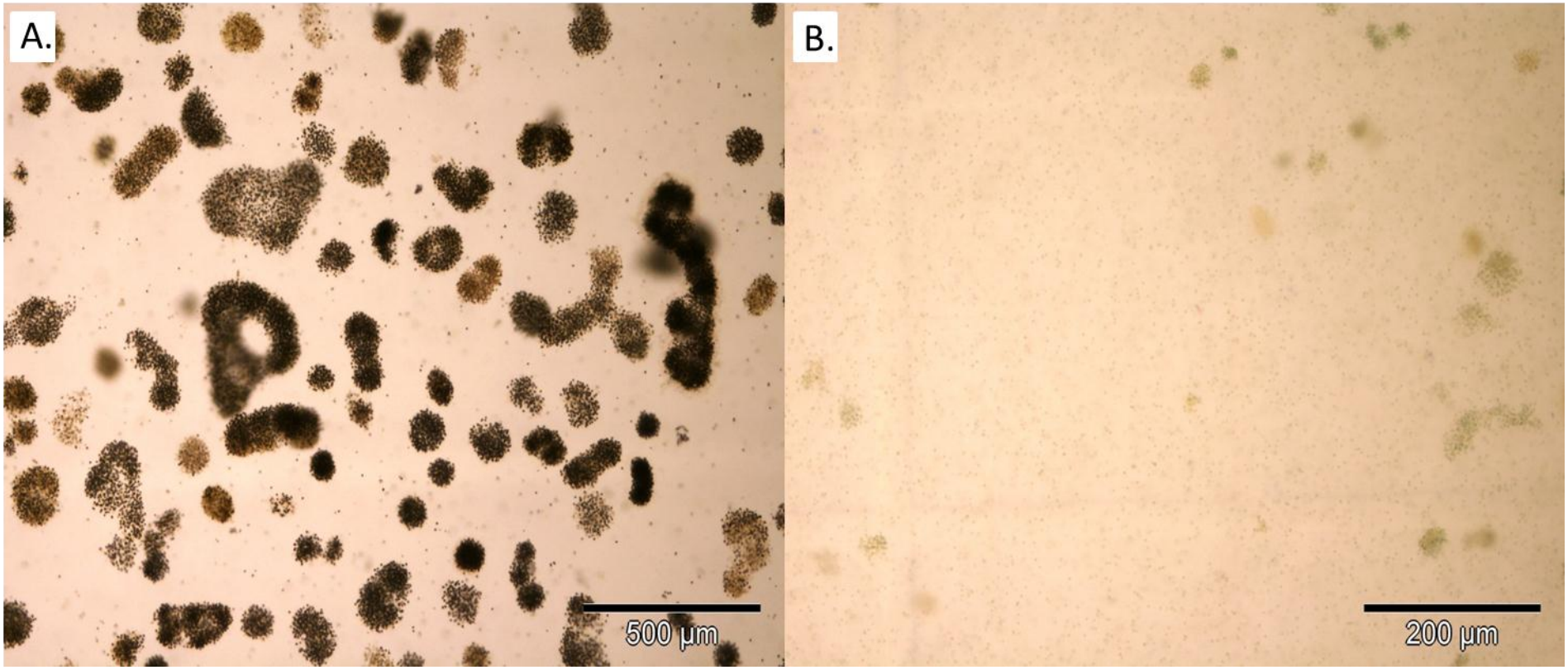


Figure 2.9. Light microscope photographs of A) *Microcystis* sp. Longham with no colonial separation technique applied (x40 mag.) and B) the same species exposed to ultra sound for 40 seconds, producing smaller colony diameters and more individual cells (x100 mag.).

2.6 Single cell physiology

The cell is a complex system which constantly responds to the environment by modifying physiological parameters and altering its function. Population dynamics of isogenic microbial populations both in nature and the laboratory are affected by the development of subpopulations, even under constant environmental conditions (Kell *et al.* 1998; Lidstrom and Konopka 2010). Establishing a method to define heterogeneity within a population is key to understanding the ecology of microbes in various environments and how they respond to stress, which is essential when developing control techniques.

Cyanobacterial species such as *Anabaena* / *Dolichospermum* display morphological heterogeneity in response to environmental fluctuations, developing specialised cells like heterocysts and akinetes (Adams and Duggan 1999). In contrast, *Microcystis* cells do not display obvious morphological heterogeneity as a response to stress. Until recently non-diazotrophic cyanobacterium like *Synechococcus* were thought to survive for long periods of time in nutrient-deplete environments by down regulating their photosynthetic apparatus (Sauer *et al.* 2001). However, in similar conditions *Microcystis* display a different phenotype within its population, whereby a small population retains 'viability' and full photosynthetic functions, compared to the dead cells that lack membrane integrity (de Abreu Meireles *et al.* 2015).

2.6.1 Classifying cell physiological states

The concept of cell viability especially in prokaryotes goes beyond the simple distinction of life and death, as they can often remain in physiological states with no measureable metabolic activity. This alternate cellular state is poorly understood and has been characterised under many different terminologies e.g. 'cryptobiotic', 'dormant', 'latent' or 'moribund' or 'quiescence' (reviewed by Davey 2011). As high heterogeneity exists in microbial populations any physiological assessment must be carried out on an individual basis. Such physiological analysis must also be non-invasive with minimal disturbance to cell biochemistry and physiology, being viewed in the context of environmental triggers (Lloyd and Hayes 1995; Gray *et al.* 2004).

FCM in conjunction with molecular probes can provide rapid and accurate ways to determine cellular physiological states, by non-invasively distinguishing a number of metabolic and biochemical processes (Shapiro 2003). The understanding of how *Microcystis* responds to stress through changes in physiological states will help develop novel control methods and contribute to the knowledge of their ecological role.

2.6.2 Molecular probes and *Microcystis*

Molecular probes are fluorescent compounds that are valuable tools for studying cellular architecture, metabolism and function on a molecular and single-cell level. For example SYTOX® probes have been used to determine membrane integrity, allowing the user to assess cell mortality under the assumption that once plasma membrane structure is compromised cellular degradation and lysis will follow (Figure 2.10). Another molecular probe, 5-chloromethylfluorescein diacetate (CMFDA) passes through cell membranes where it is cleaved by intracellular general hydrolytic enzymes (esterases) representing an active metabolism.

A number of molecular probes have been developed to study phytoplankton physiology, however, the performance of each probe varies in algal classes and culture states (Peperzak and Brussaard 2011). Whilst molecular probes have been successfully used across many disciplines of cellular biology, a limiting factor which needs to be considered is the methodological or protocol adjustment. The “off the shelf” protocols are very broad and potentially could lead to false positives or negative results. This is especially relevant as published methods need to account for FCM hardware, software, target species and the growth conditions of the microbe(s) in question (Davey 2011).

Therefore in order to use a molecular probe effectively there should be a species specific optimisation. This would include testing a number of molecular probe concentrations over an extensive incubation period against a valid control. Such biological controls are important (Section 2.2) and require appropriate methods, like heat treating cells to cause a specific membrane injury response.

A methodological approach was devised specific for *Microcystis* and SYTOX® molecular probes, confirming Peperzak and Brussaard (2011) findings that probe performances do vary. The developed method is transferable to other species and can be used as a platform for optimising protocols for other molecular probes. By demonstrating a logical sequence of steps for optimising molecular probes through FCM the resulting work was published (Chapman *et al.* 2016). The work allowed for a more effective analysis of cyanobacterial physiological states (Appendix B5) and helped address a number of aims (especially aim 2) of this research.

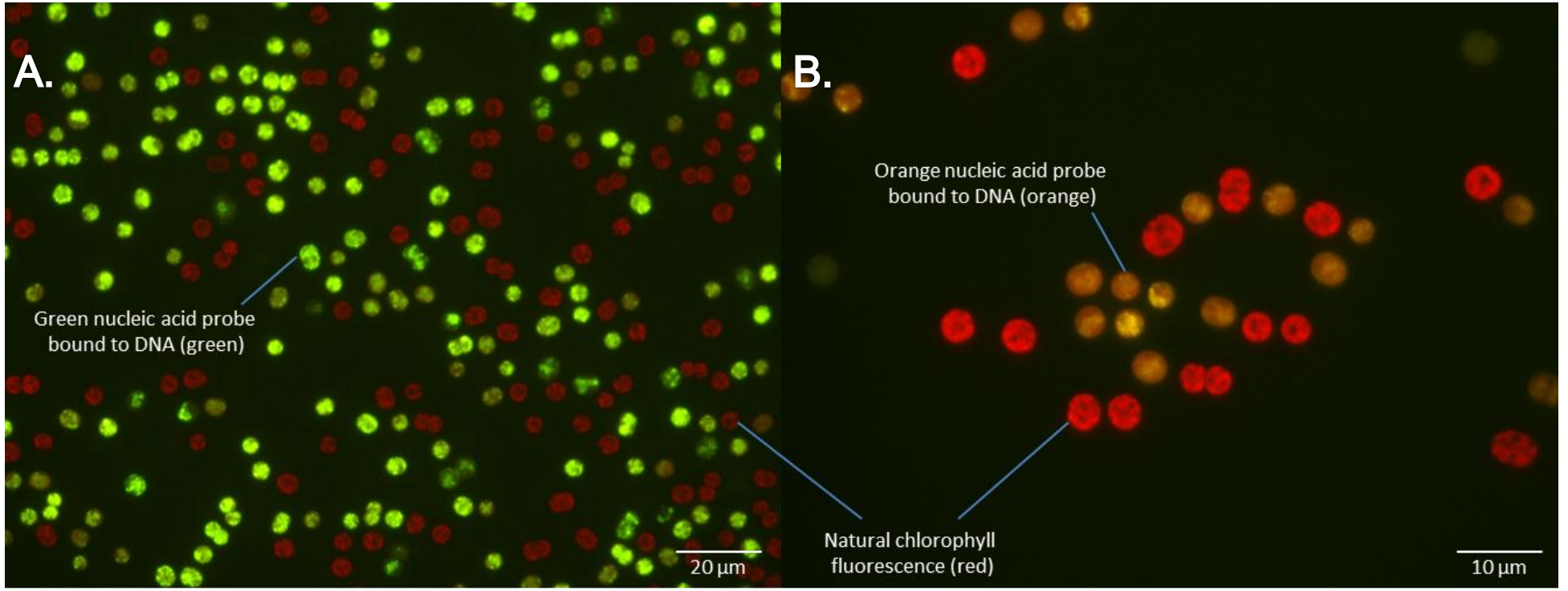


Figure 2.10. Epifluorescence microscopy image of a 50:50 live:dead (exponential phase culture and heat treated) *Microcystis aeruginosa* culture (PCC 7806, +mycB) incubated with SYTOX® A) Green (x400 mag.) and B) Orange (x1,000 mag.) nucleic acid probes (Chapman *et al.* 2016).

2.6.3 Metabolic activity, reactive oxygen species (ROS) and dual staining

Introduction

With the molecular probes SYTOX® Green and Orange being optimised at 0.5 µM after 30 min and 1 µM after 10 min respectively (Chapman *et al.* 2016) molecular probes designed for detecting general hydrolytic enzymes and ROS were also optimised to help address other parts of this research. CMFDA (5-chloromethylfluorescein diacetate) was used to detect general hydrolytic enzymes and subsequently metabolic activity and CM-H_2DCFDA (5-[and-6]-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester) was used to record ROS. In addition a novel dual stain protocol (CMFDA and SYTOX® Orange) was tested to assess two physiological parameters at the same time in a *Microcystis* sample.

CMFDA (Invitrogen, USA, C2925) is a cell tracker molecular probe that passes through cell membranes, where it is cleaved by intracellular esterases at the acetate group (Kaneshiro *et al.* 1993; Porter *et al.* 1995). A positive green fluorescence signal is recorded at an approximate light excitation of 492 nm, giving an emissions maxima of 517 nm.

CM-H_2DCFDA (Invitrogen, USA, C6827) is general oxidative stress indicator which is used to detect cellular ROS (Hakkila *et al.* 2014). CM-H_2DCFDA is membrane permeable, where it is cleaved by intracellular esterases to 2'7'-dichlorohydrofluorescin (DCFH) becoming polar and trapped in the cell. There, if present, it is oxidised by ROS to dichlorofluorescein (DCF) emitting a green fluorescence (517 - 527 nm) from an excitation source around 492 nm.

Method

CMFDA was supplied as a 1 mg powder and diluted into a 1 mM stock solution with 2.15 mL of HPLC-grade acetone (Fisher Scientific, USA), which was stored immediately at -20^{o}C. A positive control for *Microcystis* cells was set up using a heated water bath at 60^{o}C for 1 hour to denature the enzymes. The ROS (CM-H_2DCFDA) probe was supplied in 50 µg aliquots, stored at stored at -20^{o}C and freshly diluted in ethanol on the day of use to a working stock of 1 mM. Hydrogen peroxide was used as a positive ROS control for *Microcystis* cells (Sigma Aldrich, UK) at a concentration of 0.5 mM and incubated for 45 min (Figure 2.11). The concentrations and incubation times for both probes are found in Table 2.3.

Table 2.3. Concentrations (µM) and incubation times (min) used for optimisation of the molecular probes CMFDA and CM-H_2DCFDA.

Molecular probe	Concentrations (µM)	Incubation times (min)
CMFDA	0.5, 1, 5, 10, 20 & 50	1, 5, 10, 30 & 60
CM-H_2DCFDA	5, 10 & 25	10, 30, 60 & 90

For dual staining, once optimised, CMFDA and SYTOX® Orange were mixed together in a 50:50 live:dead (exponential phase culture and heat treated) culture to determine if there was a distinguishable fluorescence resolution between the two molecular probes. A two-way repeated measures (RM) ANOVA (SigmaPlot 13.0, Windows, Systat Software, San Jose, CA) with *post hoc* analysis was used to report significant differences in the percentage cell uptake of molecular probes through different concentrations and incubation times.

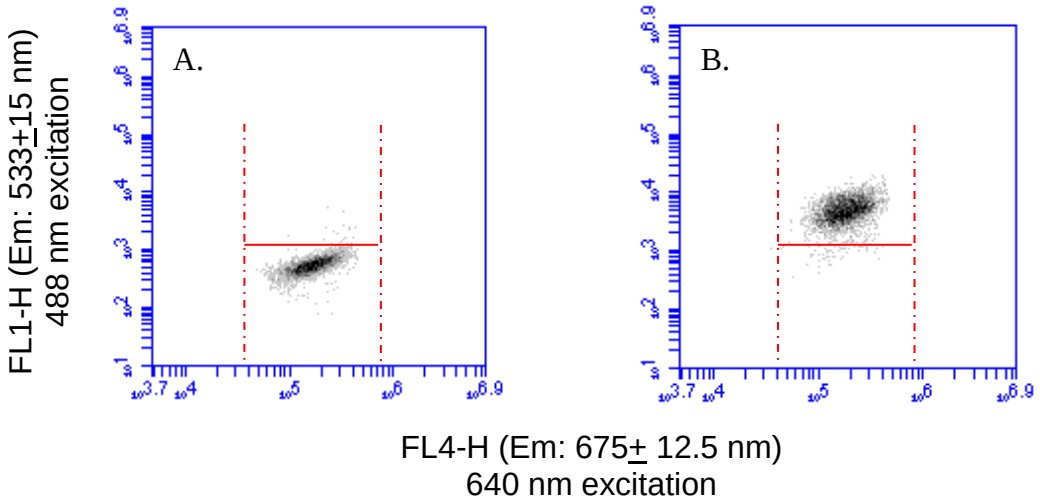


Figure 2.11. Cytogram of A) untreated and B) H_2O_2 treated (0.5 mM, 45 min) *Microcystis aeruginosa* (PCC 7806) cells. Both incubated with 25 µM of CM-H_2DCFDA in the dark for 60 min. Cytogram B) shows an increase in green fluorescence due to the increased ROS response form H_2O_2 treatment.

Results and discussion

For CMFDA and CM-H_2DCFDA a two-way RM ANOVA reported significant differences in the percentage of *Microcystis* cells that took up the molecular probes (individually) between the tested concentrations and incubation times ($p < 0.001$) (Table 2.4). Using a *post-hoc* test (Holm-Šídák method) on CMFDA results, with the exception of concentrations at 0.5 µM, over 99% of the *Microcystis* population took up the molecular probe after 30 min (Appendix B6) with 1 µM being the lowest concentration tested to do so. However, adding the molecular probe with a reasonable CMFDA handling volume would require concentrations to be increased, so the molecular probe was subsequently optimised at 5 µM (2.5 µL CMFDA for 500 µL of *Microcystis* sample) with an incubation time of 30 min.

For CM-H_2DCFDA a *post-hoc* test (Holm-Šídák method) reported the time difference was significant between two sub groups i) 10/30 min and ii) 60/90 min, with the highest amount of molecular probe cell uptake (%) in the 60 and 90 min incubation periods. For optimisation 60 min was chosen along with the concentration 25 µM as it represented the most cell percentage up take of CM-H_2DCFDA (97.1$\pm$0.6%) (Appendix B6).

Table 2.4. A two way RM ANOVA for optimisation of molecular probe uptake in *Microcystis aeruginosa* (PCC 7806) from CMFDA and CM-H_2DCFDA, conducted over a number of concentrations and incubation times (Table 2.3).

Two-way RM ANOVA	CMFDA		CM-H_2DCFDA	
	F	*p*	*F*	*p*
Concentration	35.39 ($_{5,20}$)	< 0.001	0.68 ($_{2,18}$)	< 0.001
Time	380.00 ($_{4,20}$)	< 0.001	84.69 ($_{3,18}$)	< 0.001
Concentration x time	13.73 ($_{20,48}$)	< 0.001	9.98 ($_{6,18}$)	< 0.001

Using the optimised protocols developed for SYTOX® Orange (Chapman *et al.* 2016) and CMFDA, both molecular probes can be employed in a single *Microcystis* sample with good fluorescence discrimination (Figure 2.12). Although SYTOX® Oranges optimal incubation can be as little as 10 min, 30 min will produce the same result, which allowed CMFDA to be fully taken up by the highest number of cells (Appendix B6 and Figure 2.12).

The increased number of publications using molecular probes indicates that reliable and informative data can be obtained for cell physiological states, including cyanobacteria (Shapiro 2003; Davey *et al.* 1999; Mikula *et al.* 2012). However, there is no single compound capable of analysing cell viability or other physiological parameters that is equally effective across all species with the same concentrations and incubation times (Davey 2011; Peperzak and Brussaard 2011). Even the same functional molecular probe with different fluorescence emission wavelengths (e.g. SYTOX® Green and Orange) can have different optimal concentration and incubation times (Chapman *et al.* 2016). Although the optimal development of a molecular probe protocol is very time consuming it will allow for the most accurate assessment of a target organisms physiological state, which should reflect internal and external conditions as well as appropriate controls.

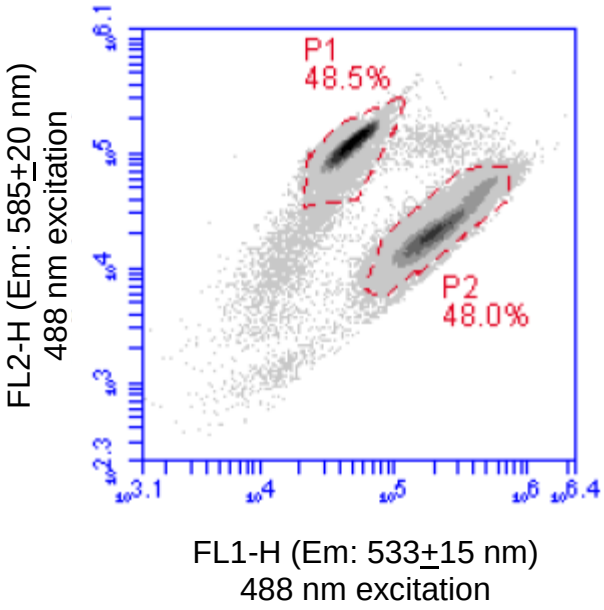


Figure 2.12. Cytogram of STYOX® Orange positive (P1, membrane permeable cells) and CMFDA positive (P2, metabolically active) cells after the optimised dual staining protocol in a live:dead 50:50 *Microcystis aeruginosa* (PCC 7806) culture (exponential phase culture and heat treated).

2.7 Cyanotoxin detection

2.7.1 Microcystins and detection

Identification and quantification is a principle component of cyanotoxin monitoring programmes in drinking water. Effective analysis can also provide an early warning system for potential toxic bloom development (Chorus and Bartram 1999). Prior research has shown that the microcystins -YR, -RR, and -LR are the most common isomers detected, with microcystin-LR being the most potent to mammals. The World Health Organisation (WHO) has set forth a water quality guideline specifying that microcystin-LR concentrations should be maintained below 1 ng/mL or 1 ug/L (WHO 2011).

A variety of physico-chemical and biochemical methods to measure cyanotoxins like microcystins are available. Each method varies in sensitivity (detection limit), technical ability and cost. Methods such as enzyme linked immunosorbent assay (ELISA) (Nagata *et al.* 1995), protein phosphatase inhibition (PPi) (Metcalf *et al.* 2001), drop-coating deposition Raman (DCDR) spectroscopy (Halvorson *et al.* 2011), high performance liquid chromatography (HPLC) (Latwon *et al.* 1994) as well as PCR-based molecular methods (Baker *et al.* 2001) have been used for screening environmental water samples for microcystins.

To assess microcystin detection and quantification in this research a new Ultra Performance Liquid Chromatography (UPLC) method was devised with Dr. Andrew Turner from the Centre for Environment, Fisheries and Aquaculture Science (CEFAS). The UPLC method was created to identify and quantify 12 microcystins and 1 nodularin from available standards.

2.7.2 UPLC MS/MS microcystin detection

Traditionally, cyanotoxins have been measured by performing extraction and concentration through solid-phase extraction (SPE) followed by high-performance liquid chromatography with ultraviolet detection (HPLC/UVD) or photodiode array detection. More recently, the analysis time has been reduced and the sensitivity improved through the use of liquid chromatography–mass spectrometry (LC-MS/MS) applying electrospray ionization (ESI).

2.7.3 UPLC-MS/MS and toxin quantitation methodological description

Microcystis samples (volume 1 mL) were filtered through a Whatman® GF/C glass microfiber disc and immediately stored at -80^{o}C. Prior to toxin extraction sample discs were subjected to three freeze (-80^{o}C) thaw (room temperature) cycles. Before UPLC-MS/MS analysis the filter discs were placed in a glass beaker containing 10 ml of 80% methanol and allowed to extract for 24 hours at 4$\pm$1^{o}C.

A Waters (Manchester, UK) Xevo TQ tandem quadrupole mass spectrometer (MS/MS) coupled to a Waters Acquity UPLC was used for LC-MS/MS analysis. Chromatography was conducted using a 1.7 µm, 2.1x50 mm Waters Acquity UPLC BEH C18 column (P/N 186002350, Lot no. 0249343351) in conjunction with a Waters VanGuard BEH C18 1.7 µm 2.1x5 mm guard cartridge (P/N 186003975, Lot no. 0245343321).

The columns were held at +60^{o}C, with samples held in the sample manager at +4^{o}C. The sample injection volume was 5 µL and the mobile phase flow rate was consistently 0.6 mL/min. Mobile phase A1 consisted of water + 0.025% formic acid, mobile phase B1 comprised of acetonitrile (MeCN) + 0.025% formic acid. The UPLC gradient was: 2% B1 initial conditions rising to 25% B1 at 0.5 min holding until 1.5 min, rising to 40% B1 at 3.0 min, increasing further to 50% B1 at 4 min, then a quick rise to 95% B1 at 4.1 min and held until 4.5 min until dropping back to 2% B1 at 5 min.

The total run time was 5.5 min. Each instrumental sequence started with a series of instrumental blanks, followed by toxin calibration standards and an extract of RM-BGA to be used as a matrix-based retention time marker and as an internal quality control (QC). Instrumental sequences finished with a water and MeCN flush, first at 60^{o}C and followed by a second at 30^{o}C. New columns were conditioned as per the manufacturer's instructions.

The Waters Xevo TQ tune parameters were as follows: 150^{o}C source temperature, 600^{o}C desolvation temperature, 600 L/hr desolvation gas flow, 0.15 mL/min collision gas flow. Capillary voltage was held at 1.0kV. Selected Reaction Monitoring (SRM) transitions were built into the MS/MS method using positive mode acquisition for each toxin. Parent and daughter ions, as well as cone and collision voltages were optimised following experiments infusing pure standards into the mass spectrometer in the mobile phase (Table 2.5).

The majority of toxins exhibited unique SRM transitions and chromatographic retention times, resulting in good separation of cyanotoxins over the 5 min run time. The exception was [Dha^{7}]-MC-LR and Asp^{3}-MC-LR, which shared the same transitions and could not be completely resolved. These two analytes are therefore reported together.

The LC-MS/MS microcystin (MC) and Nodularin (Nod) method involved the direct quantitation of cyanotoxin toxins against working standards available as certified reference standards. Quantitation was performed using external calibration and results calculated in terms of µg/L of cultures. All chemicals, standards and preparation thereof can be found in the appendices (B7).

Table 2.5. SRM transitions used for MC detection and quantitation (CE = Collision Energy).

Analyte	Mode	SRM transitions	Cone, V	CE, eV
MC-RR	+ve	519.9 > 134.9; 126.9; 102.8	30	30; 50; 70
Nod	+ve	825.5 > 135.1; 103.1	55	60; 100
MC-LA	+ve	910.1 > 135.1 ; 106.9	35	70: 80
[Dha7]-MC-LR	+ve	981.5 > 135.0; 106.8	75	75: 80
Asp^{3}-MC-LR	+ve	981.5 > 134.9; 106.9	75	70; 80
MC-LF	+ve	986.5 > 213.0; 135.0	35	60; 65
MC-LR	+ve	995.6 > 135.0; 127.0	60	70; 90
MC-LY	+ve	1002.5 > 135.0; 106.9	40	70; 90
MC-HilR	+ve	1009.7 > 134.9; 126.9; 106.9	75	75; 90; 80
MC-LW	+ve	1025.5 > 134.9; 126.8	35	65; 90
MC-YR	+ve	1045.6 > 135.0; 126.9	75	75; 90
MC-HtyR	+ve	1059.6 > 134.9; 106.9	75	70; 90
MC-WR	+ve	1068.6 > 134.9; 106.9	80	75; 100

Although there are a number of techniques to identify and quantify cyanotoxins, LC-MS/MS is one of the most sensitive (detection limits) methods, although, the system is quite expensive and requires skilled operators. The UPLC-MS/MS technique devised here was chosen as a cyanotoxin analysis for this research as reflects what local drinking water companies use to conform to UK and EU water quality standards (Wessex Water and Southern Water, personal communications).

This page intentionally left blank

Chapter III: Monitoring the seasonal dynamics of *Microcystis* in a lowland British reservoir using flow cytometry

Abstract

Determining the seasonal population dynamics and environmental drivers of nuisance phytoplankton growth is essential for water quality managers. Monitoring toxic cyanobacteria like *Microcystis* underpins initiatives designed to improve surface waters and their ecological status, such as the European Union's Water Framework Directive (WFD). Traditional identification and enumeration methods for algal species provide essential information on community structure, however, real-time multi-site analysis is not feasible without considerable investment. In this study a high throughput protocol using a relatively low-cost flow cytometer was developed to analyse a specific cyanobacterium (*Microcystis*) and for the first time establish seasonal patterns in a lowland British reservoir. Flow cytometric detection of *Microcystis*-like cells via light scatter and fluorescence was based on *Microcystis* measurements from six different culture lines and a number of other uni-algal phytoplankton cultures known to be native to the reservoir. The subsequent flow cytometric assay was used to count *Microcystis* over a two year monitoring period. *Microcystis*-like cells in the water column showed a typical temperate cyanobacterial increase during late summer and early autumn, followed by a population crash and low winter and spring abundance. Higher temperatures were found to be the most significant factor influencing *Microcystis*-like cell density increase, contributing to the wealth of evidence that climate change plays a significant role in cyanobacterial numbers. This high resolution sampling protocol was a sensitive method which can potentially be used to identify and enumerate a number of phytoplankton species, enhancing monitoring efforts in drinking water sources and benefiting aquatic ecosystem assessments.

3.1 Introduction

3.1.1 The status of water monitoring

Whilst water is relatively abundant in Europe millions of its people still live in areas under water stress, where droughts are common place (EEA 2016). The increasing demand placed on aquatic resources has been around for some considerable time, where early European legislation has introduced drinking water standards from the beginning of the 1970's. Since then European water policy has undergone reform, where an operational tool has been adopted in the form of the Water Framework Directive (WFD). The WFD aims to improve and protect current and future waters in all member states (WFD 2000/60/EC), managing this in the form of river basin districts covering all types of waters (surface, ground, transitional and coastal).

On a global scale the World Health Organisation (WHO) recognises that microorganisms have become a primary concern in drinking water, where pollutants, climatic conditions and nutrient loading have increased their threat (WHO 2008; 2011). Article 8 of the WFD specifically requires monitoring programmes to create a coherent and comprehensive overview within river basin districts (WFD 2000/60/EC). However, the European Commission (EC) recently concluded that more than one third of small water supplies were not properly monitored or delivering water of a quality standard (EC 2014).

Subsequently the "Framework for Action for the management of small drinking water supplies" was published, which refers to those suppliers providing less than 1,000 cubic meters daily or less than 5,000 people (EC 2014). Within this supplementary framework there is also an acknowledgement of concern, where overall compliance of small water supplies are reported to be around 60% when monitoring for microbiological parameters. This lack of compliance highlights the need for robust monitoring methods of microbes, where by identification and quantification of algal groups such as toxic the cyanobacteria *Microcystis* can provide data to prevent the development of hazardous blooms in our drinking water supplies (Chorus and Bartram 1999).

3.1.2 Microbial monitoring techniques in water systems

To understand the epidemiology of hazardous microbes in drinking water sources the knowledge of their life cycles during treatment and distribution is required, starting with the ability to rapidly and accurately enumerate target organisms (Hammes *et al.* 2008). There are a number of methods used for microbial quantification in drinking water such as heterotrophic plate counts (Bartram *et al.* 2003), titration of adenosine tri-phosphate by bioluminescence (Delahaye *et al.* 2003) and various molecular techniques (reviewed by Gilbride *et al.* 2006).

The most common and direct counting technique for phytoplankton in freshwater samples is through microscopy (Brierley *et al.* 2007) but the various microscopy approaches can differ in preparation times and sensitivity (detection limits) (Anderson and Throndsen 2003) (Table 3.1). In addition, due to laboratory staffing, the evaluation of a large number of samples can be prohibitive and potentially reduce precision (Bartram and Rees 2000). As cyanobacteria bloom formation can occur rapidly the monitoring of cell densities becomes essential. Flow cytometry (FCM) offers the potential to accurately enumerate toxic cyanobacterium like *Microcystis* in a fast semi-automated capacity, compared to traditional techniques like microscopy (Chapman *et al.* 2015).

Table 3.1. A summary of direct microscopy counting methods for algae (adapted from Anderson and Throndsen 2003).

Method of quantification	Counting devise	Volume (mL)	Sensitivity (cells/L)	Preparation time (min)
Compound microscopy	Sedgewick-Rafter Cell	1	1,000	15
	Palmer-Malloney Cell	0.1	10,000	15
	Drops on slide	0.02	50,000 - 100,000	1
Inverted microscopy	Uthermöhl (sediment chamber)	2 - 50	20 - 500	120 - 1440
Epifluorescence microscopy	Counting on filters	1 - 100	10 - 1,000	15

3.1.3 Freshwater biological monitoring and FCM

The use of FCM in autotrophic and heterotrophic plankton research is much more established within marine science than limnology (Gasol and del Giorgio 2000; Veldhuis and Kraay 2000). FCM is a highly sensitive method of monitoring cells and only a handful of publications have used biological calibrations based on pure algal cultures to increase monitoring accuracy in lakes (Toepel *et al.* 2005; Dennis *et al.* 2011), reservoirs (Becker *et al.* 2002) and rivers (Dubelaar *et al.* 2004; Read *et al.* 2014).

To the author's knowledge FCM has only been used in two studies to quantify potentially harmful algal groups in British freshwaters (Read *et al.* 2014; Bowes *et al.* 2016 –*unpublished*). However, Read *et al.* (2014) and Bowes *et al.* (2016 –*unpublished*) work focused on a lotic system (the River Thames) demonstrating temporal phytoplankton assemblages, which leaves FCM monitoring in British lentic systems neglected.

Read *et al.* (2014) and Bowes *et al.* (2016 –*unpublished*) also included an approach that identified and counted within algal groups, where phyla such as cyanobacteria could be profiled flow cytometrically corresponding to genera (e.g. *Synechococcus* and *Microcystis*-like cyanobacterial cells). As *Microcystis* has the potential to be a problematic species in all types of freshwater systems there is a need to develop fast and robust methods to analyse their population dynamics.

3.1.4 Influences of cyanobacteria in water supplies

Toxic cyanobacterial bloom formation has historically been a problem in drinking water supplies (Francis 1878; Codd *et al.* 1994) and it is well established that the dominance of the phyla is not a result of one significant factor but a combination of variables, both biotic and abiotic. Biological influences on *Microcystis* include predation by zooplankton and heterotrophic bacteria, infection by viruses or fungi and competition for resource from other cyanobacteria or phytoplankton (Sigee 2005; Sigee *et al.* 2007). *Microcystis* can also change trophic level dynamics, with blooms causing shifts from large-bodied daphnid cladocerans to smaller bodied cladocerans, copepods and rotifers (Fulton and Paerl 1988).

Eutrophication is regarded as one of the most influential factors leading to freshwater cyanobacterial harmful algal blooms (CHABs), although the exact role of nitrogen and phosphorus ratios continues to be debated (Smith 1983; Downing *et al.* 2001; Schindler *et al.* 2008; Pearl 2009). Growing evidence also points towards climate change for enlarging cyanobacterial densities including *Microcystis* (Carvalho and Kirika 2003; Winders and Schindler 2004; Paerl and Huisman 2008; Carey *et al.* 2012), where temperature is a positive selection pressure for a number of species (Wagner and Adrian 2009; Kosten *et al.* 2011; Rigosi *et al.* 2014).

In addition hydrodynamic process such as turbidity, wind, water column depth, mixing rates, system flushing and water residence times contribute to the vertical light gradient influencing cyanobacterial densities such as *Microcystis* (Huisman *et al.* 1999; Elliot 2010; Šejnohová and Maršálek 2012). Well mixed waters support cyanobacteria species with the lowest critical light intensity or deepest critical depth. In contrast incomplete mixing will favour cyanobacteria species that can obtain the best position in the water column like *Microcystis*, which are able to use gas vesicles (Section 1.3.2) to provide buoyancy (Huismann *et al.* 1999; Klausmeier and Litchman 2001; Huismann *et al.* 2004).

3.1.5 Predicting *Microcystis* in dynamic water bodies

Although future predictions contain large uncertainties, recent spatial-temporal studies of sediment records spanning over centuries in temperate and sub-arctic regions have revealed that temperature and nutrient concentration (N and P) are the two main drivers of cyanobacteria increase (Taranu *et al.* 2012; 2015). With widespread nutrient loading and predictions of a 2–3.5 ^{O}C temperature increase in British freshwaters by the 2080's (Hulme *et al.* 2002; Hannah and Garner 2015) there is an urgent need to increase our knowledge of seasonal patterns in toxic species like *Microcystis* through robust and accurate monitoring programmes.

Most lakes which have records of environmental factors and phytoplankton dynamics are in water bodies subject to stratification. In contrast to natural lake basins, reservoirs are often hydrodynamically complex lacking regular or strong stratification. Only a small number of studies have investigated environmental parameters on cyanobacterial populations in temperate freshwater systems which exhibited negligible stratification (Adrian *et al.* 1999; Jacoby *et al.* 1999; Gerten and Adrian 2000; Chrzanowski and Grover 2001; Grover and Chrzanowski 2006; Wagner and Adrian; 2009; Nõges *et al.* 2012; Lee *et al.* 2015).

Within those studies that have demonstrated minimal or no stratification, all were performed on large scale water bodies (surface area >0.5km^2). As there are many small shallow domestic water storage facilities in the British Isles there is a knowledge gap of how cyanobacterium (*Microcystis*) seasonal patterns are influenced by various parameters.

With specific reference to *Microcystis* spp. this study for the first time demonstrated what environmental factors strongly influenced cyanobacteria in a lowland British reservoir. *Microcystis* enumeration was achieved through a novel FCM assay, which used light scatter and fluorescent emissions discrimination from a number of uni-algal cultures found in a local reservoir, including multiple *Microcystis* strains. The devised methodology coupled with routine sampling of environmental parameters determined for the first time seasonal patterns of *Microcystis* in a lowland British reservoir.

3.2 Methodology

3.2.1 FCM and *Microcystis*

FCM data was used to enumerate *Microcystis*-like cells from a lowland British reservoir (Longham reservoirs) over an annual monitoring period. All FCM analysis was carried out by using an Accuri C6 (BD Biosciences, US), equipped with an argon ion 488nm solid state laser (blue) and a 640nm red diode laser. To determine *Microcystis*-like cell densities the default on-board light scatter and fluorescence detection channels of the Accuri C6 were used to discriminate the cyanobacterium within a diverse microbial community (Appendix B1).

A forward light scatter detector (FSC-width, 0$\pm$13^o) and a side light scatter detector (SSC-H, 0$\pm$13^o) excited by the 488 nm laser was used to identify cell size and internal complexity / granularity respectively. In addition, three fluorescence detectors were used to record photosynthetic pigment fluorescence. A yellow / orange (FL2-H, 585$\pm$20 nm) and a red fluorescence detector (FL3-H, >670 nm long pass filter) excited by a 488 nm laser was used to record pigments such as phycoerythrin (PE) / carotenoids and chlorophyll (Chl) respectively. Phycocyanin (PC), a signature light harvesting pigment native to cyanobacteria was recorded by a far red fluorescence detector (FL4-H, 675±12.5 nm) excited by a 640 nm light source. From FCM outputs, software gates were applied to discriminate algal phenotypes (BD Biosciences, US).

3.2.2 Determination of environmental *Microcystis*-like cells

To use FCM as a tool for counting *in-situ Microcystis*-like cells a number of strains were cultured to act as a 'calibration' for analysing environmental samples. Six *Microcystis* cultures (in triplicate) were grown from relatively low lag phase numbers (≈$5x10^5$ cells/mL) for 31 days in order to analyse morphological (cell size and internal complexity) and physiological parameters (pigment fluorescence) throughout one batch cycle in different media types.

Microcystis aeruginosa PCC 7806 both toxic (+mcyB) and its genetically modified non-toxic strain (-mcyB) from the Pasteur Culture Collection (PCC, Institut Pasteur, France) and *M. aeruginosa* CCAP 1450/8, *Microcystis* sp. CCAP 1450/13 and CCAP 1450/17 (previously isolated from a nearby reservoir, Hartnell *et al.* 2016) from the Culture Collection of Algae and Protozoa (CCAP, Oban, U.K.) were all analysed to understand their inherent ranges of light scatter and fluorescence emissions. In addition a *Microcystis* sp. (identified as *Microcystis* sp. Longham) was freshly isolated from a bloom at Longham reservoir 1 after environmental sampling had finished (Hartnell *et al.* 2016). *Microcystis* sp. Longham formed dense colonies and was subjected to sonication for separation in an ultrasound bath (Appendix B4).

Calibration strains of *Microcystis* were incubated at 25$\pm$1^{o}C under an irradiance range 15 – 19.5 µE m^{-2} s^{-1} (Luminex, 58 watt fluorescent cool white tube) on a 12 hour light/dark cycle (Conviron, CMP6010, Canada). All *Microcystis* cultures were maintained on BG-11 media (Sigma-Aldrich, UK) in 250mL Erlenmeyer beakers at a total volume of 100mL and agitated by hand once a day. The FCM protocol used for analysing cells was through the 'custom' setting and acquired over 5 minutes (Section 2.2.2) with samples taken approximately every three days.

Two supplementary batch cycles were also maintained on modified BG-11, one without nitrates and one without phosphates for all strains except *Microcystis* sp. Longham. The different media compositions were used to observe any differences in cell sizes, internal complexity or shift in pigment peaks. *Microcystis* were directly cultured from BG-11 samples to view any 'shock' responses that may occur from nutrients shifts which is evident through cyanobacterial densities and community shifts in British freshwaters, especially during the spring-summer transitions (Reynolds 1984; Talling *et al.* 2005).

3.2.3 Discrimination of Longham reservoirs phytoplankton community

Reservoirs contain diverse microbial communities which undergo succession and are affected by various physiochemical and biological processes. To help refine the FCM detection of *Microcystis*-like cells different phytoplankton groups that have been regularly recorded in Longham reservoirs (Table 3.2) were used as 'non-*Microcystis* calibrations'.

Table 3.2. List of algal species at Longham reservoir 1 identified over the monitoring period. This is combined data from Southern Water laboratories (Utermöhl method) and samples taken for this chapter (pelleted and observed through light microscopy).

Bacillariophyceae	Cholorphyta	Chrysophyceae
Amphora sp.	*Actinastrum* sp.	*Dinobryon* sp.
Asterionella formosa	*Ankistrodesmus* sp.	
Aulacoseira sp.	*Ankyra* sp.	Cryptophyta
Cocconeis sp.	*Chlamydomonas* sp.	*Cryptomonas* sp.
Cyclotella sp.	*Chlorella* sp.	
Cyclotella/Stephanodiscus sp.	*Chlorococcum* sp.	Cyanophyta
Cymatopleura sp.	*Cladophora* sp.	*Anabaena* sp.
Cymbella sp.	*Closteriopsis* sp.	*Aphanizomenon*/*Oscillatoria* sp.
Diatoma sp.	*Closterium* sp.	*Aphanothece*/*Aphanocapsa* sp.
Eunotia sp.	*Coelastrum* sp.	*Chroococcus* sp.
Fragilaria sp.	*Coenococcus* sp.	*Gloeotrichia* sp.
Gomphonema sp.	*Cosmarium* sp.	*Merismopedia* sp.
Gyrosigma sp.	*Crucigenia* sp.	*Microcystis* sp.
Meridion sp.	*Dictyosphaerium* sp.	*Oscillatoria* sp.
Navicula sp.	*Elakatothrix* sp.	*Pseudanabaena* sp.
Nitzschia sp.	*Euastrum* sp.	
Pinnularia sp.	*Golenkinia* sp.	Dinoflagellata
Rhopalodia sp.	*Kirchneriella* sp.	*Gymnodinium*/*Peridinium* sp.
Stauroneis sp.	*Oocystis* sp.	*Mallomonas* sp.
Surirella sp.	*Pandorina* sp.	
Synedra sp.	*Pediastrum* sp.	Euglenophyta
Tabellaria fenestrata	*Phacotus* sp.	*Euglena* sp.
	Scenedesmus sp.	*Trachelomonas* sp.
Charophyta	*Selenastrum* sp.	
Zygnema sp.	*Sphaerocystis* sp.	Xanthophyceae
	Spirogyra sp.	*Tribonema* sp.
	Staurastrum sp.	
	Tetraedron sp.	
	Ulothrix sp.	
	Volvox sp.	

The phytoplankton species chosen had very similar, as well as contrasting morphologies and pigment fluorescence to the calibration *Microcystis*. Two cyanobacteria, *Anabaena inaequalis* (CCAP 1446/1A) and *Oscillatoria animalis* (CACAP 1459/6), two green algal taxa *Euglena gracilis* (CCAP 1224/5Z) and *Chlorella vulgaris* (CCAP 211/11B), along with one commonly found diatom in Longham reservoirs *Asterionella formosa* (CCAP 1005/23) were selected to represent non-*Microcystis* cells.

All non-*Microcystis* species were incubated under the same conditions as *Microcystis* calibrations in BG-11 media (Section 3.2.2) and run through the FCM at custom setting for 5 min. Non-*Microcystis* calibrations were sampled as a standalone culture, mixed with other species (including all *Microcystis* calibrations) and incorporated (spiked) into field samples.

FSC-width (W), SSC-H and fluorescence detectors (FL2,3 and 4-H) were then used to record parameters that were common and unique to all species. FCM signatures from all uni- and multi-algal cultures were subjected to software gating. Manual software gates were constructed through isolated data points on histogram plots (Figure 3.1 A). Ranges were determined on the histogram plots to include specific outputs from *Microcystis* and exclude readings from non-*Microcystis* samples (Figure 3.1, Figure 3.5 and Figure 3.6).

The software gates were then overlaid on environmental FCM outputs to determine reservoir *Microcystis* density patterns. After the determination of *Microcystis* densities from environmental samples FCM results were compared to cell numbers obtained by an independent accredited water quality laboratory (sedimentation / Utermöhl technique).

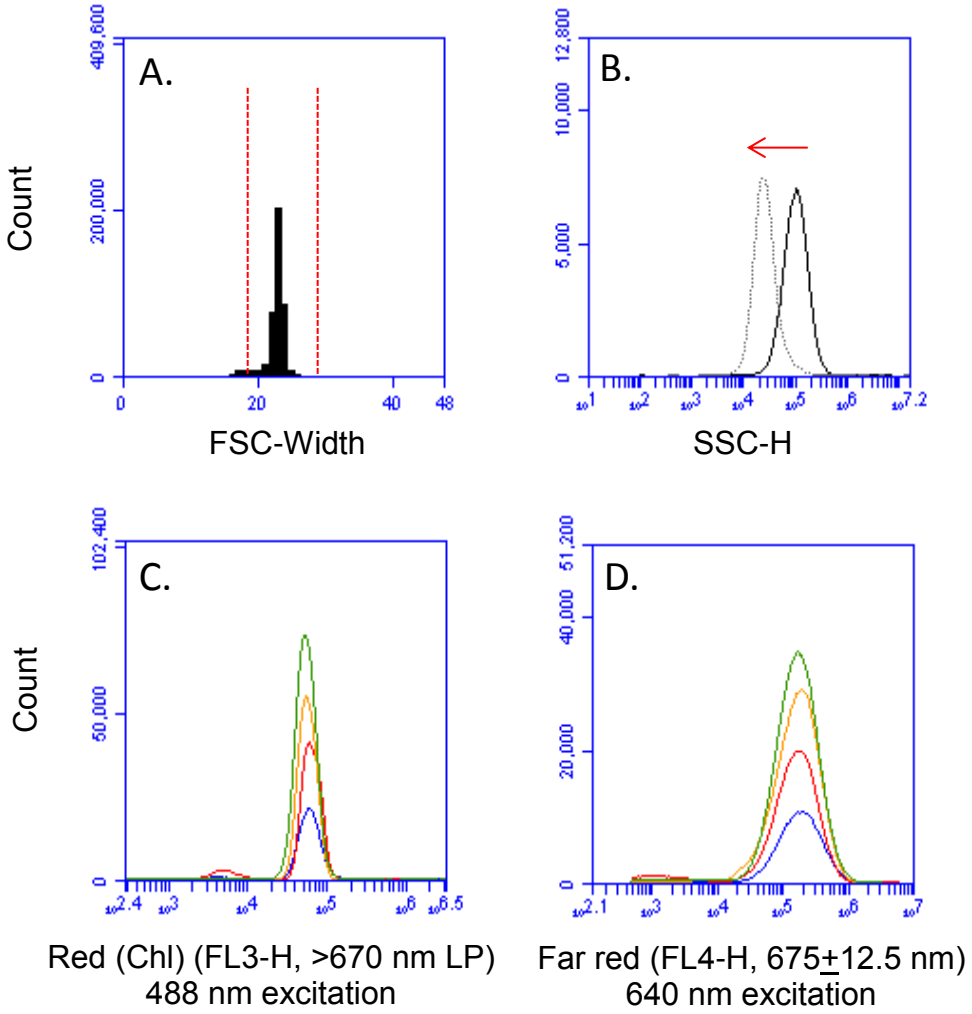


Figure 3.1. FCM outputs showing: A) *Microcystis aeruginosa* PCC 7806 (+mcyB) cell size with the calibrated range between the red dotted lines. B) CCAP 1450/17 side light scatter shift (decrease) after exposure to ultrasound (50s, 38kHz±10%, 75W). Fluorescent signatures of C) chlorophyll *a* (FL3-H) and D) phycocyanin (FL4-H) peaks, with an overlay from a number of *Microcystis* stock cultures at different densities used for *in-situ* calibration (blue = PCC 7806 (+mcyB), red = CCAP 1450/8, orange = CCAP 1450/13 and green = CCAP 1450/17).

3.2.4 Seasonal morphological characteristics of reservoir *Microcystis*-like cells

Microcystis morphological parameters were analysed over the seasons to determine changes in mean cell sizes and total *in-situ* biovolumes. Mean cell sizes (diameter) and total *Microcystis* biovolumes were calculated using sizing beads (Spherotech, US) and a spherical volume formula from FSC-W histograms (Figure 3.1 A) (Section 2.3).

As *Microcystis* form dense colonies it is sometimes necessary to break their formation to get better FCM data resolution, with ultrasound being an effective technique (Reynolds and Jaworski 1978; Wu *et al.* 2012). Although no dense *Microcystis* colonies were observed during *in-situ* sampling, by previously recording shifts in light scatter (SSC-H) from the use of sonication on colonies it was necessary to include larger SSC-H gates for *Microcystis* discrimination (Appendix B4) (ultrasonic bath 50s, 38kHz±10%, 75W, KC2, Kerry, USA).

3.2.5 Site description

Environmental samples were obtained from Longham reservoirs, Dorset, UK (50°46' N and 1°54' W), which is operated by the utilities company Bournemouth Water (Figure 3.2). The site contains two man made water bodies fed by the nearby River Stour servicing the Bournemouth and Poole area as an auxiliary water source, which is capable of sustaining the local population for approximately eight days.

Reservoir 1, the focus of this study was operational by 2003 (start of water extraction) and is a shallow polymictic water body with a perimeter of 1,400 m and an area of 97,000 m^2. It has a maritime climate characterised by intra-annual and inter-annual weather variability. It extracts water directly from the River Stour at an average of 65 ML a day and has a draw off chamber on the eastern side (Figure 3.3).

Reservoir 2, to the immediate south was operational from 2010. It is larger than reservoir 1 with a perimeter of 2,050 m and an area of 250,000 m^2. The water is not directly drawn from the River Stour but instead via an inlet pipe connected to reservoir 1 and has a draw off chamber at its southern point. The maximum depth for both lakes is approximately 14 m with an average depth of 2.9 m.

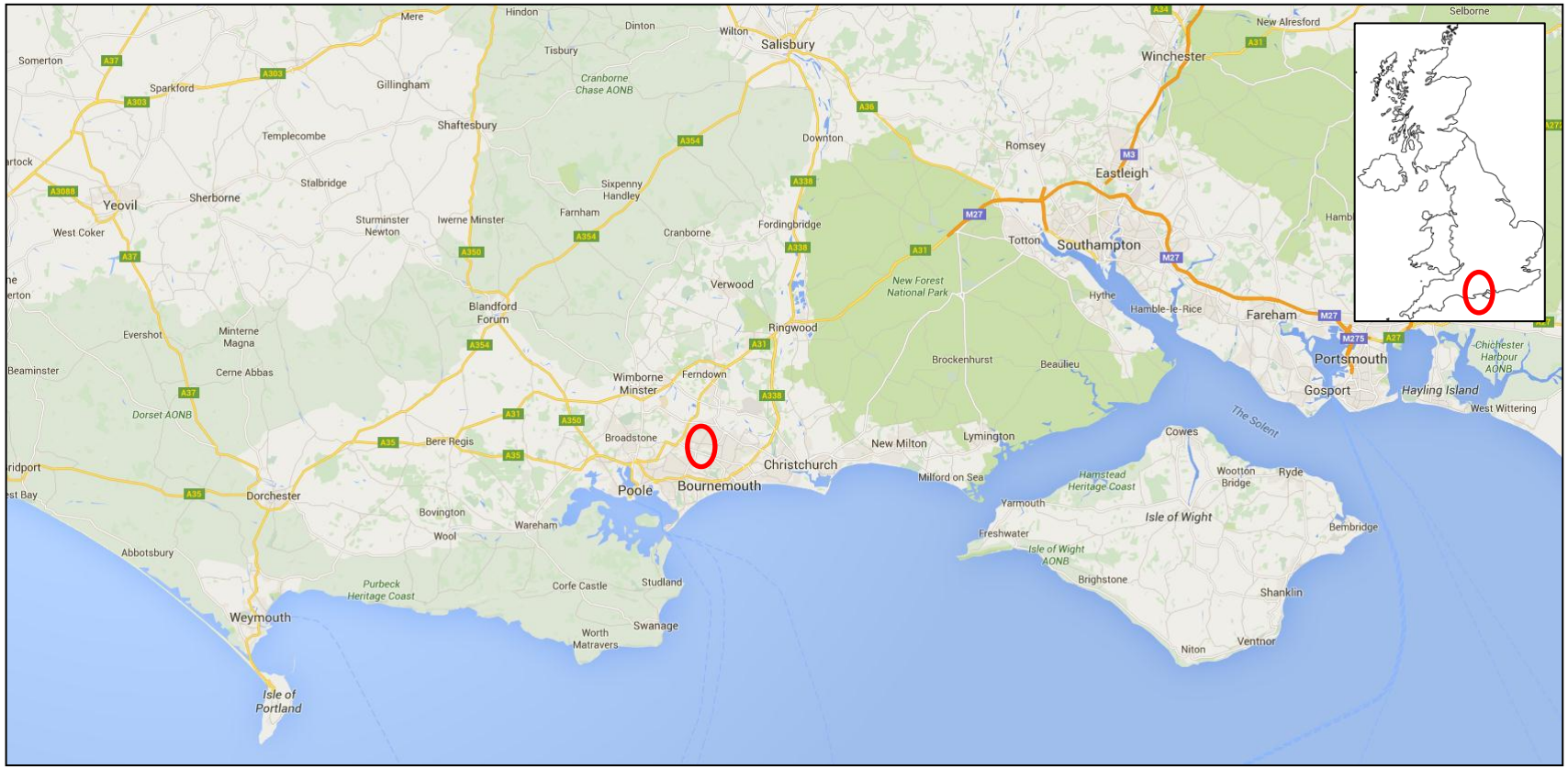


Figure 3.2. Location of *Microcystis* environmental monitoring site, Longham reservoirs, Dorset, UK.

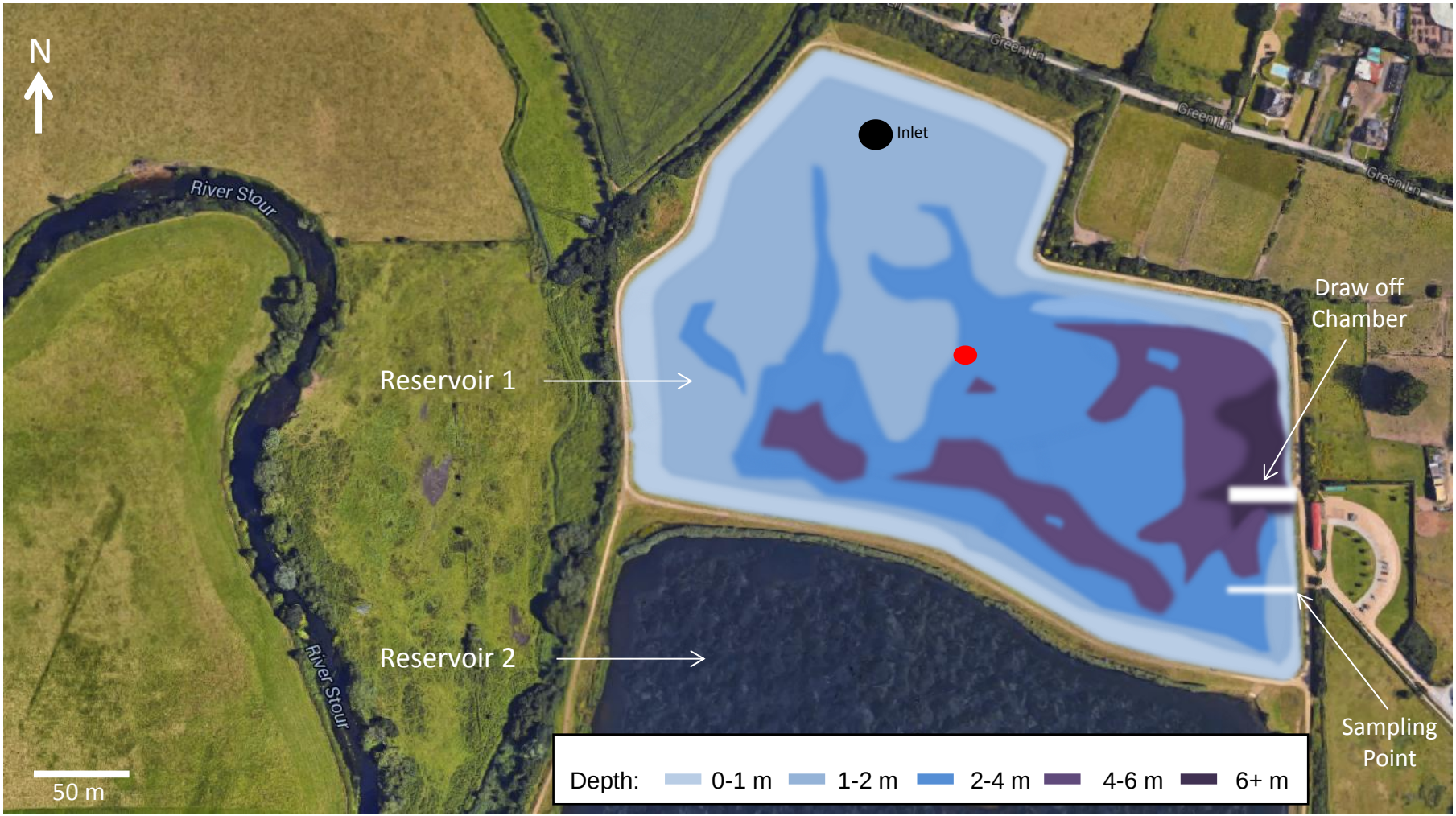


Figure 3.3. Bathymetric map of Longham reservoir 1, including the River Stour water flow inlet and sampling points. Red circle = location of comparison samples collected by boat in 2014. This map was adapted from a bathymetric map provided by Bournemouth Water (2014).

Before the site was turned into drinking water storage the area was previously a sand and gravel pit. Although Longham reservoirs have a primary function to improve water security the local authority deemed it necessary that other stake holders must be catered for.

Wildlife management was taken into consideration with a number of trees and reeds planted on the periphery, along with a small number of manmade eco-habitats placed in the reservoirs themselves. In reservoir 1 only, a number of fish species have been introduced for anglers to facilitate a mixed coarse fishery which include Carp, Bream, Skimmers, Tench, Roach, Rudd and Pike. There is only one limitation for the type of bait used which is ground cereal bait. Other types of non-cereal based and live baits are allowed with no limitation on quantities, which could potentially change the nutrient dynamics of the water.

Land management strategies have also encouraged wildlife including a number of top predator bird species such as kestrels (*Falco tinnunculus*), peregrine falcons (*Falco peregrinus*), kingfishers (*Alcedo atthis*) and great cormorants (*Phalacrocorax carbo*). In addition large mammals are often seen such as Roe deer (*Muntiacus* reevesi), badgers (*Meles meles*) and to a lesser extent otters (*Lutra lutra*), stoats (*Mustela ermine*) and weasels (*Mustela nivalis*).

3.2.6 Environmental monitoring parameters

In order to monitor the changes in *Microcystis* densities and environmental parameters samples were taken approximately every 10 days from a small jetty (Figure 3.3). The time period of this study was 22 months, which started on 01/07/2013 and finished on 22/05/2015. *In-situ* environmental monitoring was performed by a YSI 6600 V2 sonde (Yellow Springs Instrument, Xylem Inc. USA) measuring water temperature (^{O}C), specific conductivity (mS/cm), turbidity (NTU), optical dissolved oxygen (ODO %) and depth (m).

Data was collected from the surface, 1m and each proceeding meter until the bottom. At the same depths of sonde measurements water samples for *Microcystis* FCM counts were taken using a Beta Plus, horizontal, transparent, acrylic 2.2 litre water sampler (Wildco, USA). Custom FCM settings were used for 5 minutes in order to test

the highest amount of water volume reflecting site sample populations (Section 2.2). Samples were taken between 10:00am-12:00pm corresponding with highest irradiance.

Total suspended solids (mg/L), total organic carbon (mg C/L) and total phosphates (µg P/L) were analysed by an independent water quality laboratory, Southern Waters. The same laboratory also used sedimentation / Utermöhl technique to enumerate *Microcystis* which was compared against this chapters FCM records. Meteorological data was obtained from a nearby MET office UK climate data station (Hurn airport, EGHH) for mean ambient temperature (^{o}C), precipitation (mm) and length of visible sunlight (min). The length of visible light was determined by civil sunset minus the civil sunrise. Samples were also checked through microscopy to confirm the presence of *Microcystis* (Figure 3.4).

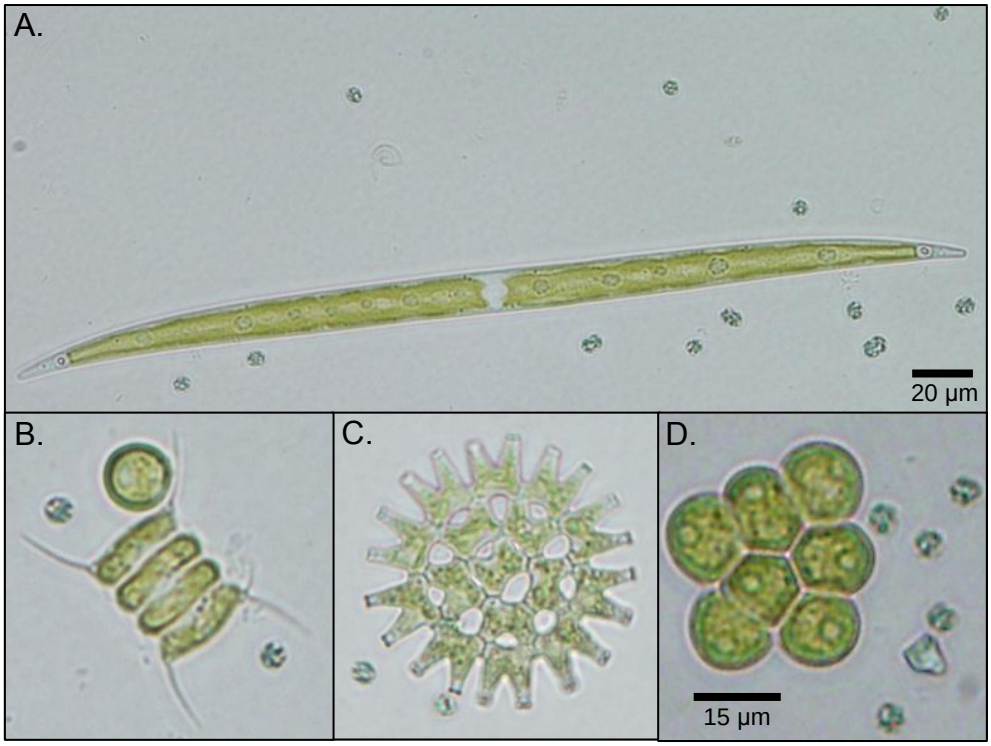


Figure 3.4. Pelleted light microscope images (Olympus BX51, Japan) of environmental water samples from Longham reservoir 1. A variety of phytoplankton were also confirmed in presence of *Microcystis* A) *Closterium* sp. B) *Scenedesmus* sp. and *Chlorella* sp. C) *Pediastrum* sp. and D) *Coelastrum* sp.

3.2.7 Statistical analysis

Microcystis-like cell calibrations

A two-way ANOVA was carried out to analyse significant differences in light scatter (FSC-W and SSC-H) and pigment fluorescence (FL2-H, FL3-H and 4-H) between *Microcystis* calibration strains that were grown in different nutrient types. The mean peaks were calculated from cumulative batch cycle data (FCM histogram plots) and used as the response factor. As the strain *Microcystis* sp. Longham was only cultivated in BG-11 it was excluded from the two-way ANOVA but all FCM parameters were still used for comparative FCM ranges. The resulting ranges were then incorporated into the discriminate analysis for specific detection of *Microcystis*-like cells from environmental samples. Non-*Microcystis* cell calibration ranges were also used to exclude data to re-fine the position of *Microcystis*-like cells on FCM plots.

Seasonal patterns of Microcystis-like cells

To identify significant changes in water column depths and between all seasons a one-way ANOVA (or the non-parametric equivalent Kruskal-Wallis one-way ANOVA on ranks) was applied to all environmental and biological *Microcystis*-like cell parameters (including cell counts) over the monitoring period. Cell counts that equalled zero were included as a value for abundance measurements and total biovolume but not cell size.

Seasons were specified by using the meteorological seasonal calendar and defined as spring (March, April and May), summer (June, July and August), autumn (September, October and November) and winter (December, January and February). A student's T-test (or Mann-Whitney *U* test) was also used to test any variance between single seasons for all environmental and biological parameters (e.g. between summer 2013 and summer 2014).

A multi linear model (LM) was run to determine which environmental variables influenced *Microcystis*-like cell densities over the monitoring period. A variance inflation factor (VIF) was used to identify colinearity amongst explanatory variables, with VIF values greater than 2.17 considered to be auto-correlated and rejected. All statistics were calculated through SigmaPlot 13.0 (Systat Software, San Jose, CA) for windows.

3.3 Results

3.3.1 *Microcystis* calibration through light scatter from six strains

Enumeration

Microcystis populations responded differently when transferred to other nutrient types. All species continued to grow in BG-11 recording relatively high final densities of ≈$10x10^7$ - $15x10^7$ cells/mL (Appendix C1). In the modified media absent of phosphates, densities reached a stationary phase between 4-11 days recording highest numbers at ≈$4x10^6$ - $6x10^6$ cells/mL (Appendix C2). *Microcystis* grown in media without nitrates recorded growth up day 4, with a maxima density between ≈$1x10^6$ - $1.7x10^6$ cells/mL. After day 4 all *Microcystis* strains exhibited a population crash with relatively minimal numbers recorded from day 11.

Forward light scatter (FSC-W)

A two-way ANOVA reported that media types and *Microcystis* strains were significant factors in cell size ($p < 0.001$) (Table 3.3). *Microcystis* sp. Longham recorded the largest cell size and CCAP 1450/8 the smallest both in BG-11, with means of 5.74±0.24 µm and 3.34±0.14 µm respectively (Appendix C3). In both modified medias PCC 7806 (+mcyB) recorded the largest *Microcystis* mean cell sizes (5.45±0.03 µm in BG-11 minus P and 5.30±0.06 µm in BG-11 minus N), significantly bigger than the smallest strain CCAP 1450/8 (4.29±0.08 µm and 3.93±0.13 µm).

As there was a significant difference in media types it is worth noting a difference between the combined means of all *Microcystis* cell sizes per media. With the largest mean cell sizes found in BG-11 with no nitrates, recording BG-11 minus N > BG-11 minus P > BG-11 (5.15±0.07 µm > 4.90±0.09 µm > 4.01±0.09 µm).

The smallest mean cell size corresponded to 3.34±0.16 µm and the largest to 5.74±0.24 µm, with an overall *Microcystis* cell size from all media types of 4.76±0.77 µm. The range of the FSC-W *Microcystis* peaks which corresponded to size (Section 2.3) all fell within 2.18 - 7.23 µm (Figure 3.1 A). In a review of European *Microcystis* (morpho) species, Komárek and Komárková (2002) reported that cell diameter (measured by light microscopy) varies through the vegetative cycle and between morpho-types, ranging from 2.0 - 8.5 µm. Komárek and Komárková (2002) cell size ranges are slightly wider than the ones found for the strains investigated here.

Table 3.3. A two-way ANOVA for mean histogram peaks of light scatter and fluorescence emissions from different *Microcystis* strains in three media types for *in-situ* calibrations. Homogeneous subgroups were defined and indicated by similar symbols (a,b,c and d) per column.

	Cell size (µm)		SSC-H (AU)		FL2-H (AU)		FL3-H (AU)		FL4-H (AU)	
Strain										
PCC 7806 (+mcyB)	a		a		a		a		a	
PCC 7806 (-mcyB)	a		b		b		b		b, d	
CCAP 1450/8	b		c		b		c		c	
CCAP 1450/13	a		d		b		a		a, c	
CCAP 1450/17	a		a		a		a		b, c, d	
Two-way ANOVA	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Media ($_{2,135}$)	185.56	< 0.001	9.39	< 0.001	9.59	< 0.001	2.69	> 0.05	9.50	< 0.001
Strain ($_{4,135}$)	71.71	< 0.001	91.26	< 0.001	7.44	< 0.001	14.57	< 0.001	20.36	< 0.001
Media x strain ($_{8,135}$)	2.52	< 0.05	9.35	< 0.001	7.43	< 0.001	4.15	< 0.001	4.85	< 0.001

However, if ultrasound is used to separate colonies there will be a change in cell size measured (Appendix B4). FCM gating of size would then agree with Komárek and Komárková (2002) which ranges from 2 - 8.5 µm or FSC-W 17.7-30.5 AU and calibrated accordingly.

Side light scatter (SSC-H)

A two-way ANOVA reported significant differences in internal complexity (side light scatter) between *Microcystis* strains in different media types ($p < 0.001$) (Table 3.3). In BG-11 *Microcystis* sp. Longham recorded the lowest SSC-H mean peak wavelength at $6.5x10^4 \pm 9.5x10^2$ AU, which was four times less than the highest SSC-H mean peak of $6.5x10^5 \pm 2.0x10^4$ AU from CCAP 1450/13 (Appendix C4). *Microcystis* in BG-11 without phosphate reported a significant difference in SSC-H ($p < 0.001$), with CCAP 1450/8 and CCAP 1450/13 recording almost double SSC-H mean peak wavelengths compared to other the strains. BG-11 without nitrates also reported a significant difference in SSC-H ($p < 0.001$), again with CCAP 1450/8 and CCAP 1450/13 having up to three times higher mean peak SSC-H wavelengths (AU) than the other populations.

The lowest mean SSC-H peaks from *Microcystis* sp. Longham had been due to the cells exposure to ultrasound for colony separation (Appendix B4). All the other strains were additionally subjected to ultrasound for 50s, resulting in a mean SSC-H decrease of 78.5±2.4% (AU), with no obvious signs of mortality observed through light microscopy. As there may be a need to break colony formation or that *Microcystis* cells may have collapsed gas vesicles at the time of sampling, the SSC-H calibration range (including gas vesicle collapse) was defined between SSC-H $7.4x10^3$ – $7.5x10^5$ AU (Figure 3.1 B).

3.3.2 *Microcystis* calibration through fluorescence emissions from six strains

Yellow / Orange fluorescence FL2-H (585±20 nm)

Excited by a 488 nm laser certain natural pigments like phycoerythrin (PE) can produce fluorescence emissions in the detection range of 585 nm. A relatively low histogram peak was recorded in all *Microcystis* strains through the FL2-H detection channel. A two-way ANOVA reported a significant difference between media types and strains on FL2-H mean peak wavelengths ($p < 0.001$) (Table 3.3). FL2-H mean peaks did have the smallest relative range of *Microcystis* native fluorescence emissions, with the largest mean peak found in PCC 7806 (-mcyB) in BG-11 minus N at 474±14.9 AU, which was double that of the lowest in BG-11, PCC 7806 (+mcyB) at 234±7.2 AU (Appendix C5). Although FL2-H mean peak shifts were significantly different the identification of a FL2-H peak range was on the lowest end of a log scale. The software gates for *Microcystis* calibration through FL2-H signatures were therefore set between 40-2,000 AU.

Red fluorescence FL3-H (long-pass filter >670 nm)

Using a 488 nm laser native fluorochromes such as the common phytopigment chlorophyll *a* (Chl) can be detected using a long pass filter above 670 nm. A clear distinct FL3-H peak can be observed in all six *Microcystis* calibration cultures (Figure 3.1 C). Statistically there was a difference between the strains ($p < 0.001$) but not between media types ($p > 0.05$).

Microcystis sp. Longham recorded the highest FL3-H mean peak at $13.6 \times 10^4 \pm 1.2 \times 10^4$ AU in BG-11, with CCAP 1450/8 in BG-11 without nitrates exhibiting the lowest at $6.3 \times 10^4 \pm 3.1 \times 10^3$ AU (Appendix C6). Although the highest peak found in *Microcystis* sp. Longham was over 4 times that of CCAP 1450/8 (BG-11 minus N), the ranges of FL3-H were relatively very narrow and overlapped in each strain (Figure 3.1 C). The FL3-H calibration for *Microcystis* was gated between a range of 1.8×10^4 – 2.2×10^5 AU.

Far red fluorescence FL4-H (675±12.5 nm)

Excited by a 640 nm laser phytopigments like phycocyanin (PC) can produce fluorescence in the detection range of 675 nm. Within the FL4-H detection channel a relatively high peak was observed in all *Microcystis* calibrations (Figure 3.1 D). A two-way ANOVA did report significant differences between FL4-H mean peak wavelengths from different *Microcystis* calibration strains in different media types ($p < 0.001$). *Microcystis* sp. Longham again recorded the highest fluorescence emissions mean peak at $1.0x10^6 \pm 9.3x10^4$ AU in BG-11 and the lowest was CCAP 1450/8 at $1.6x10^5 \pm 1.9x10^4$ AU in BG-11 minus P (Appendix C7). Despite the significant difference all strains wavelengths fell within the same range.

Compared to FL3-H, FL4-H mean peak ranges looked relatively wide. However, as the FCM output is measured on a log scale and through different fluorescence channels (with specific wavelength detectors) the *Microcystis* calibration was still well defined and identifiable (Figure 3.1 D). As such, gating FL4-H signatures for *Microcystis* calibration was set between $2.5x10^4$ - $2.0x10^6$ AU.

3.3.3 Non-*Microcystis* algal species FCM Calibration

Other cyanobacterial species

Based upon their morphology and phytopigments *Anabaena inaequalis* (CCAP 1446/1A) and *Oscillatoria animalis* (CCAP 1459/6) could be identified and gated from *Microcystis* FCM outputs. In a diluted culture mix of *Anabaena, Oscillatoria* and a '*Microcystis* stock calibration', a number of detectable light scatter and fluorescent signatures were recorded.

Individually the single-cell size of both *Anabaena* and *Oscillatoria* in British freshwater systems can fall into the size range of many *Microcystis* spp. (John *et al.* 2011). However, as they form filaments consisting of many cells, FSC-W can be used just to include the size of cells found in the *Microcystis* calibration ranges (Section 3.3.1). In contrast through SSC-H the internal complexity alone was not enough to discriminate between cyanobacterial species. All three SSC-H ranges overlapped which revealed *Anabaena* and *Oscillatoria* to both have very similar SSC-H mean peaks (Figure 3.5 A).

FL2-H fluorescence was detected at a relatively low level for all cyanobacteria, with *Anabaena* and *Oscillatoria* peaks again being very similar to each other. No overlap was seen with *Microcystis* showing a distinctive FL2-H species peak separation (Figure 3.5 B). Again using FL3-H and FL4-H channels separately fluorescence signatures of *Microcystis* could be distinguished from the other two cyanobacterial species (Figure 3.5 C). By combining separate fluorescent parameters (FL2-H and FL3-H) *Microcystis* could then be further discriminated from *Anabaena* and *Oscillatoria* and be easily enumerated in the mixed cyanobacteria culture (Figure 3.5 D).

Non-cyanobacterial species

Two green alga, one of similar size to *Microcystis* (*Chlorella vulgaris,* CCAP 211/11B) and one of very different dimensions (*Euglena gracilis,* CCAP 1224/5Z) were mixed with *Microcystis* calibration cells. Cell size parameters were very useful in discriminating against larger algal species like *Euglena,* where it was possible to see their diverse size range within that species (Figure 3.6 A). *Chlorella* on the other hand could not be separated by cell size (diameter) as they both had comparable FSC-W histogram peaks which overlapped. Similar to the other cyanobacterial-non-*Microcystis* calibrations, *Chlorella* also recorded a SSC-H histogram peak that overlapped, being analogous to *Microcystis* strains.

Looking at fluorescent signatures *Microcystis* and *Chlorella* recorded a very similar FL2-H histogram peak range which overlapped and could not be easily discriminated. *Euglena* recorded two relatively close FL2-H peaks, of which the largest overlapped both *Microcystis* and *Chlorella*. As with the FL2-H peaks *Microcystis* and *Chlorella* shared a similar FL4-H range but were much more distinguishable between the alga. *Euglena* recorded another double peak in FL4-H which was a lot more separated than FL2-H but this time had no overlap with *Microcystis* FL4-H wavelengths.

Fluorescence emissions in FL3-H were the most distinctive phytopigment signature between the three (*Microcystis, Chlorella* and *Euglena*), with no histogram peaks in the same range as *Microcystis*. Again *Euglena* produced a double peak with the highest FL3-H reading (second peak) nearly out of detection range but overlapped FL3-H outputs from *Chlorella* (Figure 3.6 B).

The diatom *Asterionella formosa* commonly found at Longham reservoir could be identified immediately from their size, where FCM outputs recorded a mean cell size of 71.3 µm, falling into the *Asterionella formosa* average cell size of 60 – 80 µm (Hartley 1996). In addition all fluorescent signatures are clearly distinguishable between both alga (Figure 3.6 C), with only side light scatter overlapping in the *Microcystis* calibration range.

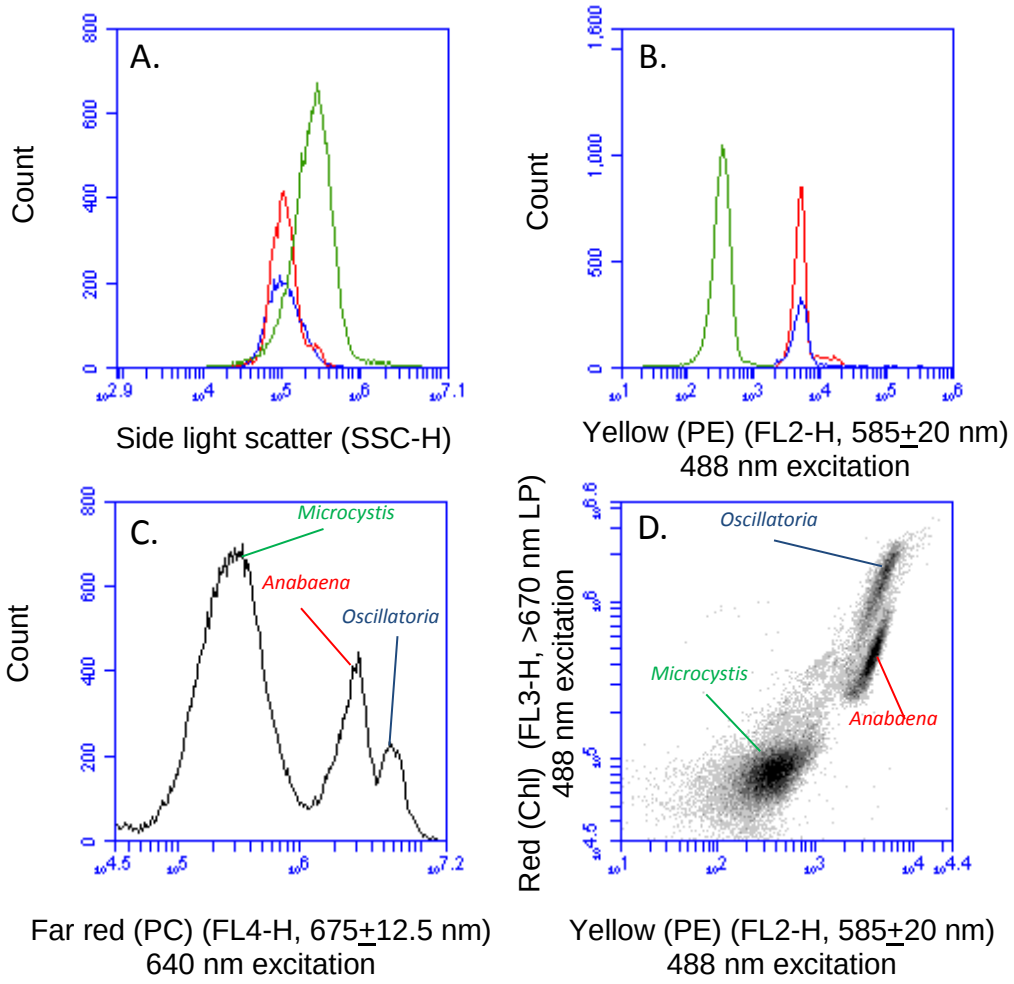


Figure 3.5. FCM outputs from individual and mixed cyanobacterial cultures at different densities; *Microcystis* calibration stock (green), *Anabaena* (red) and *Oscillatoria* (blue). A) Reveals all three have a SSC-H range that overlapped. B) Shows a distinctive FL2-H peak in *Microcystis* to *Anabaena* and *Oscillatoria*. C) Identifies distinct FL4-H fluorescent signatures between genera. D) By using dual detectors (FL2-H and FL3-H) all three cyanobacteria can be discriminated and *Microcystis* enumerated.

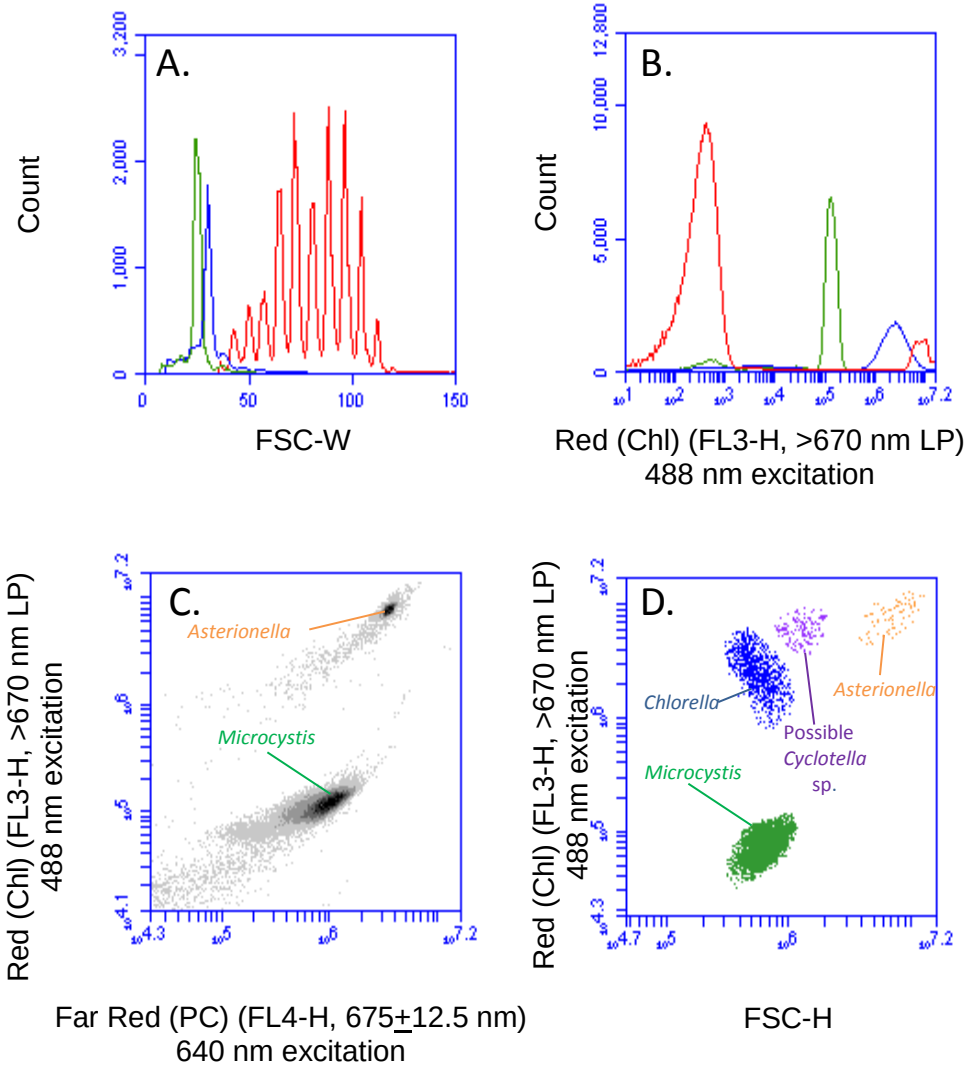


Figure 3.6. FCM outputs for mixtures of algal groups. Differences can be identified from histograms using A) FSC-width or cell size and B) FL3-H fluorescence between *Microcystis* (green), *Chlorella* (blue) and *Euglena* (red). C) A commonly found diatom in Longham *Asterionella formosa* can be distinguished from *Microcystis* relatively easily by using dual fluorescence detection due to their different FL3-H and FL4-H signatures. D) By spiking reservoir samples with calibration stocks a clearer profile of algal phenotypes can be seen. Knowledge of light scatter and fluorescence signatures then helped identify and enumerate *in-situ Microcystis*-like cells.

3.3.4 Combining FCM calibrations with natural samples

To further identify algal phenotypes a field sample which at the time had a dense population of a *Cyclotella* sp. was spiked with cultures of *Asterionella, Chlorella* and *Microcystis* (CCAP 1450/17). Each algal group could be distinguished through their previously identified light scatter and fluorescence emissions from stock calibrations (Figure 3.6 D).

By using algal stock calibrations of various species present in Longham reservoir a more accurate picture of FCM outputs can be made, facilitating the enumeration of *Microcystis* populations. However, *Microcystis* had no exclusive light scatter or fluorescence signals, with some FCM peak ranges overlapping other species from various domains of life, such as the eukaryote *Chlorella* (Figure 3.6 B).

Separation of algal groups and genera by FCM should therefore 'exclude' light scatter and fluorescent signatures identified from non-*Microcystis* calibration stocks. For example *Chlorella* had a similar FL2-H and FL4-H histogram peak range but a very different FL3-H signature. If software gating was to exclude FL3-H wavelength ranges found in *Chlorella* then cells containing that peak would not show up on other FCM outputs (e.g. FL2-H or FL4-H channels).

Another example can be found from cell sizes in *Euglena*, as FSC-W was very different to *Microcystis* (Figure 3.6 A) it can be excluded in the FL2-H channel where both species overlapped in range. The larger excluded *Euglena* cells would then reduce the number of FL2-H emitting cells (data points) within *Microcystis* range, increasing enumeration accuracy.

By including and excluding data points from calibrated algal strains / species it increased the data resolution of *Microcystis in-situ*. However, from environmental samples it would not be possible to confirm each individual cell as *Microcystis*. Using the FCM calibration ranges (Section 3.3.2 and 3.3.3) cells quantified from this novel technique would now be known as the previously stated '*Microcystis*–like cells' (Figure 3.7) a term also adopted by Read *et al.* (2014).

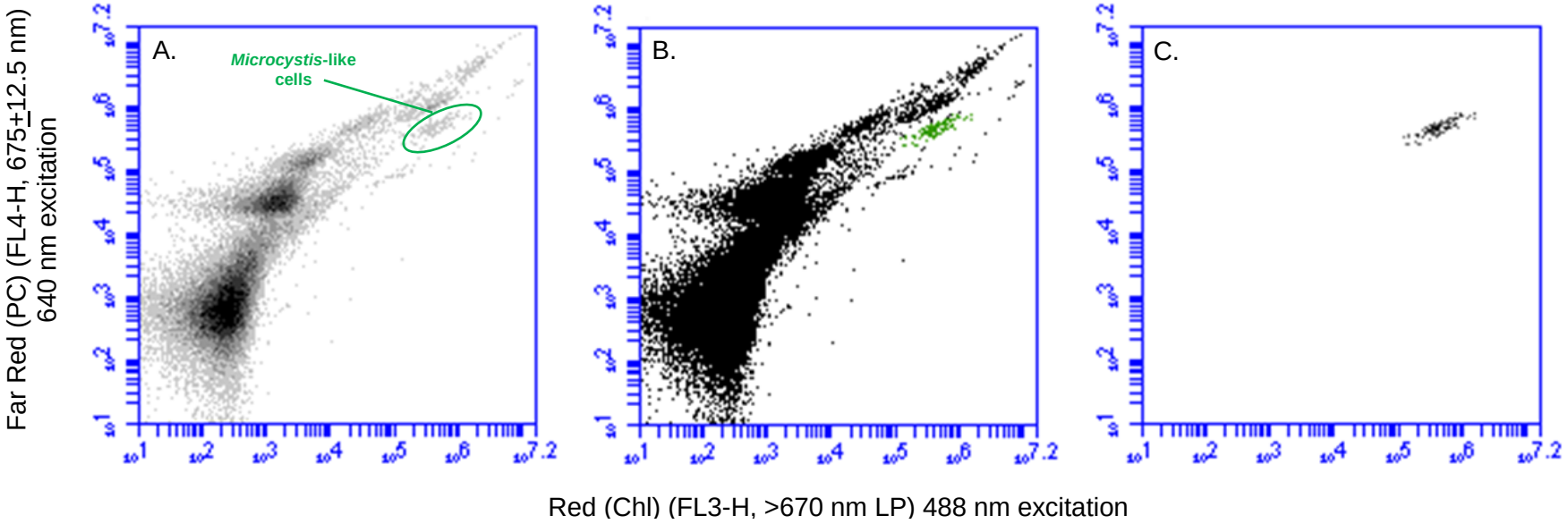


Figure 3.7. FCM data outputs from Longham reservoir using FL3-H against FL4-H detection channels represented by A) density and B) dot plots. *Microcystis*-like cells were identified by using manual software gates from the *Microcystis* calibration stocks through light scatter and phytopigment fluorescence. Once software gates were imposed the population that remained was enumerated as C) *Microcystis*-like cells. Here, these FCM outputs were taken from when *Microcystis*-like cell densities were relatively high (summer 2014). This particular sample also contained relatively high densities of *Scenedesmus* sp., *Sphaerocystis* sp., *Coelastrum* sp. and *Pandorina* sp. (Figure 3.4 B and C).

3.3.5 Environmental parameters and *Microcystis*-like cell dynamics in the water coloumn

Data collected during sampling across the whole monitoring period reported no significant differences in environmental parameters between water depths in water temperature, specific conductance, pH, turbidity and optical dissolved oxygen ($p > 0.05$). Similarly biological parameters from *Microcystis*-like cell counts, size (diameter and biovolume) and total biovolume in the water column reported no significant differences between various depths from September 2013 to May 2015 ($p > 0.05$) (Table 3.4). Due to changing water levels, the bottom depth sample had a range of 2.6 – 3.3m during the annual monitoring but was still designated the last (bottom) measurement.

Table 3.4. A Kruskal-Wallis one-way ANOVA on ranks, reporting no significant differences in environmental and *Microcystis*-like cell parameters between depths over the monitoring period.

	Parameter	H	d.f.	*p*
Environmental	Water temperature (^{O}C)	0.27	3	> 0.05
	Specific conductance (mS/cm)	0.37	3	> 0.05
	pH	3.37	3	> 0.05
	Turbidity (NTU)	6.38	3	> 0.05
	Optical dissolved oxygen (%)	1.48	3	> 0.05
Biological	*Microcystis*-like cell count (cells/mL)	3.49	3	> 0.05
	Microcystis-like cell size (µm)	0.43	3	> 0.05
	Microcystis-like cell biovolume (µm^3)	0.43	3	> 0.05
	Microcystis-like cell total biovolume (µm^3/mL)	0.87	3	> 0.05

3.3.6 Water conditions at Longham reservoir

As there were no significant differences between the depths from environmental and *Microcystis*-like cell records, a mean was taken from the water column at each date and used as the value to subsequently calculate seasonal averages (Table 3.5).

Water temperatures were significantly different between all seasons ($p < 0.001$) (Table 3.6), with summer 2013 recording the highest mean of 21.24$\pm$0.6 5^{O}C and winter 2014 the lowest mean of 6.32$\pm$0.55 ^{O}C (Figure 3.8). There were no significant water temperatures variance recorded between any of the same seasons, e.g. winter 2013 and winter 2014 ($p > 0.05$) (Table 3.7). Partial ice formation was observed in

winter 2014 (01/02/2015), mainly around the perimeter which did not cover the sampling point. The lowest water temperature was recorded 12 days after ice was seen (13/02/2015), however, in that time no parts of the reservoir was visibly frozen.

Specific conductance throughout sampling significantly differed ($p < 0.001$), with the highest mean found in winter 2014 (0.55±0.01 mS/cm) and lowest in both summers (0.43±0.01 mS/cm). There was a significant difference between the same season of winter ($p < 0.001$), with winter 2014 recording a mean of 0.07 mS/cm more than winter 2013.

The pH levels were statistically different between all sampling seasons ($p < 0.001$), showing a range of pH 7.49 in March 2015 to a pH 8.68 recorded in February 2014 (Appendix C8). There was also significant differences in pH levels between the two summer, spring (both $p < 0.05$) and winter seasons ($p < 0.001$).

Dissolved oxygen significantly differed over the monitoring period ($p < 0.001$) recording the highest mean in summer 2013 (160.09±9.75%) and lowest in winter 2013 (98.86±1.90%). A significant variance was found between the summer seasons ($p < 0.05$) with summer 2014 reporting a mean of 28.06% less than summer 2013.

Turbidity significantly differed between all the seasons sampled ($p < 0.001$), with two extremely high readings during autumn 2013 (130.98 and 201.50 NTU). There was only on four occasions that readings were above 5 NTU, with 50% of the total turbidity recordings being less than 1 NTU. Turbidity between single seasons significantly varied between both summers and autumns ($p < 0.001$).

Surface phosphorus-SRP was significantly different between all the seasons monitored ($p < 0.001$) (Appendix C9) with a number of dates showing the lowest value of 25 µg P/L, which increased threefold to a maxima of 104 µg P/L in January 2014. The summer and spring values over the entire sampling period were between 25-31 µg P/L, whereas in the autumn and winter a higher range of 50-63 µg P/L was recorded. SRP revealed no significant difference between single seasons measured (all $p > 0.05$).

Table 3.5. Seasonal means for environmental water parameters at Longham reservoirs with standard error (±S.E.). Highest and lowest values are in bold text.

Date (Season/year)	Water temperature (^{O}C)	Specific conductance (mS/cm)	pH	Turbidity (NTU)	Optical dissolved oxygen (%)	Surface phosphorus-SRP (µg P/L)	Surface total organic carbon (mg C/L)	Surface suspended solids (mg/L)
Summer 2013	**21.24** ± 0.65	**0.43** ± 0.01	**8.32** ± 0.08	2.28 ± 0.22	**160.09** ± 9.75	30.88 + 3.18	**5.01** + 0.26	**8.63** + 1.90
Autumn 2013	13.71 ± 1.14	0.46 ± 0.02	8.20 ± 0.05	**26.55** ± 17.65	109.65 ± 5.90	49.85 + 5.23	4.43 + 0.26	6.85 + 1.62
Winter 2013	7.13 ± 0.35	0.48 ± 0.01	8.26 ± 0.07	1.75 ± 0.43	**98.86** ± 1.90	**63.00** ± 9.52	4.55 ± 0.32	4.45 ± 0.77
Spring 2014	12.89 ± 0.92	0.46 ± 0.01	8.25 ± 0.05	0.94 ± 0.42	119.80 ± 4.74	**25.00** ± 0.00	4.00 ± 0.20	4.64 ± 0.84
Summer 2014	20.72 ± 0.51	**0.43** ± 0.01	8.08 ± 0.06	0.69 ± 0.14	134.77 ± 5.1	26.44 ± 0.87	3.66 ± 0.07	6.22 ± 1.72
Autumn 2014	14.31 ± 1.18	0.45 ± 0.05	8.05 ± 0.06	**0.47** ± 0.07	99.31 ± 5.86	62.33 ± 4.28	3.41 ± 0.30	**4.02** ± 0.92
Winter 2014	**6.32** ± 0.55	**0.55** ± 0.01	7.90 ± 0.04	3.88 ± 1.83	103.83 ± 3.84	58.33 ± 9.91	**3.05** ± 0.18	4.83 ± 0.94
Spring 2015	12.65 ± 1.97	0.45 ± 0.02	**7.88** ± 0.12	1.68 ± 1.30	118.16 ± 15.10	26.83 ± 1.83	4.08 ± 0.58	7.90 ± 2.84

Table 3.6.Compared means between all seasons from environmental hydrological parameters using a Kruskal-Wallis one-way ANOVA on ranks. The means, maximums and minimums with standard deviation (± S.D.) are also included from the whole 22 months of recording data at Longham reservoir (* denotes a one-way ANOVA for parametric data sets).

Parameter	X^2	d.f.	*p*	Mean ±S.D.	Max (dd/mm/yy)	Min (dd/mm/yy)
Temperature (^{O}C)	50.39	7	< 0.001	**14.10** ± 5.62	**24.12** (22/07/13)	**5.12** (13/02/15)
Specific Conductance (mS/cm)	29.9	7	< 0.001	**0.46** ± 0.07	**0.57** (13/02/2015)	**0.06** (03/10/2014)
pH	5.16*	7,60	< 0.001	**8.14** ± 0.23	**8.68** (24/02/2014)	**7.49** (20/03/2015)
Optical dissolved oxygen (%)	33.72	7	< 0.001	**117.85** ± 27.05	**194.63** (17/07/2013)	**81.93** (22/05/2015)
Turbidity (NTU)	30.62	7	< 0.001	**6.31** ± 28.76	**201.50** (14/10/2013)	**0 .00** (22/05/2015)
Phosphorus-SRP (µg P/L)	39.53	7	< 0.001	**43.25** ± 21.45	**104.00** (13/01/2014)	**25.00** (various)
Total organic carbon (mg C/L)	32.18	7	< 0.001	**4.05** ± 0.92	**6.80** (05/03/2015)	**2.40** (09/10/2014)
Suspended solids (mg/L)	9.84	7	> 0.05	**5.88** ± 4.65	**22.90** (07/10/2013)	**2.50** (various)

Table 3.7. Comparative means between single seasons (2013 – 2015) through a students T-test for water conditions at Longham reservoir (* denotes a Mann-Whitney *U* test for non-parametric data).

Season	Temp (^{O}C)		SpCond (mS/cm)		pH		Turbidity (NTU)		ODO (%)	
	t (d.f.)	*p*	*t* (d.f.)	*p*	*t* (d.f.)	*p*	*t* (d.f.)	*p*	*t* (d.f.)	*p*
Summer	1.25 (17)	> 0.05	41*	> 0.05	2.30 (17)	< 0.05	2*	< 0.001	2.76 (17)	< 0.05
Autumn	-0.36 (20)	> 0.05	56*	> 0.05	1.99 (20)	> 0.05	11*	< 0.001	35*	> 0.05
Winter	1.30 (12)	> 0.05	-4.69 (12)	< 0.001	3.90 (12)	< 0.001	15*	> 0.05	-1.26 (12)	> 0.05
Spring	0.12 (11)	> 0.05	20*	> 0.05	3.00 (11)	< 0.05	20.5*	> 0.05	0.11 (11)	> 0.05

Season	SRP (µg P/L)		TOC (mg C/L)		SS (mg/L)	
	t (d.f.)	*p*	*t* (d.f.)	*p*	*t* (d.f.)	*p*
Summer	35*	> 0.05	0*	< 0.001	20*	< 0.05
Autumn	-1.72 (20)	> 0.05	3.21 (20)	< 0.001	33*	> 0.05
Winter	0.33 (12)	> 0.05	3.70 (12)	< 0.001	20.5*	> 0.05
Spring	17.5*	> 0.05	16*	> 0.05	-1.18 (11)	> 0.05

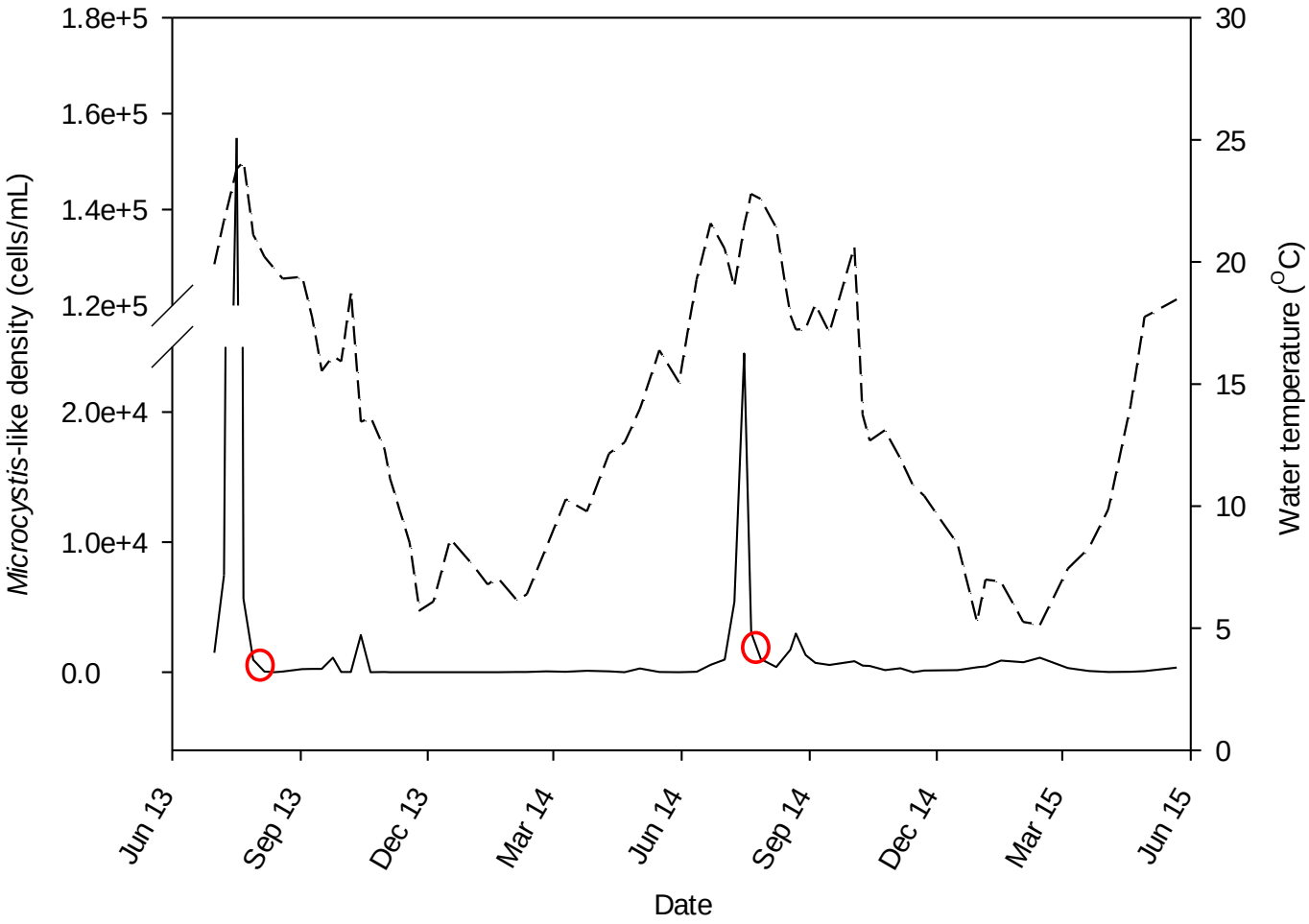


Figure 3.8. Temporal variation of *Microcystis*–like cell populations (solid line) and mean water temperature (dashed line) from environmental monitoring. The red circles denote when the contracted lab identified and enumerated *Microcystis* sp.

Total organic carbon measurements were also significantly different between all seasons ($p < 0.001$), revealing a range of 2.4 mg C/L (09/10/2014) to 6.8 mg C/L (05/03/2015). The highest mean values were found in summer 2013 at 5.01±0.26 mg C/L and the lowest in winter 2014 with 3.05±0.18 mg C/L (Table 3.5). There was a difference between all single seasons ($p < 0.05$), except between both springs.

Suspended solids was the only parameter that reported no significant difference throughout environmental sampling ($p > 0.05$), with a mean of 5.5±0.6 mg/L (Appendix C9). However, a statistical difference was reported between single seasons in both summer months, where summer 2014 recorded 35.69% less small solid particles than the previous year.

3.3.7 Ambient environmental conditions at Longham reservoir

When recorded on the days of sampling, air temperature significantly changed between the monitored seasons ($p < 0.001$) (Table 3.8), with a mean high of 17.6±1.1^{O}C in summer 2013 to a mean low of 4.3±1.5^{O}C during winter 2015. The highest recorded daily temperature was 22^{O}C in July 2013 and the lowest was 0^{O}C in February 2015, seeing parts of the lake frozen (Appendix C10).

Precipitation on the days of monitoring significantly changed between all seasons during the sampling period ($p < 0.001$) (Appendix C9). The autumn and winter of 2014 exhibited daily mean rainfall of 2mm or more, in contrast to zero (0mm) recorded in summer 2013 (Appendix C11). No rain (0mm) was recorded on 50% of the days which monitoring was carried out.

Visible light when sampling was significantly different over all seasons ($p < 0.001$), with a mean decrease of 41% in both winters compared to both summers (Appendix C9). Sunlight exposure means were calculated from the days of sampling so the true measure is closer to a 50% decrease but still reflective of the temperate climate in southern Britain. No variance for all ambient environmental conditions were found between single seasons when monitoring ($p > 0.05$) (Appendix C11).

Table 3.8. A Kruskal-Wallis one-way ANOVA on ranks for statistical analysis of ambient environmental conditions on the days of which monitoring occurred at Longham reservoir. The means, maximums and minimums with standard deviation (± S.D.) are also included from the whole 22 months data set (* denotes a one-way ANOVA for a parametric data).

Parameter	X^2	d.f.	*p*	Mean ± S.D.	Max (dd/mm/yy)	Min (dd/mm/yy)
Mean daily temperature (^{O}C)	17.18*	7,60	< 0.001	11.16 ± 5.09	22 (22/07/2013)	0 (01/02/2015)
Mean daily precipitation (mm)	24.59	7	< 0.001	0.86 ± 1.93	12 (09/10/2014)	0 (various)
Mean length of daily visible sunlight (min)	53.49	7	< 0.001	808.54 ± 175.21	1082 (22/06/2014)	556 (17/12/2013)

3.3.8 *Microcystis*-like cell density and morphology at Longham reservoir

Microcystis-like cell densities were significantly different between all seasons at Longham reservoir ($p < 0.001$) (Table 3.9). Summer 2013 recorded the highest mean of 21,316±19,111 cells/mL, with 17/07/2013 recording the most *Microcystis*-like cells at 154,918 cells/mL (Figure 3.8). Winter 2013 recorded the lowest mean of 11±8 cells/mL with two dates showing no *Microcystis*-like cells in the water column (17/12/2013 and 02/01/2014). Autumn 2014 also produced a third no count, two weeks before winter. There was only a significant difference between the same single season in both winters ($p < 0.001$) (Appendix C12), with winter 2014 having on average 611 cells/mL more per sample.

Both summer 2013 and 2014 seasons saw a very distinct large peak (154,918 and 24,498 cells/mL respectively), where numbers increased rapidly within approximately one week and crashed at a similar rate. After the summer 2013 peak, two smaller peaks were recorded in the proceeding autumn (1,112 and 2,863 cells/mL). The second and largest autumn 2013 peak coincided with a peak in water temperature. Again after the highest summer 2014 peak cell count there was also another peak increase in *Microcystis*-like cell populations (2,970 cells/mL) a week before autumn. Due to the *Microcystis*-like cells rapidly increasing to peak populations from small numbers and crashing at a similar rate, large seasonal standard errors were calculated.

Microcystis-like cell sizes (diameter) between seasons were significantly different over the monitoring period ($p < 0.05$). The mean size of *Microcystis*-like cells over the 22 months was 5.17±0.13 µm with a FCM (FSC-W) histogram range of

2.93 - 7.62 µm. The largest mean cells recorded were in summer 2014 (6.08±0.36 µm) and the smallest were found in autumn and winter 2014 (4.66±0.09 and 4.69±0.20 µm) (Appendix C12). Between single seasons there was a significant difference in both summers and both winters ($p < 0.05$).

As the individual biovolumes of the spherical *Microcystis*-like cells are dependent on cell diameter (linear regression, $F_{1,63} = 966.32$, $p < 0.001$, $R^2 = 0.94$), there was again a significant difference between all the seasons ($p < 0.05$). The largest mean individual biovolumes were found in summer 2014 at 129.8±21.8 µm^3, which were 14 and 13 times larger than cells in autumn and winter 2014 respectively. As with cell size, mean individual biovolumes reported a statistical difference between both summer and both winters ($p < 0.05$).

Table 3.9. A Kruskal-Wallis one-way ANOVA on ranks for *Microcystis*-like cell densities and morphologies over the complete sampling period at Longham reservoir. The means, maximums and minimums with standard deviation (± S.D.) are also included from the whole data set.

Parameter	X^2	d.f.	p	Mean ± S.D.	Max (dd/mm/yy)	Min (dd/mm/yy)
Cell density (cells/mL)	33.77	7	< 0.001	3,325 ± 18,929	154,918 (17/07/2013)	0 (various)
Cell size (µm)	2.74	7,57	< 0.05	5.17 ± 1.03	7.62 (21/07/2014)	2.93 (11/11/2013)
Cell biovolume (µm^3)	18.42	7	< 0.01	81.27 ± 50.67	231.79 (21/07/2014)	13.12 (11/11/2013)
Total biovolume (µm^3)	32.02	7	< 0.001	185,179 ± 771,496	5,655,904 (16/07/2014)	0 (various)

The total *Microcystis*–like cell biovolume (being dependent on cell numbers and individual biovolumes, multi-linear regression, $F_{2,62} = 23.14$, $p < 0.001$, $R^2 = 0.43$), again reported a significant difference over the monitoring period showing seasonal variances ($p < 0.001$).

The largest mean total biovolume of *Microcystis*–like cells was recorded in summer 2014 with 7.5x10^5±5.0x10^5 µm^3/mL, where the single largest total biovolume was found at 5.7x10^6±5.0x10^5 µm^3/mL (16/07/2014). The lowest total biovolume of *Microcystis*-like cells was 0 µm^3 found on three occasions (17/12/2013, 02/01/2015 and 14/11/2014) as there were no *Microcystis*-like cells detected. Only between the two winters were total biovolumes significantly different ($p < 0.001$) recording 25 times more total biovolume in winter 2014 than the previous year.

3.3.9 Ecological variables that affect *Microcystis*-like cells in a lowland British reservoir

A multi linear model analysed significant correlations that influenced *Microcystis*-like cell densities at Longham reservoir during annual monitoring. A variance inflation factor (VIF) was used to qualify any multicollinearity, by which factors with a VIF higher than 2.17 were removed along with parameters that revealed no significant difference over all the seasons.

A significant regression equation was reported ($F_{7,60}$=2.36, R^2 = 0.22, p < 0.05), where water temperature (p < 0.05) and total organic carbon (p < 0.001) were significant factors influencing *Microcystis*-like cell numbers (Table 3.10).

Table 3.10. Results from a multiple linear model reporting the environmental interactions that influenced *Microcystis*–like cell populations at Longham reservoir over the monitoring period.

Parameter	Coefficient	Error	*t* value	*p* value
Water temperature (^{O}C)	1260.5	524.0	2.4	< 0.05
Specific conductance (mS/cm)	33609.7	38553.5	0.9	-
pH	-19193.7	11043.5	-1.7	-
Turbidity (NTU)	59.8	86.3	0.7	-
Surface phosphorus-SRP (µg P/L)	14.7	120.6	0.1	-
Surface total organic carbon (mg C/L)	8916.7	2694.2	3.3	< 0.001
Precipitation (mm)	474.9	1183.9	0.4	-

3.3.10 Comparison of *Microcystis*-like cell densities from flow cytometric analysis to contracted laboratory counts

During the monitoring period at Longham reservoir only two incidences of *Microcystis* sp. were detected by the independent laboratory. The contracted laboratory recorded 1,971 cells/mL in summer 2013 (09/08/2013) and 6,209 cell/mL in summer 2014 (21/07/2014). Both of these identifications and enumerations were made after the peak measurements by FCM, at the end of a population crash (Figure 3.8). The independent count was nearly 50 times the FCM enumeration in summer 2013 (40 cells/mL) and double that of summer 2014 (3,034 cell/mL). *Microcystis*-like cell numbers obtained by the independent laboratory cannot be statistically compared as there were only two incidence of detection.

3.4 Discussion

3.4.1 Light scatter discrimination of *Microcystis*-like cells via FCM

In terms of forward light scatter (FSC-width) cell size was a very usefully parameter for identifying *Microcystis*–like cells in environmental samples, restricting the final counts to a range of 2 - 8.5 µm. Calibrations for *Microcystis* did see a mean range of 3.34 – 5.74 µm from the six culture lines using various media types, indicative of *Microcystis* eco-types found in European temperate climates (Komárek and Komárková, 2002). However, caution must be taken when using pre-treatment techniques like ultrasound, as cell size (diameter) in *Microcystis* recorded a 22-44% increase after 50s using an ultrasonic bath (38kHz±10%, 75W, KC2, Kerry, USA) (Appendix B4). Therefore any pre-treatment techniques must be factored into the final calibration of a target organism size range.

Although useful, cell size alone cannot determine a target organism from environmental samples through FCM. There are limitations when certain alga form colonies exhibiting either irregular, spherical or filamentous morphologies. As *Microcystis* can form irregular / spherical colonies it may be necessary to use ultrasound (Appendix B4), as single-cells need to be analysed to give the most accurate counts. Filamentous forming cyanobacteria like *Anabaena* and *Oscillatoria* which cannot be easily separated will produce large data spreads in FSC-W (size) FCM outputs, so for these genera fluorescence traits would be heavily relied on for *in-situ* detection (Figure 3.5).

Depending on the cell cycle of a *Microcystis*, cell division may also influence FSC-W recordings, with reproduction through binary fission resulting in doublets (Figure 2.7). Doublets can be easily discriminated by detecting the disproportions between forward light scatter pulse; height, width and area (Section 2.3). Doublets will have double the pulse area and width values of a single-cell, whilst height will be approximately the same. Future work carried out could involve looking at the frequency of dividing cells (FDC) between seasons in pelagic waters, something which has only been carried out with benthic *Microcystis* in European reservoirs (Latour *et al.* 2004). FDC would be based upon the relationship between *in-situ Microcystis* growth rates and the number of cells in a given stage of division. Testing water column *Microcystis* FDC could support work by Verspagen *et al.* (2005) and Cires *et al.* (2013) in that pelagic *Microcystis* are the main drivers of blooms (high growth rates) rather than the benthic populations.

Cyanobacteria also exhibit light dependent regulation of morphogenesis, where environmental cues induce adjustments to cellular dimensions (Lidstrom and Konopka 2010; Montgomery 2015). Although only a single light intensity was used to grow all algal calibration cultures in this study, size will vary under changing environmental conditions (as seen in the modified medias) and cell cycle stages. Cell size dynamics should therefore be taken into account when optimising FCM protocols by using the knowledge of a target organisms size range, understanding the stresses that will alter morphological characteristics and an accurate size calibration curve.

3.4.2 Light scatter discrimination of non-*Microcystis* cells via FCM

A well-known limitation of FCM is based on the assumption of analysing spherical cells / particles. *Microcystis* falls within this assumption where accurate biovolumes can be calculated but other phytoplankton species (especially eukaryotes) like *Euglena* (Figure 3.6 A) have non-spherical dimensions. Irregular shapes may record wider FSC-W histogram peaks depending which way the organism is aligned (length or width) when being interrogated by the laser. Although not an exact proxy FSC-H (height) has provided strong relationships with biovolume through laboratory species, despite the morphological shape not being uniform (Becker *et al.* 2002).

Detection limits will also be determined by individual FCM systems, where the aperture of flow cells, nozzle tips and settings used will have an impact on data resolution. A good example of this would be found in Section 2.2.2 where light scatter peaks shift when using different fluidic speeds and core sizes.

Smaller target organisms such as *Synechococcus* will need smaller FSC thresholds to record data accurately but there is potential to include background electronic noise and debris. Small thresholds can be overcome by using triggers for native phytopigments like Chl in FL3-H or PC in FL4-H detection channels. As the *Microcystis* size range is above background noise values, by using the set threshold (Section 2.2.1) it was possible to increase data sensitivity.

The maximum particle size which can be analysed by the Accuri C6 is reported to be 40 µm, however, personal communications with a number of researchers using the same model along with the *Asterionella formosa* and *Euglena gracilis* FSC-W

outputs (Section 3.3.3 and Figure 3.6 A) puts the maxima in the region of 100 µm. Samples that are too large can block FCM instruments or increase widths of peaks in in a number of light scatter and fluorescence channels, so pre-filtering samples along with sonication may be advised before running samples.

Side light scatter (SSC-H) is a very good parameter for differentiating between vacuolated and non-vacuolated cells, although peak shifts can occur in vacuolated organisms from the collapsing of gas vesicles as seen in *Microcystis* (Figure 3.1 B). SSC-H shifts were evident from exposure to ultrasound (Figure 3.1 B and Appendix B4) and may also occur through mutation (CCAP 1450/14), physiological processes or from machinery (e.g. water extraction equipment found at reservoirs), which all need to be taken into account when analysing phytoplankton data through FCM SSC outputs.

Forward light scatter was beneficial for refining data to identify *Microcystis*-like cells, however, for SSC-H the other algal genera tested all recorded similar ranges which overlapped, despite not containing gas vesicles. As a standalone or even in a dual combination these two parameters were not enough to accurately identify a cell / data point as *Microcystis*(-like cell). Nonetheless both were used in the process of 'including or excluding' data ranges for successfully gating a *Microcystis*-like cell / population.

3.4.3 Native fluorescence discrimination of *Microcystis*-like and other cyanobacterial cells via FCM

In this study the target organism *Microcystis* produced both distinctive and similar fluorescent wavelengths to other cyanobacteria and phytoplankton groups. After calibration from stock *Microcystis* culture lines, *in-situ Microcystis*-like cells could be identified and enumerated through their native phytopigments, facilitated by gating from light scatter histograms.

The autofluorescence recorded within the three cyanobacterial genera (*Anabaena*, *Oscillatoria* and *Microcystis*) provided a good basis for discriminative analysis. Although some of the detectable fluorescence did overlap enough relative wavelengths were recorded to identify each species in a mixed population and from spiked field samples (Figure 3.5 and Figure 3.6).

The differences in FCM outputs detecting native fluorescence can be attributed to the great structural variability and ratios of light harvesting complexes within species. During the light reactions of cyanobacterial oxygenic photosynthesis a number specialised extra-membranous antenna structures known as phycobilisomes (PBS) (Figure 1.5) are involved with the energy transfer to reaction centres found in PSII and sometimes to PSI (Sidler 1994). In addition carotenoids are found to harvest light at other spectral wavelengths, which produce fluorescence emissions at a number of wavelengths.

PBS are composed of phycobiliproteins (e.g. phycocyanin and phycoerythrin), where absorption properties originate from various side chains of tetrapyrrole proesthic groups and a number of conjugated double bonds, known as phycobilins (Sidler, 1994). PBS absorb photosynthetically active radiation (PAR) light from a relatively wide wavelength range (450-665 nm), extending the photosynthetic spectral range to red and blue wavelengths not utilised by chlorophyll.

Excited by a 488 nm laser FL3-H fluorescence detection is heavily associated with chlorophyll *a* (Chl) detection. Although the FL3-H/Chl mean peaks did show a relative shift in strain types, the range selected for *Microcystis* calibrations was very narrow and stable. Chlorophyll *a* is a commonly used parameter in FCM to detect and count uni-algal *Microcystis* species under laboratory conditions (Latour *et al.* 2004; Mikula *et al.* 2012).

Here there was a distinct difference between the Chl signatures of the three cyanobacteria. Chl peak shifts between the cyanobacteria could be attributed to the photochemical quenching and excitation pressure from cellular PBS content, where composition and content can influence fluorescence yields (Campbell *et al.* 1998). The composition of some cyanobacteria PBS is regulated by light with a structural response known as chromatic adaptation (Bogorad 1975; De Marsac 1977). Chromatic adaption could potentially reduce the precision for identifying species through specific photosynthetic fluorescence. However, *Microcystis* is a genus that reports no photosynthetic pigment composition change due to light intensity (Raps 1985).

The phytopigment fluorescence recorded through *Microcystis* calibrations also supports the lack of chromatic change due to nutrient concentrations, as FCM outputs stayed within a set range when grown in media lacking both nitrogen and phosphorus. In addition, *Microcystis/Microcystis*-like cells have also been successfully monitored through FCM over different time periods (Dennis *et al.* 2011; Read *et al.* 2014).

3.4.4 Native fluorescence discrimination of non-*Microcystis* cells via FCM

Although single fluorescence parameters could identify algal groups, an appropriate combination of excitation wavelengths and detectors are needed when recording *in-situ* genus specific data. Microbial communities of (semi-) natural aquatic bodies are very diverse, which requires a working knowledge of FCM optical signatures from phytoplankton found at monitoring sites, as seen in this study where there was overlap from one or more light scatter or fluorescence emissions.

FL3-H recordings from a blue 488 nm laser is often associated with Chl fluorescence and the FL4-H recordings from a red 640 nm laser associated with the commonly found cyanobacterial phytopigment phycocyanin (PC). However, the detection channels will also include any phytopigments that can be excited by an appropriate light source. This can be seen in Figure 3.6 C where the diatom *Asterionella formosa* records a very high signal in the FL4-H / PC detection range. Although *Asterionella formosa* does not contain PC, diatoms synthesis other pigments such as Chl c, which in its secondary absorption maxima peak is excited by a red light source ≈ 630 nm (Stauber and Jeffrey, 1988; Scheer, 2006; Kuczynska *et al.* 2015) and picked up by a higher wavelength detector (e.g. FL4-H 675±12.5 nm).

Once a reference library / data base of local microbes is established software gating becomes much easier for the target organisms identification and enumeration. The refining of data points are also increased by excluding flow cytometric parameters from non-target organism. To further improve *Microcystis* monitoring FCM with imaging (FlowCam) could be utilised to enhance genus detection, where software can even be trained to identify biomass in the form of colonies / filaments (Sieracki *et al.* 1998; Poulton 2006; Dashkova *et al.* 2016). FCM sorting of cells and then re-running samples along with microscope work can also validate gated regions of phytoplankton groups.

The discriminate analysis in this study demonstrates a high resolution protocol sensitive enough to monitor *Microcystis*-like cells in a reservoir, providing essential water quality data. The calibrated light scatter and fluorescence signatures from a number of *Microcystis* and non-*Microcystis* species enabled this study to quantify *Microcystis*-like cell populations, establishing for the first time seasonal patterns in a lowland British reservoir.

3.4.5 *Microcystis*-like cell seasonal patterns

The data collected from *Microcystis*-like cells in a temperate lowland reservoir agrees with many studies observing freshwater cyanobacterial populations, where by an increase in (water) temperature subsequently increases blue green alga (Reynolds 1984; Carvalho and Kirika 2003; Paerl and Huisman 2008; Wagner and Adrian 2009; Becker *et al.* 2010; Tarnau *et al.* 2012; 2015). Again supported by the results from this study typical *Microcystis* seasonal patterns were observed. *Microcystis*-like cell densities had rapidly increased in the late summer / early autumn period, followed by a population crash and low winter / spring counts (Reynolds 1981; Dennis *et al.* 2011; Read *et al.* 2014).

In addition to water temperature the total organic carbon (TOC) was also a significant factor that influenced *Microcystis*-like cell seasonal numbers. However, unlike temperature TOC correlation cannot completely imply causation. As a biological parameter TOC is associated with extracellular matrix production in *Microcystis* and other cyanobacterial species (Knappe *et al.* 2004; Aoki *et al.* 2014) and not a clear independent factor like temperature. TOC may also be dependent on other planktonic organism and could be explained by other algal population increases and mortality, especially after bloom crashes (Sigee *et al.* 2007).

Nutrient data in terms of mean surface phosphorus levels (SRP) at Longham over the monitoring period was 42.35$\pm$2.60 µg P/L, which is in line with WFD ecological status of good to high (UKTAG 2008). This was quite stable apart from a few higher levels recorded during winter seasons but did not significantly influence *Microcystis*-like cell numbers. Therefore, results from annual monitoring of *Microcystis*-like cells in a reservoir confirm temperature as the significant factor but cannot completely rule out nutrients, as total organic carbon could not be determined as an independent factor in addition to the lack of nitrogen data.

From the body of literature it does seem clear that continued climate change will increase cyanobacterial populations, highlighting the need for rapid and accurate ways of monitoring. Routine monitoring by the FCM protocol developed here will aid water management strategies through early warming detection of potentially toxic populations of *Microcystis*. As *Microcystis* densities are likely to increase with climate change putting more stress on our freshwater supplies so a better understanding of their ecology will be needed. To improve our knowledge of *Microcystis* dynamics in water bodies future viability studies should be carried out. Determining where and when the most viable cells are present would be very useful information, as it would allow management strategies to be more effective when treatment processes are applied.

3.4.6 Monitoring at Longham reservoir

Longham reservoir can be classed as a small water body due to its physical dimensions and hydrology. As a shallow reservoir (mean depth < 3 m) Longham has insufficient depth for thermal profiling compared to larger stratified lakes / reservoirs. Heat absorption by sediments and its subsequent transfer around the site is also very small compared to direct solar absorption.

As a result of the low heat storage capacity in shallow water bodies winter planktonic communities are very short lived (Adrian *et al.* 1999; Wetzel 2001). This is true for Longham and *Microcystis*-like cells but the population dynamics may change in larger (deeper) and stratifying water bodies, thereby recording a different seasonal phytoplankton community composition (Nixdorf *et al.* 2003).

Intense flushing events have an impact on cyanobacteria in temperate freshwaters (Reynolds *et al.* 2001; Elliot 2006), however, the parameter was not included in the statistical models as Longham extraction rates were relatively constant throughout the monitoring period with a mean extraction of 65 mega litres a day (personal communication, Bournemouth Water).

The WFD requires the ecological status of waters to be assessed on the conditions of their biological quality elements (WFD 2000/60/EC), however, Carvahlo *et al.* (2013) raises a valid point which is not included, in where samples are taken. Hydrological and biological parameters can easily change at different sampling locations (e.g. between water extraction points, the periphery and middle of water bodies).

During autumn 2014 a boat was available to compare parameters from the sampling point and the middle of the reservoir at five different dates. A point was located (Figure 3.3) where comparable depths were measured. No environmental parameters or *Microcystis*-like cell counts significantly differed in the middle of the reservoir both in the water column (one-way ANOVA, $p > 0.05$) or between that location and the original sampling point (Mann-Whitney U test, $p > 0.05$) (Appendix C13). Although not a long period of comparison it was enough to confirm that the sampling point was relatively representative of other parts in the reservoir, which may not occur in a larger or stratifying reservoirs / lakes.

Microcystis does include a benthic part of its life cycle during the winter and early spring, where the benthic densities can be used to re-inoculate the water column (Reynolds *et al.* 1981, Latour *et al.* 2004; 2007). However, recent evidence suggests the smaller populations that reside in the water column throughout the year play a more significant role in summer / autumn numbers. It is the higher growth rates of nekton *Microcystis* that influence blooms compared to the benthic cells, where the sediment is used more as a sink than a source (Verspagen *et al.* 2005; Cires *et al.* 2013).

Along with the majority of *Microcystis in-situ* monitoring publications here the benthic population was neglected. This is because water column numbers are more relevant for utilities companies, as extraction sources are commonly situated above sediment levels to reduce particulates for filtration. Water column measurements are also adequate to address the aims of this study, which kept in mind national monitoring programmes frequency of sampling and restricted budgets.

3.4.7 *Microcystis*-like cell counts compared to the independent laboratory results

With comparable findings to Becker *et al.* (2002) for independent algal analysis, FCM is found to be more accurate than microscope counts (Chapman *et al.* 2015). The independent laboratory used to detect algal species only identified two incidences of *Microcystis*. Both incidences came after peak FCM densities where a population crash was observed (Figure 3.8).

This highlights a major concern, as the time it takes for samples to be collected, transported, prepared, analysed (sedimentation / Utermöhl technique) and information finally disseminate to site managers it could take many days or even over a week. With rapid growth rates of cyanobacteria it may be too late to implement the most effective control technique(s), especially if large populations are not identified quickly enough. FCM assays can therefore bring strong benefits to aquatic ecosystem health assessments and increase human water security.

3.5 Conclusion

The FCM protocol developed in this study provides steps towards an adaptive framework for managing small drinking water supplies, as high resolution data sets can be obtained quickly and accurately for enumerating *Microcystis* sp. Once established, FCM protocols can process multiple samples from multiple sites at a relatively low cost with minimal specialist training. As FCM can rapidly quantify nuisance algae management strategies can be effectively put into place to mitigate negative impacts on drinking water sources. Since the genus *Microcystis* contains both toxic and non-toxic strains there is potential to combine this protocol with rapid toxin detection equipment, such as a portable Raman spectrophotometer, where toxin analysis will further provide crucial information for ecosystem managers and drinking water providers.

With reference to established cultures lines and fresh isolations distinct groups of phytoplankton could be identified and enumerated through FCM from their respective light scatter and fluorescence emissions. A *Microcystis*-like cell sp. was specifically detected form this novel protocol, successfully determining for the first time seasonal patterns in a lowland British reservoir.

Chapter IV: Grazing of *Blepharisma americanum* on toxic and non-toxic *Microcystis aeruginosa* cells

Abstract

The ecological role in cyanobacterial bloom management is complex and poorly understood, even with a long history of research using biomanipulation from higher trophic levels to reduce algal populations. In addition protozoan-cyanobacterium interactions have also been severely neglected through grazing experiments, providing contradictory evidence when toxic prey items such as *Microcystis* are involved. Here using *Microcystis aeruginosa* containing the microcystin-LR gene (toxic) and the same strain with an inactivated microcystin-LR gene (non-toxic) this study investigated the impacts of a novel predator-prey interaction between the cyanobacterium and a microphagus filter feeder, *Blepharisma americanum*. Through flow cytometric analysis the non-toxic strain recorded a significant decrease in growth when exposed to *B. americanum*, where grazing subsequently sustained ciliate populations. In contrast, *B. americanum* predation of toxic *M. aeruginosa* showed no significant control of cyanobacterial growth and a complete reduction in ciliate numbers. These results indicated that cyanotoxin production (MC-LR) may play a role in the anti-grazing behaviour of *B. americanum*. Furthermore there was no induction of ciliate encystment or cannibalistic behaviour (gigantism). Individual *B. americanum* morphological responses (biovolume and width) also recorded a significant decrease when sustained on a diet of non-toxic *M. aeruginosa,* supporting previous studies that cyanobacteria are a relatively poor source of nutrients. This chapter reveals that ciliates like *B. americanum* can be effective at supressing potential bloom-forming cyanobacteria, providing further insight to their ecological roles in the microbial loop. However, bottom-up effects of aquatic trophic interactions can be significantly altered by the presence of cyanotoxins (MC-LR), which will not only affect the zooplankton community but also the autotrophic assemblage in their dominance and persistence.

4.1 Introduction

4.1.1 Non-invasive approach to water treatment

Water treatment strategies that use physical or chemical processes to control phytoplankton communities often require high energy inputs (Appendix A) or apply very invasive algalcides. Certain chemicals especially copper based compounds are extremely toxic to other organisms and may accumulate in sediments, with the potential to cause long term problems for the local aquatic ecology (Haughey *et al.* 2000; Nyström *et al.* 2002). Even if algalcides were phylum specific, the lysing of cells as found in *Microcystis* can release cyanotoxins (microcystins) into the surrounding environment (Cooke *et al.* 2005; Lürling *et al.* 2014).

Despite continuing attempts to control nuisance algal species (e.g. *Microcystis*) there is an urgent need to develop non-invasive water management practices to sustainably control cyanobacterial hazardous algal blooms (CHABs). Biological manipulation, where balances in aquatic ecosystems are maintained can potentially be one sustainable approach to alleviate factors which enhance cyanobacterial growth. Biomanipulation, a term originally founded for water quality restoration in lakes is based on biological interventions between food web levels (Shapiro *et al.* 1975). Biomanipulation in contemporary limnology is viewed as a top-down control of microbial grazers by piscivores and through a bottom-up approach of nutrient cycling to control algal populations, which can also be supported by benthivorous fish (Shapiro *et al.* 1975; Shapiro 1990).

4.1.2 Biomanipulation, a tool for water treatment?

As nutrient loading mitigation requires co-operation from a number of stakeholders and takes a relatively long time for results to be established, short term water management can feasibly manipulate trophic cascades by reducing the feeding pressures of fish on zooplankton. Although a number of reviews have provided evidence of controlling algal biomass by changing fish communities the success is limited, where by a number of specific conditions have to be fulfilled or combined with other physico-chemical control techniques (Shapiro 1994; Jeppesen *et al.* 2012; Triest *et al.* 2015).

Biomanipulation also cannot be viewed as a routine method since aquatic ecosystems are very dynamic, with no one organism able to control the vast numbers of indigenous phytoplankton species. In addition the number of factors that need to be controlled would require constant maintenance by skilled limnologists (Cooke *et al.* 2005). Although cyanobacterial control is a target of water management, the process of biomanipulation is one aspect of that particular resource planning, where applied research is needed to identify any species-specific interactions relating to their wider ecological function (Sigee *et al.* 1999; Tillmann 2004). Zooplankton grazing is one major factor that modifies autotrophic biomass but is complex and poorly described when cyanobacteria are involved.

4.1.3 Zooplankton and cyanobacterial interactions

Although most zooplankton-cyanobacteria interaction research has been focused on crustacean and metazoan grazers (Ger *et al.* 2014) protozoan predators can account for a high percentage of a zooplankton community in lakes and grazing rates therein (Davis *et al.* 2012). However, the well-established concept of the microbial loop focusing on protozoan-cyanobacterium trophic relationships stands out as a significant knowledge gap (Ger *et al.* 2016).

Cyanobacterial blooms are associated with low grazer biomass and shifts in zooplankton communities. Ecological paradigms dictate that high primary production, in this case cyanobacteria, will lead to an increase in predator densities, subsequently reducing prey numbers and controlling bloom formation (Molles 2012). Despite this traditional concept the formation of cyanobacterial blooms exerts a number of constraints on zooplankton consumers.

4.1.4 Factors effecting zooplankton grazing on cyanobacteria and bloom formation

The main factors attributed to cyanobacterial bloom formation, dominance and persistence point towards their morphology, nutritional value and intracellular secondary metabolite production (Wilson *et al.* 2006). Bloom forming cyanobacteria exhibit large spherical, irregular or filamentous colonies where the size, shape and extracellular matrices play an important role in the indigestibility by suspension / filter feeding zooplankton.

The cell mechanics of any predator will therefore be crucial in determining their grazing success, as handling and filtration may make some prey impossible to consume (Lynch 1980; Lampert 1987; Wilson *et al.* 2006). If consumption of a prey item is possible the maximum clearance rates will be determined by an optimal food particle size (Fenchel 1980).

It is also well recognised that cyanobacteria are low in nutritional value and are poorly assimilated due to the lack of essential compounds e.g. amino acids and lipids (PUFAs) (Holm *et al.* 1984; Fink *et al.* 2011; Von Elert *et al.* 2014). Additionally the production of secondary metabolites, in particular cyanotoxins have recorded reduction in zooplankton (*Daphnia*) growth and fecundity (Infante and Abella 1985) but their exact role as anti-herbivore molecules is still debated requiring further study (Carmichael 1992; Babica *et al.* 2006).

If the above factors contribute to the lack of cyanobacterial predation by zooplankton the subsequent grazing selection pressures can result in adaptation or genotypic changes within populations for both predators and prey (Fenchel 1982; Sarnelle and Wilson 2005; Van Wichelen *et al.* 2010; Chislock *et al.* 2013). Changes on a population level have been recorded in *Microcystis* when exposed to predation (*Ochromonas* sp.) through the induction of colonies from single-celled cultures (Burket *et al.* 2001; Yang *et al.* 2008). As the knowledge of protozoan control in cyanobacterial bloom formation is limited any use of a potential biological control must be viewed in the context of biotic interactions governing the microbial loop (Sommer *et al.* 2012).

4.1.5 Testing a model ciliate for cyanobacterial biomanipulation

Protozoan species particularly ciliates are the largest and most complex of micro-organism which significantly influence zooplankton trophic interactions. Ciliates in testament to their efficient grazing abilities can be found in a wide range of aquatic habitats. Recorded in both freshwater and marine ecosystems ciliates are distributed throughout the water column and the benthos, surviving in a variety of oxygen levels including anaerobic environments (Finlay and Esteban 1998; Esteban *et al.* 2015). Being a ubiquitous grazer of primary producers the impacts of ciliates as a predator on toxic cyanobacteria have been severely neglected requiring further investigation.

One model ciliate, *Blepharisma,* is a genus that has global distribution being observed on all continents including Antarctica, with representative species found in fresh, brackish and marine environments (Roberts *et al.* 2004; Petz *et al.* 2007). When environmental conditions become unfavourable and / or during nutrient limitation *Blepharisma* boast two important survival strategies, cannibalism and encystment (Giese 1973). When conditions become more suitable *Blepharisma* will re-emerge and continue its life cycle. With such a sustainable approach to survival and their ubiquity *Blepharisma* makes an ideal candidate to test as a potential biological control of nuisance cyanobacteria like *Microcystis.*

4.1.6 *Blepharisma* and their role in the microbial loop

Regarded as a microphagous bacterial filter feeder *Blepharisma* is often found in nature amongst decomposing vegetation, with laboratory experiments commonly using a number of heterotrophic bacteria as a nutrient source (Giese 1973). Being a filter feeder and omnivorous (cannibalistic) *Blepharisma* has the potential to strongly influence *Microcystis* populations through direct consumption. If *Microcystis* are not ingested, which to date has not been comprehensively studied, *Blepharisma* may indirectly manipulate aquatic food web architecture through the consumption of other prey items shifting the microbial community assemblage.

Blepharisma is a light sensitive ciliated protozoan, pyriform in shape with the anterior apex often curing over a well-defined peristome (Figure 4.1) (Carey 1992). As a genus *Blepharisma* are not highly contractile but remarkably flexible, recording a wide range in body lengths (50 – 750 µm). When food availability is limited *Blepharisma* may become elongated (flatten spindle shape), encyst or can induce gigantism through cannibalism (Giese 1938; 1973). As the microbial loop encompasses a holistic perceptive on trophic interactions the influence of cyanobacteria on a potential predator must also be observed. With *Microcystis* producing cyanotoxins, the effect of a toxic and a non-toxic strain on *Blepharisma* through cell numbers and morphology will provide a novel insight into this grazing interaction, including potential impacts on bloom formation.

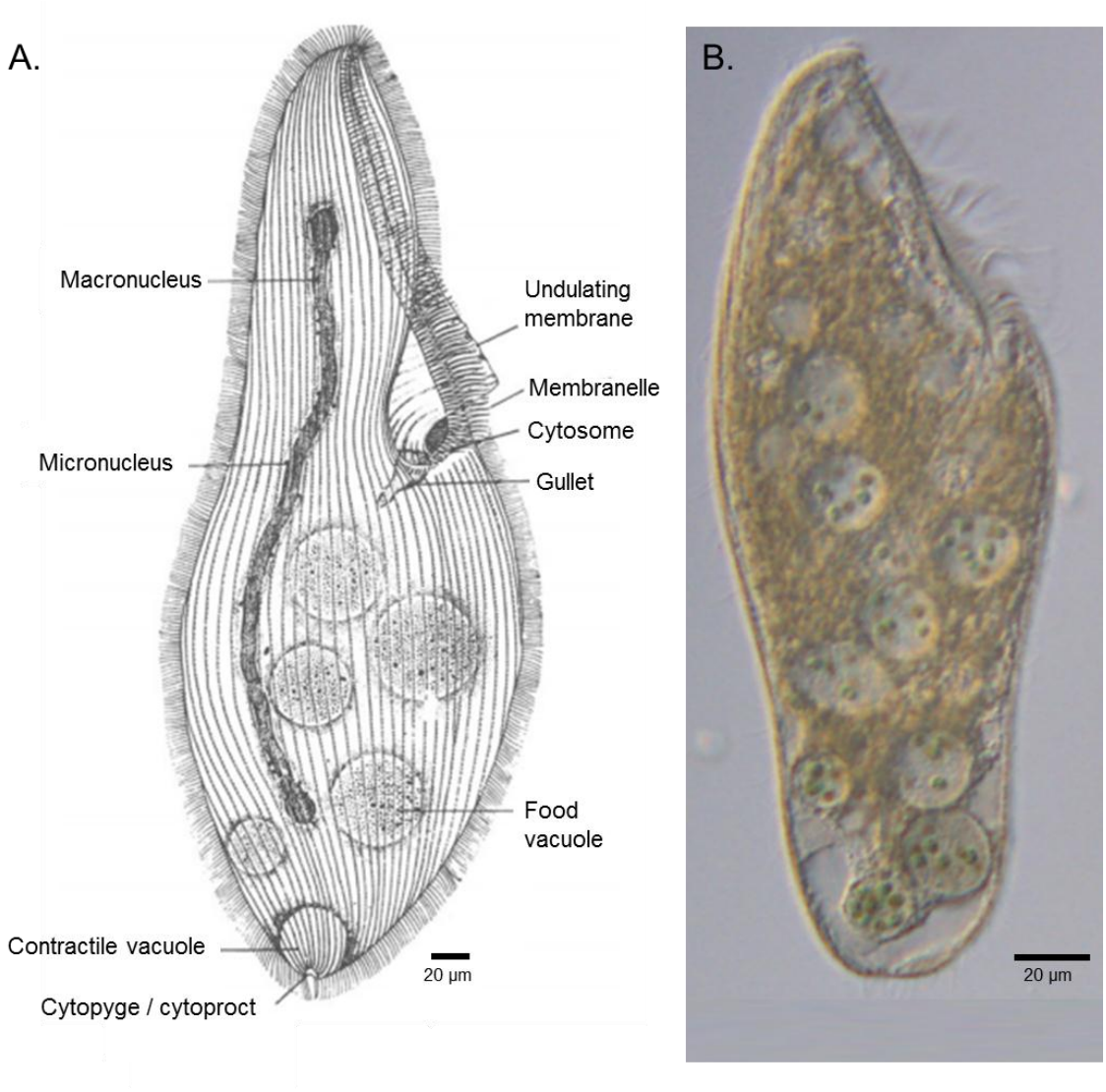


Figure 4.1. A) Cell structure of *Blepharosma japonicum* in vegetative form (adapted from Suzuki 1951). B) Light microscope image of a smaller *Blepharisma* (*B. americanum*) with toxic *Microcystis aeruginosa* contained within its food vacuoles (x400 magnification).

To the best of the authors knowledge, despite ciliates playing a significant role in aquatic ecosystems through the reduction of cyanobacterial biomass (Finlay and Esteban 1998) only one study has looked at the impact and fate of a highly toxic secondary metabolite (microcystin) from a cyanobacteria (*Planktothrix agardhii*) on laboratory ciliates (*Nassula* sp.) (Combes *et al.* 2013). Here the grazing of a *Blepharisma* sp. on the microcystin-producing cyanobacterium *Microcystis aeruginosa* (PCC 7806) and its non-toxic mutant is studied. The experiment analysed the potential impact of such a grazer on bloom formation and the intra-cellular cyanotoxin response to predation pressure. The interaction on a population and morphological level was also assessed which subsequently developed a clearer understanding of that specific trophic association, contributing to their role in the microbial loop.

4.2 Methodology

4.2.1 Cyanobacteria, ciliate strains and culture conditions

Two axenic *Microcystis aeruginosa* culture lines were used in this experiment i) the toxic PCC 7806 (+mycB) wild type and ii) the non-toxic PCC 7806 (-mcyB) mutant type which were both provided by the Pasteur Culture Collection of Cyanobacteria (Institut Pasteur of Paris). Strains were pre-cultivated in 'cap vented' Corning® cell culture flasks (Sigma-Aldrich, UK) with a 1:1 mixture of BG-11 media (Sigma-Aldrich, UK) and Volvic® mineral water, which was sterile filtered through a 0.2µm cellulose acetate filter (Minisart® plus, Sigma-Aldrich, UK). Non-toxic *M. aeruginosa* cultures were also supplemented with 5 µg of chloramphenicol mL^{-1} (Sigma-Aldrich, UK) as the strain was generated by insertion of a chloramphenicol resistance cassette into the mcyB gene required for microcystin-LR biosynthesis (Dittman *et al.* 1997). All cultures used in this chapter contained a total volume of 30mL.

Blepharisma americanum was obtained through the Culture Collection of Algae and Protozoa (CCAP 1607/1, UK). The ciliate cultures were initially established with a 1:1 mixture of BG-11 media and Volvic® mineral water in Corning® cell culture flasks. To this half a crushed boiled wheat grain was added to promote growth of indigenous heterotrophic bacteria. All cultures in this experiment were incubated under a single 58 watt cool-white fluorescent tube, illuminated at 7 ± 2 µE m^{-2} s^{-1} on a 12 hour light / dark cycle at $25 \pm 1^{o}C$ (Conviron, CMP6010, Canada) and agitated gently by hand once a day. Light intensities were lower than other experimental chapters for *Microcystis* as *B. americanum* is a light sensitive protozoan (Giese 1973).

4.2.2 Experimental design

To identify the grazing habits of *B. americanum* on *M. aeruginosa* and the cyanobacterial response to predation, ciliates were introduced to separate toxic and non-toxic *M. aeruginosa* cultures at two different cell densities. *B. americanum* cell numbers, morphological dimensions (lengths, widths and total biovolumes) and cyst formation were recorded over a 32 day period, along with *M. aeruginosa* cell numbers, sizes (diameters) and intra-cellular toxin content.

Blepharisma

To initiate the grazing experiment *B. americanum* first needed to be isolated from its primary food source and starved. When reared on heterotrophic bacteria *B. americanum* regularly recorded up to 8,000 cells/mL. Through a combination of repeated dilutions in fresh 1:1 BG-11:Volvic® mineral water and micro-pipetting, ciliate densities were reduced to 1,000 cells/mL then starved for 48 hours. Before being introduced to the control or test cultures, samples were checked through light microscopy to confirm the absence of heterotrophic bacteria in the media and ciliate food vacuoles.

B. americanum are known to be cannibalistic which leads to gigantism (Giese 1973). To observe if that feeding habit existed under experimental conditions two control groups were set up (in triplicate). In the same media and incubation conditions as described in Section 4.2.1. the 1,000 cell/mL densities of *B. americanum* were transferred to new culture flasks and diluted to 100 cells/mL (30mL total sample volume) with no additional nutrient source (prokaryotes). One control group was also supplemented with 5µg of chloramphenicol mL^{-1} to observe any potential effects that could occur from the antibiotic when experimenting with non-toxic populations.

Microcystis

For the cyanobacterium control groups, pre-cultivated toxic and non-toxic *M. aeruginosa* were taken from stock cultures at a high exponential phase ($8x10^6$ cells/mL) and diluted to $1x10^5$ cells/mL in triplicate (30mL total). When cultures reached approximately $4x10^5$ (low) or $2x10^6$ (high) cells/mL *M. aeruginosa* cell counts, sizes (diameters) and intra-cellular toxin levels were recorded. The cell densities were grown from relatively low numbers (lag phase) and chosen to experiment on as parameters like growth rates, morphology and toxin content per cell can change during different phases of a batch cycle, especially under nutrient stress (Long *et al.* 2001; Li *et al.* 2013; Appendix C2).

Both toxic and non-toxic *M. aeruginosa* test groups (exposed to *B. americanum*) were set up in the same way as the control groups but with sample volumes of 27 mL, allowing for 3 mL of *B. americanum* (1,000 cells/mL) for a final sample volume of 30 mL with 100 ciliates/mL. All control and test groups were kept under the same conditions as the pre-cultivated samples and gently agitated once a day (Section 4.2.1).

4.2.3 *Microcystis aeruginosa* enumeration and sizing through flow cytometry

Total cyanobacterium cell counts were measured approximately every 3 days by flow cytometry (FCM), using an Accuri C6 and its in built Accuri C6 software (BD Biosciences, US). Detection of *M. aeruginosa* was defined through forward angle light scatter (FSC-W) and the endogenous fluorescent species phycocyanin (FL4-H). Counts were acquired from the slow settings and sampled for 2 min as described in Chapter 2.

Cell diameter was calculated through a 6-point calibration curve from flow cytometry grade polystyrene sizing beads ranging from 2.0 - 14.5µm (Spherotech, US), with biovolume determination based on the volume of a sphere (eq. 1), V = Volume and r = radius (Section 2.3).

$$V = 4/3\ \pi\ r^3 \qquad \text{(eq. 1)}$$

4.2.4 *Blepharisma americanum* enumeration, sizing and cyst counts

Samples (0.5 mL) were fixed with Lugol's iodine (final concentration 0.0175% v/v) to 1mL. Ciliate cell and cyst numbers were determined through light microscopy (BX51 microscope, Olympus, Japan) at x40 magnification in a Sedgwick Rafter Chamber at the same time as the *M. aeruginosa* counts. Ciliate cell sizes (length and width) were measured using the same samples at x100 magnification and a semi-automatic image analysis system (Camera DP70, Software package Cell ^F, both Olympus, Japan).

Biovolumes of *B. americanum* were assumed to be a rotational ellipsoid (Olenina *et al.* 2006) (Figure 4.2) where V = volume, d = diameter and h = height (eq. 2). A minimum of 10 ciliates were measured for morphological parameters.

$$V = \pi/6 * d2 * h \qquad \text{(eq. 2)}$$

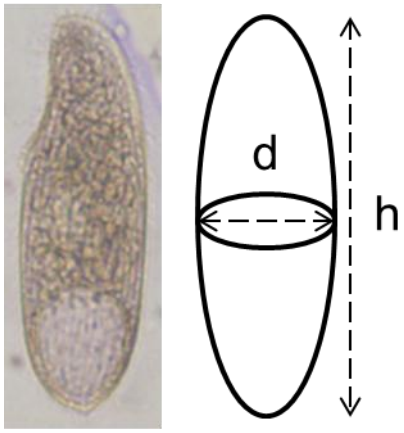


Figure 4.2. The assumed rotational ellipsoid shape of *Blepharisma americanum* with biovolumes calculated from V = π/6*d2*h (eq. 2).

4.2.5 Toxin analysis

Samples were filtered through a Whatman® GF/C glass microfiber disc at a volume of 1 mL approximately every 6 days. These were immediately stored at -80^{o}C and freeze thawed 3 times prior to extraction (room temperature). Filter discs were placed in a glass beaker containing 10 ml of 80% methanol and allowed to extract for 24 hours at (4±1^{o}C) before UPLC-MS/MS analysis. The novel methodology used to identify and quantify cyanotoxins (microcystins) is described in Section 2.7.

4.2.6 Data analysis

Microcystis

To test the effects of *B. americanum* on toxic and non-toxic *M. aeruginosa* populations a two-way repeated measures (RM) ANOVA was performed on cell densities, cell sizes (diameter), total sample biovolume and intracellular cyanotoxins, using sampling time points and cultures conditions as factors. *M. aeruginosa* culture conditions were set as control (-ve ciliates) or test (+ve ciliates). The time points tested where over a 32 day period or a week after the last live ciliate was recorded. If ciliate recordings did stop before 32 days due to absolute population mortality observation were still conducted until the end of the experiment, as *B .americanum* has the potential to re-emerge from an encystment phase. A linear regression was run if an intra-cellular toxin changed over the experiment to determine a secondary metabolite response to predation.

Blepharisma

B. americanum and cyst densities were also run through a two-way RM ANOVA, reporting significant differences at $p < 0.05$ over time and between all conditions. Conditions for *B. americanum* included the grazing on toxic and non-toxic *M. aeruginosa* cultures at both initial cyanobacterium densities and controls. The two controls consisted of *B. americanum* with no nutrients and another same culture lacking a food source supplemented with 5 µg of chloramphenicol mL^{-1}.

To assess how a diet of *M. aeruginosa* affects *B. americanum* morphology the dimensions of ciliates were measured when *M. aeruginosa* test cultures continued to support populations throughout the experimental period. A two-way RM ANOVA was performed to find significant changes in individual ciliate total biovolumes, lengths and widths between test cyanobacterium densities and over time. When a morphological parameter was found to significantly change over time a linear regression analysis was performed in order determine if *M. aeruginosa* is an adequate food source. All statistical analyses were carried out with SigmaPlot 13.0 (Systat Software, San Jose, CA) for windows.

4.3 Results

4.3.1 Effect of *Blepharisma* on *Microcystis* cell numbers

A two-way RM ANOVA reported that ciliates introduced to toxic cultures had no significant impact on *M. aeruginosa* cell numbers compared to control samples, in both high and low starting cyanobacterium densities ($p > 0.05$) (Table 4.1). Both the control and experimental populations continued to grow significantly over time ($p < 0.001$), reaching cell numbers between 7.0 - 7.5×10^6 cells/mL and 2.4 - 2.6×10^6 cells/mL for high and low initial *M. aeruginosa* densities respectively (Figure 4.3).

In contrast, *B. americanum* was found to significantly reduce non-toxic *M.aeruginosa* cell numbers when introduced to test cultures in both high and low starting cyanobacterium densities ($p < 0.001$) (Table 4.2). When introduced to non-toxic *M. aeruginosa B. americanum* reduced cyanobacterium cell numbers by $75.6 \pm 1.7\%$ and $87.3 \pm 1.3\%$ in both high and low initial densities respectively by the end of the experiment (Figure 4.4).

Table 4.1. A two-way RM ANOVA of cell counts and morphological parameters for toxic *Microcystis aeruginosa* between control and test cultures, from both high and low starting cyanobacterium densities.

Two-way RM ANOVA	Cell count (cells/mL)		Cell size (µm)		Total biovolume (µm^3)	
	F	*p*	*F*	*p*	*F*	*p*
High						
Condition ($F_{1,24}$)	4.73	> 0.05	0.05	> 0.05	3.83	> 0.05
Time ($F_{6,24}$)	669.34	< 0.001	24.80	< 0.001	161.22	< 0.001
Condition x time ($F_{6,24}$)	4.35	< 0.01	5.71	< 0.001	5.03	< 0.01
Low						
Condition ($F_{1,24}$)	3.43	> 0.05	3.10	> 0.05	1.68	> 0.05
Time ($F_{6,24}$)	762.30	< 0.001	183.64	< 0.001	469.16	< 0.001
Condition x time ($F_{6,24}$)	8.49	< 0.001	34.41	< 0.001	30.92	< 0.001

Table 4.2. A two-way RM ANOVA of cell counts and morphological parameters for non-toxic *Microcystis aeruginosa* between control and test cultures, from both high and low starting cyanobacterium densities.

Two-way RM ANOVA	Cell count (cells/mL)		Cell size (µm)		Total biovolume (µm^3)	
	F	*p*	*F*	*p*	*F*	*p*
High						
Condition ($F_{1,52}$)	534.31	< 0.001	14.31	< 0.05	561.45	< 0.001
Time ($F_{63,52}$)	407.86	< 0.001	127.95	< 0.001	147.13	< 0.001
Condition x time ($F_{13,52}$)	196.06	< 0.001	45.7	< 0.001	108.88	< 0.001
Low						
Condition ($F_{1,52}$)	55.68	< 0.01	4.07	> 0.05	16.86	< 0.05
Time ($F_{63,52}$)	75.99	< 0.001	50.32	< 0.001	55.1	< 0.001
Condition x time ($F_{13,52}$)	46.44	< 0.001	4.85	< 0.001	41.82	< 0.001

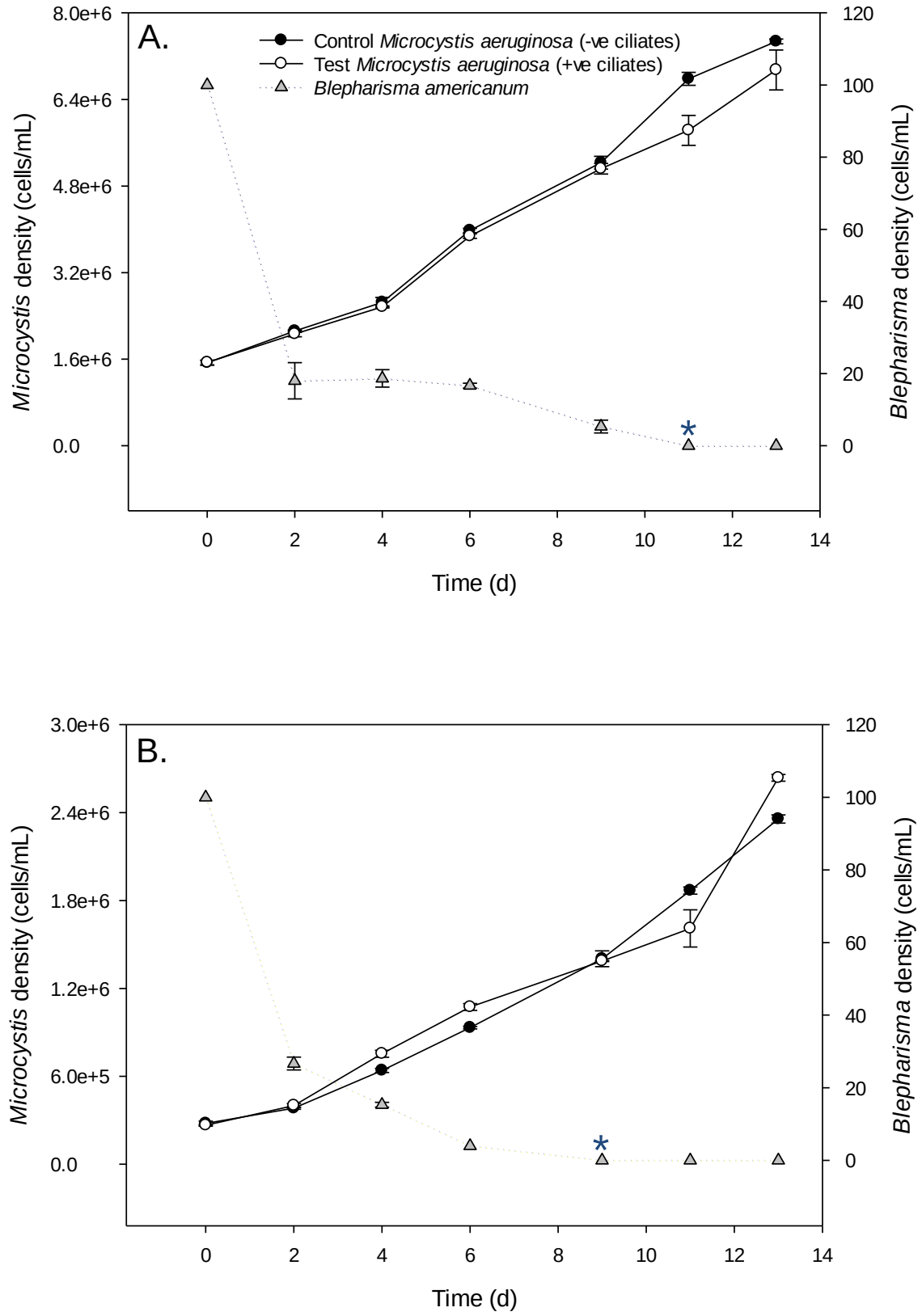


Figure 4.3. Toxic *Microcystis aeruginosa* cell numbers from cultures containing ciliates (test) and without ciliates (control) at both A) high and B) low starting densities of the cyanobacterium. *Blepharisma americanum* cell densities are also included on each graph with zero counts (*) recorded from days 9 and 11 in low and high starting densities respectively.

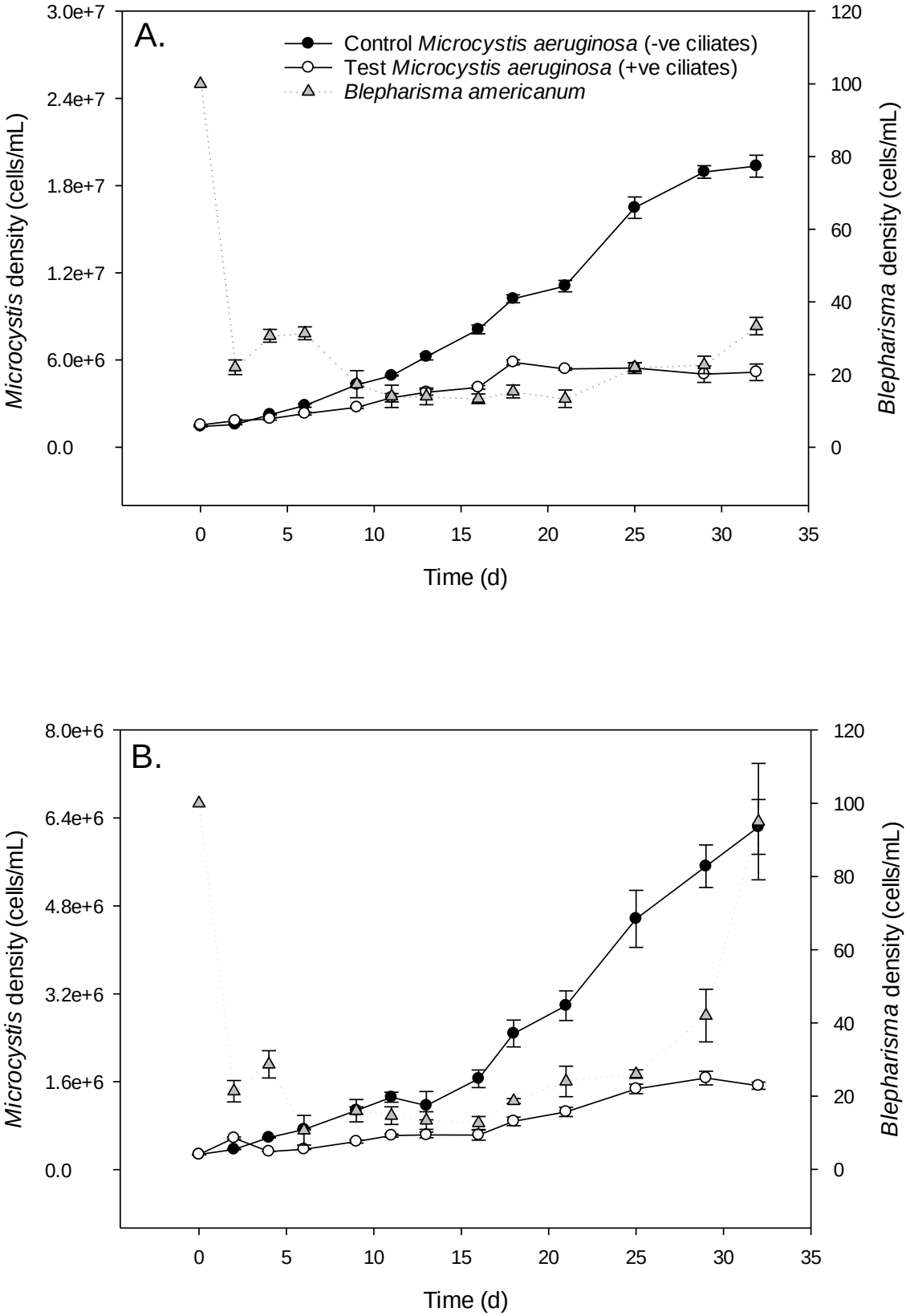


Figure 4.4. Non-toxic *Microcystis aeruginosa* cell numbers from cultures containing ciliates (test) and without ciliates (control) at both A) high and B) low starting densities of the cyanobacterium. *Blepharisma americanum* cell densities are also included.

4.3.2 Effect of *Blepharisma* on *Microcystis* morphology

Analysis through a two-way RM ANOVA reported that *B. americanum* had no significant effect on cell size (diameter) or total cyanobacterium biovolume when incubated with toxic *M. aeruginosa* at either high or low starting cyanobacterium densities ($p > 0.05$) (Table 4.1). The mean cell diameter over the experiment was 4.98±0.05 µm for high and 5.16±0.10 µm for low starting toxic *M. aeruginosa* densities (Appendix D1 and D2).

As there was no significant difference in toxic *M. aeruginosa* total biovolume between conditions (control and test) a combined mean recorded an increase of 1,266.6±33.0% in high and 2,544.5±13.6.1% in low initial cyanobacterium densities from day 0-32.

When *B. americanum* was incubated with non-toxic *M. aeruginosa* there was a significant difference in cell sizes between the control and test samples for high ($p < 0.05$) but not low initial cyanobacterium densities ($p > 0.05$) (Table 4.2). However, the difference in mean non-toxic *M. aeruginosa* cell diameters was very small, with the control recording a mean of 3.70±0.10µm, only 2.4±2.9% smaller than the test cultures in high initial densities (Appendix D3).

As cell numbers and sizes (diameter) are directly correlated with spherical *M. aeruginosa* biovolumes there was also a significant difference in the total non-toxic *M. aeruginosa* biovolumes when grazed on by *B. americanum* in both high ($p < 0.001$) and low ($p < 0.05$) initial cyanobacterium densities. Total biovolume reduction was very large in both high (76.3±0.9%) and low initial densities (89.4±0.8%) by the end of the experiment when compared to the controls (Appendix D4).

4.3.3 Ciliate abundance

B. americanum densities did significantly change in numbers between all culture conditions (controls and tests cultures) over the experimental period (two-way RM ANOVA, $p < 0.001$) (Table 4.3). Compared to the initial count of 100 cells/mL, ciliate densities in all conditions decreased over the first week (Appendix D5-D7).

No *B. americanum* were recorded from day 9 in the two controls and the low initial density toxic *M. aeruginosa* test cultures. Additionally no ciliates were observed from day 11 in toxic *M .aeruginosa* cultures with high initial densities (Figure 4.3). These groups with total cell mortality between days 9-11 were identified in the same sub group when a *post hoc* test (Holm-Šídák) was performed (Table 4.3).

Table 4.3. Two-way RM ANOVA of ciliate and cyst counts for all *Blepharisma americanum* controls and tests (both and high and low initial cyanobacterium densities). Homogeneous subgroups were defined and indicated by similar symbols (a, b and c) per column.

Condition	Ciliate count (cells/mL) Holm-Šídák		Cyst count (cysts/mL) Holm-Šídák	
Control	a		a	
Control + chloramphenicol	a		a	
Toxic high density	a		a	
Toxic low density	a		a	
Non-toxic high density	b		a	
Non-toxic low density	c		a	
Two-way RM ANOVA	*F*	*p*	*F*	*p*
Condition ($F_{5,144}$)	122.69	< 0.001	0.25	> 0.05
Time ($F_{12,144}$)	612.23	< 0.001	26.37	< 0.001
Condition x time ($F_{60,24}$)	16.84	< 0.001	3.75	< 0.001

In contrast, *B. americanum* populations survived in all non-toxic *M. aeruginosa* test cultures. Both conditions recorded a steady decrease until approximately 3 weeks into the experiment, where after ciliate populations started to increase (Figure 4.4). A *post hoc* analysis (Holm-Šídák) identified both ciliate populations had different mean densities over the experiment with 20±1 cells/mL recorded in high initial non-toxic *M. aeruginosa*, 20% less than in low starting cyanobacterium densities (25±4 cells/mL). Although both ciliate populations were similar in mean densities over the experimental period, it was statistically different due to the last ciliate count (day 32) in the low initial densities, where numbers increased dramatically by over 53±12% from the previous count on day 29 (Figure 4.4 and Appendix D7).

Although toxic *M. aeruginosa* cells were not reduced with the introduction of *B. americanum*, it was observed through light microscopy that ingestion of the toxic cells occurred (Figure 4.1 and Figure 4.5). However, despite toxic *M. aeruginosa* being evident in food vacuoles these digestive organelles migrated relatively quickly to the posterior end of *B. americanum*, where cells were then expelled through the cytoproct between 5-10 min of initial ingestion (Figure 4.6).

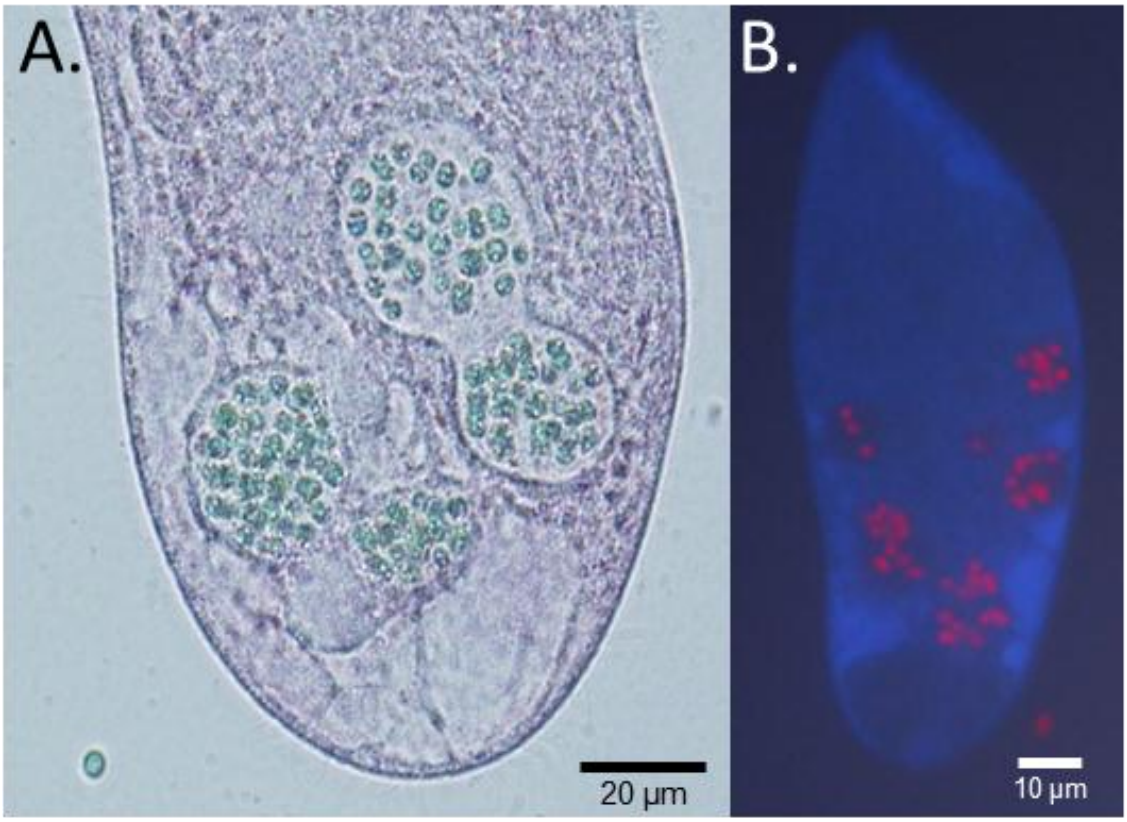


Figure 4.5. A) Light microscope image of *Blepharisma americanum* with toxic *Microcystis aeruginosa* cells in its food vacuoles (x400 mag.). B) An epifluorescence microscopy image of *B. americanum* (x200 mag.) with ingested toxic *M. aeruginosa* cells, viability was assumed through active chlorophyll fluorescence (red cells).

4.3.4 Cyst formation abundance

The formation of cysts and count thereof did not significantly change between all conditions ($p > 0.05$) (Table 4.3) but did change over time ($p < 0.001$). A general decrease in cyst count was observed for all conditions, as over half of all the total cysts (59%) were recorded in the first week at a combined mean of 4.4±0.7 cysts/mL per sample analysed and approximately a third of the total cyst counts were observed during the second week (3.4±0.5 cysts/mL per sample analysed). For all cultures containing *B. americanum*, cysts were rarely observed after day 16 (Appendix D5 - D7).

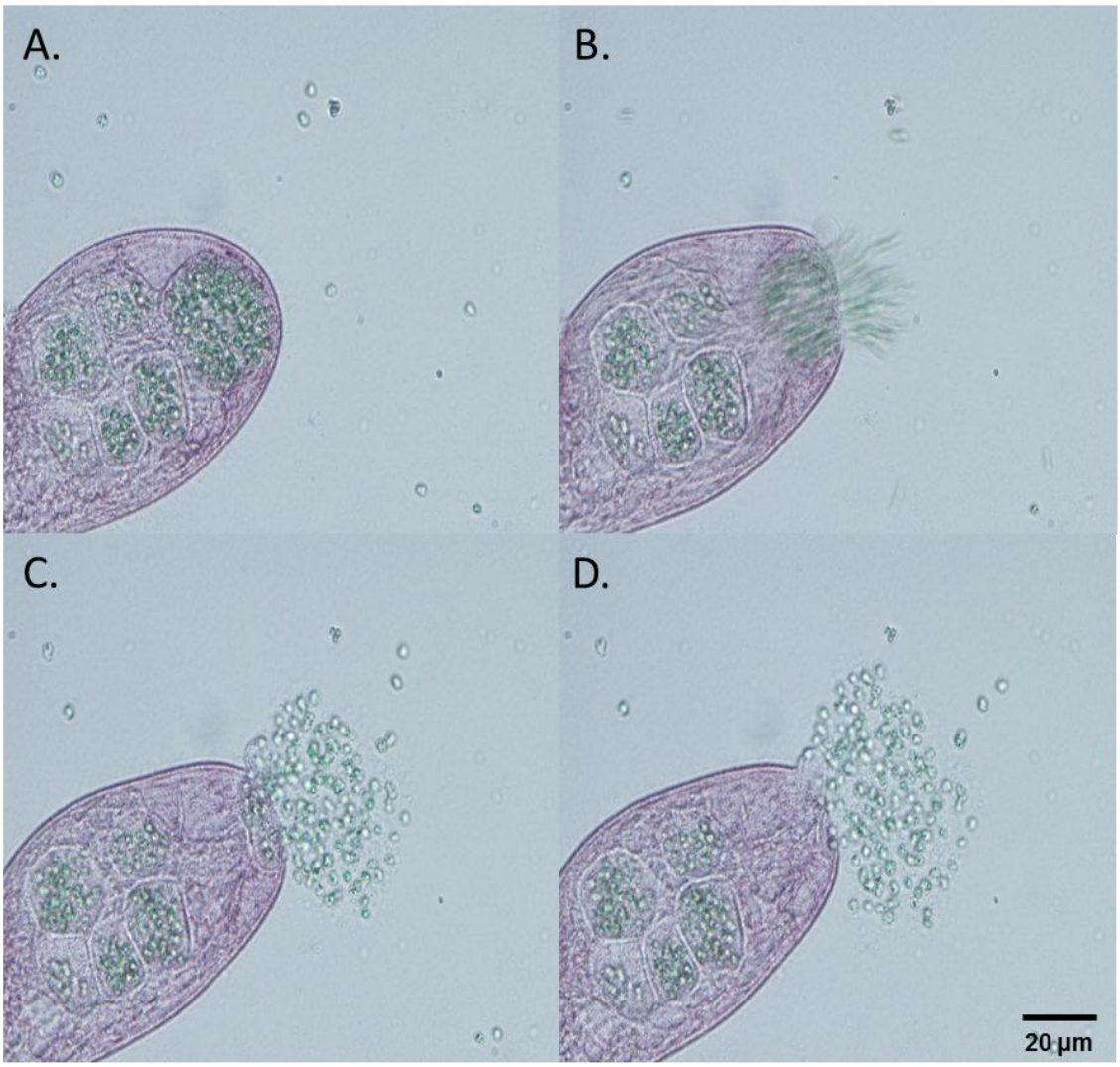


Figure 4.6. A fast frame capture of *Blepharisma americanum* expelling toxic *Microcystis aeruginosa* through its cytoproct into the immediate surroundings without digestion, time sequence starts at A) and runs through to D). Observations recorded that food vacuoles took between 5-10 minutes to release toxic *M. aeruginosa* cells after initial ingestion.

4.3.5 Ciliate morphology

Only *B. americanum* that feed on non-toxic *M. aeruginosa* survived with cell division observed. A two-way RM ANOVA reported that the biovolumes of *B. americanum* calculated from the shape of a rotational ellipsoid (eq. 2) which grazed on non-toxic *M. aeruginosa* did not significant differ between high and low initial cyanobacterium densities ($p > 0.05$) but both changed over time ($p < 0.001$) (Table 4.4). A linear regression analysis reported that mean *B. americanum* biomass decreased the longer they grazed on non-toxic *M. aeruginosa* ($F_{1,259} = 64.03$, $R^2 = 0.20$, $p < 0.001$) (Figure 4.7). The combined mean biovolumes of *B. americanum* cells in both non-toxic test cultures on the first day of sampling was $1.2 \times 10^5 \pm 1.5 \times 10^4$ µm^3, double that of *B. americanum* on day 32.

The mean lengths of *B. americanum* did not significantly change between either non-toxic *M. aeruginosa* initial densities or over the course of the experiment (both $p > 0.05$) (Table 4.4). A combined mean length of 121.2±1.6 µm was recorded for ciliates with a range of 107.2 – 147.7 µm.

In contrast, mean *B. americanum* width did significantly change over time ($p < 0.001$) but not between ciliates grazing on high or low starting densities ($p > 0.05$). Ciliate grazers in both non-toxic *M. aeruginosa* initial densities recorded a reduction in width over the experiment from a combine mean of 39.7±1.6 µm at day 0 to 28.4±1.1 µm on day 32 (Appendix D7). A linear regression confirmed that *B. americanum* significantly decreased in width from the start to the end of experiment ($F_{1,259} = 119.94$, $R^2 = 0.32$, $p < 0.001$) (Figure 4.7).

Table 4.4. Two-way RM ANOVA of ciliate morphologies for all *Blepharisma americanum* controls and tests (both and high and low initial cyanobacterium densities). Homogeneous subgroups were defined and indicated by symbols (a) per column.

Condition	Ciliate biovolume (µm^3)		Ciliate length (µm)		Ciliate width (µm)	
	Holm-Šídák		Holm-Šídák		Holm-Šídák	
Non-toxic high density	a		a		a	
Non-toxic low density	a		a		a	
Two-way ANOVA	*F*	*p*	*F*	*p*	*F*	*p*
Condition ($F_{1,216}$)	0.20	> 0.05	0.14	> 0.05	1.03	> 0.05
Time ($F_{12,216}$)	7.61	< 0.001	1.44	> 0.05	17.28	< 0.001
Condition x time ($F_{12,216}$)	0.08	> 0.05	0.20	> 0.05	0.21	> 0.05

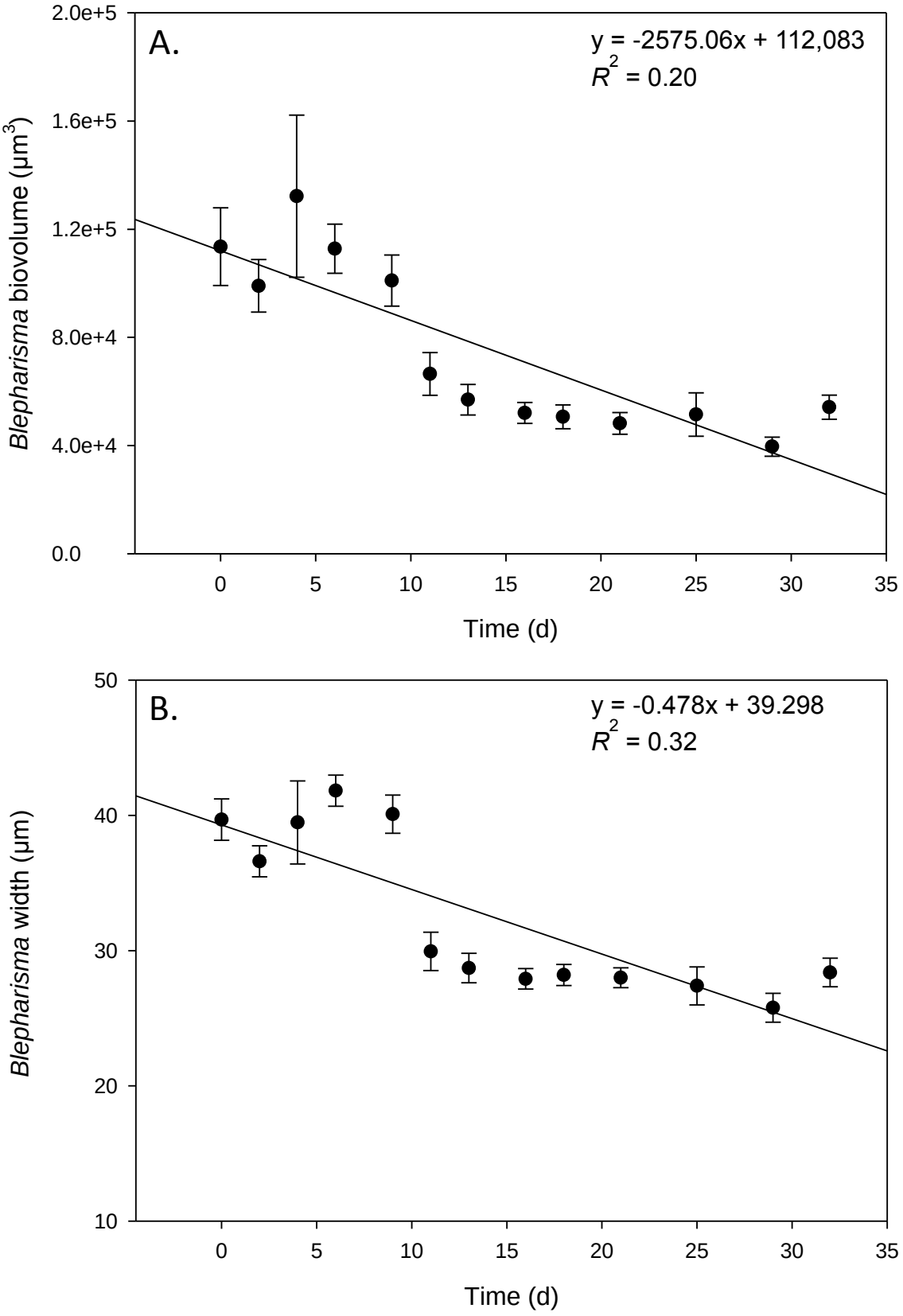


Figure 4.7. Regression analysis of *Blepharisma americanum* A) individual biovolumes and B) widths over the experiment when grazing on a diet of non-toxic *Microcystis aeruginosa* using both initial densities data.

4.3.6 Toxin levels

Toxic *M. aeruginosa* was the only culture sample that recorded any cyanotoxins, with microcystin-LR (MC-LR) and Asp^3-MC-LR detected from the 13 standards tested for (Section 2.7). Total intracellular MC-LR (MC-LR + Asp^3-MC-LR) did significantly change between culture conditions and over time (RM two-way ANOVA, all $p < 0.001$) (Table 4.5). However a *post hoc* test (Holm-Šídák) reported that a difference did not exist between the controls at both high and low initial staring *M. aeruginosa* densities ($p > 0.05$) (Table 4.5).

Table 4.5. A two-way RM ANOVA for intracellular MC-LR results from toxic *Microcystis aeruginosa* between all conditions and initial cyanobacterium densities over the experimental period. Homogeneous subgroups were defined and indicated by symbols (a, b and c) per column.

Two-way RM ANOVA	Total intra-cellular microcystin content (fg/cell) Holm-Šídák	
Control high density	a	
Test high density	b	
Control low density	a	
Test low density	c	
	F	*p*
Condition ($F_{3,48}$)	35.81	< 0.001
Time ($F_{6,48}$)	15.71	< 0.001
Condition x time ($F_{18,48}$)	4.74	< 0.001

The mean total intracellular MC-LR (fg/cell) was 2.62±0.15 fg/cell for the high density control and 2.65±0.14 fg/cell for the low density control (Appendix D8). This was 20.6±0.2% and 43.2±0.2% higher than both cultures containing ciliates for high and low initial *M. aeruginosa* densities respectively (Figure 4.8).

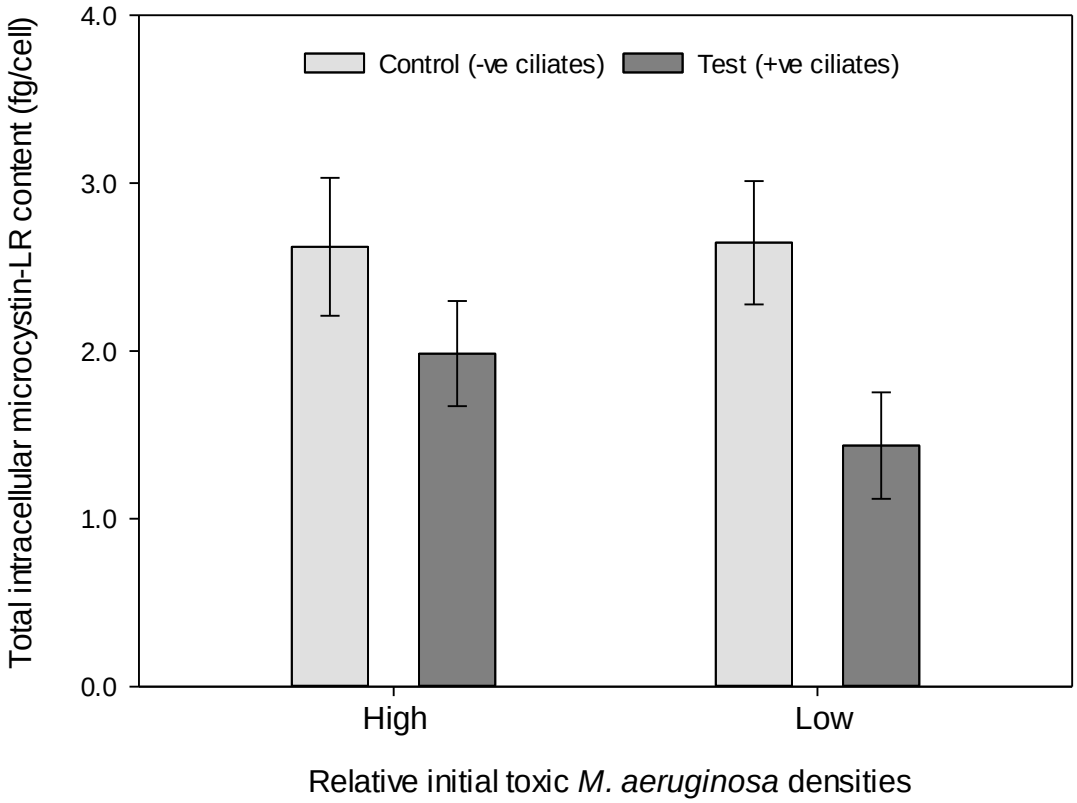


Figure 4.8. Mean intracellular total MC-LR content for control and test cultures between both high and low initial starting *Microcystis aeruginosa* densities.

Although there was a significant difference in total MC-LR intracellular content over time a linear regression analysis revealed that there was no way to predict total MC-LR content, as cyanotoxins were variable throughout. Representing all culture conditions total MC-LR ranged from 0.70 - 4.04 fg/cell both increasing and decreasing against preceding sample points.

Table 4.6. Linear regression analysis with one-way ANOVA outputs from total intracellular MC-LR content for both culture conditions and densities.

	One-way ANOVA			Model Summary
Condition	*F*	d.f.	*p*	R^2
High control	1.15	1,19	> 0.05	0.06
High test	2.15	1,19	> 0.05	0.10
Low control	0.17	1,19	> 0.05	0.01
Low test	8.00	1,19	> 0.05	0.30

4.4 Discussion

4.4.1 *Microcystis* cell growth and morphological response to *Blepharisma* predation

Ingestion of *M. aeruginosa* PCC 7806 by *Blepharisma* was observed in both toxic and non-toxic forms (Figure 4.1 and Figure 4.5). However, in toxic cultures *B. americanum* was seen to be actively expelling the cyanobacterium on a constant basis from food vacuoles to the culture media via the cytoproct (Figure 4.6). Toxic producing *M. aeruginosa* cell densities and their resulting total biomasses subsequently did not differ significantly between the two initial test densities and their controls, providing evidence that *B. americanum* cannot actively digest MC producing cyanobacteria. In contrast non-toxic *M. aeruginosa* growth patterns at both initial densities were significantly different when exposed to *B. americanum*. This suggests that *M. aeruginosa* cannot be selectively handled as a prey item and only discriminated when it has entered the digestion system of *B. americanum.*

Both toxic and non-toxic *M. aeruginosa* PCC 7806 exist as single-cells and do not form colonies. However, in previous work two strains of *M. aeruginosa* incubated with a flagellate grazer (*Ochromonas* sp.) induced colonies which was not obvious in this experiment (CCAP 1450/1-Burket *et al.* 2001; PCC7820-Yang *et al.* 2008). Although colony formation was not obvious through light microscopy in the PCC 7806 strain, future work on ciliate grazing with *Microcystis* should include polysaccharide assays. Recording a polysaccharide increase in cultures will suggest a morphological response to ciliate predation. *M. aeruginosa* grazing could adapt by either secreting extracellular polysaccharide (EPS) for cell aggregation or bound extracellular polysaccharide (bEPS) resulting in thicker cell walls as seen in Yang *et al.* (2008).

As both *M. aeruginosa* PCC 7806 cultures were unicellular in densities the mean cell sizes (diameters) could be recorded relatively easily through FCM. Fenchel (1980) suggests that the maximum clearing rate of prey items as a function of cells size by *B. americanum* is optimal with 6 µm diameter cells. Here the non-toxic *M. aeruginosa* population were smaller by a mean of over 1 µm compared to the toxic cells with an average of 3.8$\pm$0.1 µm. These smaller cells could be attributed to the addition of chloramphenicol, as it is a protein inhibitor required for MC synthesis inhibition (mcyB gene) (Dittman *et al.* 1997). As maximum clearance rates in terms of prey cell sizes need to be increased by 57.9% to match Fenchel (1980) optimal size of 6 µm this could suggest why non-toxic densities were mitigated in densities rather than recording a complete reduction of cyanobacterium populations (Figure 4.4).

4.4.2 *Microcystis* intracellular toxin production during grazing

In this study only toxic *M. aeruginosa* produced toxins that were identified and quantified through the standards tested for (MC-LR and Asp^3-MC-LR). There was a difference between controls and tests cultures with total intracellular MC-LR having a higher mean concentration when no ciliates were present, which could point towards a metabolic release due to predation.

However, the fate of cell bound MC-LR was not determined during the experiment but can be reasonably assumed it was expelled by the cells into the media. In addition as there was such a degree of variability in MC-LR results potentially other factors such as small differences in light irradiance due to the culture position in the incubator or a response to a virus could affect cyanotoxin synthesis (Paerl and Otten 2013). Nonetheless the presence or absences of intracellular MC-LR dose have a significant role in the consumption and control of *M. aeruginosa* densities through *B. americanum*.

To consolidate the idea that extracellular release of MC-LR was induced by the presence of potential predators in *M. aeruginosa* populations future work could record the extracellular MC-LR content. In addition grazed densities of toxic *M. aeruginosa* could be spiked with MC-LR. If no cell bound MC-LR was released due to increased MC-LR levels it would confer that MC-LR was a density dependent info-chemical (quorum sensing) and would further suggest that the cyanotoxin is an anti-grazer molecule.

However, genetic evidence has shown toxic secondary metabolites in cyanobacteria like microcystins pre-dates any potential grazers (Rantala *et al.* 2004). Since there are high levels of sequence divergence and the sporadic distribution of microcystin synthesis genes in modern cyanobacteria (Rantala *et al.* 2004) it may underline why the role of the cyanotoxins are so contradictory.

4.4.3 Ciliate response to *Microcystis* grazing

B. americanum densities

Constant active grazing was only observed on non-toxic *Microcystis* cultures allowing *B. americanum* populations to survive and reproduce. The number of *B. americanum* in both initial non-toxic prey densities dropped by three quarters within the first five days and stayed very low at around 14% (from the original 100 cell/mL) up until week three.

After three weeks on a non-toxic *Microcystis* diet the ciliate population did start to increase. This seems to indicate an adaptation to the food source by a small number *B. americanum,* which agrees with some studies of grazers on *Microcystis* that certain local adaptions or genotypes can be favoured (Fenchel 1982; Sarnelle and Wilson 2005; Chislock *et al.* 2013).

In contrast, no ciliates were observed in the toxic *M. aeruginosa* cultures after 9-11 days of incubation. Despite being seen in the food vacuoles evidence of acute toxicity cannot be concluded as expulsion of toxic cells were observed (Figure 4.6) and the recordings of ciliates stopped at the same time as the nutrient-starved controls. Therefore the presence of microcystins (MC-LR) during *B. americanum* grazing indirectly reduces this particular ciliates population, as the absence of its production (as found in non-toxic cells) allows the ciliates to feed and absorb enough nutrients to maintain a population.

Further investigation into a mixed *M. aeruginosa* population containing both toxin and non-toxin cells would determine if there was a type of internal selectivity for non-toxic prey items and if *B. americanum* were capable of surviving in a heterogeneous toxic cyanobacterial community. Analysing different toxic populations could be achieved through molecular techniques such as qPRC or a combination of adding a fluorescent protein to an active mcy gene and then running it through FCM.

Cyanotoxin and nutrient response

The understanding of how MC-LR directly influences *B. americanum* physiology remains unclear and indeed what triggers the release of intracellular MC-LR containing cells. It is commonly thought that MC-LR released form *Microcystis* is induced by the decomposition or lysis of a cell (Watanabe *et al.* 1992). Enzymes in the food vacuoles of *B. americanum* could initiate cell wall break down of toxic *M. aeruginosa* causing the release of MC-LR. MC-LR within food vacuoles could then generate an expulsion response as seen in this study with minimal impact on overall toxic *M. aeruginosa* populations. If MC-LR also acts a cell signalling info-chemical there would be a cumulative response when enclosed in a food vacuole, as concentrations would not be as diluted compared levels in the sample media promoting an increased synthesis rate and / or release of the cyanotoxin.

The nutritional value of cyanobacterial for grazers is perceived to be very low (Holm *et al.* 1984; Fink *et al.* 2011; Von Elert *et al.* 2014). When sustained on a diet of non-toxic *Microcystis* the mean width and consequently total biovolume of individual *B. americanum* decreased over time. As seen in nutrient deficient *B. americanum* the cell shape and overall biovolume became elongated or spindle shaped (Giese 1973).

B. americanum morphology

Non-toxic *M. aeruginosa* at both initial densities were controlled but no total population mortality was observed. This would indicate that the doubling time of the cyanobacterium was faster than the rate at which they were being consumed. However, towards the end of the experiment both non-toxic test cultures started to decrease corresponding to the increase of ciliate numbers. There may a number of underlining reasons for the relative slow response of *B. americanum* grazing and subsequent reproduction rates.

The first could be from the onset of starvation at the start of the experiment, leading to a decrease in metabolic rates as found in heterotrophic microflagellates (Fenchel 1982). The second could be down to a relatively slow feeding behaviour, as *B. americanum* ingestion rates have been recorded to be no more than 10% of its own volume per hour (Fenchel 1980).

Fenchel (1980) also described the optimal food particle size for *B. americanum* as 6 µm from biovolumes of 10^5 μm^3. As non-toxic *M. aeruginosa* test cultures were smaller than 6 µm (mean 3.8 ± 0.1 µm) ingestion rates would be sub-optimal, resulting in only a control of *M. aeruginosa* densities rather than population mortality as seen here. This in turn could have also played a part in the increased numbers of ciliates towards the end of the experiment, as the cyanobacterium would have been a more optimal size for smaller *B. americanum* which were recorded after a month on this type of prey item.

Gigantism and encystment

Unfavourable conditions such as nutrient limitation, temperature, pH and oxygen levels have induced a dormant stage or gigantism (cannibalism) in many protozoa, including *B. americanum.* Although put under nutrient stress no sign of cannibalism or increase in encystment took place. Giese (1973) suggests that not forming giants or cysts can be part of adaptive behaviour, so if vegetative cells have not been exposed to regular temporal or seasonal stresses they will not show rapid physiological heterogeneity. The *B. americanum* used during this experiment had been kept under constant stable conditions (environmental and nutrient) since 1951 (CCAP 1607/1, UK), where as a lineage it would have faced relatively little stress. When kept in optimal conditions the adaption to cannibalism or encystment maybe lost or take a longer period of time (Giese 1973), which could also account for the time needed in populations to adapt to the non-toxic food source and start to reproduce (Figure 4.4) and why the control two cultures recorded a complete population mortality with no encystment after the first week.

4.5 Implications for the microbial loop

Omnivores like ciliates are pivotal components to food webs and can dominate consumptive relationships in natural food webs (Finlay and Esteban 1998; Davis *et al.* 2012; Esteban *et al.* 2015). However, under laboratory settings distinguishing between the nutrient qualities of microbes is not that clear and usually limited to comparing predatory responses (densities) of diets with the addition or absence certain prey items (e.g. cyanobacteria). The design of this experiment cannot accommodate the role of all alternative determinants found in natural settings and the author does acknowledge that a large variety of physiological and morphological differences between cyanobacteria exists.

The population morphology of environmental *Microcystis* when in mass bloom densities can usually be found as a colonial form. The configuration of such blooms can mechanically interfere with filtering appendages (Lynch 1980; Lampert 1987; Wilson *et al.* 2006), raising questions about the suitability of the strain used in this study as it failed to develop into colonies existing only as a uni-algal culture. However, ciliated protozoans such as *Blepharisma* and their ecological significances go beyond the mass uptake of prey items. Before an algal bloom such as *Microcystis* can develop the growth rates must exceed the sum of all population losses.

Recent research reveals that the most important biological driver in annual *Microcystis* bloom formation is their growth rates from over wintering water column populations. Although very small in proportion to benthic populations, controlling these non-mass bloomed densities can suppress or at least delay summer blooms (Verspagen *et al.* 2005; Cirés *et al.* 2013). In addition not all *M. aeruginosa* natural densities that are in relatively high numbers form blooms, as evident in the environmental monitoring of *Microcystis*-like cells at Longham reservoir (Dorset, UK) (Chapter 3). This problem can be overcome by using freshly isolated species (Hartnell *et al.* 2016), but various phytoplankton (like here) can lose their colonial formation once isolated (John Day, Culture Collection of Algae and Protozoa, pers. comm.) and may not have comparable toxic and non-toxic populations.

During this study it was evident that intracellular cyanotoxins (MC-LR) played an important role in the growth rates of *M. aeruginosa* and ciliate sustainability. Despite the ecological function of MC-LR is still in debate (Babica *et al.* 2006) and many contradictory studies on *Microcystis* grazing, it would seem that an anti-predatory molecule / bio-control may need to be indigenous to that of the problematic microbe and local environment (Sigee *et al.* 1999). This can be supported by this chapters results from the adaptive nature or phenotypes that existed for successful *Microcystis* mitigation from ciliates and conversely by the toxin content of cyanobacterial cells.

Here even though the assumed initial nutrition values of cyanobacteria were relatively low *B. americanum* was observed to graze sustainability on *Microcystis* cells in the absence of intracellular microcystin. The ability to only control one genotype suggests that primary consumption of non-toxic *Microcystis* can shift the balance in a species population and possibly the phytoplankton community. As in this experiment if a certain algal species is difficult to consume and more importantly to digest (e.g. contains MC-LR) the reduced grazing pressure will favour bloom development (Sigee *et al.* 1999; Tillmann 2004), in this case toxic *Microcystis*.

4.6 Conclusion

The grazing of zooplankton on cyanobacteria is an essential part of aquatic community dynamics. This work reveals that ciliates like *B. americanum* can be very effective at supressing a population of cyanobacteria. However, bottom-up effects of aquatic trophic interactions can be significantly altered by the presence of cyanotoxins (MC-LR), which will not only affect the zooplankton community but also the autotrophic assemblage in their dominance and persistence.

Before biomanipulation and subsequent mass culturing of successful grazers (e.g. ciliates) can be successfully applied in the field, more research like the one conducted here is needed to understand species-specific interactions (Tillmann 2004). As there is no one biological solution to control cyanobacterial species like *M. aeruginosa* there may need to be a multiphasic approach in controlling densities. This can be from both bottom-up and top-down process of biomanipulation. A combination of removing fish that feed on zooplankton and reducing nutrients in an aquatic system can encourage other cyanobacterial grazers, which can potentially mitigate problem algal like *Microcystis* that degrade water quality.

This page intentionally left blank

Chapter V: Comparing hydrogen peroxide (an established phytoplankton chemical control agent) with acetic acid, a novel control agent for the cyanobacterium *Microcystis*

Abstract

Microcystis and its toxins can occur in water sources designated for human consumption, aquaculture and amenity use. However, only a few rapid control measures exist for suppressing these nuisance cyanobacteria which are not harmful to other organisms and detrimental to the environment. This chapter reports on the application and cellular consequences of a proven *Microcystis* chemical control hydrogen peroxide (H_2O_2) and a novel agent acetic acid, which is cheap to produce and has demonstrated control of terrestrial weeds. H_2O_2 and acetic acid was tested on a fresh *Microcystis* isolate (CCAP 1450/17) in order to investigate any differential effects compared to the long-established culture lines used in previous studies. H_2O_2 cell analysis through flow cytometry recorded significant *Microcystis* density reduction at concentrations of $\geq$ 4 mg/L in light conditions but needed much higher concentrations under constant darkness ($\geq$ 40 mg/L). Acetic acid also initiated a reduction of biomass at $\geq$ 40 mg/L, but light irradiance only facilitated density reduction at 72 h in comparable 40 mg/L concentrations. Both H_2O_2 and acetic acid induced the formation of reactive oxygen species (ROS) (CM-H_2DCFDA labelling) and increased membrane permeability (SYTOX® Green labelling) during the period of cell number decline. This is the first time that the efficacy of acetic acid in causing *Microcystis* mortality has been investigated, with observations also documenting pigment change. The use of the recently isolated *Microcystis* strain indicated that any conclusions about potential chemical control concentrations need to be assessed with environmental phenotypes. As here the fresh isolate was considerably less sensitive to H_2O_2, requiring $\geq$ 40 mg/L to cause complete population mortality compared to ≥ 4 mg/L as reported in long established *Microcystis* strains tested in previous studies.

5.1 Introduction

5.1.1 Chemical control of cyanobacteria

Cyanobacteria especially *Microcystis* have been well documented to cause problems when they form super-abundant populations in drinking water supplies (Chorus and Bartram 1999; Codd 2000; Codd *et al.* 2005), with nutrient reduction being arguably the best control strategy in freshwater ecosystems (Conley *et al.* 2009; Schindler *et al.* 2009; Paerl and Otten 2013). However, results from nutrient manipulation are only evident after many years of sustained management and requires full co-operation from all land users.

Artificial mixing (Huisman *et al.* 2004), flushing (Mitrovic *et al.* 2011) and coagulating nutrients for sedimentation, e.g. phosphate binding (Van Oosterhout and Lürling 2011) are additional methods that have also proved successful. Although in today's economic climate nutrient restrictions and advanced mechanical controls are currently too expensive or unfeasible in most areas across the world (Jančula and Maršálek 2011), highlighting the need for cheap, efficient alternatives.

Although biological (biomanipulation of grazers) and biochemical (auto-inhibition) controls of *Microcystis* have shown promise (Chapters IV and VI), the demands on water resources are extremely high (drinking water, aquaculture, industry etc.) and often require fast results. The use of cyanobacterial / algalcides is seen as a relatively rapid control technique, however, the detriment to aquatic ecosystems is potentially very high due to oxygen depletion and / or toxin release from lysed cells (Cook *et al.* 2005; Lürling *et al.* 2014).

The most toxic chemical treatments for microbes include a number of metals and their ions such as aluminium, iron, silver, copper, calcium and potassium. Halogen elements like chlorine and fluoride, photosensitisers and commercial herbicides have also reported success in reducing general phytoplankton biomass. However, again these chemicals induce photophosphorylation and cell lysis which can release toxins and in turn affect water quality (WHO 1999; Cook *et al.* 2005). Additionally many commercial herbicides are not species specific causing mortality to other microorganisms and can accumulate in the benthos resulting in long term damage to aquatic ecology (Mason 2002). There are many positives and negatives associated with chemical agents in water management, which subsequently determines the precise control measures that can be implemented (summarised in Table 5.1), with the concentration / exposure time (CET) relationship an important consideration (Getsinger 1998).

5.1.2 Hydrogen peroxide as a photodynamic control measure

A number of studies have suggested photosensitisers show high anti-cyanobacterial potency in waste water ponds and open systems (Barrington and Ghadounai 2008; Matthijs *et al.* 2012; Barrington *et al.* 2013). Photosensitisers are chemicals that are facilitated by light irradiance and generate reactive oxygen species (ROS). These ROS agents can produce singlet oxygen, hydroxyl and peroxyl radicals which are easily biodegradable. Hydrogen peroxide (H_2O_2) is a strong oxidising agent and has been studied as a potential algalcide for cyanobacteria since the 1980's (Barroin and Feuillade 1986). H_2O_2 has also been used as a disinfectant and anti-fungal agent for several years, playing an important part in aquaculture (Rach *et al.* 1998; Avendaño-Herrera *et al.* 2006).

ROS agents producing singlet oxygen can cause universal and non-selective toxicity towards aquatic biota, with invertebrates having a higher resistance than green algae and cyanobacteria (Jančula *et al.* 2008). Chemical compounds that mainly produce hydroxyl radicals (e.g. H_2O_2) $_{also}$ show more specificity towards phytoplankton groups, with cyanobacteria mortality being more than ten times that of green algae (Drábková *et al.* 2007*a*; 2007*b*; 2007*c*).

H_2O_2 is produced naturally in surface and ground waters by photolysis from UV radiation on dissolved organic matter (Copper and Zike 1983) and biologically as a bi-product of metabolic processes, which can also act as a signalling molecule (Apel and Hirt 2004; Asada 2006; Veal *et al.* 2007). A number of recent laboratory studies have looked at H_2O_2 as a *Microcystis* control method with a focus on intracellular mechanisms (Table 5.2).

Table 5.1. Advantages and disadvantages of using chemical control measures on cyanobacterial populations.

Mode of Action	Compound / element	Advantages	Disadvantages
Cell toxicity	• Copper sulphate (Crance 1963) • Fluoride (Nichol *et al.* 1987) • Potassium (Parker *et al.*1997) • Chlorine (Daly *et al.* 2007) • Silver (Choi *et al.* 2008) • Sodium and copper chloride (Deniz *et al.* 2011) • Nickle-chloride (Nohomovich *et al.* 2013)	• Relatively very low in price • Very accessible • Can degrade some toxins (e.g. microcystins)	• Accumulates in the environment • Release toxins via cell lysis • Toxic to non-target species • Human consumption danger
Coagulation agents	• Calcium hydroxide and calcite (Zhang and Prepas 1996; Prepas *et al.* 2001) • Ferric chloride (Chow *et al.* 1998) • Aluminium salts (Mohamed 2001; Jančula and Maršálek 2012)	• Relatively very low in price • Can remove phosphorus • Some record long term effects with high water residence times • Low toxicity against non-target phytoplankton	• Some record short term effects with low water residence times • Can influence pH levels
Photosensitisers	• Titanium dioxide (Ohko *et al.* 2009) • Phthalocyanines (Jančula *et al.* 2008; Drábková *et al.* 2007a) •Hydrogen peroxide (Table 5.2)	• Relatively low in price • Low toxicity against non-target phytoplankton • Selective to photoautotrophs • Photoautotrophic ROS production • Degrades fast • Biodegradable	• Short term effect • Can be insoluble in water • Unknown toxicity in macrophytes & higher organisms
Commercial herbicides	• Reglone A (Lam *et al.* 1995) (diquat, 1,1-ethylene-2, 2-dipyridilium dibromide) • Simazine (Lam *et al.* 1995) (2-chloro-4,6-bis(ethylamino)-s-triazine) • Endothall (Ameel *et al.* 1997) (7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid) • Diuron (Giacomazzi and Cochet 2004) (3-[3,4-dichlorophenyl]-1,1-dimethyl-urea)	• Relative low in price • Toxic to photoautotrophs	• Accumulates in the environment • Toxic to non-target species • Release toxins via cell lysis • Leaves toxic residues

Laboratory experiments have used many different strains of *Microcystis* spp. with varying concentrations of H_2O_2, resulting in biomass reductions recorded as low as 0.27 mg/L (7.94 µM) (Table 5.2). However, a study using freshly isolated *Microcystis* from Chaohu Lake (China) reports that a minimum of 60 mg/L was needed reduce environmental densities (Wang *et al.* 2012). As *Microcystis* has a phenotypic response to environmental conditions (ecotypes) it highlights the need to test freshly isolated species, rather than use stock cultures that have been exposed to relatively little stress over large numbers of generations (Lakeman *et al.* 2009; Wang *et al.* 2012).

As H_2O_2 is a photo reactive agent the toxicity increases when exposed to continuous light compared to continuous dark (Mikula *et al.* 2012). The potency of H_2O_2 to *Microcystis* also rises when irradiance increases, seeing a 5-fold toxicity in relative low light (10 µmol m^{-2} s^{-1}) and up to 20 times that in relative high light (500 µmol m^{-2} s^{-1}) when compared to that of dark periods (Drábková *et al.* 2007*b*). Within a cell H_2O_2 induced intracellular ROS starts an apoptotic like cell death by degrading and condensing DNA and increasing caspase-like activity (Bouchard and Purdie 2011; Ding *et al.* 2012; Huo *et al.* 2015). Subsequently *Microcystis* cells undergo physiological and morphological responses through down regulating transcription genes, cytoplasmic vacuolisation, disintegration of thylakoids, decreasing photosynthetic efficiency, reducing general hydrolytic enzyme activity and ultimately cell membrane rupture (Drábková *et al.* 2007*b*; Qian *et al.* 2010; Bouchard and Purdie 2011; Ding *et al.* 2012; Mikula *et al.* 2012; Huo *et al.* 2015).

Although H_2O_2 is effective at reducing *Microcystis* biomass, the consequent lysising of cells provides a new problem through toxic microcystin release into the immediate environment (Lürling *et al.* 2014; Huo *et al.* 2015). In addition if the application of H_2O_2 concentrations is unregulated it can lead to developmental damage or acute toxicity in higher organisms, such as fish and humans (Rach *et al.* 1997; Gaikowski *et al.* 1999; Halliwell *et al.* 2000).

In the United States H_2O_2 can be limited up to 100 mg/L depending on its usage and state, where it is designated for functions such as re-oxygenation of treated water, oxidation of organic residues and oxidation of any reduced products such as Fe(II) and sulphides (NSF/ANSI 60, USA, 2011). Here in the U.K. the Environmental Agency (EA) has set an operational H_2O_2 residual limit of 20 mg/L for isolated pollution incidents or recommends that an average concentration does not exceed applications of 1 mg/L during a 24 hour period (EA, Litton and Henry 1999).

Table 5.2. Summary of *Microcystis* H_2O_2 experiments with different strains and culture parameters. H_2O_2 represent the lowest concentrations (mg/L) that growth was inhibited and time (h) indicates first observations of significant change (* start times of data collection varies between studies).

Author	*Microcystis* strain	Media	Light irradiance µmol m^{-2} s^{-1}	Light cycle Light:dark	Temperature ^{o}C	Initial density cells / mL	H_2O_2 conc. mg/L (µM)	Time* h	Physiological changes (*PS = photosynthetic)*
Drábková *et al.* (2007*b*)	PCC 7806	1:1 Z:BB	10, 40, 120, 500 & 1000	16:8	26 - 28	$5x10^5$	0.27 (7.94)	3	• Reduced PS yield • Decreased oxygen evolution
Qian *et al.* (2010)	Academy of Sciences China (Code: 905)	BG-11	56	14:10	Unknown	Unknown	0.85 (25)	6	• Enhanced ROS production • Down regulated transcription • Reduced anti-oxidant enzymes • Decreased PS pigments
Bouchard and Purdie (2011)	CCAP 1450/16	BG-11	30	12:12	25	$9.7x10^4$	17 (500) *only tested*	24*	• Reduced PS yield • Decreased PS pigments • Induced ROS & membrane injury • Increased caspase like proteins • DNA fragmentation
Ding *et al.* (2012)	FACHB-905	BG-11	20 - 25	12:12	25	$8x10^6$	5.1 (150)	3	• Membrane deformation • Disintegration of thylakoids • Nucleoid condensation • Increased caspase like proteins • DNA fragmentation
Mikula *et al.* (2012)	UTEX B 2667	1:1 Z:BB	140	24:00 (*continuous light and dark*)	21	$1x10^6$	2 (58.8)	6	• Induced membrane injury • Decreased hydrolytic enzymes • Reduced PS yield
Lürling *et al.* (2014)	PCC 7820	WC	100	12:12	23	$3.8x10^6$	4 (117.6)	4	• Reduced PS yield • Increased dissolved toxins • Change toxin composition
Huo *et al.* (2015)	PCC 7820	ASM	103	12:12	25	$6x10^4$ - $7x10^5$	10 (293.9)	2.5	• Induced ROS & membrane injury • Released toxins

5.1.3 Allelopathy and natural compounds controlling *Microcystis* growth

The discovery of compounds derived from natural sources can potentially be an environmentally friendly substitute for chemical agents containing toxic metals, such as copper and nickel (Table 5.1). Natural compounds released by organisms as found in the decomposition components of barley straw (*Hordeum vulgare*) have recorded impacts on *Microcystis* growth (Newman and Barrett 1993; Martin and Ridge 1999; Ball *et al.* 2001; Ferrier *et al.* 2005; Purcell *et al.* 2013), albeit the inhibitory effect is more algalstatic rather than algalcidal (Oberholster *et al.* 2008; Murray *et al.* 2010).

The exact compound(s) or cellular responses in barley straw that lead to lower *Microcystis* growth rates have yet to be characterised. So far identification of allelochemicals ranges from phenolics and quinones to carboxylic acids (Pillinger *et al.* 1994; Everall and Lees 1997; Nakai *et al.* 2001; Waybright *et al.* 2009). Lignins, a class of organic polymers (an important structural material in seed producing plants) are claimed to be the main source of phenolic acids and quinones (Ó hUallacháin and Fenton 2010), whereby oxidative breakdown from plant material like barley straw is a key component for inhibiting growth in phytoplankton (Everall and Lees 1997; Martin and Ridge 1999).

From Everall and Lees (1997) *in-situ reservoir* experiment (Linacre Bottom, Derbyshire, UK) after 12 days a number of trace organic compounds were specifically identified from decomposing barley straw (Table 5.3). Although a number of those compounds were found to be phenols, numerous carboxyl acids were featured, including acetic acid. Despite the push for natural allelopathic algalcides there is always a need to balance the economical aspect, especially when dealing in large quantities of fresh water for numerous stakeholders.

As acetic acid is relatively cheap and has been effective in controlling agricultural weeds (Fausey 2003; Spencer *et al.* 2003; Moran and Greensburg 2008; Abouziena *et al.* 2009) it has the potential to be a chemical control agent against photosynthetic microbes in drinking water sources. To the authors knowledge no comprehensive literature has be found on any carboxyl acid and its effect on phytoplankton populations. Nor has there been any evidence found of a cellular response in cyanobacteria to exposures of acetic acid.

Table 5.3. Trace organic compounds identified from 12 day old decomposing barley straw in Linacre Bottom reservoir, Derbyshire, UK (including acetic acid), adapted from Everall and Lees (1997).

Compound	Maximum concentration (µg / kg)	Compound	Maximum concentration (µg / kg)
Hexanoic acid	1000	**Acetic Acid**	**100**
Octanoic acid	1000	Heptanoic acid	100
Nonanoic acid	1000	(1, 1-Dimethylethyl)-4-methoxyphenol	100
Decanoic acid	1000	2-Methylbutanoic acid	100
Dodecanoic acid	1000	3-Methylbutanoic acid	100
Tetradecanoic acid	1000		
Hexadecanoic acid	1000	*11 unidentified organics*	1000
1-Methylnaphthalene	1000		
2-(1, 1-Dimethylethyl) phenol	1000		
2,6-Dimethoxy-4-(2-propenyl) phenol	1000		
2,3-Dihydrobenzofuron	1000		
5,6,7,7A-Tetrahydro-4,4,7A -trimethyl-2 (4H) benzofuranone	1000		
1,1,4,4-Tetramethyl-2,6 -bis(methylene) cyclohexone	1000		
1 -Hexacosene	1000		

5.1.4 Acetic acid as a potential *Microcystis* control

Acetic acid or ethanoic acid (CH_3COOH) is a colourless organic compound and is the second simplest carboxylic acid, consisting of an acetyl and hydroxyl group. In its purest form anhydrous (water free) acetic acid (> 99.8%) is referred to as glacial acetic acid, which solidifies at below room temperature (16.75^{o}C) to a colourless crystalline solid (Le Berre *et al.* 2014).

Acetic acid is produced synthetically or by bacterial fermentation through converting carbohydrates directly to acetic acid or indirectly via ethanol metabolism. A variety of bacteria can produce acetic acid, with the genus *Acetobacter* (*Gluconacetobacter*) being the main producer in aerobic fermentation and *Clostridia* via the anaerobic process (Raspor and Goranovič, 2008). Although microbial fermentation is essential in the production for human consumption most of this organic compound used in industry is produced synthetically. Synthetic routes include liquid phase oxidation of acetaldehyde, butane and naphtha or the main choice for large scale production, methanol carbonylation (Le Berre *et al.* 2014).

Acetic acid is an important chemical reagent, descaling agent, food additive (human and animals) and condiment, approved by many countries including the United States and large governing bodies like the European Union (EC, No. 1831/2003; EC, No. 1333/2008; FDA, USA, 2015). In the water industry acetic acid has been used to treat aquatic systems with high pH levels. These 'acid injections' lower the pH of water and can eliminate bad tastes.

Acetic acid is preferred to other high pH substance like alum and citric acid because of its expense and to other compounds like sulphuric or hydrochloric acid as they are hazardous and require specialist training. High pH and alkaline environments play an important role in the distribution and dominance of cyanobacterial genera such as *Microcystis* (Carvalho *et al.* 2011; Maileht *et al.* 2013). Therefore reducing pH levels could potentially inhibit *Microcystis* growth or reduce their persistence / dominance.

In the United States acetic acid has been certified for drinking water treatment to function as a reducing agent in biologically active drinking water systems (NSF/ANSI 60, USA, 2011), with concentrations ranging from 150 – 300 mg/L. Acetic acid also makes it on the list of approved products to use in UK public water supplies, with its defined usage as '*a carbon source for biological denitrification'* but gives no guidelines on concentrations for application (DWI 2016). Acetic acid easily dissociates in water, especially compared to other plant decomposition compounds such as phenols. It rapidly degrades in the environment where it can evaporate from aquatic sources, making it a potential low-residing *Microcystis* control chemical.

5.1.5 Acetic acid as a photo-toxic compound

Acetic acid has been proven to be a successful herbicide against bryozoans and higher plants such as liverwort, marchantia (*Marchantia polymorpha*), silver thread moss (*Bryum arenteum*), American pondweed (*Potamogeton nodsus*), sunflower (*Helianthus annus*), Palmer amaranth (*Amaranthus palmeri*), common purslane (*Portulaca oleracea*) and a number of broadleaf weeds (Fausey 2003; Spencer *et al.* 2003; Moran and Greensburg 2008; Abouziena *et al.* 2009). However, effectiveness varied in species through concentration, volume and application timing, in addition to recording a lack of selectivity reducing non-targeted terrestrial crop plants (Moran and Greensburg 2008). The effectiveness of acetic acid on phytoplankton is unknown and could potentially reduce algal growth as it has done in terrestrial autotrophs. As no cyanobacterial studies have provided evidence of growth inhibition from acetic acid, any potential mortality mechanisms also remains unclear.

Antimicrobial action from organic acids is believed to be the ability of a dissociated acid to cross biological membranes and release protons within a cell (Salmond *et al.* 1984; Brul and Coote 1999). Having lowered cellular pH levels high energy would be required to remove protons (excessive ATP usage), resulting in mortality (Booth 1985; Axe and Bailey 1995). Alternatively, acid anions may accumulate inside a cell altering the intracellular ionic strength, leading to a disruption of primary metabolic activity (Russell 1992; Diez-Gonzalez and Russell 1997). Although the previous examples centre on heterotrophic bacteria, especially *Escherichia coli*, other micro-organisms like yeast (*Saccharomyces cerevisiae*) have seen a ROS response from acetic acid inducing programmed cell death (PCD) (Ludovico *et al.* 2001; Guaragnella *et al.* 2007).

As acetic acid can naturally degrade in environmental water bodies and has been proven to affect higher plants, it can potentially provide a novel chemical control measure for cyanobacteria. Here a study assessed the growth inhibition of *Microcystis* through exposure to various acetic acid concentrations and compared it to another established control method, hydrogen peroxide. In addition to biomass changes, functional cell markers helped identify modes of death through the FCM probes SYTOX® Green and CM-H_2DCFDA (Section 2.6). For the first time in cyanobacteria, detection of cell membrane permeation and reactive oxygen species characterised the mechanics of mortality induced by acetic acid.

5.2 Methodology

5.2.1 Culture conditions

As there have been questions surrounding the effectiveness of H_2O_2 on environmental *Microcystis* compared to established stock culture lines (Lakeman *et al.* 2009; Wang *et al.* 2012) this study used the freshly isolated *Microcystis* sp. CCAP 1450/17 (Hartnell *et al.* 2016). All samples were cultivated in 250ml Erlenmeyer flasks (triplicates) at a total volume of 100mL in BG-11 cyanobacterial growth media (Sigma-Aldrich, UK) and agitated by hand once a day.

The pre-sampled stock cultures were kept at exponential phase densities (≈$1x10^6$ - $8x10^6$ cells/mL) and exposed to incubated conditions on a 12 hour light / dark cycle at 25±1°C (Conviron, CMP6010, Canada). The irradiance source was a single 58 watt cool-white fluorescent tube, illuminated at a 49±2 µE m^{-2} s^{-1}, which was measured by a PAR scalar irradiance sensor (Biospherical Instruments Inc., US).

5.2.2 Experimental design

To assess the effects of H_2O_2 and acetic acid on a cyanobacterium species both compounds were introduced to *Microcystis* sp. CCAP 1450/17 during a short term experiment. Initial populations at exponential levels (approximately $2x10^6$ cells/mL) were exposed to a number of concentrations under two different light conditions. Irradiance cycle i) represented a diurnal light regime, which had a 12 hour light dark cycle as per parameters in Section 5.2.1 and irradiance cycle number ii) which had a continuous dark cycle, where samples were placed into a homemade dark box under the same incubated conditions as Section 5.2.1.

H_2O_2 (30%, Fisher Scientific, UK) and acetic acid (99.7%, Sigma-Aldrich, UK) were added to *Microcystis* cultures to obtain various concentrations (similar to Mikula *et al.* 2012) under both light and dark conditions (Table 5.4). Ultra-pure filtered water (0.22µm) was added to the control sample at a volume of 4.92mL, corresponding to the total volume that each chemical was diluted for both H_2O_2 and acetic acid experiments. The effects of both chemicals were investigated over three days with cell numbers, membrane integrity and reactive oxygen species (ROS) recorded over periods of 0, 2, 8, 24, 48 and 72 hours.

Table 5.4. Concentrations of H_2O_2 and acetic acid used in this study. *Microcystis* sp. (CCAP 1450/17) samples were exposed to 0, 1, 4, 10 and 40 mg/L under light conditions and multiplied by a factor of ten for continuous dark conditions.

Concentration (mg/L)	H_2O_2 (µM)	Acetic Acid (µM)
0 (control)	0	0
1	29.4	16.65
4	117.6	66.61
10	293.99	166.53
40	1175.96	666.11
100	2939.91	1665.28
400	11759.62	6661.12

5.2.3 Cell quantification and physiological state analysis

During preliminary experiments exposure to acetic acid at high concentrations caused *Microcystis* cells to exhibit a visible degradation of green pigment (assumed to be chlorophyll *a*), leaving a light blue coloured population (Figure 5.1). As the study incorporated molecular probes and there was the potential for natural fluorochrome degradation (e.g. chlorophyll *a*, FL3-H or phycocyanin, FL4-H) only FSC-H and SSC-H discrimination was used for cell quantification, with an analysis time of 2 min on the slow setting (Chapter 2).

To measure cell membrane integrity a nucleic acid indicator SYTOX® Green (Invitrogen, USA, S7020) was used at a concentration of 5 µM incubated under dark for 30mins (Chapman *et al.* 2016). In a separate test CM-H_2DCFDA (Invitrogen, USA, C6827) a general oxidative stress indicator was used and incubated in the dark for 60 min at concentrations of 25 µM (Section 2.6). Culture pH levels were also tested at each time point (IP67 3-point calibration, 0.1 pH resolution, Hach, USA).

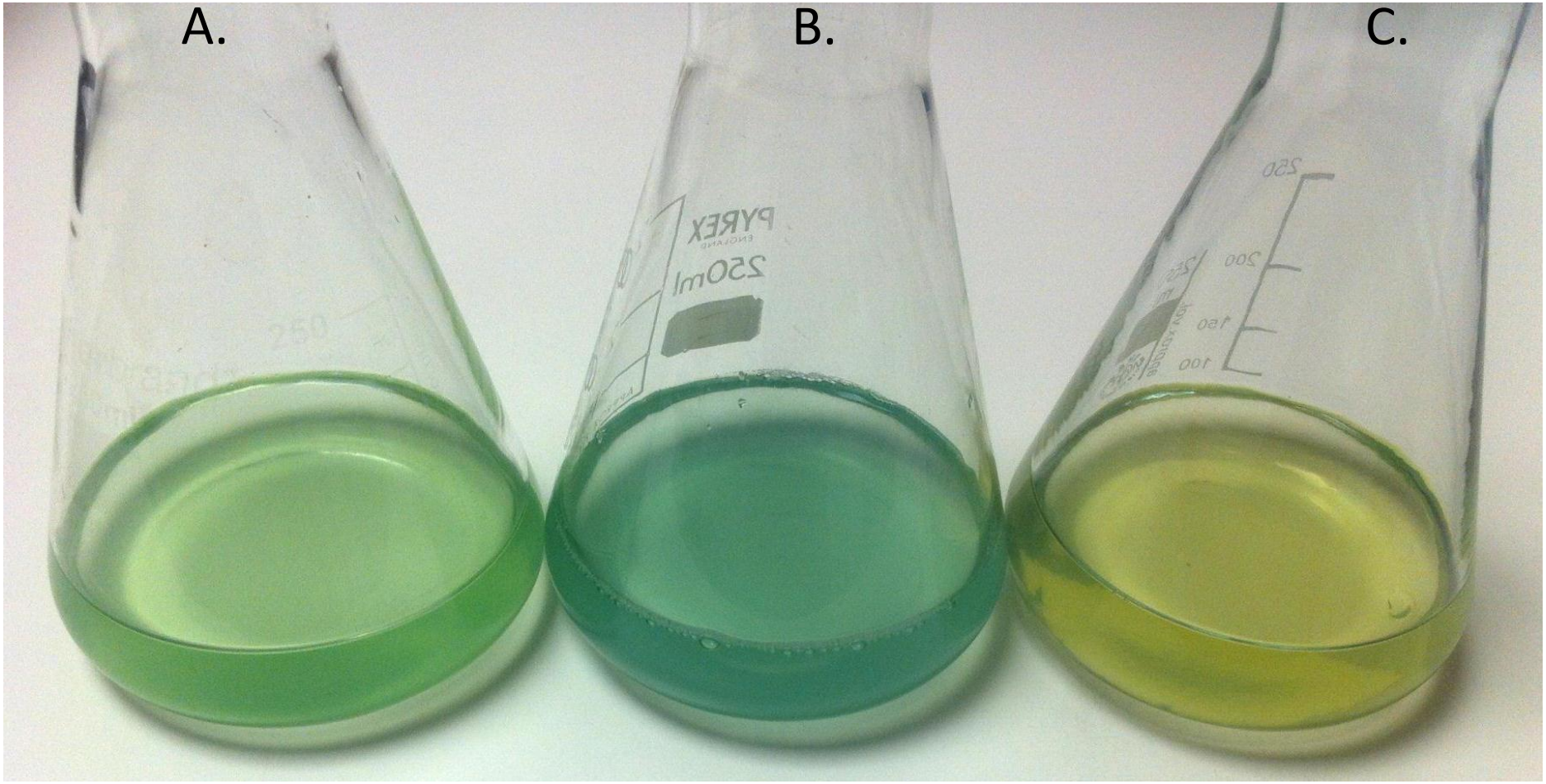


Figure 5.1. *Microcystis* cultures after exposure to A) 0 mg/L of chemicals (control), B) 40 mg/L of acetic acid and C) 40 mg/L of H_2O_2, under light conditions after 72 hours.

5.2.4 Statistical analysis

To analyse the effect of H_2O_2 and acetic acid a Kruskal-Wallis one-way ANOVA on ranks was applied to cell numbers, the % of SYTOX® Green labelled cells and the % of CM-H_2DCFDA labelled cells between all concentrations (including control), using the results from over all the experimental time periods (0-72 h). Any differences found between the means were evaluated by a *post hoc* analysis at $p < 0.05$. All data was processed using SigmaPlot 13.0 (Systat Software, San Jose, CA) for windows.

5.3 Results

5.3.1 Hydrogen peroxide and cell counts

Light conditions

A Kruskal-Wallis One-Way ANOVA on ranks reported a statistical difference in cell numbers over the experimental period (0-72 h) between concentrations under light condition ($p < 0.001$) (Table 5.5). A pairwise multiple comparison *post hoc* test revealed two sub groups i) 0 and 1 mg/L and ii) 4, 10 and 40 mg/L.

Table 5.5. Kruskal-Wallis one-way ANOVA results for differences in cell densities, the % of molecular probe labelled cells and pH levels between all concentrations of H_2O_2 in light conditions. Homogeneous subgroups were reported through a Tukey's *post hoc* test and indicated by alphabetical symbols (a, b and c).

Concentration (mg/L)	Cell count (cells/mL)		CM-H_2DCFDA-labelled (% population)		SYTOX®-labelled (% population)		pH	
0	a		a		a		a	
1	a		a		a		a	
4	b		a		a, b		a, b	
10	b		b		b, c		b, c	
40	b		b		c		c	
One-way ANOVA	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
(d.f. = 4)	49.42	< 0.001	35.84	< 0.001	33.09	< 0.001	36.93	< 0.001

Cell numbers increased by 178% and 172% over 72 h in both 0 and 1 mg/L cultures respectively, but decreased by a combined mean of 83±4% in 4, 10 and 40 mg/L of H_2O_2 (Figure 5.2 A). Although 4, 10 and 40 mg/L concentrations did reduce *Microcystis* numbers, 40 mg/L had the highest mortality rate of 99±0.4% compared to a combined mean 75.1±1.3% for both 4 and 10 mg/L (Appendix E1). In all concentrations, cell numbers increased after 2 h and it was only after 8 h of H_2O_2 incubation that the concentrations inhibiting *Microcystis* growth (4, 10 and 40 mg/L) started to reduce biomass (Figure 5.2 A).

During testing in light conditions 40 mg/L of H_2O_2 turned cultures from a green to a yellow colour (Figure 5.1), however, in 4 and 10 mg/L which also recorded a reduction of *Microcystis* biomass no colour change was observed.

Dark conditions

Under constant darkness there was a significant difference in cell numbers between concentrations over the experiment ($p < 0.001$) (Table 5.6). A pairwise multiple comparison analysis again reported two sub groups i) 0 and 10 mg/L and ii) 40, 100 and 400 mg/L.

Table 5.6. Kruskal-Wallis one-way ANOVA results for differences in cell densities, the % of molecular probe labelled cells and pH levels between all concentrations of H_2O_2 in dark conditions. Homogeneous subgroups were reported through a Tukey's *post hoc* test and indicated by alphabetical symbols (a, b and c).

Concentration	Cell count		CM-H_2DCFDA-labelled		SYTOX®-labelled		pH	
(mg/L)	(cells/mL)		(% population)		(% population)			
0	a		a		a		a	
10	a		a		a		a	
40	b		b		b		a	
100	b		c		b		a	
400	b		c		b		a	
One-way ANOVA	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
(d.f. = 4)	28.39	< 0.001	28.99	< 0.001	39.79	< 0.001	3.73	> 0.05

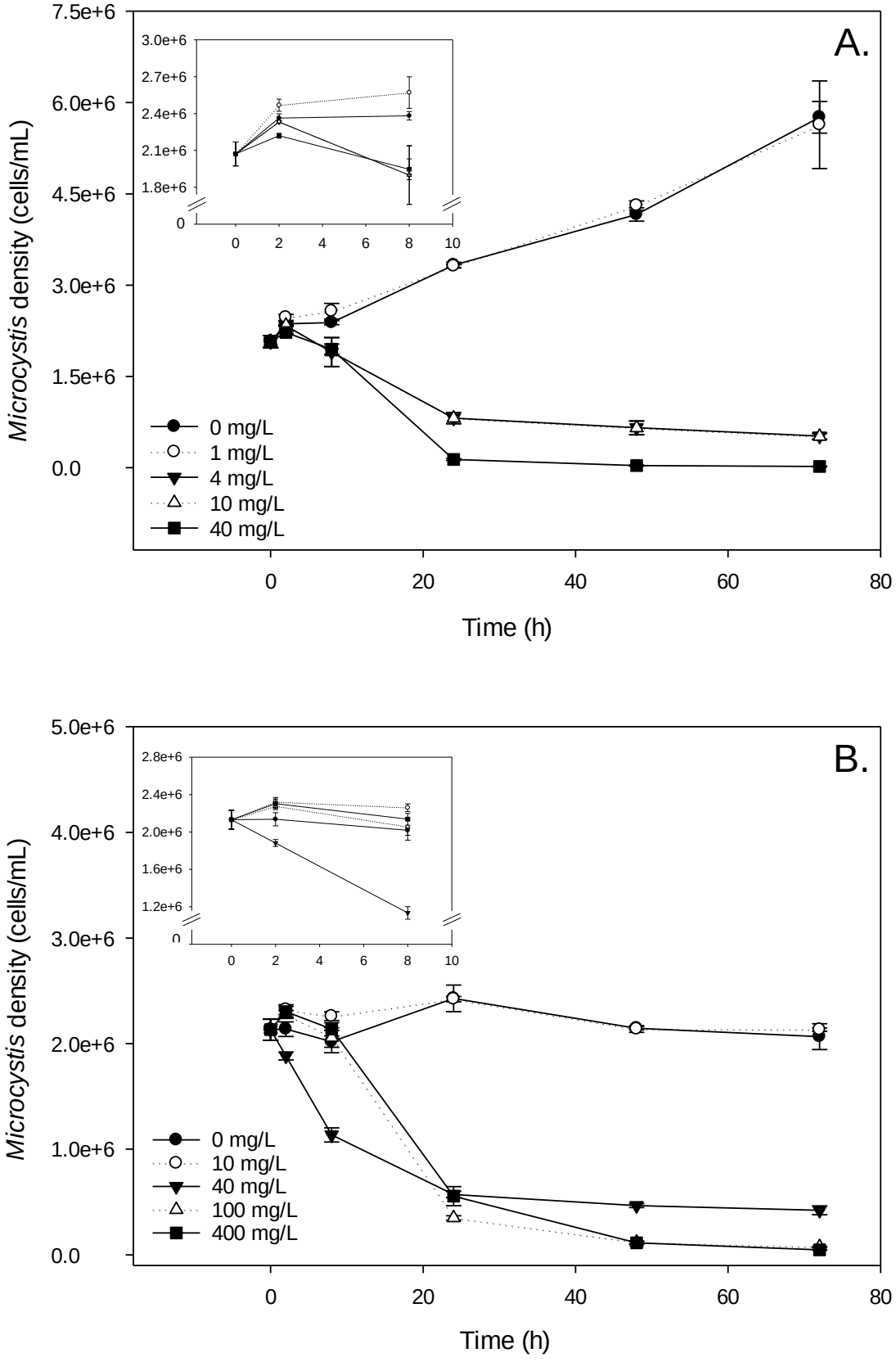


Figure 5.2. *Microcystis* cell counts (± S.E. bars) after exposure to different concentrations of H_2O_2 under A) light and B) continuous dark conditions over 72 h. The smaller inserts represent the first 8 h of H_2O_2 incubation.

Cell numbers for 0 and 10 mg/L were relatively unchanged over 72 h with a 3-4% decrease in *Microcystis* densities (Figure 5.2 B). The higher concentrations (40, 100 and 400 mg/L) recorded a greater reduction in *Microcystis* numbers with 40 mg/L recording at decrease of 80±2.6% and 100 and 400 mg/L both having over 96% less cells at 72 h compared to 0 h (Appendix E2). An increase in cell numbers after 2 h was recorded in all but 40 mg/L. After 8 h the two highest concentrations (100 and 400 mg/L) recorded a small decrease between 2–5%, with 40 mg/L exhibiting the largest decrease, 39.5±4.4% (Figure 5.2 B).

The lowest concentrations (0 and 10 mg/L) recorded an increase at 24 h, before a slight decrease until the end of the experiment. Whereas the higher concentrations (40, 100 and 400 mg/L) continued to see a reduction in cell numbers, with 100 and 400 mg/L nearly recording a complete population mortality at 72 h. As with the light experiment *Microcystis* cultures turned from a green to a yellow colour in constant darkness. However, under continuous dark conditions it took ≥ 100 mg/L H_2O_2 to confer a colour change, instead of 40 mg/L as found when cultures were incubated under light.

5.3.2 Hydrogen peroxide and reactive oxygen species

Light conditions

To determine the percentage of cells in a population with ROS, CM-H_2DCFDA was used to examine the effect of H_2O_2 on *Microcystis* along a concentration gradient. In light conditions the percentage of cells with ROS significantly differed between concentrations of H_2O_2 over 72 h ($p < 0.001$) (Table 5.5). Using a pairwise multiple comparison *post hoc* test reported two sub groups with i) 0 and 1 mg/L and ii) 4, 10 and 40 mg/L concentrations.

For the lowest concentrations ROS activity started at 4 mg/L after 8 h where the % of labelled cells was 11.1 time higher than the control, CM-H_2DCFDA signals decreased at 24 h then further increased at 48 and 72 h, recording a final total of 6.3±0.6% (Appendix E1). A similar pattern was observed in 10 mg/L, with 22.7±1.2% of the total population recording ROS activity at 72 h. The highest percentage population of ROS was recorded at 2 h in 40 mg/L with 32.1±4.5%, which decreased to 15.0±0.8% at 8 h and declined further until the end of the experiment as most *Microcystis* cells were dead (Figure 5.3 A).

Dark conditions

Under constant dark conditions there was a significant difference in the ROS % signals between concentrations over 72 h ($p < 0.001$) (Table 5.6). Compared to cultures with irradiance the induction of ROS required much higher concentrations. It was only in 40 mg/L at 8 h that ROS was seen to record a relative increase compared to the control (44 times higher), but it only represented 4.1±1.6% of the total population (Appendix E2). The highest level of ROS activity as % population was recorded in 400 mg/L at 2 h with 18.03±0.36% (Figure 5.3 B), however, this peak ROS activity was still 44% less than cultures under light conditions at ten times the concentration.

5.3.3 Hydrogen peroxide and membrane integrity

Light conditions

FCM provided measurements that cell membrane permeability can be detected in *Microcystis* when exposed to H_2O_2 using SYTOX® Green. In light conditions there was a significant difference in the membrane permeable population (%) between different concentrations of H_2O_2 over 72 h ($p < 0.001$) (Table 5.5). The highest recordings of % SYTOX® Green labelled cells under light conditions was found during exposures to 40 mg/L at 8 h with 89.5±1.4%, where membrane permeation was induced at 2 h with over a third (36.8±6.27%) of the population recording a positive fluorescence signal (Figure 5.3 C).

Dark conditions

During the dark experiment the % of SYTOX® Green labelled cells significantly differed between concentrations of H_2O_2 over 72 h ($p < 0.001$) (Table 5.6). For the lowest concentrations there was a 5.9 times higher % of SYTOX® Green labelled cells in 10 mg/L compared to the control at 24 h (1.67±1.14% total population) but it wasn't until 24 h in 40 mg/L that recorded a dramatic increase in cell membrane permeation (66.52±3.08%), which subsequently declined to 18.23±1.05% at 72 h (Figure 5.3 D). The highest % of SYTOX® labelled cells were found in 100 mg/L and 400mg/L concentrations at 8 h and 2 h respectively, with approximately 77% of the population recording a positive fluorescence signal (Appendix E2).

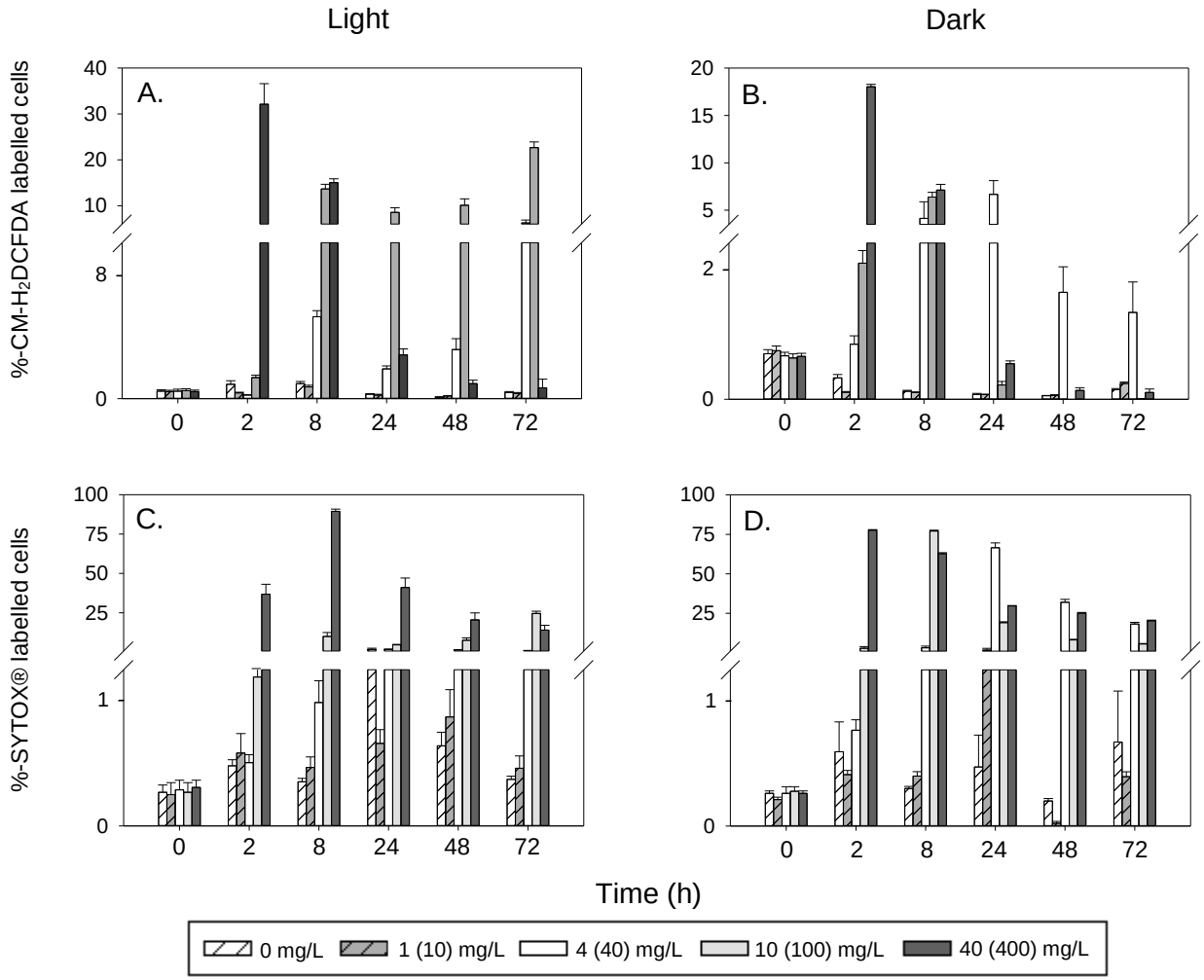


Figure 5.3. Positive ROS fluorescence from *Microcystis* when exposed to H_2O_2 under A) light and B) continuous dark conditions and SYTOX® Green under C) light and D) continuous dark conditions. Figures in brackets are from the dark experiments at tenfold concentrations.

5.3.4 Acetic acid and cell counts

Light conditions

Exposing *Microcystis* to different concentrations of acetic acid during light conditions did report a significant difference in cell numbers over 72 h ($p < 0.001$) (Table 5.7). A pairwise multiple comparison revealed two sub groups with i) the control and three lowest concentration (0, 1, 4, and 10 mg/L) and ii) 40 mg/L.

Microcystis densities showed a combined mean increase of 166±6.1% in all but 40 mg/L at 72 h. The application of 40 mg/L was the only concentration to see a cell number decrease with an 87.4±3.5% reduction from its original density by the end of the experiment (Appendix E3). All concentrations recorded an initial increase after 2 h which continued to 8 h in concentrations between 0 – 10 mg/L (8.7±1.2% - 13.4±2.4%) and to a lesser extent in 40 mg/L (0.15±2.9%) (Figure 5.4 A). It was only after 24 h of acetic acid exposure that *Microcystis* numbers started to reduce in concentrations of 40 mg/L, where populations continued to decrease until the end of the experiment with a final count of $2.7x10^5 \pm 7.0x10^4$ cells/mL.

Table 5.7. Kruskal-Wallis one-way ANOVA results for differences in cell densities, the % of molecular probe labelled cells and pH levels between all concentrations of acetic acid in light conditions. Homogeneous subgroups were reported through a Tukey's *post hoc* test and indicated by alphabetical symbols (a, b and c).

Concentration (mg/L)	Cell count (cells/mL)		CM-H_2DCFDA-labelled (% population)		SYTOX®-labelled (% population)		pH	
0	a		a		a		a	
1	a		a		a		a	
4	a		a		a		a	
10	a		b		a, b		a	
40	b		b		b		b	
One-way ANOVA	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
(d.f. = 4)	27.71	< 0.001	61.92	< 0.001	26.41	< 0.001	34.62	< 0.001

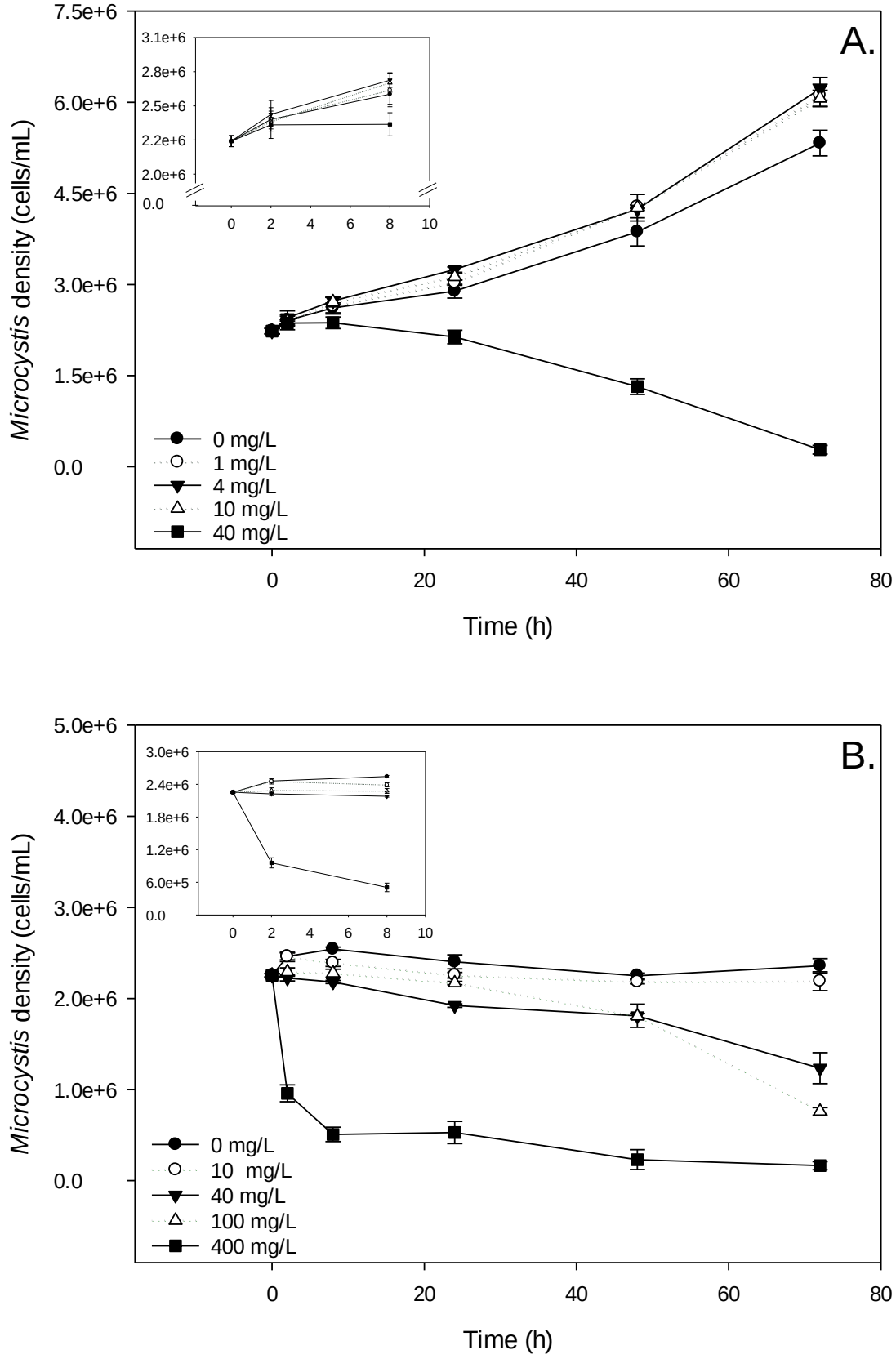


Figure 5.4. *Microcystis* cell counts (± S.E. bars) after exposure to different concentrations of acetic acid under A) light and B) continuous dark conditions over 72 h. The smaller inserts represent the first 8 h of H_2O_2 incubation.

Dark conditions

During continuous darkness cell numbers significantly differed between acetic acid concentrations over the experiment ($p < 0.001$) (Table 5.8). Similar to the light conditions only $\geq$ 40 mg/L of acetic acid had an impact on *Microcystis* growth (Figure 5.4 B). At 72 h only the control (0 mg/L) showed an increase (4.7±4.3%), with 10 mg/L exhibiting a relatively small decrease of 2.9±4.2% and 40, 100 and 400 mg/L recording a much larger decrease of 45.3±7.6%, 66.3±2.1% and 81.4±1.2% respectively (Appendix E4).

Table 5.8. Kruskal-Wallis one-way ANOVA results for differences in cell densities, the % of molecular probe labelled cells and pH levels between all concentrations of acetic acid in dark conditions. Homogeneous subgroups were reported through a Tukey's *post hoc* test and indicated by alphabetical symbols (a, b and c).

Concentration (mg/L)	Cell count (cells/mL)		CM-H_2DCFDA-labelled (% population)		SYTOX®-labelled (% population)		pH	
0	a		a		a		a	
10	a		a		a		a	
40	b		b		b		b	
100	b		b, c		b		b	
400	b		c		b		b	
One-way ANOVA	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
(d.f. = 4)	48.15	< 0.001	48.65	< 0.001	57.95	< 0.001	14.85	< 0.001

At 2 h only 400 mg/L saw a substantial decrease with a reduction of 56.7±4.1% from its initial densities. All other concentrations (0 - 40 mg/L) actually recorded a mean increase with a combined 4.7±1.9% growth in numbers. It was only after 8 h that 40 and 100 mg/L showed a continuous drop in cell numbers compared to their original densities. An acetic acid concentration of 10 mg/L did show an eventual decrease in numbers at 72 h, although only 2.6±1.8% less than that of 0 h (Figure 5.4 B). In both light and dark conditions only $\geq$ 40 mg/L inhibited *Microcystis* growth. As with H_2O_2, acetic acid also showed a culture colour change with exposures of $\geq$ 40 mg/L turning samples from a green colour to a light blue colour (Figure 5.1).

5.3.5 Acetic acid and reactive oxygen species

Light conditions

As with H_2O_2, CM-H_2DCFDA was used to record the percentage of a *Microcystis* population that had generated ROS when exposed to acetic acid. Under light conditions the percentage of ROS per *Microcystis* population over the experiment was significantly different between concentrations of acetic acid ($p < 0.001$) (Table 5.7), with a *post hoc* test reporting two sub groups i) 0, 1 and 4 mg/L and ii) 10 and 40 mg/L.

The first sign of ROS generation with a significant % of the population labelled (>10%) was seen at 2 h from 10 and 40 mg/L. Acetic acid concentrations at 40 mg/L recorded the highest %-CM-H_2DCFDA labelled cells with 71.3±3.0% at 2 h which decreased until 72 h. Exposures of 0, 1 and 4 mg/L did not record > 2% of ROS signals during any point of the experiment (Appendix E3).

Dark conditions

During continuous darkness, unlike H_2O_2, acetic acid did not require a higher concentration to record an increase in ROS generation. There were significant differences in the %-CM-H_2DCFDA labelled cells between the concentrations over 72 h ($p < 0.001$) (Appendix E4). At 40 mg/L it took 2 h to record a relatively high increase compared to the control, equating to 64.0±0.9% of the total population. The ROS % population then decreased to approximately 37% at both 8 h and 24 h, which continued to decline until the end of the experiment (Figure 5.5 B). Interestingly there was only a relatively small amount of ROS at higher concentrations of acetic acid, recording the highest total %-CM-H_2DCFDA labelled cells of 12.6±0.2% at 2 h for 100mg/L and 7.1±1.2% at 48 h for 400 mg/L (Appendix E4).

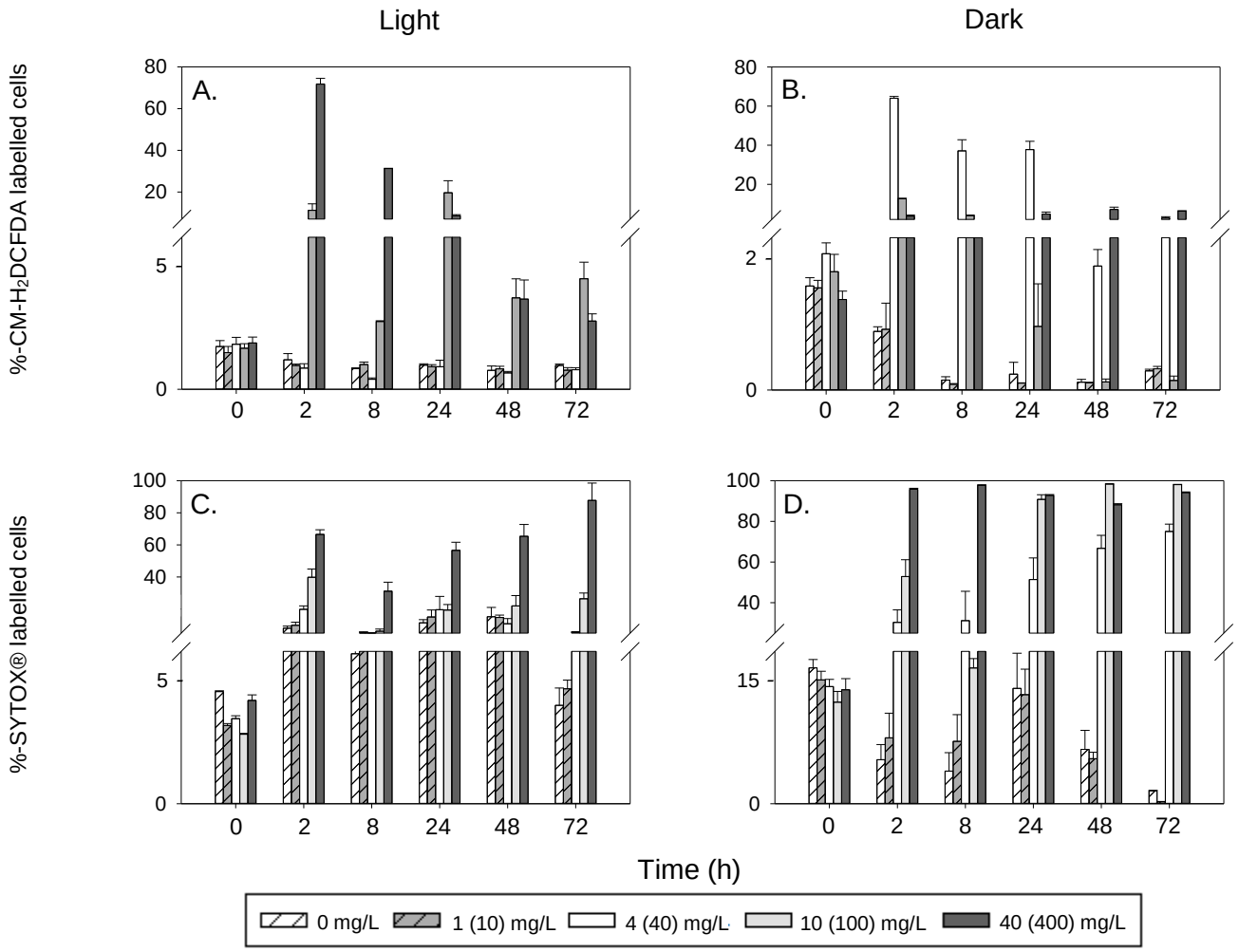


Figure 5.5. Positive ROS fluorescence from *Microcystis* when exposed to acetic acid under A) light and B) continuous dark conditions and SYTOX® Green under C) light and D) continuous dark conditions. Figures in brackets are from the dark experiments at tenfold concentrations

5.3.6 Acetic acid and membrane integrity

Light condition

When exposed to acetic acid SYTOX® Green was able to produce good fluorescent discrimination for membrane permeated *Microcystis* compared to membrane intact cells. Under light conditions the population of membrane permeated cells (%-SYTOX® labelled cells) was significantly different between concentrations over the experiment ($p < 0.001$) (Appendix E3).

Membrane permeability analysed through SYTOX® Green labelling increased with acetic acid concentration. The control (0 mg/L) had a total mean SYTOX® Green population of 7.6±1.4% which increased with concentration to a maxima total mean of 51.8±7.0% recorded in 40 mg/L (Appendix E3).

Compared to the control SYTOX® Green fluorescence recorded a relative increase at 2 h in 4, 10 and 40 mg/L acetic acid concentrations. At 8 h the membrane permeated populations decreased in all concentrations but still remained high in 40 mg/L, representing 31.2±5.7% of the total population (Figure 5.5 C). The highest % of SYTOX® labelled cells was recorded in 40 mg/L at 72 h with 87.8±10.8%.

Dark conditions

Under continuous dark conditions there was a significant difference between concentrations of acetic acid on membrane permeability over 72 h ($p < 0.001$) (Table 5.8). Only in concentrations of ≥ 40 mg/L did SYTOX® Green signals increase beyond that of the control (Appendix E4). The highest incidence of SYTOX® Green signals in 40, 100 and 400 mg/L was at 72 h, where SYTOX® Green populations were 47, 61 and 58 times higher than the control respectively. The mean %-SYTOX® Green labelled cells again appeared to increase with concentration reporting that 40, 100 and 400 mg/L had means of 45.3±5.8%, 62.3±8.7% and 81.0±7.0% respectively across the experiment (Figure 5.5 D).

5.3.7 pH during chemical treatment

H_2O_2 exposure

Hydrogen peroxide exposure significantly changed the pH levels between concentrations during light conditions over 72 h ($p < 0.001$) (Figure 5.5). The control cultures (0 mg/L) initially recorded a pH of 8.5±0.1 which increased to 10.3±0.2 at 72 h (Appendix E5). The higher concentrations of 10 mg/L and 40 mg/L did not exceed pH levels of 8.7±0.1 at any point during the experiment, recording pH means of ≤ 1.8 compared to 0, 1 and 4 mg/L over a 72 h period (Figure 5.6 A).

Under constant darkness pH levels did not significantly change across H_2O_2 concentrations ($p > 0.05$). Initial pH levels in the control declined by 0.9 from a pH of 8.6±0.1 at 2 h, which continued to drop until its lowest pH level of 7.0±0.1 at 72 h (Figure 5.6 B). A similar trend was recorded in the other concentrations by 72 h, all of which were much lower than tests carried out under light conditions (Appendix E5).

Acetic acid exposure

In experiments using acetic acid during light conditions it was reported that a significant difference was found between concentrations in pH levels over the experiment ($p < 0.001$) (Table 5.7). A *post hoc* test identified two sub groups, i) 40 mg/L and ii) all other concentrations (including the control). The control initially started at a pH level of 8.6±0.1 and increased to 10.5±0.0, with a similar pattern observed in 1, 4 and 10 mg/L (Figure 5.6 C). It was only the highest concentration under light conditions (40 mg/L) that showed a pH decrease, with pH 4.8±0.2 recorded at 72 h (Appendix E5).

Under continuous darkness there was a significant difference in pH levels between concentrations over 72 h ($p < 0.001$) (Table 5.8). All cultures containing acetic acid recorded a decrease in pH levels at 72 h in a dose dependent manner, with 400 mg/L recording the lowest pH levels of 2.8±0.0 at 48 h (Figure 5.6 D). The control, like hydrogen peroxide also continued to decline to a pH level of 7.0±0.1.

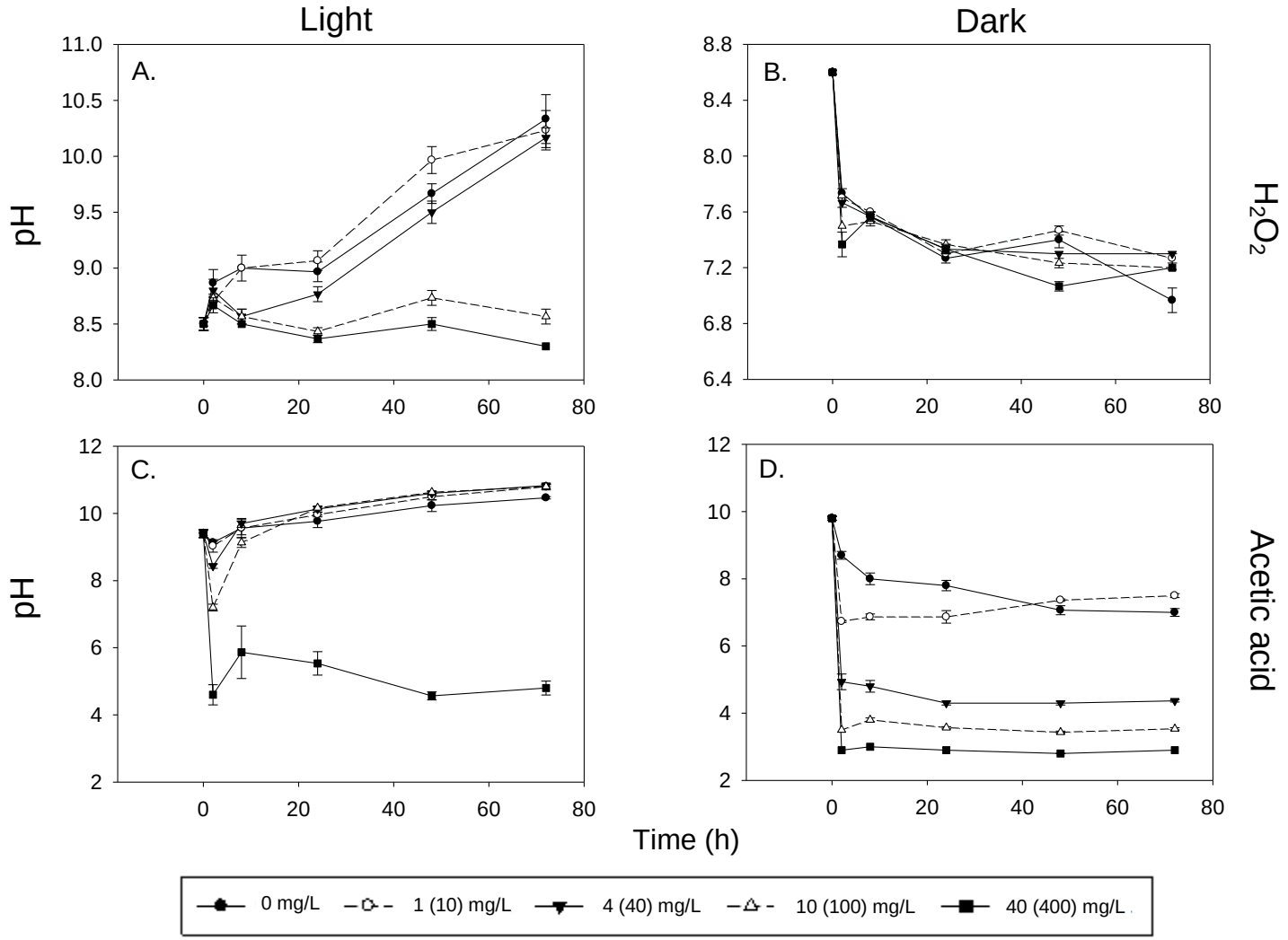


Figure 5.6. pH levels during the experiment when *Microcystis* cultures were exposed to H_2O_2 during A) light and B) continuous dark conditions and acetic acid during C) light and D) continuous dark conditions. Figures in brackets are from the dark experiment at tenfold concentrations.

5.4 Discussion

5.4.1 *Microcystis* growth inhibition in hydrogen peroxide under light conditions

Results from this study show that both hydrogen peroxide and acetic acid did inhibit growth of *Microcystis* populations. The effectiveness of both chemicals depended on dose and for H_2O_2 enhanced by light. Under light conditions in the recently isolated *Microcystis* culture exposure to H_2O_2 recorded toxicity at $\geq$ 4 mg/L. Although biomass was reduced at H_2O_2 concentrations of 4 mg/L and 10 mg/L (≈75% at 72 h) it could be deemed algalstatic instead of algalcidal. Not reducing enough density may present a potential problem, as populations could become tolerant to low H_2O_2 concentrations and eventually recover cell numbers (Qian *et al.* 2010; Mikula *et al.* 2012).

The clarification of what is an acceptable commercial control in terms of biomass reduction is very open to interpretation (FAO 2006). As concentrations of 4 and 10 mg/L H_2O_2 may be designated algalstatic rather than algalcidal in *Microcystis* an extended experimental time period would give clearer results in terms of low concentrations and their efficiencies. Nevertheless, as water supplies can be extremely limited *Microcystis* mortality rates from chemical treatments should have a fast response time (e.g. $\leq$ 24 hours) and any long term studies could be redundant when testing single doses of photosensitising chemicals like H_2O_2, as they would naturally degrade when exposed to light.

In a number of laboratory studies H_2O_2 concentrations ≤10 mg/L have seen complete reduction of *Microcystis* numbers (Table 5.2). However, this study confirms with Wang *et al.* (2012) that recently isolated *Microcystis* spp. need higher concentrations of H_2O_2 than established lines from culture collections. Wang *et al.* (2012) reported that 60 mg/L of H_2O_2 caused population mortality, here densities were reduced to > 93% of initial numbers by using 40 mg/L at 24 h.

Environmental *Microcystis* may have an adaptive advantage against chemical controls through excretion of extracellular polymeric substances (cyanobacterial mats), as it can buffer environmental stresses and also be used as a carbon source (Flemming and Wingender 2010; Stuart *et al.* 2015). Therefore a recommendation of no less 40 mg/L of H_2O_2 from a single application would be needed for effective chemical control. However, 40 mg/L would exceed the limit 1 mg/L per 24 hours or even 20 mg/L for isolated incidences recommended by the EA (Litton and Henry 1999).

5.4.2 *Microcystis* growth inhibition in acetic acid under light conditions

Under light conditions acetic acid did have an inhibitory effect on *Microcystis* growth but only in higher concentrations compared to H_2O_2. Although higher concentrations were needed acetic acid did reduce *Microcystis* biomass by 87.4±3.5% at 72 h using the recommended same dose as H_2O_2 (40 mg/L) under experimental conditions.

Acetic acid compared to H_2O_2 may need higher concentrations (≥ 40 mg/L) to reduce *Microcystis* numbers due to a number of factors, i) H_2O_2 is a comparably stronger biological oxidation agent inducing cell death at lower concentrations, as seen in other micro-organisms (Ribeiro *et al.* 2006), ii) A lower pH is needed for acetic acid to dissociate across membranes and alter intracellular ionic strength (Section 5.1.5) or iii) *Microcystis* has a higher tolerance to acetic acid.

In regards to tolerance aldehyde dehydrogenase (ADH) is a very important enzyme contributing to acetic acid resistance in bacteria (Wang *et al.* 2015). Homologous sequences have been found in *Microcystis*, with ADH increasing when exposed to longer light periods (Moezelaar *et al.* 1997; Vidal *et al.* 2009). One or a combination of the previous three factors could be the underlying cause for requiring higher concentrations compared to H_2O_2.

5.4.3 *Microcystis* growth inhibition in hydrogen peroxide under dark conditions

Under continuous darkness H_2O_2 and acetic acid both saw a reduction in *Microcystis* biomass. The concentrations needed for H_2O_2 exposure inhibition was however ten times that of experiments under light conditions. Like previous experiments this study confirms H_2O_2 toxicity on *Microcystis* is highly light dependent (Drábková *et al.* 2007*b*; Mikula *et al.* 2012). As hydroxyl radicals can be generated from photolysis of H_2O_2, one reason for less growth inhibition under dark conditions might be due to the lack of irradiance needed to induce ROS. Another explanation for the lack of H_2O_2 sensitivity in the dark may be facilitated in light conditions during photosynthesis by the Mehler reaction.

The Mehler reaction reduces O_2 to superoxide radicals during photosynthesis and is further converted to H_2O_2 catalysed by superoxide dismutase, subsequently accumulating in cells. The cumulative concentration of H_2O_2 could in theory increase internal oxidative stress leading to acute toxicity (Drábková *et al.* 2007*b*). Therefore, in the absence of light there will be a limitation on the photoreduction of bi-products from

photosynthesis, resulting potentially in a smaller growth inhibition on *Microcystis* populations.

However, recent evidence in *Synechocystis* indicates the Mehler reaction does not produce H_2O_2 or other ROS but is done through highly conserved A-type flavoproteins (Helman *et al.* 2003). Further investigation into cyanobacterial reduction of O_2 to H_2O_2 is therefore necessary on other cyanobacteria (including *Microcystis*) to discount any co-action facilitating acute toxicity from H_2O_2 through the Mehler reaction.

5.4.4 *Microcystis* growth inhibition in acetic acid under dark conditions

Microcystis growth inhibition in constant darkness did occur upon acetic acid exposure but to a lesser extent than in light where 40 mg/L recorded a decrease of 45.3±7.6% compared to 87.4±3.5% under light at 72 h, which may suggest that acetic acid to some extent acts on the electron transfer chain. Further examination of acetic acid on photosynthetic efficiency through different light regimes should be tested using measurements from PSII photochemistry e.g. fluorometry (Fv/Fm). Acetic acid testing on heterotrophic bacteria or mutant cyanobacteria which lack proteins essential for photosystem II (e.g. the TD34 mutant *Synechocystis* PCC 6803 grown heterotrophically) also can determine if photosynthetic apparatus mediates cell damage (Williams 1988).

5.4.5 Physiological responses from hydrogen peroxide exposure

Oxidative stress in cells generally occurs when environmental conditions alter the dynamics between oxidants and the antioxidant defence system. In this present study ROS was recorded when *Microcystis* was exposed to H_2O_2 in both light and continuous dark conditions. *Microcystis* populations under a light cycle was observed to increase ROS (%) from ≥ 4 mg/L in a dose dependent manner, where ROS activity was also induced faster.

The ROS percentage population in continuous dark conditions from CM-H_2DCFDA fluorescence was recorded far less than that under light experiments (< ten times). As H_2O_2 is a photosensitiser molecule decomposition leading to ROS will dramatically lower if cultures were not exposed to irradiance, conferring with previous studies that the irradiance is critical factor determining the usefulness of H_2O_2 for *Microcystis* control (Drábková *et al.* 2007*b*; Mikula *et al.* 2012).

Membrane permeability (% of population), as observed through SYTOX® Green labelling was always highest when ROS signals peaked in both light and dark experiments. The correlation of *Microcystis* membrane permeation to ROS increase is logical, as cellular rupture proceeds in cells that have undergone molecular and physiological changes from unbalanced intracellular oxidants (Table 5.2). Again as in recorded positive ROS signals, SYTOX® Green fluorescence was only observed in continuous darkness at higher concentrations, which can be attributed to the lower photoreduction of H_2O_2.

5.4.6 Physiological responses from acetic acid exposure

The present study reports that *Microcystis* exposure to acetic acid in concentrations of 10 and 40 mg/L under light and dark conditions respectively induces significant levels ROS ($\gtrsim$ 20%). Although 10 mg/L induce intracellular ROS it wasn't until $\geq$ 40 mg/L that *Microcystis* cell number declined. This is the first time that ROS generation has been recorded in the cyanobacterium *Microcystis* when exposed to acetic acid.

However, under dark conditions at relatively high acetic acid concentrations (100 and 400 mg/L) ROS % population was lower than 40 mg/L but recorded a larger reduction of *Microcystis* cells. The lack of CM-H_2DCFDA fluorescence at these concentrations may be due to the pH stability of the molecular probe. CM-H_2DCFDA has a recommended pH working level of approximately 7.0, however, much lower pH levels were recorded in 100 and 400 mg/L ($\leq$ pH 4.2) potentially destabilising the molecule making fluorescence undetectable. Questions may therefore be raised over the use and validity of a single step ROS probe application with lower than recommended pH levels. Nevertheless, gating methods (Chapter 2) confirm that ROS is present in *Microcystis* cells when exposed to acetic acid concentrations up to 40 mg/L. Future metabolic profiling could include buffering samples raising pH levels to around 7 before adding CM-H_2DCFDA, although this may alter the immediate physiological state of *Microcystis* as optimal incubation time is 60 min (Section 2.6.3).

As with H_2O_2 exposure and growth inhibition membrane permeability increased with significant ROS detection using acetic acid, under both light and dark conditions. As ROS was confirmed in *Microcystis* cells at 40 mg/L but not higher, further work on the mechanics of mortality should be undertaken. A demonstration of enhanced caspase like protein activity and a TUNEL assay could shed further light on the mode of cell death associated with acetic acid.

5.4.7 Acetic acid and pigment response

With acetic acid a unique physiological characteristic appeared when growth inhibition occurred at ≥ 40 mg/L. The cultures turned a light blue colour which indicated decomposition of photosynthetic pigments (Figure 5.1). The colour change observed was similar to Arii *et al.* (2015) experiment when pH levels decreased chlorophyll *a* and β-carotene, leaving predominantly phycocyanin.

Phycobilipigments such as phycocyanin degrade or 'bleach' at a much slower rate compared to other photosynthetic pigments (Simis and Kauko 2012), highlighting that standard techniques like spectrophotometry (Chl *a* detection) may not always be the most accurate method for quantify cyanobacterial cells in short term experiments. The pigment degradation from acetic acid indicates that a longer time is needed for the compound to be effective on *Microcystis*.

5.4.8 Overview of chemical applications for *Microcystis*

Aquatic chemical controls are commonly developed for terrestrial weeds, mainly in agriculture (Cooke *et al.* 2005). Land use herbicide exposure times are not usually part of management plans unless there was a meteorological event washing out the effective compound(s). In contrast water management strategies must adopt a dynamic approach especially when trying to prevent cyanobacterial hazardous algal blooms in reservoirs, lakes and rivers, which have various resident times and exposed to constantly changing environmental conditions. Successful chemical compounds for treating nuisance algal species like *Microcystis* are not only dependent on effective doses but also environmental factors, such as UV-irradiance.

The concentration / exposure time (CET) relationship is critical for controlling target organisms, as an effective concentration can be achieved using high doses and a short contact time or a low dose over a long contact time (Getsinger 1998). The result from this study does indicate that H_2O_2 is dose for dose a more effective growth inhibitor on *Microcystis* populations than acetic acid, especially during light conditions. However, H_2O_2 operational limits are exceeded in the recommended dosage for *Microcystis* control in UK waters. Understanding of the CET is therefore particularly important when having to manage aquatic sites if short term clean water is in need of immediate consumption, but limited in supply.

Although CET is an appropriate guideline to aquatic herbicide application, Getsinger (1998) also highlights product efficacy ranges between species. Whilst the controls for target organisms do have a narrow spectrum, intra species efficacy also needs to be understood. Here a relatively new isolate of *Microcystis* required higher concentrations of H_2O_2 (approximately ten times) for high population mortality than recorded in previous laboratory experiments (Table 5.2). The need for higher concentrations to control environmental *Microcystis* agrees with Wang *et al.*'s (2012) findings from H_2O_2, whereby environmental phenotypes need to be taken into account during water management strategies. In addition great care must be adopted when short term approaches are considered, as lysis of cyanobacterial cells may increase toxins like microcystins (Lürling *et al.* 2014; Huo *et al.* 2015), deplete oxygen levels and / or cause shifts in the phytoplankton community (Cook *et al.* 2005).

5.5 Conclusion

Although not as effective as H_2O_2, acetic acid did produce an algalcidal response and revealed a mode of mortality in a cyanobacterium through ROS the degradation of photosynthetic pigments and ROS induction. However, reducing the pH in the long term (which was only observed in acetic acid treatment) may prevent or stop *Microcystis* becoming locally dominant, as cyanobacterial distribution correlates with more alkaline water bodies (Carvalho *et al.* 2011). Acetic acid may also have selectivity as found with H_2O_2 in cyanobacteria (Drábková *et al.* 2007*a*, 2007*b*, 2007*c*) and should be tested on other phytoplankton groups, e.g. green algae or diatoms.

When comparing both concentrations in terms of biomass reduction it can be concluded that H_2O_2 is a better chemical control method than acetic acid, as it requires a smaller concentration which causes faster *Microcystis* mortality rates. However, as the H_2O_2 concentrations need to be higher than the limits set out by the EA in the UK, acetic acid could be more viable option once tested on a number of organisms.

This page intentionally left blank

Chapter VI: An evaluation of *Microcystis* auto-inhibition and cell mortality from a chlorotic, cell free media extract

Abstract

Toxic cyanobacteria bloom formation in drinking water sources are of global concern. Increasing with frequency and dominance species like *Microcystis* can have negative impacts on human health and aquatic ecology. The aim of this chapter was to examine a potential biochemical *Microcystis* control which had been reported to inhibit growth rates through applications of a conditioned media (CM). CM is a cell free medium extracted from a chlorotic *Microcystis* (PCC 7806) population, induced by nutrient limitation under light conditions. *Microcystis* cell counts, membrane integrity and hydrolytic enzyme activity were analysed through flow cytometry and a novel dual staining molecular probe technique (SYTOX® Orange and CMFDA). Applications were assessed against a number of concentrations in three batch cycle phases (lag, exponential and stationary) under both a light / dark cycle and continuous darkness. CM caused an initial toxic effect in lag and exponential phase cultures, where cell counts and enzyme activity decreased (CMFDA) and membrane injury (SYTOX® Orange) increased. CM was only effective under light conditions in a density dependent manner, drawing parallels to quorum sensing. A heterogeneous CM sensitivity was recorded between *Microcystis* test cultures with percentages of populations showing both viable and non-viable cells. As there was also a distinct viable population in the chlorotic biomass used to generate the original CM it suggested different *Microcystis* phenotypes existed. CM application to stationary phase cultures did not affect cell numbers, as potentially damage alleviation occurred by increased anti-oxidant activity from high densities. This study confirms an anti-microbial action on *Microcystis* exists in CM media by reducing enzyme activity and increasing membrane permeability, whereby the responsible compound(s) needs to be isolated. This chapters results also highlight the need to test potential control compounds under various concentrations, growth phases and irradiance conditions, thereby making any water treatment process more effective.

6.1 Introduction

6.1.1 Natural products from microbes

Micro-organisms are an important source of bioactive chemicals with a number of compounds producing anti-tumour, anti-mitotic, hypocholesterolemic, enzyme inhibiting and immunosuppressive agents (Demain 1999). In addition to these vital medicinal properties microbial secondary metabolites can also serve as antibiotics causing antagonistic effects to viruses, bacteria, fungi, algae, amoebas and higher organism such as insects (Demain and Fang 2000). Over the past few decades most of these anti-microbials and sources of new drugs have been isolated from terrestrial actinomycetes and filamentous fungi (Newman and Cragg 2007).

Cyanobacteria on the other hand, although a rich source of natural products, have not been exploited to such an extent for their potential pharmacological and biotechnological applications. As the existence of cyanobacteria has been dated back to ≈3.5 billion years (Schopf 2000; 2006) a complex chemical response to their evolution with other organisms and environmental factors has produced an array of secondary metabolites (Leão *et al.* 2012). Several secondary metabolites including the potent anti-proliferative agent largazole has been isolated from a marine cyanobacterium *Symploca* sp., which reports selectively towards cancer cells (Taori *et al.* 2008).

However, research into freshwater cyanobacterial secondary metabolites has mainly focused on cyanotoxins such as microcystins from *Microcystis* sp., which can cause serious deterioration of water quality impacting human and environmental health (Chorus and Bartram 1999; Codd *et al.* 2005). Further research into freshwater cyanobacterial species is required to find potential anti-microbial compounds that could prevent toxic alga proliferation in drinking water supplies (e.g. *Microcystis*).

6.1.2 Cyanobacterial allelopathy

Community dynamics of phytoplankton are not only affected by the physical and chemical nature of environments but also by the interactions between species within their immediate surroundings. One complex chemical signalling interaction in particular, also known as allelopathy, can regulate growth rates (positive or negative) of other species through the production of secondary metabolites. Interspecific cyanobacterial allelopathy against *Microcystis* has been found from *Cylindrospermopsis* and

Fischerella species effecting growth rates, metabolic activity and inducing colony formation (Etchegaray *et al.* 2004; Mello *et al.* 2012; Rzymski *et al.* 2014). Although allelopathy is a well characterised response between species, intra-species regulation of cell densities is less well defined.

6.1.3 Cyanobacterial quorum sensing and adaption to environmental stress

To ensure an organisms existence adaptation to the immediate environment is essential for survival. Many prokaryotes have sensing mechanisms and intracellular signalling systems by which biochemicals are excreted. These biochemical metabolites can lead to a communal response which facilitates adaptation through physiological changes (Shapiro 1998). This cell-to-cell communication is known as quorum sensing or auto-induction, where the regulation of a cell gene expression is dependent on cell densities and subsequent metabolite production (Nealson *et al.* 1970; Fuqua *et al.* 1994). Until recently quorum sensing had not been characterised in cyanobacteria. However, a diverse array of *N*-acylhomoserine lactones / auto-inducers has been isolated from cyanobacterial extracellular matrices which can change photosynthetic composition, biofilm formation and growth rates on native populations (Sharif *et al.* 2008; Decho *et al.* 2009; Schatz *et al.* 2013).

In unfavourable conditions (e.g. nutrient limitation) some species of cyanobacteria can develop specialised resting cells, such as heterocysts and akinetes (Adams and Duggan 1999). Other adaptation techniques include desiccation tolerance and chlorosis (reduction of chlorophyll), where populations can recover when environmental parameters become suitable again (Satho *et al.* 2002; Saha *et al.* 2003; Potts *et al.* 2005). Chlorosis has often been reported as an adaption to long term survival in non-diazotrophic cyanobacteria where under stress several genera become chlorotic, with non-pigmented cells surviving nutrient starvation in light conditions (Allen and Smith 1969; Stevens *et al.* 1981; Saha *et al.* 2003). The proteolytic degradation of phycobiliproteins allows cyanobacterial some genera such as *Synechococcus* to enter a fully differentiated cellular state which can be reversed when adequate growth conditions return, with cell division occurring after 4-5 days (Görl *et al.* 1998; Sauer *et al.* 2001).

6.1.4 *Microcystis* in chlorosis

In contrast to other genera of cyanobacteria *Microcystis* (PCC 7806) respond very differently during chlorosis that is induced by nutrient limitation under light conditions. Instead of a homogenous dismantling and reassembling of photosynthetic apparatus as seen in *Synechococcus*, *Microcystis* populations exist as two different phenotypes (de Abreu Meireles *et al.* 2015). The majority of *Microcystis* that enter a chlorotic state exhibit no autofluorescence (chlorophyll), have permeable membranes and degraded DNA. Only a very small amount of a *Microcystis* population (≈1%) keep their chlorophyll content, membrane integrity and DNA viability, consequently representing the cells responsible for population recovery when conditions are favourable (de Abreu Meireles *et al.* 2015).

6.1.5 Can cyanobacterial auto-inducers be found in chlorotic environments?

Various factors can result in an abrupt termination of phytoplankton blooms including biotic interactions (e.g. viruses, grazers and allelopathic compounds) and abiotic stressors (e.g. irradiance or nutrient limitations). However, relatively little is known about cyanobacterial mortality and growth regulation by conspecific populations. Two recent studies have provided evidence that biochemical products or secondary metabolites released from collapsed cyanobacterial cultures can inhibit growth when applied to the same species, one of which was *Microcystis* (Dagnino *et al.* 2006; Cohen *et al.* 2014).

Cohen *et al.* (2014) demonstrated that media from a collapsing or chlorotic culture of *Synechococcus* (PCC6803) had an anti-algal effect on the growth of a number of species including itself. This media also known as conditioned media (CM) was extracted by centrifuging chlorotic cultures, filtering the supernatant and storing it at -20^{O}C before use. The CM was found to contain a light and density dependent cytotoxic compound, which ruptured membranes and degraded photosynthetic pigment via oxidative stress.

A second study reports that CM from *Microcystis aeruginosa* (PCC 7806) inhibits *Microcystis* growth rates with no strain specificity (Dagnino *et al.* 2006). CM was collected from *Microcystis* in the same way as Cohen *et al.* (2014) minus the filtration step. Observations between 3-5 days reported that cell density increase reduced, with healthy cells (chlorophyll containing) losing their characteristic green colour through thylakoid membrane disorganisation turning chlorotic (Dagnino *et al.* 2006).

6.1.6 Examining conditioned media from *Microcystis* for potential auto-inducers or anti-*Microcystis* compounds

Dagnino *et al.* (2006) reported that CM inhibited *Microcystis* cell proliferation but the effects were more algalstatic than algalcidal. Cohen *et al.* (2014) on the other hand recorded cell mortality in a density dependent manner, as they conducted their experiment on a number of initial cell densities from an exponential growing batch culture. As Dagnino *et al.* (2006) incubated their *Microcystis* cells in a 1:1 volume ratio of CM there is the potential that any auto-inducers or anti-*Microcystis* compound can be more effective at different (higher) concentrations, conferring quorum sensing.

In addition Dagnino *et al.* (2006) claimed that CM worked on all stages of growth with cell densities measured through OD_{750}. Despite OD_{750} indicating a relative change in culture biomass the exact number of cells could not be verified from the publication. In higher cell densities there is potential for many other metabolites to increase as seen in *Synechococcus*, where stationary phase cultures produced higher quantities of antioxidants which postulated the density dependent CM resistance in *Synechococcus* (Cohen *et al.* 2014). To confirm the efficacy of CM in *Microcystis,* exposure to different stages of a batch cycle must be conducted, as micro-organisms metabolite composition is a function of growth rates and environmental conditions (Tweeddale *et al.* 1998).

To further the knowledge of how any anti-proliferation/*Microcystis* CM compound works observations over a number of time periods will be needed. Dagnino *et al.* (2014) started recording only after 3 days and the effects of *Synechococcus* CM inhibition was only recorded after a single 24 hour point (Cohen *et al.* 2014). To understand the short term effects and observe any potential population recovery of *Microcystis* CM recordings will need to be taken immediately after applications (at least 24 hours) and regularly through-out a short term experiment.

Interestingly Cohen *et al.* (2014) did not observe any CM growth inhibition on *Synechococcus* under dark conditions. *Synechococcus* CM was also tested on heterotrophic (non-photosynthetic) bacteria with no response, where CM mortality was then linked to the photosynthetic electron transport chain. As Dagnino *et al.* (2006) only examined CM under a light / dark cycle a greater understanding of how CM interacts with *Microcystis* on a physiological level will need to be assessed under various light regimes, as any growth inhibiting CM compound could be identified as photoactive like hydrogen peroxide (Mikula *et al.* 2012).

To gain a better understanding of a potential growth inhibitor or a *Microcystis* mortality inducing compound from CM the work set out by Dagnino *et al.* (2006) was extended. This was done by testing CM on the three main batch cycle phases (lag, exponential and stationary) at a number of concentrations, in both light and constant dark conditions. The physiological and metabolic responses of *Microcystis* were also assessed by a novel method of molecular probe dual staining, analysing membrane integrity (SYTOX® Orange) and hydrolytic enzyme activity (CMFDA) through flow cytometry (FCM).

6.2 Methodology

6.2.1 Culture conditions and generation of conditioned media

Experiments were carried out with an axenic cyanobacterium strain *Microcystis aeruginosa* (PCC 7806, +mcyB) supplied by Pasteur Culture Collection (France). Cultures were incubated at 25±1^{o}C under an irradiance range 15–19.5 µE m^{-2} s^{-1} (Luminex, 58 watt fluorescent cool white tube) on a 12 hour light/dark cycle (Conviron, CMP6010, Canada). All cultures were maintained on BG-11 media (Sigma-Aldrich, UK) and swirled by hand (conditioned media) or vortexed (stock and experimental cultures) once a day.

The chlorotic conditioned media (CM) was generated by inoculating *M. aeruginosa* to a total of 2.1L from an exponential phase density (≈2x10^{6} cells/mL) in a large 10 L Duran bottle. The CM culture was left under the same conditions as above for a period of seven weeks until a population crash occurred, resulting in a pale, milky coloured media (Figure 6.1).

Cell counts and molecular probe analysis for CM were taken approximately every 4 days. The expired culture (CM) was then centrifuged at 1710 x g for 5 min. The supernatant was extracted using a sterile 0.2 µm syringe filter tip, aliquoted in 50 mL centrifuged tubes and frozen (-20^{o}C). Prior to use the conditioned media was left to thaw overnight at 25±1^{o}C.

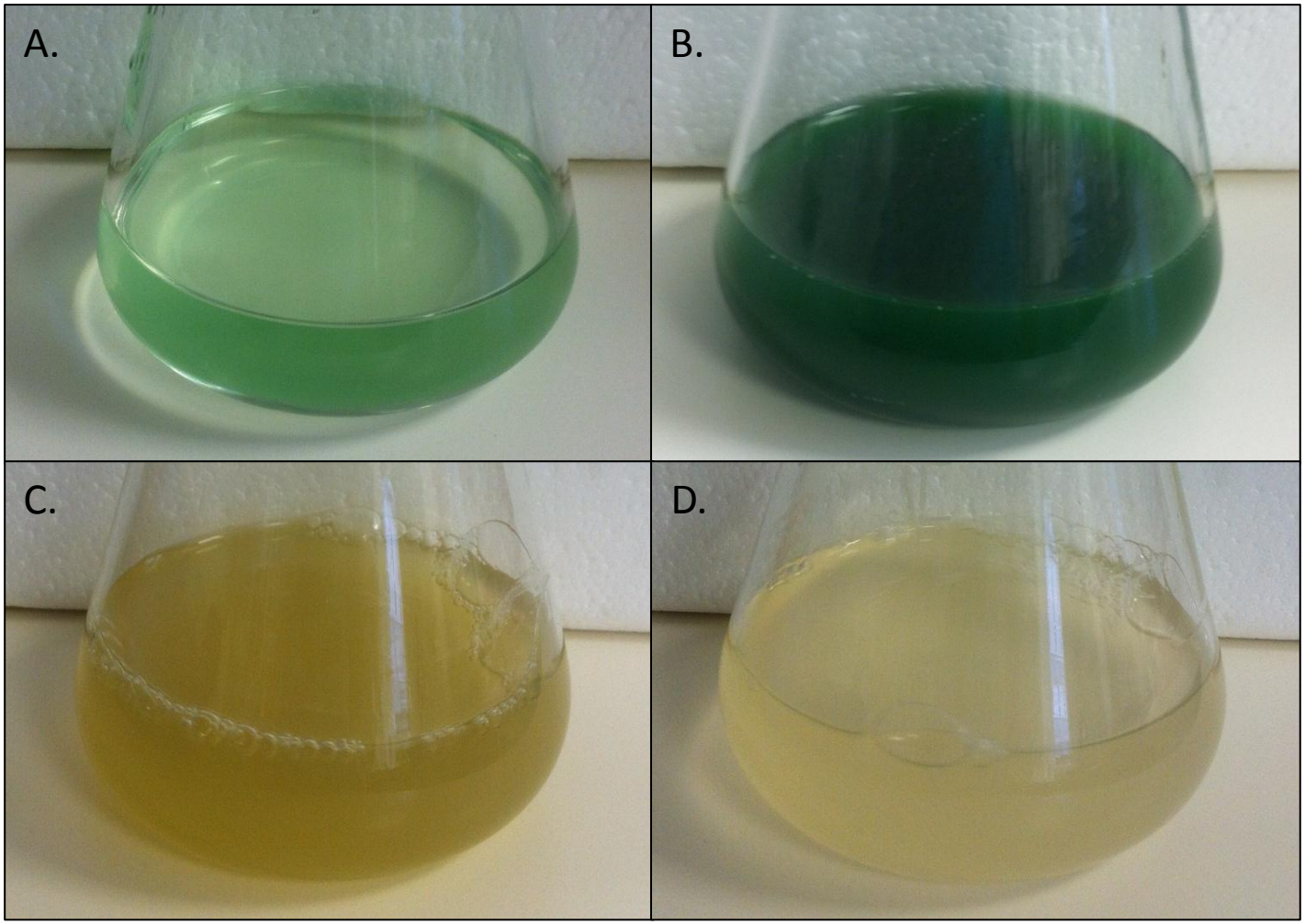


Figure 6.1. Samples from the *Microcystis aeruginosa* culture used to generate the 'conditioned media', with distinguishable colour changes through batch cycle phases. A) Exponential – light green B) Stationary – dark blue-green C) Collapsing – green / yellow and D) Crashed culture – pale, milky white.

6.2.2 Experimental Design

M. aeruginosa used as stock cultures for the experiment were grown to three different densities representing the start of a lag, exponential and stationary phase. These phases of a *M. aeruginosa* batch cycle were determined by routine measurements of stock cultures under the same culture conditions as Section 6.2.1 and designated as ≈$4x10^5$, $2x10^6$ and $14x10^6$ cells/mL for lag, exponential and stationary phase respectively. Stock cultures were kept in 50 mL glass tubes at a total volume of 26 mL to simulate the culture conditions which experimental *M. aeruginosa* were exposed to. Twenty glass tubes containing stock *M. aeruginosa* per batch cycle phase (60 in total) were cultured to collect enough biomass for testing.

For the experiment 26 mL, 19.5 mL, 13 mL and 6.5 mL of *M. aeruginosa* was transferred from stock cultures to new 50 mL glass tubes (in triplicate) for each batch cycle phase. Each tube was then filled to a final volume of 26 mL with conditioned media for a v/v% dose increase (0% as a control, 25%, 50% and 75%). This experimental culture set-up was then duplicated with another set of identical cultures and conditions but this time samples were placed into a homemade dark box (Figure 6.2).

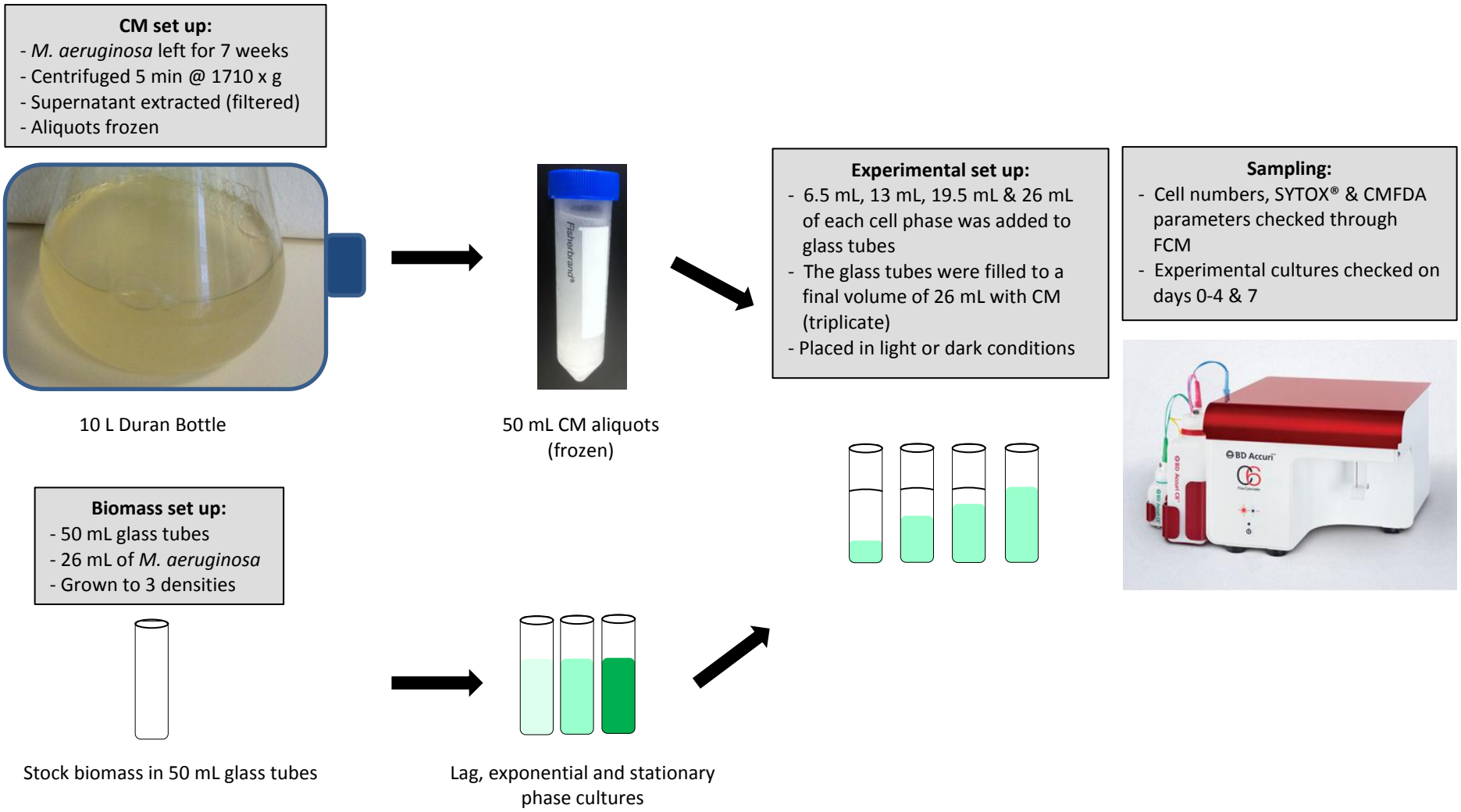


Figure 6.2. Schematic diagram of the autoinhibition experiment

6.2.3 Cell counts and physiological assessment

Cell densities and physiological states for CM and experimental cultures were assessed through flow cytometry (FCM). Cell counts, membrane integrity and metabolic activity (hydrolytic enzymes presence per cell) were taken from the CM stock culture approximately every 4 days and for experimental cultures between days 0-4 and then again at day 7. Cell counts were obtained through FCM forward light scatter (FSC-W) and phycocyanin fluorescence (FL4-H) discrimination, being acquired through the slow fluidic settings and run for 2 min (Chapter 2). To measure cell membrane integrity and metabolic activity a novel dual staining technique was employed through SYTOX® Orange (FL2-H) and CMFDA (FL1-H) at concentrations of 1 µM and 5 µM respectively, under an incubation period of 30 min in the dark (Chapter 2).

6.2.4 Statistical analysis

To measure if CM had an effect on *M. aeruginosa* changes in cell numbers, membrane integrity and metabolic activity were analysed. As the experiment used a v/v% dilution initial *M. aeruginosa* densities would be different, so the percentage change in cell numbers from the previous time point was used to determine significant differences in cell counts.

For each culture phase (lag, exponential and stationary) a two-way repeated measures (RM) ANOVA tested the percentage difference in cell numbers, the % of SYTOX® Orange labelled cells and the % of CMFDA labelled cells between dose concentrations (0, 25, 50 and 75%) and between the days which sampling took place (day 0-4 and 7). The same statistical analysis was performed for both light and continuous dark conditions. All data was processed using SigmaPlot 13.0 (Systat Software, San Jose, CA) for windows.

6.3 Results

6.3.1 Generation of the conditioned media

Initially inoculated with an exponential number of cells (≈$2x10^6$ cells/mL) the *M. aeruginosa* culture grown to generate CM entered a stationary phase after 8 days, with maximum cell density of ≈$3.5x10^7$ cells/mL on day 30 (Table 6.1). This stationary phase lasted 4 weeks before a population crashed occurred (Figure 6.3 A). The CM culture exhibited a colour change during its batch cycle, starting off light green in exponential phase then turning to a distinguishable dark blue-green shade typically associated with cyanobacteria. The dark blue-green (stationary phase) persisted for 28 days then transitioned to a 'greenish/yellowish' colour. This 'greenish / yellowish' colour lasted for 2-3 days then a pale white, milky colour was observed (Figure 6.1). The final CM was left to stand for a further 10 days before it was centrifuged, filtered and frozen before use on experimental cultures (Figure 6.4).

The physiological state of *M. aeruginosa* cells did change over batch culture phases. The membrane permeable population or %-SYTOX® Orange labelled cells initially started off at 2.3% (exponential phase) which increased through to the stationary and population crash phases, where a maximum 87.1% was recorded on the last day of sampling (day 48) (Table 6.1). The hydrolytic enzyme activity or %-CMFDA labelled cells had inverse recordings compared to %-SYTOX® labelled cells, where initial exponential phase results were 96.8%, dropping by approximately two thirds during the highest cell densities (day 30) and finally reporting a minimum of 5.6% on day 48 (Figure 6.3 B).

Table 6.1. Cell counts and molecular probe data for membrane integrity (SYTOX® Orange) and hydrolytic enzyme activity (CMFDA) from the generation of CM. As there was only a single large volume of CM generated only one replicate per sampling day was taken.

Time (d)	Cell count (cells/mL)	SYTOX®-labelled (% population)	CMFDA-labelled (% population)
0	1,907,488	2.3	96.8
4	7,205,648	4.5	92.3
8	23,987,643	12.4	82.3
12	28,886,311	17.8	72.1
15	31,772,946	27.8	62.7
20	33,831,265	33.8	48.5
25	32,664,935	55.6	41.2
30	34,715,487	64.2	31.8
35	17,825,594	72.2	15.2
38	6,896,921	77.9	12.8
40	2,321,487	80.4	9.4
48	4,263	87.1	5.6

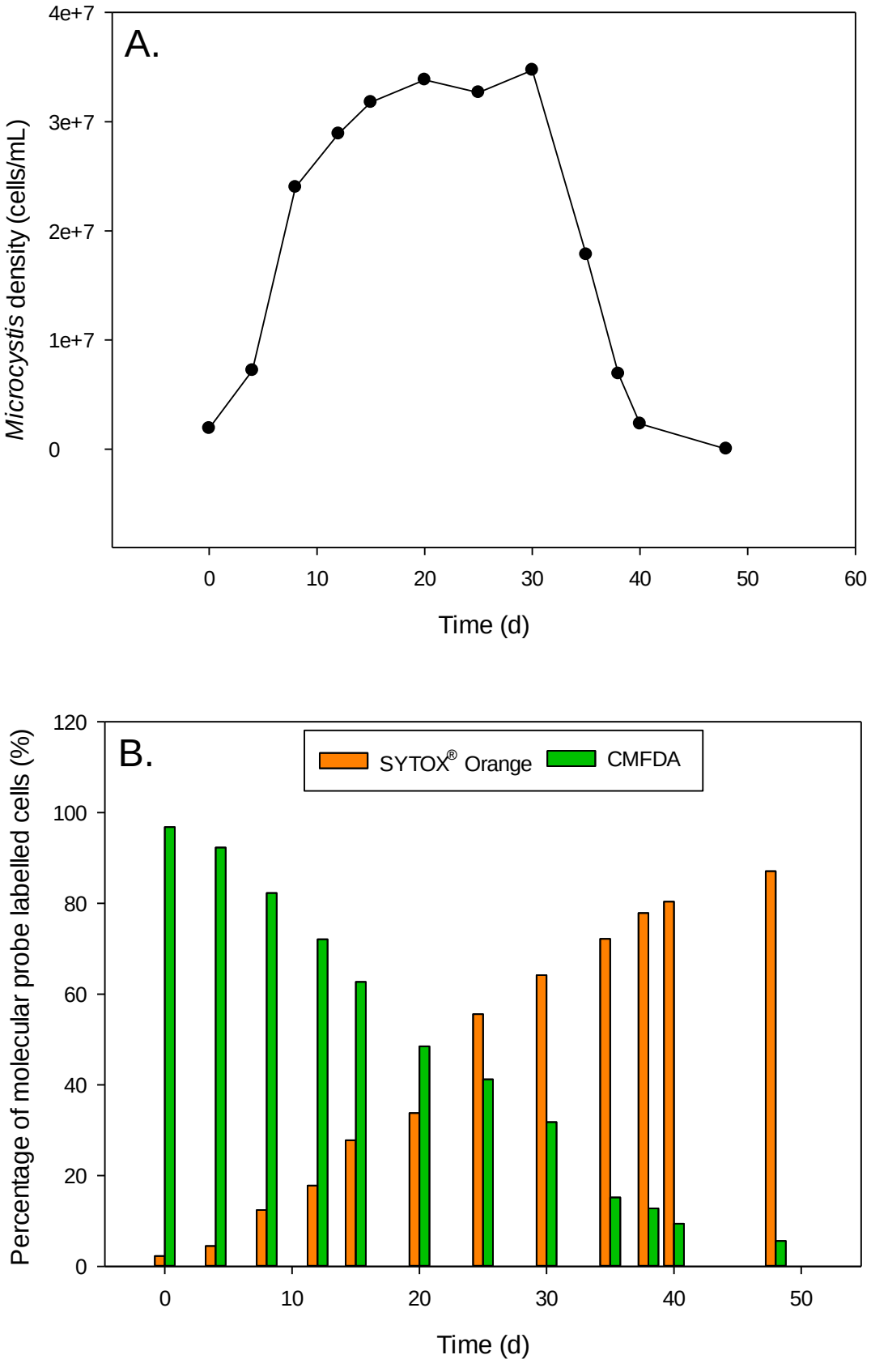


Figure 6.3. A) Cell counts taken over the generation of CM recording an exponential phase between days 0-8, a stationary phase between days 8-30 and a subsequent population crash from day 30. B) % of cells labelled with the molecular probes reporting an inverse relationship (only one replicate was taken from CM samples so no error bars were calculated).

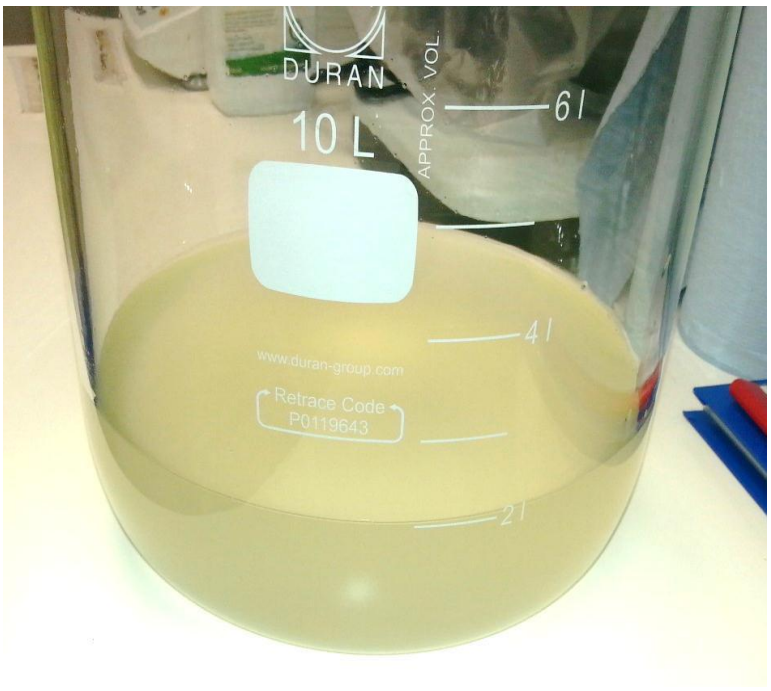


Figure 6.4. Photo of the CM at day 40 where the *Microcystis aeruginosa* culture had changed to a pale, milky white colour.

6.3.2 Effect of conditioned media on lag phase *Microcystis aeruginosa*

Cell density

Under light conditions a two-way RM ANOVA examined the effects of CM concentrations and the time between sampling periods (days 0-4 and 7) on the percentage change in cell numbers from the previous days counts. Both the main effects of CM concentration and time, as well as their interactions were significant factors in the percentage change in cell numbers (all $p < 0.001$) (Table 6.2). Incubated under light all lag phase experimental cultures exposed to CM saw an initial percentage drop in cell numbers over the first day, with the control (0% CM) recording an increase (Table 6.3). Cultures that exhibited a decrease over the first day did so in a dose dependent manner with increased CM concentrations. A v/v% addition of 25% CM reduced densities by 29.4±2.5%, with 50% and 75% CM decreasing populations by 33.9±6.4% and 45.0±7.3% respectively.

After day 1, recovery of cell numbers were observed in all CM experiments expect 75% CM. All populations that increased in cell numbers did so until the end of sampling (including the control), whereas 75% CM continued to decline with no *M. aeruginosa* recorded on day 7 (Figure 6.5 A). In continuous dark conditions there was no significant differences in the percentage change of cell numbers ($p > 0.05$) (Table 6.4) between CM concentrations (Figure 6.5 B) and across the experiment (Table 6.5).

Table 6.2. Two-way RM ANOVA results from the percentage change of cell numbers and % of molecular probe labelled cells between CM concentrations across 7 days under light conditions.

Two-way RM ANOVA	Δ Cell density (%)		SYTOX®-labelled cells (%)		CMFDA-labelled cells (%)	
	F	*p*	*F*	*p*	*F*	*p*
CM concentration effect ($F_{3,24}$)	46.96	< 0.001	211.63	< 0.001	705.88	< 0.001
Time effect ($F_{4,24}$)	20.14	< 0.001	30.86	< 0.001	170.25	< 0.001
CM concentration x time interaction ($F_{12,24}$)	12.19	< 0.001	40.08	< 0.001	145.16	< 0.001

Table 6.3. Results from the lag phase cultures in different CM v/v% concentrations under light conditions for cell counts, the % population density change and the % of molecular probe labelled cells.

75% CM

Day	Cells/mL	S.E.	Δ Density (%)	S.E.	SYTOX®%	S.E.	CMFDA%	S.E.
0	94,691	10,626	-	-	4.0	0.8	94.3	0.9
1	51,025	5,809	-45.0	7.3	15.9	2.1	80.5	0.8
2	32,802	7,092	-34.9	15.3	28.9	2.6	62.8	4.0
3	20,704	4,593	-37.2	1.6	48.0	4.3	34.4	1.8
4	19,000	2,445	-1.3	18.3	66.1	7.7	13.1	2.9
7	0	0	-100.0	0.0	0.0	0.0	0.0	0.0

50% CM

Day	Cells/mL	S.E.	Δ Density (%)	S.E.	SYTOX®%	S.E.	CMFDA%	S.E.
0	187,963	8,152	-	-	4.1	0.3	93.6	1.0
1	123,235	6,468	-33.9	6.4	10.6	0.3	83.4	0.9
2	129,086	7,166	5.8	10.2	10.4	1.0	83.4	0.6
3	155,333	15,394	19.8	5.6	8.4	1.4	86.5	1.7
4	191,235	32,990	21.5	8.6	7.3	1.5	85.3	1.3
7	546,444	234,067	164.8	70.9	5.1	1.8	89.3	3.4

25% CM

Day	Cells/mL	S.E.	Δ Density (%)	S.E.	SYTOX®%	S.E.	CMFDA%	S.E.
0	275,160	9,010	-	-	5.3	0.8	94.0	0.7
1	196,259	3,945	-29.4	2.5	9.0	0.1	88.7	0.7
2	232,272	916	19.9	0.8	9.0	1.1	85.2	1.4
3	422,741	7,661	82.0	4.0	5.5	0.5	90.1	1.0
4	738,086	7,117	74.8	4.8	4.3	0.3	88.6	2.3
7	2,809,074	115,998	280.5	13.5	1.8	0.1	94.5	2.1

0% CM

Day	Cells/mL	S.E.	Δ Density (%)	S.E.	SYTOX®%	S.E.	CMFDA%	S.E.
0	407,259	16,979	-	-	5.1	1.1	94.6	1.2
1	707,723	44,543	74.0	10.6	3.3	0.7	96.3	0.7
2	1,022,210	68,423	44.5	3.7	2.5	0.4	97.3	0.4
3	1,826,605	127,046	78.7	3.2	2.0	0.2	97.8	0.2
4	4,142,494	265,007	127.0	2.3	1.1	0.2	98.6	0.2
7	8,053,901	364,897	95.0	5.6	1.5	0.5	97.5	0.5

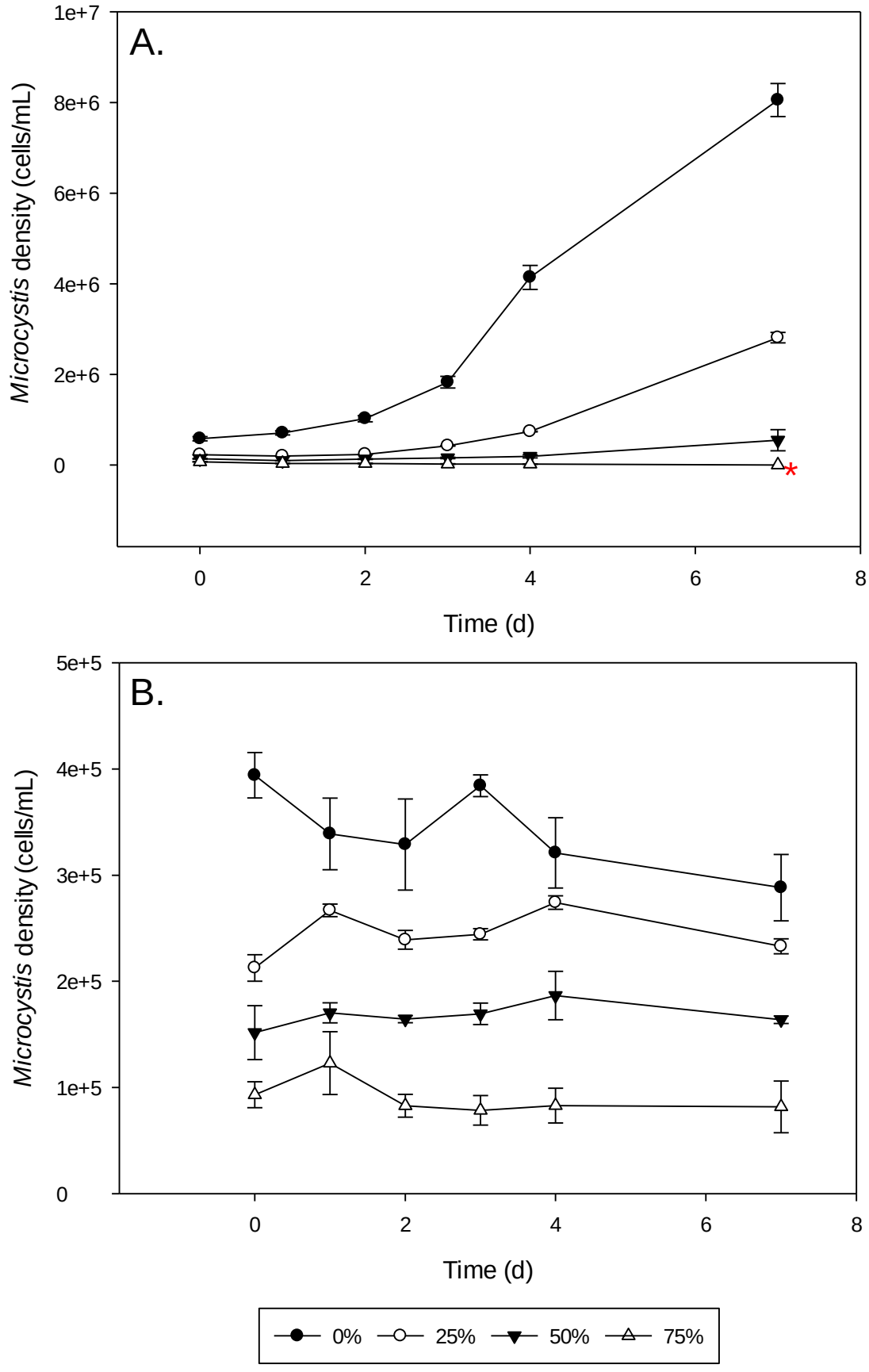


Figure 6.5. Cell numbers from all concentrations of CM (v/v%) in lag phase batch culture cycles under A) light and B) continuous darkness (* zero cell count for day 7 in light conditions).

Table 6.4. Two-Way RM ANOVA results from the percentage change of cell numbers and % of molecular probe labelled cells between CM concentrations across 7 days under dark conditions.

Two-Way RM ANOVA	Δ Cell density (%)		SYTOX®-labelled cells (%)		CMFDA-labelled cells (%)	
	F	*p*	*F*	*p*	*F*	*p*
CM concentration effect ($F_{3,24}$)	0.87	> 0.05	9.51	< 0.05	10.17	< 0.01
Time effect ($F_{4,24}$)	2.23	> 0.05	10.14	< 0.001	21.39	< 0.001
CM concentration x time interaction ($F_{12,24}$)	1.06	> 0.05	2.38	< 0.05	2.15	< 0.05

Table 6.5. Results from the lag phase cultures in different CM v/v% concentrations under dark conditions for cell counts, the % population density change and the % of molecular probe labelled cells.

75% CM

Day	Cells/mL	S.E.	Δ Density (%)	S.E.	SYTOX®%	S.E.	CMFDA%	S.E.
0	93,099	12,232	-	-	4.1	0.3	93.6	1.0
1	122,877	29,579	41.2	47.9	7.0	1.7	91.5	2.2
2	82,790	10,765	-24.9	18.2	6.4	1.4	92.6	1.4
3	78,420	13,892	-6.3	4.1	6.5	1.2	92.6	1.5
4	82,914	16,310	5.3	5.7	6.4	2.0	92.4	2.7
7	81,735	24,300	-4.6	9.9	8.7	3.7	77.2	2.9

50% CM

Day	Cells/mL	S.E.	Δ Density (%)	S.E.	SYTOX®%	S.E.	CMFDA%	S.E.
0	187,481	7,378	-	-	4.0	0.8	94.3	0.9
1	197,457	2,991	5.6	3.5	12.7	0.9	85.6	0.2
2	164,333	3,423	-16.8	1.7	15.5	0.4	82.9	0.8
3	169,333	10,058	2.9	4.1	22.6	3.6	76.5	3.6
4	186,519	22,768	9.5	7.8	35.5	13.6	61.9	12.8
7	163,790	3,511	-9.4	11.6	27.6	2.4	69.7	3.3

25% CM

Day	Cells/mL	S.E.	Δ Density (%)	S.E.	SYTOX®%	S.E.	CMFDA%	S.E.
0	274,370	5,027	-	-	5.1	1.1	94.6	1.2
1	291,593	19,528	6.1	5.2	10.7	0.2	86.8	0.9
2	239,173	8,794	-17.4	5.0	11.1	0.5	87.8	0.5
3	244,420	5,316	2.6	5.7	11.3	0.3	87.5	0.6
4	274,309	6,322	12.5	5.1	14.2	1.6	84.3	2.0
7	233,012	7,039	-14.9	4.0	10.1	0.2	73.7	1.5

0% CM

Day	Cells/mL	S.E.	Δ Density (%)	S.E.	SYTOX®%	S.E.	CMFDA%	S.E.
0	394,111	21,383	-	-	5.3	0.8	94.0	0.7
1	338,951	33,713	-13.8	8.4	10.5	0.9	89.0	0.9
2	328,864	42,868	-24.9	18.2	18.1	1.6	81.1	1.9
3	384,296	10,236	20.1	12.9	19.1	1.2	80.2	1.0
4	321,037	33,130	-16.7	6.8	25.7	7.9	72.5	7.1
7	288,321	31,196	-4.6	9.9	29.7	6.3	65.0	5.5

Cell physiology

The %-SYTOX® Orange labelled cells (membrane permeable) under light conditions significantly differed between experimental cultures over 7 days, where CM concentrations, exposure times and both their interactions were all significant factors (all $p < 0.001$) (Table 6.2). All experimental cultures except the control recorded an increase in %-SYTOX® Orange labelled cells after the first day. As with the change in cell numbers the difference of membrane permeable cells was also dose dependent on CM concentrations. Between day 0 and 1 the 25% CM increased %-SYTOX® Orange labelled cells to 9.0±0.1%, with 50% and 75% recording an increase of 10.6±6.6% and 15.9±2.1% respectively. After day 1 all CM experiments reduced %-SYTOX® Orange labelled cells until the end of the experiment, except for 75% CM which continued to increase until no SYTOX® Orange fluorescence was recorded on day 7, as *M. aeruginosa* cell count was zero (Figure 6.6).

Under dark conditions CM concentrations, exposure times and their main interactions were significant factors in %-SYTOX® Orange labelled cells over 7 days (all $p < 0.01$) (Table 6.4). All populations had increased their %-SYTOX® Orange labelled cells by at least double on day 7 compared to their initial densities (Figure 6.7).

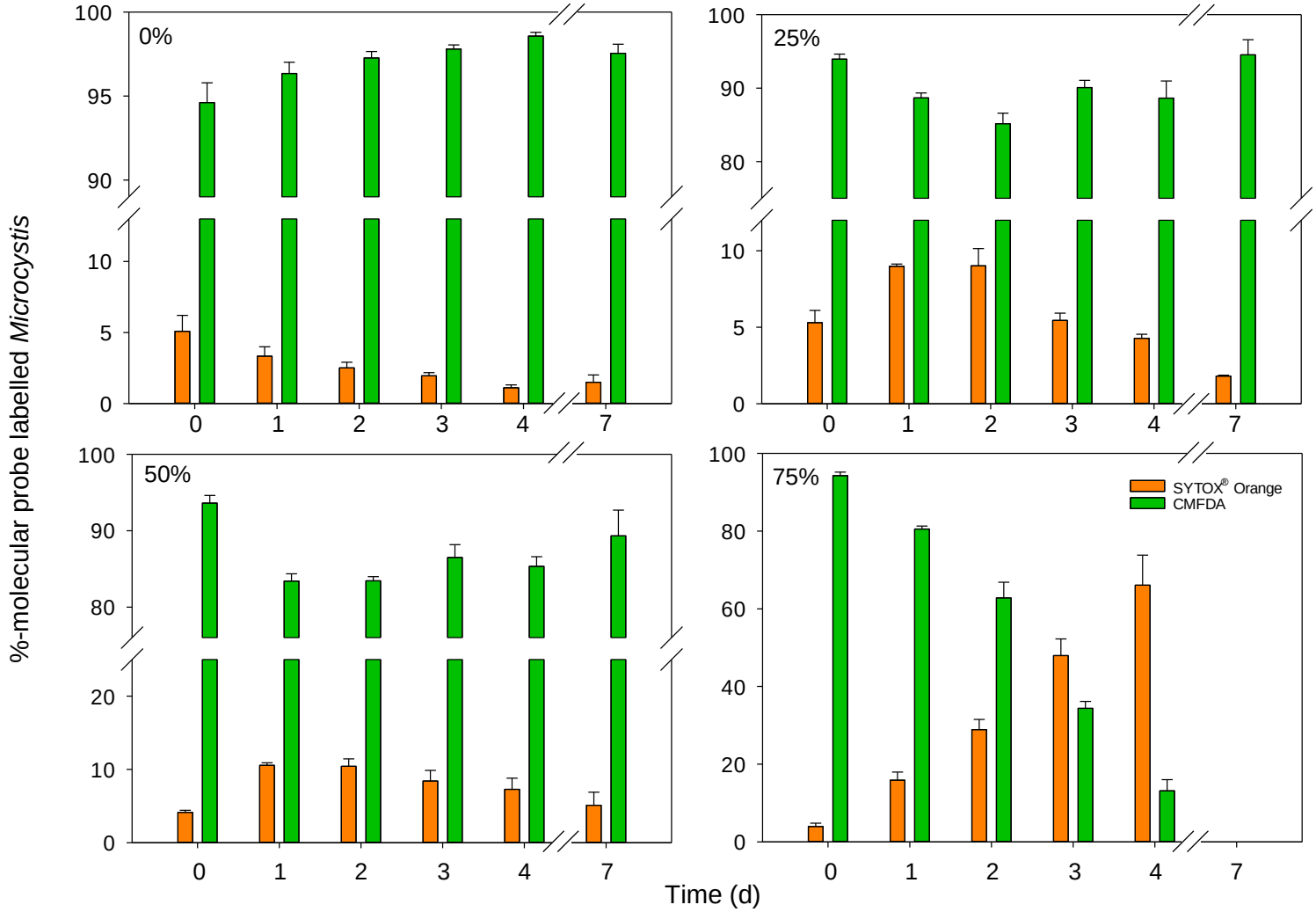


Figure 6.6. Molecular probe data from SYTOX® Orange (membrane injured cells) and CMFDA (hydrolytic enzyme activity) for the population % of labelled cells. Results include all concentrations for lag phase experiments over 7 days under light conditions, 75% CM on day 7 recorded zero cell counts.

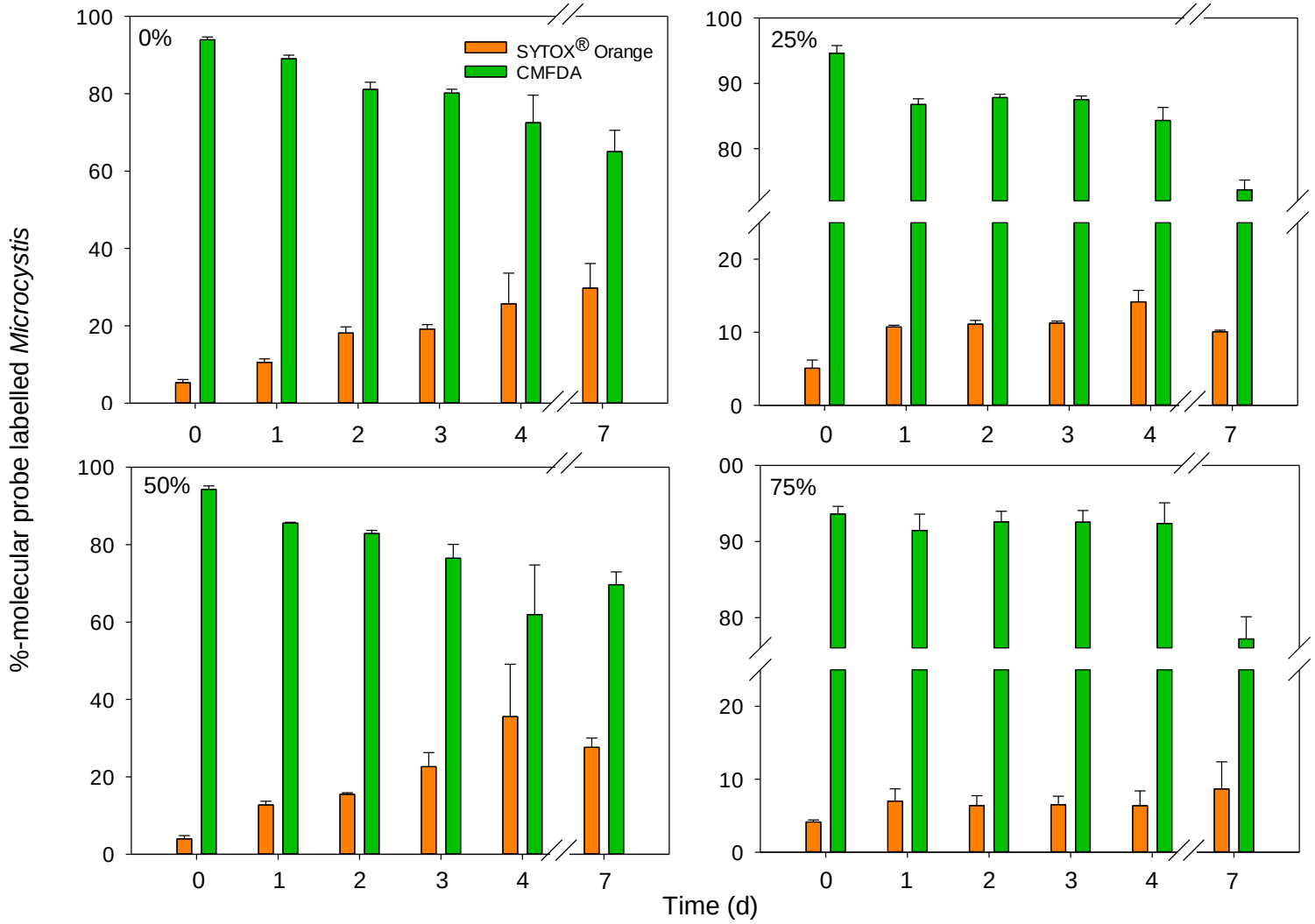


Figure 6.7. Molecular probe data from SYTOX® Orange (membrane injured cells) and CMFDA (hydrolytic enzyme activity) for the population % of labelled cells. Results include all concentrations for lag phase experiments over 7 days under dark conditions.

6.3.3 Effect of conditioned media on exponential phase *Microcystis aeruginosa*

Cell density

In light conditions analysis from a two-way RM ANOVA indicated CM concentrations ($p < 0.001$), exposure times ($p < 0.001$) and their interactions ($p < 0.01$) were significant to the percentage change in cell numbers from the previous days counts (Table 6.6). A dose dependent response from the reduction in cell numbers was recorded when CM concentrations increased over the first day (day 0-1), with mean decreases of 57.4±3.6%, 50.95±2.2% and 38.9±2.3% from CM 75%, 50% and 25% respectively. Cultures exposed to CM declined in densities until population recovery was observed near the end of the experiment (Figure 6.8 A). The control (0% CM) recorded a continued increased in *M. aeruginosa* cell numbers throughout the experiment (Appendix F1).

Experiments under constant darkness indicated that different CM concentrations had no impact on the percentage change in cell numbers from the previous days counts ($p > 0.05$) but differed significantly with time ($p < 0.001$) (Table 6.6 and Figure 6.8 B).

Table 6.6. Two-way RM ANOVA results from the percentage change of cell numbers and % of molecular probe labelled cells between all CM concentrations in an exponential phase over days 0-4 and 7.

Two-Way ANOVA	Δ Cell density (%)		SYTOX® labelled cells (%)		CMFDA labelled cells (%)	
	F	*p*	*F*	*p*	*F*	*p*
Light						
CM concentration effect ($F_{3,24}$)	41.77	< 0.001	284.38	< 0.001	316.03	< 0.001
Time effect ($F_{4,24}$)	22.79	< 0.001	88.44	< 0.001	118.45	< 0.001
CM concentration x time interaction ($F_{12,24}$)	3.43	< 0.001	50.96	< 0.001	45.2	< 0.001
Dark						
CM concentration effect ($F_{3,24}$)	3.56	> 0.05	49.69	< 0.001	38.21	< 0.001
Time effect ($F_{4,24}$)	13.02	< 0.001	108.19	< 0.001	190.26	< 0.001
CM concentration x time interaction ($F_{12,24}$)	1.26	> 0.05	19.95	< 0.001	11.94	< 0.001

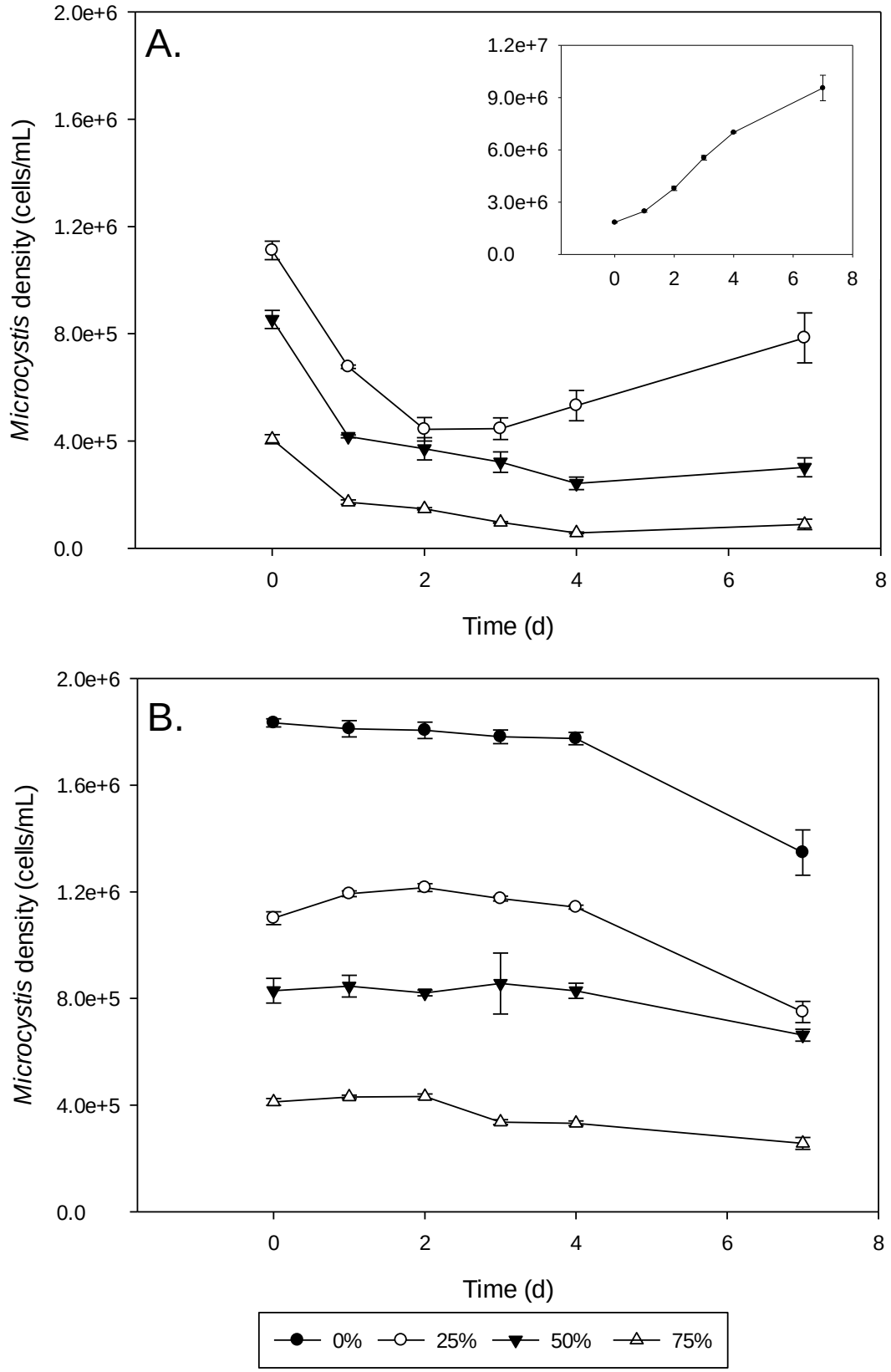


Figure 6.8. Cell numbers from all concentrations of CM (v/v%) in exponential phase batch culture cycles under A) light, with the top right hand insert showing the control (0% CM) and B) continuous dark conditions.

Cell physiology

The %-SYTOX® Orange and %-CMFDA labelled cells both differed significantly under light conditions with CM concentration, exposures time and their interactions being main factors over the experiment (all, $p < 0.001$) (Table 6.6). There was an inverse dose dependent pattern between both molecular probe signals over the 7 days. The higher the CM concentration the higher the mean %-SYTOX® Orange labelled cells were recorded, whereas the mean %-CMFDA labelled cells decreased (Appendix F1). In general the %-SYTOX® Orange labelled cells increased between 0-4 but decreased on day 7 after cell populations recovered, with the inverse being reported for %-CMFDA labelled cells (Appendix F2).

Under continuous dark conditions both %-SYTOX® Orange and %-CMFDA labelled cells differed significantly across CM concentrations, exposure times and their interaction ($p < 0.001$) (Table 6.6). Compared to the light experiments *M. aeruginosa* cells under dark conditions exposed to CM (excluding control) recorded a lower mean %-SYTOX® Orange and higher mean %-CMFDA labelled cells (Appendix F3 and Appendix F4).

6.3.4 Effect of conditioned media on stationary phase *Microcystis aeruginosa*

Cell density

A two-way RM ANOVA reported that under light conditions there were no significant differences with the percentage change in cell numbers from the previous days count ($p > 0.05$) (Table 6.7), all cultures increased in densities over 7 days (Figure 6.9 A). The percentage change in cell numbers also did not significantly differ under continuous dark conditions ($p > 0.05$) (Table 6.7), with all cultures exhibiting a decrease in densities over the experiment at a combined mean of 62.1±1.4% after 7 days from initial staring numbers (Figure 6.9 B).

Cell physiology

The %-SYTOX® Orange labelled cells did not significantly differ under light conditions over the experiment when CM was introduce to stationary phase cultures ($p > 0.05$) (Table 6.7) but they change with time ($p < 0.001$) (Appendix F5 and Appendix F6). The %-CMFDA labelled cells did on the other hand significantly differ with CM concentrations ($p < 0.05$) and exposure times ($p < 0.001$). Under dark condition both %-SYTOX® Orange ($p < 0.001$) and %-CMFDA labelled cells ($p < 0.05$) significantly differed between all CM concentrations and exposure times (Appendix F7 and Appendix F8).

Table 6.7. Two-way RM ANOVA results from the percentage change of cell numbers and % of molecular probe labelled cells between all CM concentrations in an stationary phase over days 0-4 and 7.

Two-Way ANOVA	Δ Cell density (%)		SYTOX® labelled cells (%)		CMFDA labelled cells (%)	
	F	*p*	*F*	*p*	*F*	*p*
Light						
CM concentration effect ($F_{3,24}$)	3.24	> 0.05	4.96	> 0.05	8.49	< 0.05
Time effect ($F_{4,24}$)	0.93	> 0.05	46.3	< 0.001	83.88	< 0.001
CM concentration x time interaction ($F_{12,24}$)	1.20	> 0.05	3.68	< 0.001	15.08	< 0.001
Dark						
CM concentration effect ($F_{3,24}$)	1.00	> 0.05	103.92	< 0.001	8.82	< 0.05
Time effect ($F_{4,24}$)	134.69	< 0.001	106.4	< 0.001	96.51	< 0.001
CM concentration x time interaction ($F_{12,24}$)	26.58	< 0.001	29.28	< 0.001	11.59	< 0.001

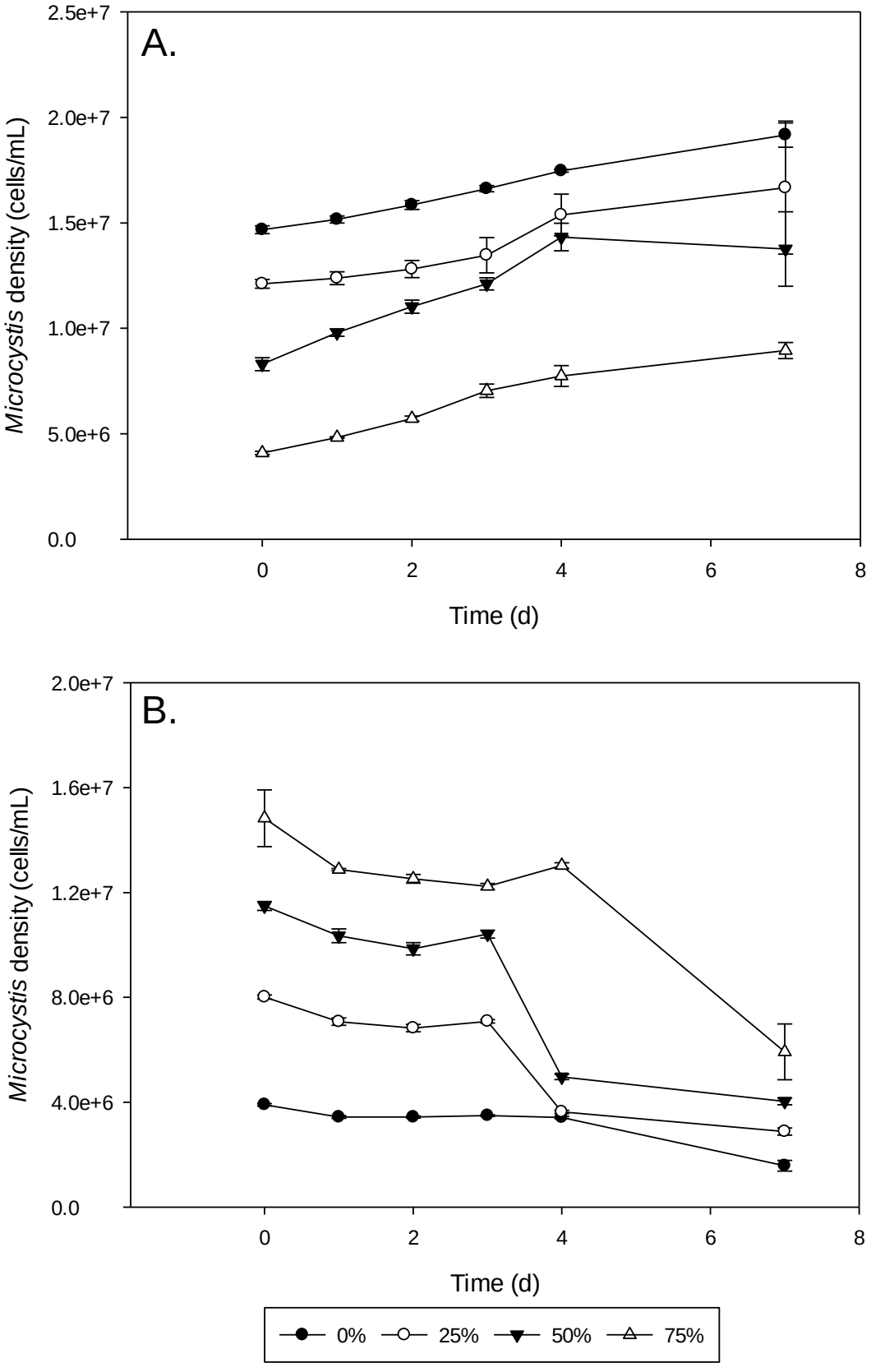


Figure 6.9. Cell numbers from all concentrations of CM (v/v%) in stationary phase batch culture cycles under A) light and B) continuous dark conditions (some S.E. bars are too small to visualise).

6.4 Discussion

6.4.1 Collapsing culture of *Microcystis aeruginosa*

Environmental conditions such as temperature, nutrient availability, light intensity and duration have all been found to affect *M. aeruginosa* growth rates and cyanotoxin production (Watanabe and Oishi 1985; Wiedner *et al.* 2003), consequently altering gene transcription and secondary compounds like microcystins (Lee *et al.* 2000; Kuniyoshi *et al.* 2013). After seven weeks from initial exponential phase densities *M. aeruginosa* was observed to go through a stationary phase, where after the population crash occurred the culture turned 'chlorotic' evident from cell counts and pigment change.

Similar to Dagnino *et al.* (2006) the CM media took seven weeks to transition from the highest productive phase (exponential growth) to the lowest density forming the resulting CM (Figure 6.1 and Figure 6.4). Although the time took for the CM to be generated is approximately the same in both studies, environmental conditions could have affected the nature of the CM in terms of molecular composition. Dagnino *et al.* (2006) inoculated *M. aeruginosa* in 200 mL of ASM-1(x2) to a final OD_{750} of 0.1 using a 1L Erlenmeyer flask. Their CM culture was placed on a rotary shaker, under a light intensity of 30–45 µE m^{-2} s^{-1} at 23^{o}C with a 16:8 light dark photo period, different to the conditions here (Section 6.2.1) potentially altering any anti-proliferation/anti-*Microcystis* compounds.

The timing of CM population mortality is assumed to be from the relative lack of nutrients compared to the initial BG-11 inoculated media. Although nutrient levels were not recorded through-out it can be reasonably concluded as cell reduction had increased due to modified media (2x ASM-1 without a N source) in de Abreu Meireles *et al.* (2015) and from the *M. aeruginosa* cultured on modified media (BG-11 without a P and N source) in Chapter 3 (Appendix C3).

However from both Dagnino *et al.* (2006) and in this experiment only one culture vessel was used to generate CM, whereas in Cohen *et al.* (2014) despite being a different genus the fifteen cyanobacterial CM generated cultures 'collapsed' at different rates. When swapped from ASM-1 to BG-11 media other cyanobacteria like *Anabaena / Dolichospermum* reduce toxin production to undetectable levels, with toxin stimulation being suggested as a product of growth rates decrease through major nutrient limitation (N and P) (Carmichael 1986). The media Dagnino *et al.* (2006) used was double strength ASM-1 (Gorham *et al.* 1964) which contains 44x less nitrates and

5.4x less phosphates than the BG-11 media used in this study (Stanier *et al.* 1971), potentially altering the composition and quantity of any compound(s) released.

The initial starting *M. aeruginosa* density which underwent starvation for CM generation from Dagnino *et al.* (2006) again differed from this study. By using spectrophotometry (Varian, Cary 50, UV/Vis, Varian, USA) the OD_{750} was found to be comparable to $4.75x10^6$ cells/mL when normalised through FCM counts (Figure 6.10). This meant that the starting *M. aeruginosa* density was double to that of this study, impacting on not only nutrient uptake but also on the biosynthesised compound composition and release rates of any secondary metabolites.

To improve on the methodological design of this chapter the generation of CM cultures should be further analysed in the way of studying multiple initial growth medias (both replete and deplete nutrients), under different light regimes and from different starting densities of *M. aeruginosa*.

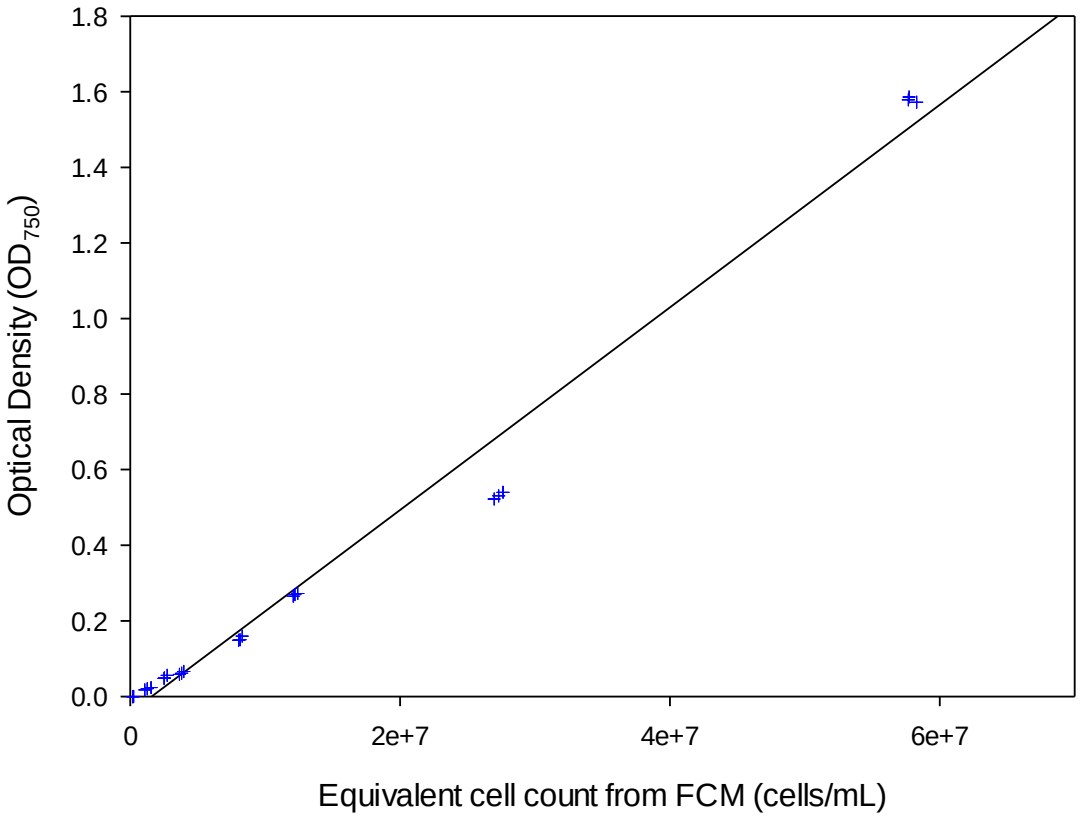


Figure 6.10. Normalising OD_{750} to *Microcystis aeruginosa* cell counts through FCM for comparing starting densities with Dagnino *et al.* (2006) (R^2 = 0.98, y = $3*10^{-8}$x – 0.042).

6.4.2 Physiological cellular states of *Microcystis aeruginosa* during CM generation

M. aeruginosa physiological cell states during Dagnino *et al.* (2006) CM generation was initially reported to have higher than 90% viability (LIVE/DEAD BacLight – epifluorescence microscopy) which stayed at the same level for over 1 year. However, the same group using the same strain, incubated under the same conditions, later reported that only 1% of cells were viable (de Abreu Meireles *et al.* 2015).

The use of LIVE/DEAD BacLight assumed viability if cell membranes have not been damaged but it may not be an indicator that cells could reproduce. Here SYTOX® Orange, which is membrane impermeant, binds to nucleic acids once membrane damage has occurred. Once cellular membrane injury transpires cell death is then assumed based on the leakage of intracellular components into the surroundings, DNA degradation and the lack of evidence to date showing that *Microcystis* can repair cell wall / membranes. In addition viable cells are more likely to record positive CMFDA signals detecting hydrolytic enzymes, which was used during this chapter analysis revealing an active metabolism.

De Abreu Meireles *et al.* (2015) viability assessment worked on the basis that the small population of *M. aeruginosa* (1%) which retained their red autoflourecence (chlorophyll *a*) increased in numbers when transferred to nutrient replete conditions. Heterogeneity in microbial metabolic activity could also affect the final composition of the CM (Kell *et al.* 1998; Lidstrom and Konopka 2010), as here only 5.6% of *Microcystis* used to generate CM were reported to be viable (Table 6.1 and Figure 6.3) and like in de Abreu Meireles *et al.* (2015) could suggest a difference in phenotypes.

Future work could focus on the viable cells left in when generating CM, as isolating these individuals (which can be achieved through FCM sorting) could provide valuble information on the persistence of *Microcystis* or resistance to anti-proliferation compounds.

6.4.3 Effect of CM on *Microcystis aeruginosa* growth rates

This study demonstrates that CM from a collapsed / crashed culture of *M. aeruginosa* (PCC 7806) can inhibit cell proliferation and induce mortality. A population decline was observed when CM was introduced during lag and exponential *M. aeruginosa* growth under light conditions. Similar to *Synechococcus* (Cohen *et al.* 2014), growth rates were only affected when CM was applied to low cell numbers, with no effect occurring at high densities. Conversely, Dagnino *et al.* (2006) did not draw the same conclusion with *M. aeruginosa,* suggesting that CM acted equally on cells from all batch cycle stages. Here, high cell numbers in a stationary phase were calculated at ≈$14x10^6$ cells/mL, whereas in Dagnino *et al.* (2006) cell maxima could not be determined from the publication, which could be much lower than numbers seen in this study.

It is possible that like *Synechococcus* in large numbers *M. aeruginosa* produced high quantities of metabolites that reduced the harmful effects of CM. In Cohen *et al.* (2014) damage by oxidative stress through *Synechococcus* CM was alleviated by antioxidants (glutathione or N-acetyl cysteine), which in high densities was postulated to enable CM resistance. This was also confirmed by a high density *Synechococcus* KatG mutant (lacking in catalase-peroxidase) being sensitive to CM compared to the wild type. CM sensitivity was however induced in high densities of *Synechococcus* through chloramphenicol treatment, indicating that *de novo* synthesis of protein is required for cell damage prevention (Cohen *et al.* 2014). Superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST) and glutathione (GSH) are antioxidant metabolites that have responded to oxidative stress induced by nonylhenols and pyrogallol in *Microcystis* (Wang *et al.* 2007; Shao *et al.* 2009). Extended work could concentrate on anti-oxidant increase or reactive oxygen species detection to help determine further the mode of mortality from CM.

Both Dagnino *et al.* (2006) and Cohen *et al.* (2014) recorded a growth rate inhibition where cell death occurred but not significantly enough to cause total population mortality. Here, under light conditions *M. aeruginosa* numbers in lag and exponential phases decreased after 24 hours in a CM dose dependent manner. However, population recovery occurred after 24 hours in all except the highest concentration of CM (75%) during the lag phase culture cycle. Evidence between CM concentration and cell numbers indicates that the CM requires high concentrations to have a sustaining effect.

However the complete reduction of *M. aeruginosa* in 75% CM may be due to a couple of underlying factors. The first could be from diluting initial experimental numbers too low, as stock cultures were usually kept at ≈4x10^5 cells/mL and once diluted to 75% CM levels cell counts would be ≈1x10^5 cells/mL. Here, the stock *M. aeruginosa* (PCC 7806) had been kept as a culture line for a long time, which may see relatively low populations unable to increase from such starting densities. Nevertheless *M. aeruginosa* did survive in dark conditions incubated with 75% CM, which may also suggest that the high mortality rates after day one under light experimentation were a too greater loss to recover from.

A second factor could be to do with the change in pH during the addition of CM. As cyanobacteria such as *Microcystis* are found in more alkaline waters (Carvalho *et al.* 2011; Maileht *et al.* 2013) decreasing the pH or increasing the acidity may result in population mortality, as suggested in Chapter 5 where certain concentrations of acetic acid reduced the pH in test cultures. Absence of pH recordings was an oversight that ideally should have been conducted during this experiment. However, pH levels were also neglected during the work carried out by Dagnino *et al.* (2006) and Cohen *et al.* (2015), in addition to observations taken by de Abreu Meireles *et al.* (2015).

6.4.4 Light dependent toxic effect of CM

As CM sensitivity only occurred during light conditions it can be implied as in Cohen *et al.* (2014) involvement of the photosynthetic electron transport chain. Although Dagnino *et al.* (2006) provides evidence through TEM that CM effects the organisation of photosynthetic apparatus, it cannot be ruled out that the compound is photoactive like hydrogen peroxide (Mikula *et al.* 2012). Despite no detailed analysis of the photosynthetic apparatus was recorded, there were definite visible signs of de-pigmentation recorded in lag phase experimental conditions (CM 75%) under light conditions (Appendix F9).

By using TEM (Dagnino *et al.* 2006) the CM effects on photosynthetic apparatus can be observed. Although, a more feasible way of recording photosynthetic activity could be done through fluorometry to analyse photosynthetic efficiency over the experiment. If the CM contains an oxidative agent, ROS detection could help identify the mode of mortality and be tested by the molecular probe CM-H_2DCFDA (a general oxidative stress indicator) through FCM (Section 2.6 and Chapter 5).

6.4.5 Effects of CM on *Microcystis aeruginosa* membrane integrity and metabolism

Through SYTOX® Orange and CMFDA molecular probe analysis a potential phenotypic response was observed, as both viable and non-viable cells were recorded in test cultures sensitive to CM. In addition a small viable cell population was also found when generating the original CM under nutrient and light stress after 7 weeks (Figure 6.3 A). Potentially the viable heterogeneity in *M. aeruginosa* exposed to CM allowed the resistant populations to recover after initial reduction of densities, thereby increasing survival rates. This was supported by a strong inverse relationship in *M. aeruginosa* physiological cell states to all CM concentrations (Appendix F11) after both initial cell reduction and when populations were recovering in numbers.

A similar phenotypic *M. aeruginosa* response had been previously observed from chlorosis induced by nutrient depletion under light conditions (de Abreu Meireles *et al.* 2015), indicating that a mortality process would have an eventual population-level benefit (Franklin *et al.* 2006) especially when there is nutrient limitation. Although programmed cell death in cyanobacteria remains unclear (Franklin *et al.* 2014) further research could involve testing for caspase-specific activity and metacaspase gene expression to identify the exact mode of cellular mortality.

Despite recording viable cells after the initial decrease and subsequent increase of experimental cultures a number of factors could have contributed to the heterogeneity of *M. aeruginosa* physiological cell states (Tweeddale *et al.* 1998; Lidstrom and Konopka 2010). Temperature in particular is a positive selection for cyanobacterial species (Wagner and Adrian 2009; Kosten *et al.* 2011; Rigosi *et al.* 2014), including *M. aeruginosa* (Chapter 3).

In this study, like many others that test on *Microcystis,* optimal parameters are usually set up for incubation and experimentation. Temperature is very important for single cell physiology and ultimately the growth rates of populations and here like the previous *M. aeruginosa* CM experiments (Dagnino *et al.* 2006; de Abreu Meireles *et al.* 2015) temperatures were kept between 23^{O}C and 29^{O}C. Those constant higher temperatures are not usually reflective of temperate climate as found in the U.K. and could possible interfere with the efficacy of CM on *Microcystis* and should be closer examined.

Although a volume to volume concentration was conducted (v/v%) perhaps a better design type for testing concentrations would have been to examine the mass percentage of CM concentrations, with ultra-pure filtered water as a control. Mass percentage concentrations would also be more valuable for the applied aspect in the environment / industry as adding relative water volumes to large aquatic bodies such as lakes and reservoirs is impractical.

6.4.6 Characterisation of potential *Microcystis* auto-inducing compounds

Only under light conditions did a significant correlation between membrane rupture increase and metabolic decrease correlate with cell count reduction, indicating that *M. aeruginosa* CM contains a photoactive cytotoxic compound. Further studies should also expose *M. aeruginosa* CM to other genera as described by Cohen *et al.* (2014) to confer a photosensitizer compound, such as other common freshwater toxin producing cyanobacteria (e.g. *Anabaena* / *Dolichospermum* sp.), non-photosynthetic microbes like heterotrophic bacteria, and / or non-photosynthesising cyanobacterium mutants (e.g. the TD34-mutant of *Synechocystis*, which lacks a functional photosystem II reaction center and grown in an enhanced glucose medium).

Initial characterisation of a cyanobacteria CM growth inhibitor seems to indicate a very robust substance, as boiling and or freezing (as found in this study) still induced a reduction in cell growth (Dagnino *et al.* 2006; Cohen *et al.* 2014). Although Dagnino *et al.* (2006) gave sound reasoning that the compound could be a low weight, heat stable, relatively apolar molecular, it cannot be dismissed that a number of molecules / compounds could have caused *M. aeruginosa* growth inhibition. Collective activity of compounds such as polyphenols have been reported to synergistically inhibit growth of *M. aeruginosa* from higher plant allelopathy*,* so potentially CM could have a number of compounds interacting at multiple sites (Nakai *et al.* 2000; Gniazdowska and Bogatek 2005).

The use of techniques such as solid phase microextraction (SPME) – gas chromatography and proton nuclear magnetic resonance (^{1}H NMR) in conjunction with real time PCR could result in single metabolites being isolated and quantified. If there is an up or down regulation of gene expression due to the compounds released in a density dependent manner it would also indicate that auto-induction occurs on growth rates in *M. aeruginosa*, giving a further insight to environmental toxic *Microcystis* blooms.

6.5 Conclusion

This study demonstrates that a bi product from a nutrient-deplete culture of *M. aeruginosa* can auto induce a sub lethal and lethal effect on conspecific populations, which can be strongly linked to quorum sensing through its density dependent mode of action. The death of *M. aeruginosa* cells through CM could have important ramifications in biogeochemical dynamics and food web structures of aquatic environments. The production of compound(s) from *M. aeruginosa* at collapsing densities could potentially alter aquatic microorganism species composition through *M. aeruginosa* mortality, shifting in phytoplankton community composition or the selection of a phenotype that has higher fitness (Lidstrom and Konopka 2010; Schatz *et al.* 2013; de Abreu Meireles *et al.* 2015).

Extended experiments on auto-inducing products could yield anti-proliferation products capable of controlling nuisance cyanobacteria, such as *Microcystis* as found here. This study also highlights the need for comprehensive testing of any potential cyanobacterial control substance. As the knowledge of when to apply and to what target population density would make any application more efficient for water treatment processes.

Chapter VII: Overview

7.1 Overview of research

Microbes are the most frequent and prevalent health risk associated with drinking water, where cyanobacteria densities can have a significant impact on water quality designated for human consumption. The research undertaken for this thesis adopted a holistic approach to a particular nuisance algal species, *Microcystis*. Here a real-time monitoring protocol was developed to identify and establish seasonal patterns of *Microcystis* in a British reservoir (allocated for drinking water) through flow cytometry (FCM). Operational monitoring of novel control techniques were also devised through biological, chemical, and biochemical means, which were discussed in the context of their ecological function and in water treatment processes. The developed FCM protocols also allowed analysis of population and single-cell physiology through optimisation of molecular probes.

As there are knowledge gaps in cyanobacterial physiological cell states undergoing stress and mortality *Microcystis* membrane integrity, metabolic activity (hydrolytic enzymes) and detection of intracellular reactive oxygen species (ROS) were assessed during the testing of control techniques. To ensure control measures had the potential to be applied to real world situations / problems both toxic and non-toxic *Microcystis* were experimented on, along with a fresh isolation. *Microcystis* analysis for both *in-situ* monitoring and testing of control measures were also carried out through various densities, batch cycles, nutrient and environmental (light) conditions to ensure accurate detection and assessment of the most effective application. To address the aims proposed in this thesis each working chapter will be summarised incorporating the main results, limitations, potential future work and contributions to *Microcystis* ecology and biology.

7.2 Chapter II - Methodological development

Enumerating and assessing morphology of Microcystis through FCM

Early on researchers have recognised the need to automate cell counts, as larger data sets can significantly increase our knowledge of a phytoplankton community especially in drinking water sources (Marie *et al.* 2006). *Microcystis* densities and cell dimensions (diameter and volume) were successfully analysed through the use of light scatter and fluorescent photosynthetic pigments, which were subsequently used for *in-situ* monitoring and laboratory experiments. Although, to obtain the most accurate and precise cell counts an understanding of both FCM instrumentation and target organism was essential. By running a series of different FCM settings any background (electronic) noise and sample debris was eliminated, subsequently refining histogram peaks for a higher resolution data set (Figure 2.2). The knowledge of correct thresholds, fluidic speeds, core sizes and acquisition times of *Microcystis* increased the accuracy of cell counts. However, changing these settings led to shifts in light scatter and fluorescence outputs which was most notable on the forward light scatter (FSC) when determining cell size.

Although the FCM used during these experiments (Accuri C6) has a microprocessor-controlled peristaltic pump system that allows sample volume per run to be determined and the proper data acquisition procedures were performed (thresholds, triggers, identification of particles of interest and data acquisition rates), caution is still needed when following flow rates. As seen in Section 2.2.2 where slight differences in cell counts were made when measuring *Microcystis* samples.

To run a quality check on flow rates counting beads can be used as a calibration, where either a set number from a stock concentration is calculated against a direct volume or counted alongside other enumeration equipment e.g. Beckman Coulter counter. Here a volumetric test was routinely performed using deionised filtered water, where the total reported sample volume acquired was checked against the difference in weight both before and after each run.

Although there may be slight variations when using different settings, cell concentrations, volumes and viscosity between samples, FCM has actually a lower error rate compared to traditional microscopy and various automated counting software (Chapman *et al*. 2015). With this in mind and the speed that FCM can analyse samples the developed methods for enumerating certain toxic microbes such as *Microcystis* can be vital to research and industry.

By using FCM as a potential pre-screening for water quality checks it would reduce the cost of labour and time, allowing certain samples to be prioritised if gated areas are found to contain high amounts of cells that could be nuisance algal species. With further technological advances in hardware and software such as imaging (FlowCam) there could be further automation, providing water managers with an earlier warning system for potential CHAB formation so control measures can be implemented.

In addition, FCM can contribute to our knowledge of cyanobacterial ecology, as more high frequency data sets can be acquired (hourly) in freshwater systems, allowing the analysis of patterns which may have been missed if sampling was less regular e.g. weekly or even daily (Bowes *et al.* 2016 –*unpublished*).

Microcystis cell sizing

FCM not only enumerates cells but can also give important information on cell characteristics such as cell diameter and biovolume. By using a set of flow cytometric grade polystyrene sizing beads a calibration curve was constructed allowing the measurement of cell diameter through forward light scatter (FSC). As above the understanding of FCM settings is crucial, as changing fluidic speeds can result in light scatter frequency shifts due to laser Doppler Anemometry (LDA) (Durst *et al.* 1981), which was recorded when using a higher custom setting compared to a lower fluidic speed in the slow settings (Figure 2.8).

Accurate cell sizing through FSC is on the assumption that the cells / particles are spherical in nature, as light scatter will be proportional to the surface area of the cell passing through a laser. Colony forming algal such as the filamentous *Anabaena* sp. / *Dolichospermum* sp. would therefore be hard to size individual cells (and enumerate) without breaking up colonial structures. Here both laboratory culture strains and the environmental *Microcystis* detected for reservoir monitoring did not form large colonies (Figure 1.4 and Figure 3.4). Nevertheless, an optimal technique to fragment *Microcystis* colonies was developed through ultrasound if bloom formation occurred, being a pre-requisite for FCM analysis.

Microcystis colony fragmentation

As *Microcystis* can form large irregular shaped colonies many pre-treatment steps have been used to separate individuals for accurate counting (Section 2.5 and Appendix B4). From a colonial forming *Microcystis* isolate (Longham sp.) a low (vortex) and high (ultrasound) impact technique was used to analysis colonial fragmentation, cell counts and morphologies through FCM (size – FSC and internal granularity – SSC). Only exposure to 50s of ultrasound (sonication bath 38kHz±10%, 75W, KC2, Kerry, USA) resulted in colonial separation good enough to enumerate cells (Appendix B4).

However, from the resulting exposure to ultrasound *Microcystis* cells recorded a higher FSC and lower SSC output due to an increase in size and collapse of gas vesicles. The change in FSC and SSC outputs highlight the need to understand each target species and how it can be affected by potential stressors. Although the ultra sound technique was good enough to obtain accurate cell counts from a colony forming species, caution should be exerted when trying to obtain physiological results such as cell sizing or membrane integrity, as cells did rupture after prolonged sonication (≥60s).

The use of colony fragmentation can further our knowledge of cyanobacterial eco-physiology as it can provide information on how extracellular matrices can adjust to environmental dynamics affecting the microbial community (antagonistic or symbiotic), conspecific communication (quorum sensing), protection and nutrient acquisition and assimilation (Sharif *et al*. 2008; Helm and Potts 2012; Schatz *et al*. 2013).

Isolation

The physiological representativeness of *Microcystis* laboratory strains which may have been maintained under artificial conditions for years or even decades can be questioned, providing impetus for the development of simple protocols to isolate fresh cultures from natural populations (Lynch 1991; Lakeman *et al.* 2009). To address aim 1 environmental cultures containing *Microcystis* biomass were modified in accordance with eco-physiological requirements. After centrifugal washing the environmental samples containing *Microcystis* were exposed to increased temperatures and decreased nutrients, which opposed optimal conditions of a co-occurring *Synechococcus* sp*.* resulting in a fresh uni-algal *Microcystis* isolate (Hartnell *et al.* 2016).

Although extremely relevant for *in-situ* detection and testing control measures the isolation and purification of the cyanobacterium was a time consuming process and relatively labour intensive. In addition, after a couple of months the isolate (CCAP 1450/17) did lose its colony formation resulting in a unicellular culture. As colony fragmentation eventually occurred future work should focus on the mechanisms that destabilise mass bloom formation, as it could potentially reveal a method to prevent the dominance and persistence of *Microcystis* that degrades drinking water quality.

Microcystis physiology and molecular probe optimisation

Microbial subpopulations in field and laboratory studies have been shown to display high physiological heterogeneity (Kell *et al*. 1998; Lidstrom and Konopka 2010). As such the use of molecular probes through FCM to identify parameters like membrane integrity, metabolic activity (hydrolytic enzymes) and reactive oxygen species (ROS), as conducted in this research, can be very useful in determining the response of microbes to particular stresses. However, organisms vary in terms of molecular probe uptake and efflux due to different pores and pumps in cellular walls and membranes, which have led to a number of molecular probe designs and protocols (Shapiro 2003; Davey 2011).

To address aim 2 the development of a methodology which could be transferred to a number of organisms for a number of molecular probes was devised. Here the testing of molecular probes at different concentrations and incubation times determined optimal protocols for SYTOX® Green and Orange (membrane integrity), CMFDA (hydrolytic enzyme activity) and CM-H_2DCFDA (ROS detector). The optimised molecular probes were subsequently used to identify the physiological responses of *Microcystis* undergoing control measures in the proceeding chapters (Section 2.6).

Simulating the causes of natural cell death is extremely difficult as ingestion by grazers, viral lysis or programmed cell death cannot always be achieved under laboratory conditions. Experimental modes of mortality are often questioned as they may not be equivalent to stresses induced from the environment, such as heat killing or exposure to hydrogen peroxide (H_2O_2). To avoid such a paradox the operational definition by which the experiments measured must be clearly established as not to lead to confusion, easing normalisation between studies.

This study also reduced the application of molecular probes to a single step to avoid washing processes that could potentially be counter intuitive by causing cell death in itself. Future work on molecular probe optimisation should look at natural isolates, as extra cellular matrices would affect the uptake of compounds. The accurate analysis of population physiological states during bloom formation could also be applied to understanding patterns of cell death better (Sigee *et al.* 2007), thereby contributing to *Microcystis* ecological roles and identifying stress factors that cause cyanobacterial mortality.

Toxin analysis

As a principle component of monitoring programmes in drinking water sources the identification and quantification of toxins are essential (Chorus and Bartram 1999). A new Ultra Performance Liquid Chromatography (UPLC) method was created here with Dr. Andrew Turner from the Centre for Environment, Fisheries and Aquaculture Science (CEFAS) to increase the sensitively of microcystin (MC) detection when testing toxic strains. Although very successful, as a whole the preparation and run times were relatively long and labour intensive, which required modern high tech equipment in a laboratory setting. In addition the specialist training and cost of equipment to run samples was again relatively high and not always accessible, especially in areas of economic instability.

Despite already being an industry standard for cyanotoxin detection liquid chromatography (LC) equipment cannot be taken into the field. The portability of LC can be problematic as variation in cyanotoxins such as MC-LR is attributed to genotypes and acclimation to the immediate environment, whereby toxins levels can differ from source to the laboratory (Dittman and Börner 2005). Future work should combine more portable detection devices such as Raman spectroscopy, enabling drinking water managers to have more accurate real time data and increase our knowledge on the dynamics of toxic blooms.

7.3 Chapter III - Monitoring the seasonal dynamics of *Microcystis* in a lowland British reservoir using flow cytometry

Owing to its complex and sensitive nature the detection and the time taken to obtain results when testing for specific microbes is generally limited to raw water quality (WHO 2008). Major governing bodies that legislate to protect our water sources (in particular for human consumption) such as the European Union (EU) require microbial monitoring programmes of all its waters (WFD 2000/60/EC). In this chapter a real time, rapid and accurate FCM assay was developed to detect and enumerate *Microcystis*-like cells at a local reservoir (Longham reservoir, Dorset, UK) which is used as a drinking water storage facility.

By using a number of *Microcystis* cultures lines (including two fresh isolates) and a number of non-*Microcystis* species commonly found at the reservoir, FCM references for light scatter and fluorescence emissions for the local phytoplankton community was established (Figure 3.5 and Figure 3.6). These FCM calibrations were then used as a discriminative analysis to count *Microcystis*-like cells addressing aim 3. As no research has been carried out in shallow British lowland reservoirs for *Microcystis* seasonal patterns the developed protocol for aim 3 was subsequently used to answer aim 4. The novel FCM assay was implemented to monitor *Microcystis*-like cells for 22 months, where (water) temperature was reported to be the most significant factor influencing the cyanobacterium population.

Although a very robust and fast way of obtaining *Microcystis*-like cell counts there could potentially be problems when mass bloom formation occurs. High numbers of cells in a colony increases the spread of FCM histogram points, thereby decreasing the accuracy of sample analysis. To overcome this problem for *Microcystis* fragmentation of colonies through ultrasound can be achieved, where cells can then be individually and accurately counted (Section 2.4). However, to transfer this protocol to other cyanobacterium species such as *Anabaena* sp. / *Dolichospermum* sp. it presents some challenges, as it is extremely difficult to separate individuals from a filamentous colony.

The total volume for each environmental sample run may also come into question as here 500 µL was analysed, which may seem unrepresentative of a water body, even a non-stratifying small reservoir. The standard method for counting phytoplankton (Utermöhl method – inverted microscope) can analyse approximately between 5 – 50 mL depending on the sediment chamber unit used and was adopted by the contracted laboratory to estimate algal numbers in the reservoir. However, phytoplankton samples usually require 24 – 48 h of settling before analysis can take place.

The FCM results as provided in this chapter can be obtained in as little as 5 minutes, where the short run times can offer a number of repeats or even allow for higher volumes to be processed, reducing labour and time needed for sediment chambers and offsetting disadvantages from small volume sub sampling.

Despite FCM light scatter and fluorescence emission(s) calibrations from *Microcystis* cultures, in addition to light microscopy confirmation from environmental samples, it would not always be possible to confirm each individual cell as *Microcystis.* With the other tested groups of phytoplankton there were very distinctive and also very similar light scatter and fluorescent emissions that over lapped the *Microcystis* calibration ranges, which may raise the issues of accuracy, nevertheless by discriminative analysis *Microcystis*-like cells were enumerated.

To eliminate any doubt of *Microcystis* count validity FCMs with sorter capabilities can separate organisms from the set *Microcystis* calibrated ranges, be re-sampled then viewed through light microscopy as a form of quality control. To develop this novel technique further the incorporation of imaging (FlowCam) into FCM could enhance genus / species detection, where software can be trained to identify large biomass in the form of colonies / filaments (Sieracki *et al.* 1998; Poulton 2006; Dashkova *et al.* 2016). Although, FCMs with sorting and imaging options are usually more expensive than the desktop analyser used through-out this thesis. However, FCMs without sorters can still be a great indicator of increased cyanobacterial numbers (phycocyanin increase, FL4-H channel), which can be used as a pre-screening step to prioritise samples potentially allowing for quicker dissemination of cyanobacterial data for water managers.

Whilst flow cytometry holds many advantages and exciting opportunities for aquatic microbiological enumeration the start-up cost may seem quite high, especially for economically limited areas. Nevertheless short comings of costs may be addressed by the potential high through put and reduced labour required for numerous samples. Once developed the protocols for FCM analysis of phytoplankton can have a positive impact on human water security and can also contribute to the understanding of phytoplankton community structures and CHAB development.

7.4 Chapter IV - Grazing of *Blepharisma americanum* on toxic and non-toxic *Microcystis aeruginosa* cells

Ciliates in their ubiquity offer a natural solution to cyanobacterial reduction but the understanding of these predator prey interactions is complex and unclear (Finlay and Esteban 1998; Esteban *et al.* 2015) and most notably rarely tested as an anti-*Microcystis* solution. This chapter addressed aim 5 by providing evidence that a model ciliate *Blepharisma americanum* can graze on *Microcystis* contributing to their role in the microbial loop.

B. americanum was incubated with a toxic and non-toxic *M. aeruginosa* strain (PCC 7806) at two different densities to observe their trophic interactions. *B. americanum* controlled non-toxic *M. aeruginosa* growth but recorded a decrease in ciliate biovolume, potentially due to the nutritional value of cyanobacteria (Holm *et al.* 1984; Fink *et al.* 2011; Von Elert *et al.* 2014). In contrast toxic *Microcystis* did not reduce in numbers when grazed on by *B. americanum.* However toxic cells were observed to be ingested but later expelled through the cytoproct without being digested (Figure 4.6). Compared to the control, mean intracellular toxin levels (MC-LR) over the experiment significantly decreased in the lower initial density grazed cultures, indicating a release associated with predation.

Even though toxic *M. aeruginosa* densities were not supressed the ecological significance goes beyond quantitative cyanobacterial uptake, as it both selected for non-toxic *Microcystis* and recorded a smaller phenotype of ciliate, contributing information to their trophic relationship in the microbial loop.

However, the use of *M. aeruginosa* strain PCC 7806 may be questioned due to its non-colonial formation and the length of time it has been an established deposited culture line. Filter feeders like ciliates may not have the physiological mechanics to feed on

colonial or filamentous cyanobacteria, however, not all *Microcystis* populations form colonies in the wild (Chapter 3). Further grazing experiments with *B. americanum* could use freshly isolated colony forming *Microcystis* but it would difficult to i) establish two wild strains that have toxic and non-toxic production capability from the same water source and ii) have a colony that is completely uni-algal, as other microorganisms like heterotrophic bacteria are associated with the mucilage or extracellular polysaccharides (EPS) formed in blooms (Brunberg 1999) which would affect predatory behaviour.

The ecological relevance of such a grazer as *B. americanum* must also be viewed in terms of their presence, potential numbers, location and sustainability in areas of *Microcystis* bloom formation. Further information on what happens to dense *in-situ* populations of the ciliate is needed as i) in turn they may be heavily preyed upon and be limited in their cyanobacteria grazing, ii) be predominantly found only near decomposing vegetation or areas with limited irradiance or iii) may not adapt to local environmental conditions e.g. encystment phase for successful generations (Giese 1973).

Nevertheless *Microcystis* blooms are influenced by wind mixing and will often be found on the peripheries of water bodies (Šejnohová and Maršálek 2012; Whitton and Potts 2012) near vegetation. Even though this cyanobacterium-protozoan relationship may not be directly strong the understanding of what influence *B. americanum* has on *Microcystis* is extremely important, as here it could suggest such a predator can significantly alter the microbial community architecture by shifting from non-toxin cyanobacteria densities to toxin producing populations.

This chapter provided evidence of effective ciliate grazing on a bloom forming cyanobacteria where previous information has been limited. Subsequent experiments could also expand the number of ciliate genera tested (including other *Blepharisma* species) to decrease the knowledge gap of trophic interactions between protozoans and cyanobacteria in the microbial loop.

7.5 Chapter V - Comparing hydrogen peroxide (an established chemical control agent) with acetic acid, a novel control agent for the cyanobacterium *Microcystis*

The use of chemical agents can reduce phytoplankton numbers rapidly in water sources that are limited and in demand. Consequently the search for natural algalcides which balance economical and ecosystem dynamics is highly sort after. In this chapter following on from chemicals identified in decomposing barley straw, which has recorded *Microcystis* biomass reduction (Everall and Lees 1997), acetic acid was tested alongside an established chemical control H_2O_2. To address aim 6 a fresh isolate of *Microcystis* was exposed to different concentrations of acetic acid and H_2O_2 under both light and continuous dark conditions, where with cell numbers and physiological states (membrane integrity and ROS detection) were analysed.

Both chemicals were effective at decreasing *Microcystis* numbers, however, H_2O_2 required smaller concentrations but acted as a photosensitiser compound requiring light to facilitate any effect unlike acetic acid. Both acetic acid and H_2O_2 induced ROS and increased membrane injury along with photosynthetic pigment changes (Figure 5.1). The fresh isolate was also less sensitive to H_2O_2, requiring 40 mg/L to record a large final cell reduction (> 99%) significantly more than *Microcystis* strains tested on in previous studies (Table 5.2).

During this experiment it was noted that acetic acid needed low pH levels to cause *Microcystis* biomass reduction, which was not required for H_2O_2 applications. Low pH levels may not always be feasible in certain water bodies, due to ecosystem requirements or corrosion of equipment. However, *Microcystis* and most temperate freshwater cyanobacteria are distributed and dominate in high alkaline areas (Carvalho *et al.* 2011; Maileht *et al.* 2013), so lowering pH levels may mitigate populations.

The low pH levels when higher concentrations of acetic acid were used in this experiment may have interfered with the results obtained by both molecular probes. For example CM-H_2DCFDA fluorescence at 100 and 400 mg/L exhibited significantly less positive signals compared to the lower concentrations of 40 mg/L under continuous dark conditions. A single step ROS probe application for acetic acid $\geq$ 40 mg/L may not be suitable to obtain reliable results, although simply increasing the pH by using a an appropriate buffer solution could overcome this. Nevertheless the concentrations for working molecular probe pH levels ($\leq$ 40 mg/L) did report ROS

activity, which to date been has not been recorded as a mortality factor in cyanobacteria through exposures of carboxyl acids.

The use of acetic acid on *Microcystis* as a potential control contributes the lack of experimentation through exposures of cheap, accessible, naturally degrading chemicals. Although hydrogen peroxide was comparably a better density control agent the restrictions on applications in UK waters requires other chemicals to be tested. The use of chemical controls will always depend on the end management of catchment waters, as acetic acid may be detrimental to other organisms *in-situ*, but may be suitable for aquatic bodies used as pre-treatment storage of drinking water (e.g. small reservoirs).

An extension to the work done in this chapter should concentrate on testing acetic acid on other phytoplankton groups, as certain compounds like H_2O_2 work better on some algal groups (cyanobacteria) than others (green algae) (Drábková *et al.* 2007*a*, 2007*b*, 2007*c*). On a larger scale the efficacy of acetic acid should also be tested such as waste waters, where acetic acid may be more economically viable and a safer option compared to other chemicals like alum or hydrochloric acid. In addition peracetic acid (also known as peroxyacetic acid), a mixture of acetic acid and hydrogen peroxide, could be tested on *Microcystis,* as its biocidal activity has been reported not to affect pH, water hardness or produce toxic bi-products (Kramer 1997). In addition peracetic acid has been recorded to enhance the photocatalytic and sonophotocatalytic inactivation of coliforms (*E. coli*) in aqueous suspensions (Drosou *et al*. 2010).

7.6 Chapter VI - An evaluation of *Microcystis* auto-inhibition and cell mortality from a chlorotic, cell free media extract

Until recently cell-to-cell communication or quorum sensing in cyanobacteria had not been characterised. A study into the addition of conditioned media (CM) obtained from a filtered, nutrient-deplete, cell free *M. aeruginosa* culture reported auto-inducing effects on co-specific densities (Dagnino *et al.* 2006). In this chapter the work on *M. aeruginosa* auto-inhibition from CM was examined further to address aim 7, as *Synechococcus* CM auto inhibition had reported contrasting effects under different conditions (densities and irradiance) (Cohen *et al.* 2014) which were not factored into the Dagnino *et al.* (2006) study. In addition to different CM concentrations under both light and continuous dark conditions *M. aeruginosa* was incubated with CM using

various batch cycles (lag, exponential and stationary), and for the first time in the cyanobacterium a dual molecular probe protocol was designed to analyse membrane integrity (SYTOX® Orange) and metabolic activity (CMFDA-hydrolytic enzyme detector).

CM recorded an initial *M. aeruginosa* cell reduction in the lag and exponential phase cultures with increased membrane injury (SYTOX® Orange) and decreased enzyme activity (CMFDA). Like Cohen *et al.* (2014) CM was only effective under light conditions in a density dependent manner attributing the compound(s) to quorum sensing and the photosynthetic electron transport chain, but more importantly confirmed to be anti-microbial to *M. aeruginosa*. This chapter also supported de Abreu Meireles *et al.* (2015) study in that *M. aeruginosa* surviving cells exhibited a different phenotypic response to CM, through their resistance and distinct physiological states (Figure 6.5 and Figure 6.6).

However, the doses in terms of a volume to volume mix (v/v%) used in this experiment which conferred *M. aeruginosa* cell decrease would require a large amount of toxic algae to be cultivated, consequently resulting in storage and time constraints. To improve on this chapter's methodological design a mass percentage of concentration increase should be implemented, as direct cell counts can then be compared. Nevertheless as the CM worked after being frozen and is very heat stable (Dagnino *et al.* 2006) the isolated auto-inducing compound(s) could potentially be synthesised and stored more efficiently. Future work to identify the exact compound(s) that caused *M. aeruginosa* biomass reduction can be conducted through techniques like solid phase microextractions (SPME) – gas chromatography and proton nuclear magnetic resonance (^{1}H NMR). In addition other strains of *M. aeruginosa* or species of the same genus could provide further evidence of a *Microcystis* control and other CM compounds.

The conditions at which *M. aeruginosa* were under to both produce and be experimentally incubated with CM can have an effect on single cell physiology and subsequent populations. Absent here were pH, nutrient and microcystin recordings which could have significantly been altered with high v/v% (e.g. 75% CM) and changed cell counts. It is also not (like a lot of other *M. aeruginosa* experiments) too indicative of environmental settings when testing under laboratory conditions, especially using constant optimal parameters (e.g. light and temperature) which are very dynamic in temperate regions like Britain.

This chapter has recorded density and cellular physiological state changes when a bi-product of chlorosis had been incubated with experimental lag and exponential phase *M. aeruginosa* populations. The results point towards the recently recognised quorum sensing action in cyanobacteria, as CM effects increased with concentration (v/v%) and *Microcystis* densities (lag, exponential and stationary). The findings contribute not only to the knowledge of conspecific relationships and phenotypes in *Microcystis* (which could alter aquatic chemistry and phytoplankton community architecture) but also (in conjugation with the other experimental chapters in this thesis) highlights the need for comprehensive testing on preventing potential bloom forming cyanobacteria, where initial densities / life cycles of nuisance microbes need to be understood for control measures to be effective.

7.7 Conclusion

With increased nutrient loading and climate shifts favouring *Microcystis* growth the restoration of water sources affected by cyanobacterial hazardous algal blooms (CHABs) has become increasingly obvious. This research takes a holistic approach on tackling one nuisance toxic cyanobacterial species, *Microcystis*. Firstly a new isolation technique was developed, allowing for the most representative testing of some control measures and environmental detection. Then successful enumeration of *Microcystis* was achieved by a semi-automated approach through flow cytometry, which also allowed when molecular probe optimisation was established population and single-cell physiological analysis.

A novel FCM real time detection protocol was developed to monitor *in-situ Microcystis*, acting as an early warning system for potential CHABs and enabling water resources managers to implement treatment strategies more effectively. In addition biological, chemical and biochemical control measures were tested to broaden how we tackle nuisance cyanobacteria and discussed within an ecological context. This thesis set out to limit the negative impacts of a hazardous microbe in drinking water supplies by “developing new approaches for monitoring and controlling the toxic cyanobacterium *Microcystis* through flow-cytometric analysis”, whereby the aims were addressed to improve drinking water quality and aquatic ecosystem health.

References

Abouziena, H.F., Omar, A.A., Sharma, S.D. and Singh, M., 2009. Efficacy comparison of some new natural-product herbicides for weed control at two growth stages. *Weed Technology*, 23 (3), 431-437.

Adams, D.G. and Duggan, P.S., 1999. Tansley Review No. 107. Heterocyst and akinete differentiation in cyanobacteria. *New Phytologist*, 144 (1), 3-33.

Adrian, R., Walz, N., Hintze, T., Hoeg, S. and Rusche, R., 1999. Effects of ice duration on plankton succession during spring in a shallow polymictic lake. *Freshwater Biology*, 41 (3), 621-634.

Allen, M.M. and Smith, A.J., 1969. Nitrogen chlorosis in blue-green algae. *Archiv für Mikrobiologie*, 69 (2), 114-120.

Ameel, J.J., Axler, R.P., Owen, C.J., Johnson, K.E. and Guyton, B., 1997. The determination of Hydrothol 191 (amine salt of endothall) in eutrophic wastewater ponds. *Chemosphere*, 34 (3), 641-654.

Andersen, P. and Throndsen, J., 2003. *Estimating cell numbers. Manual on harmful marine microalgae*. Paris: UNESCO Publishing.

Aoki, S., Ohara, S., Kimura, K., Mizuguchi, H., Fuse, Y. and Yamada, E., 2008. Characterization of dissolved organic matter released from *Microcystis aeruginosa*. *Analytical Sciences*, 24 (3), 389-394.

Apel, K. and Hirt, H., 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annual Review of Plant Biology*, 55, 373-399.

Arii, S., Tsuji, K., Tomita, K., Hasegawa, M., Bober, B. and Harada, K.I., 2015. Cyanobacterial blue color formation during lysis under natural conditions. *Applied and Environmental Microbiology*, 81 (8), 2667-2675.

Asada, K., 2006. Production and scavenging of reactive oxygen species in chloroplasts and their functions. *Plant Physiology*, 141 (2), 391-396.

Avendaño-Herrera, R., Magariños, B., Irgang, R. and Toranzo, A.E., 2006. Use of hydrogen peroxide against the fish pathogen Tenacibaculum maritimum and its effect on infected turbot (*Scophthalmus maximus*).*Aquaculture*, 257 (1), 104-110.

Axe, D.D. and Bailey, J.E., 1995. Transport of lactate and acetate through the energized cytoplasmic membrane of Escherichia coli. *Biotechnology and Bioengineering*, 47 (1), 8-19.

Babica, P., Bláha, L. and Maršálek, B., 2006. Exploring the natural role of microcystins - a review of effects on photoautotrophic organisms. *Journal of Phycology*, 42 (1), 9-20.

Baker, J.A., Neilan, B.A., Entsch, B. and McKay, D.B., 2001. Identification of cyanobacteria and their toxigenicity in environmental samples by rapid molecular analysis. *Environmental Toxicology*, 16 (6), 472-482.

Ball, A.S., Williams, M., Vincent, D. and Robinson, J., 2001. Algal growth control by a barley straw extract. *Bioresource Technology*, 77 (2), 177-181.

Barrington, D.J. and Ghadouani, A., 2008. Application of hydrogen peroxide for the removal of toxic cyanobacteria and other phytoplankton from wastewater. *Environmental Science & Technology*, 42 (23), 8916-8921.

Barrington, D.J., Reichwaldt, E.S. and Ghadouani, A., 2013. The use of hydrogen peroxide to remove cyanobacteria and microcystins from waste stabilization ponds and hypereutrophic systems. *Ecological Engineering*, 50, 86-94.

Barroin, G. and Feuillade, M., 1986. Hydrogen peroxide as a potential algicide for *Oscillatoria rubescens* DC. *Water Research*, 20 (5), 619-623.

Bartram, J. and Rees, G., eds., 2000. *Monitoring bathing waters: a practical guide to the design and implementation of assessments and monitoring programmes*. Published on behalf of the World Health Organization, Commission of the European Communities and US Environmental Protection Agency. London: E & FN Spon.

Becker, A., Meister, A. and Wilhelm, C., 2002. Flow cytometric discrimination of various phycobilin-containing phytoplankton groups in a hypertrophic reservoir. *Cytometry*, 48 (1), 45-57.

Biosciences, B.D., 2000. Introduction to flow cytometry: a learning guide. Manual Part 1. Available from: http://www.bdbiosciences.com/immunocytometry_systems/support//training //flow_cytometry_learning_guide.pdf [Accessed 3rd July 2014].

Bogorad, L., 1975. Phycobiliproteins and complementary chromatic adaptation. *Annual Review of Plant Physiology*, 26 (1), 369-401.

Booth, I.R., 1985. Regulation of cytoplasmic pH in bacteria. *Microbiological Reviews*, 49 (4), 359.

Bouchard, J.N. and Purdie, D.A., 2011. Effect of elevated temperature, darkness, and hydrogen peroxide treatment on oxidative stress and cell death in the bloom-forming toxic cyanobacterium *Microcystis aeruginosa*. *Journal of Phycology*, 47 (6), 1316-1325.

Bowes, M., Read, D.S., McCall, S., Freeman, A., Gozzard, E., Armstrong, L., Wickham, H., Nicholls, D., and Loewenthal, M., 2016. The impact of nutrient concentrations on algal growth rate and community composition in UK rivers. *British Phycological Society 64th Annual Meeting*. Bournemouth, U.K., 21st-23rd June 2016.

Brierley, B., Carvalho, L., Davies, S. and Krokowski, J., 2007. Guidance on the quantitative analysis of phytoplankton in freshwater samples. Report to SNIFFER (Project WFD80), Edinburgh, December 2007.

Brookes, J.D. and Ganf, G.G., 2001. Variations in the buoyancy response of *Microcystis aeruginosa* to nitrogen, phosphorus and light. *Journal of Plankton Research*, 23 (12), 1399-1411.

Brul, S. and Coote, P., 1999. Preservative agents in foods: mode of action and microbial resistance mechanisms. *International Journal of Food Microbiology*, 50 (1), 1-17.

Brunberg, A.K., 1999. Contribution of bacteria in the mucilage of *Microcystis* spp. (Cyanobacteria) to benthic and pelagic bacterial production in a hypereutrophic lake. *FEMS Microbiology Ecology*, 29 (1), 13-22.

Burkert, U., Hyenstrand, P., Drakare, S. and Blomqvist, P., 2001. Effects of the mixotrophic flagellate *Ochromonas* sp. on colony formation in *Microcystis aeruginosa*. *Aquatic Ecology*, 35 (1), 11-17.

Campbell, D., Hurry, V., Clarke, A.K., Gustafsson, P. and Öquist, G., 1998. Chlorophyll fluorescence analysis of cyanobacterial photosynthesis and acclimation. *Microbiology and Molecular Biology Reviews*, 62 (3), 667-683.

Carey, P., 1992. Marine interstitial ciliates—an illustrated key. Springer Netherlands.

Carmichael, W.W., 1986. Algal toxins. *In*: Callow, E.A., ed., *Advances in botanical research*. Vol. 12. London: Academic Press, 47-101.

Carmichael, W.W., 1992. Cyanobacteria secondary metabolites—the cyanotoxins. *Journal of Applied Bacteriology*, 72 (6), 445-459.

Carvalho, L. and Kirika, A., 2003. Changes in shallow lake functioning: response to climate change and nutrient reduction. *Hydrobiologia*, 506 (1-3), 789-796.

Carvalho, L., Miller, C.A., Scott, E.M., Codd, G.A., Davies, P.S. and Tyler, A.N., 2011. Cyanobacterial blooms: statistical models describing risk factors for national-scale lake assessment and lake management. *Science of the Total Environment*, 409 (24), 5353-5358.

Carvalho, L., Poikane, S., Solheim, A.L., Phillips, G., Borics, G., Catalan, J., de Hoyos, C., Drakare, S., Dudley, B.J., Järvinen, M. and Laplace-Treyture, C., 2013. Strength and uncertainty of phytoplankton metrics for assessing eutrophication impacts in lakes. *Hydrobiologia*, 704 (1), 127-140.

Chapman, I.J., Esteban, G.F., and Franklin, D.J., 2015. Optimising the flow cytometric detection of the cyanobacteria *Microcystis* in a lowland British reservoir. *Association for the Sciences of Limnology & Oceanography, 2015 Aquatic Sciences Meeting, Aquatic Sciences: Global And Regional Perspectives — North Meets South*. Granada, Spain, 22nd-27th February 2015.

Chapman, I.J., Esteban, G.F. and Franklin, D.J., 2016. Molecular probe optimization to determine cell mortality in a photosynthetic organism (*Microcystis aeruginosa*) using flow cytometry. *JoVE (Journal of Visualized Experiments)*, 107, e53036-e53036.

Chislock, M.F., Sarnelle, O., Jernigan, L.M. and Wilson, A.E., 2013. Do high concentrations of microcystin prevent *Daphnia* control of phytoplankton? *Water Research*, 47 (6), 1961-1970.

Choi, O., Deng, K.K., Kim, N.J., Ross, L., Surampalli, R.Y. and Hu, Z., 2008. The inhibitory effects of silver nanoparticles, silver ions, and silver chloride colloids on microbial growth. *Water Research*, 42 (12), 3066-3074.

Chorus, I., 2005. Water safety plans. *In*: Huisman, J., Matthijs, H.C. and Visser, P.M., eds., *Harmful cyanobacteria*. Dordrecht: Springer Science & Business Media, 201-227.

Chorus, I., ed., 2012. *Cyanotoxins: occurrence, causes, consequences*. Dordrecht: Springer Science & Business Media.

Chorus, I. and Bartram J., eds., 1999. *Toxic cyanobacteria in water: a guide to their public health consequences, monitoring and management*. London: E & FN Spon.

Chow, C.W.K., House, J., Velzeboer, R.M.A., Drikas, M., Burch, M.D. and Steffensen, D.A., 1998. The effect of ferric chloride flocculation on cyanobacterial cells. *Water Research*, 32 (3), 808-814.

Chrzanowski, T.H. and Grover, J.P., 2001. Effects of mineral nutrients on the growth of bacteria and phytoplankton in two southern reservoirs. *Limnology and Oceanography*, 46 (6), 1319-1330.

Cirés, S., Wörmer, L., Agha, R. and Quesada, A., 2013. Overwintering populations of *Anabaena*, *Aphanizomenon* and *Microcystis* as potential inocula for summer blooms. *Journal of Plankton Research*, fbt081.

Codd, G.A., 2000. Cyanobacterial toxins, the perception of water quality, and the prioritisation of eutrophication control. *Ecological engineering*, 16 (1), 51-60.

Codd, G.A., Bell, S.G., Kaya, K., Ward, C.J., Beattie, K.A. and Metcalf, J.S., 1999. Cyanobacterial toxins, exposure routes and human health. *European Journal of Phycology*, 34 (04), 405-415.

Codd, G.A., Krokowski, J.T. and Bowling, L.C., 2016. Widening changes in cyanobacterial taxonomy and nomenclature: implications for water body - and risk management. *British Phycological Society 64th Annual Meeting*. Bournemouth, U.K., 21st-23rd June 2016.

Codd, G.A., Lindsay, J., Young, F.M., Morrison, L.F. and Metcalf, J.S., 2005. Harmful cyanobacteria. *In*: Huisman, J., Matthijs, H.C. and Visser, P.M., eds., *Harmful cyanobacteria*. Dordrecht: Springer Science & Business Media, 1-23.

Codd, G.A., Steffensen, D.A., Burch, M.D. and Baker, P.D., 1994. Toxic blooms of cyanobacteria in Lake Alexandrina, South Australia - learning from history. *Marine and Freshwater Research*, 45 (5), 731-736.

Cohen, A., Sendersky, E., Carmeli, S. and Schwarz, R., 2014. Collapsing aged culture of the cyanobacterium *Synechococcus elongatus* produces compound(s) toxic to photosynthetic organisms. *PloS one*, 9 (6), e100747.

Combes, A., Dellinger, M., Cadel-six, S., Amand, S. and Comte, K., 2013. Ciliate *Nassula* sp. grazing on a microcystin-producing cyanobacterium (*Planktothrix agardhii*): impact on cell growth and in the microcystin fractions. *Aquatic Toxicology*, 126, 435-441.

Conley, D.J., Paerl, H.W., Howarth, R.W., Boesch, D.F., Seitzinger, S.P., Havens, K.E., Lancelot, C. and Likens, G.E., 2009. Controlling eutrophication: nitrogen and phosphorus. *Science*, 323 (5917), 1014-1015.

Cooke, G.D., Welch, E.B., Peterson, S. and Nichols, S.A., 2005. *Restoration and management of lakes and reservoirs*. 3rd edition. Florida: CRC Press.

Cooper, W.J. and Zika, R.G., 1983. Photochemical formation of hydrogen peroxide in surface and ground waters exposed to sunlight. *Science (Washington)*, 220 (4598), 711-712.

Crance, J.H., 1963. The effects of copper sulfate on *Microcystis* and zooplankton in ponds. *The Progressive Fish-Culturist*, 25 (4),198-202.

Dagnino, D., de Abreu Meireles, D. and de Aquino Almeida, J.C., 2006. Growth of nutrient-replete *Microcystis* PCC 7806 cultures is inhibited by an extracellular signal produced by chlorotic cultures. *Environmental Microbiology*, 8 (1), 30-36.

Daly, R.I., Ho, L. and Brookes, J.D., 2007. Effect of chlorination on *Microcystis aeruginosa* cell integrity and subsequent microcystin release and degradation. *Environmental Science & Technology*, 41 (12), 4447-4453.

Dashkova, V., Malashenkov, D., Poulton, N., Vorobjev, I. and Barteneva, N.S., 2016. Imaging flow cytometry for phytoplankton analysis. *Methods*. In Press. Available from: http://dx.doi.org/10.1016/j.ymeth.2016.05.007 [Accessed 27th May 2016].

Davey, H.M., 2011. Life, death, and in-between: meanings and methods in microbiology. *Applied and Environmental Microbiology*, 77 (16), 5571-5576.

Davey, H.M., Kaprelyants, A.S., Weichart, D.H. and Kell, D.B., 1999. Approaches to the estimation of microbial viability using flow cytometry. *In*: Robinson, J.P., ed., *Current protocols in cytometry*. Volume 11: Microbial Cytometry. New York: Wiley, 11.3.1-11.3.20.

Davis, T.W., Koch, F., Marcoval, M.A., Wilhelm, S.W. and Gobler, C.J., 2012. Mesozooplankton and microzooplankton grazing during cyanobacterial blooms in the western basin of Lake Erie. *Harmful Algae*, 15, 26-35.

De Abreu Meireles, D., Schripsema, J., Arnholdt, A.C.V. and Dagnino, D., 2015. Persistence of only a minute viable population in chlorotic *Microcystis aeruginosa* PCC 7806 cultures obtained by nutrient limitation. *PloS one*, 10 (7), e0133075.

De Marsac, N.T., 1977. Occurrence and nature of chromatic adaptation in cyanobacteria. *Journal of Bacteriology*, 130 (1), 82-91.

Decho, A.W., Visscher, P.T., Ferry, J., Kawaguchi, T., He, L., Przekop, K.M., Norman, R.S. and Reid, R.P., 2009. Autoinducers extracted from microbial mats reveal a surprising diversity of N-acylhomoserine lactones (AHLs) and abundance changes that may relate to diel pH. *Environmental Microbiology*, 11 (2), 409-420.

Delahaye, E., Welte, B., Levi, Y., Leblon, G. and Montiel, A., 2003. An ATP-based method for monitoring the microbiological drinking water quality in a distribution network. *Water Research*, 37 (15), 3689-3696.

Demain, A.L., 1999. Pharmaceutically active secondary metabolites of microorganisms. *Applied Microbiology and Biotechnology*, 52 (4), 455-463.

Demain, A.L. and Fang, A., 2000. The natural functions of secondary metabolites. *In*: Fiechter, A., ed., *History of modern biotechnology I,* advances in biochemical engineering/biotechnology. Vol. 69. Berlin: Springer, 1-39.

Deniz, F., Saygideger, S.D. and Karaman, S., 2011. Response to copper and sodium chloride excess in *Spirulina* sp. (cyanobacteria). *Bulletin of Environmental Contamination and Toxicology*, 87 (1), 11-15.

Dennis, M.A., Landman, M., Wood, S.A. and Hamilton, D., 2011. Application of flow cytometry for examining phytoplankton succession in two eutrophic lakes. *Water Science and Technology*, 64 (4), 999-1008.

Diez-Gonzalez, F. and Russell, J.B., 1997. The ability of Escherichia coli O157: H7 to decrease its intracellular pH and resist the toxicity of acetic acid. *Microbiology*, 143 (4), 1175-1180.

Ding, Y., Gan, N., Li, J., Sedmak, B. and Song, L., 2012. Hydrogen peroxide induces apoptotic-like cell death in *Microcystis aeruginosa* (*Chroococcales*, Cyanobacteria) in a dose-dependent manner. *Phycologia*, 51 (5), 567-575.

Dittmann, E. and Börner, T., 2005. Genetic contributions to the risk assessment of microcystin in the environment. *Toxicology and Applied Pharmacology*, 203 (3), 192-200.

Dittmann, E., Neilan, B.A., Erhard, M., Von Döhren, H. and Börner, T., 1997. Insertional mutagenesis of a peptide synthetase gene that is responsible for hepatotoxin production in the cyanobacterium *Microcystis aeruginosa* PCC 7806. *Molecular microbiology*, 26 (4), 779-787.

Downing, J.A., Watson, S.B. and McCauley, E., 2001. Predicting cyanobacteria dominance in lakes. *Canadian Journal of Fisheries and Aquatic Sciences*, 58 (10), 1905-1908.

Drábková, M., Admiraal, W. and Maršálek, B., 2007*a*. Combined exposure to hydrogen peroxide and light selective effects on cyanobacteria, green algae, and diatoms. *Environmental Science & Technology*, 41 (1), 309-314.

Drábková, M., Maršálek, B. and Admiraal, W., 2007*b*. Photodynamic therapy against cyanobacteria. *Environmental Toxicology*, 22 (1), 112-115.

Drábková, M., Matthijs, H.C.P., Admiraal, W. and Maršálek, B., 2007*c*. Selective effects of H_2O_2 on cyanobacterial photosynthesis. *Photosynthetica*, 45 (3), 363-369.

Drinking Water Inspectorate – DWI, 2016. *List of approved products for use in public water supply in the United Kingdom*. Available from: http://www.dwi.gov.uk/drinking-water-products/approved-products/soslistcurrent.pdf [Accessed 7th June 2016].

Drosou, C., Coz, A., Xekoukoulotakis, N.P., Moya, A., Vergara, Y. and Mantzavinos, D., 2010. Peracetic acid-enhanced photocatalytic and sonophotocatalytic inactivation of *E. coli* in aqueous suspensions. *Journal of chemical technology and biotechnology*, 85 (8), pp.1049-1053.

Dubelaar, G.B., Geerders, P.J. and Jonker, R.R., 2004. High frequency monitoring reveals phytoplankton dynamics. *Journal of Environmental Monitoring*, 6 (12), 946-952.

Durst, F., Melling, A. and Whitelaw, J.H., 1981. *Principles and practice of laser-Doppler anemometry*. New York: Academic Press.

Elert, E.V., Martin-Creuzburg, D. and Le Coz, J.R., 2003. Absence of sterols constrains carbon transfer between cyanobacteria and a freshwater herbivore (*Daphnia galeata*). *Proceedings of the Royal Society of London B: Biological Sciences*, 270 (1520), 1209-1214.

Elliott, J., 2010. The seasonal sensitivity of cyanobacteria and other phytoplankton to changes in flushing rate and water temperature. *Global Change Biology*, 16 (2), 864-876.

Elliott, J.A., Jones, I.D. and Thackeray, S.J., 2006. Testing the sensitivity of phytoplankton communities to changes in water temperature and nutrient load, in a temperate lake. *Hydrobiologia*, 559 (1), 401-411.

Esteban, G.F., Finlay, B.J. and Warren, A., 2015. Free-living protozoa. *In*: Thorp, J.H. and Rogers, D.C., eds., *Thorp and Covich´s freshwater invertebrates*. Elsevier, 113-132.

Etchegaray, A., Rabello, E., Dieckmann, R., Moon, D.H., Fiore, M.F., Von Döhren, H., Tsai, S.M. and Neilan, B.A., 2004. Algicide production by the filamentous cyanobacterium *Fischerella* sp. CENA 19. *Journal of Applied Phycology*, 16 (3), 237-243.

European Commission, 2003. EC Regulation No. 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. *Official Journal of the European Union,* OJ L268/29.

European Commission, 2008. EC Regulation No. 1333/2008 of the European parliament and the Council of 16 December 2008 on food additives. *Official Journal of the European Union,* OJ L354/16.

European Commission, 2014. *Framework for Action for the management of small drinking water supplies*. Luxembourg: Office for Official Publications of the European Communities.

Everall, N.C. and Lees, D.R., 1997. The identification and significance of chemicals released from decomposing barley straw during reservoir algal control. *Water Research*, 31 (3), 614-620.

Falconer, I.R., 1991. Tumor promotion and liver injury caused by oral consumption of cyanobacteria. *Environmental Toxicology and Water Quality*, 6 (2), 177-184.

Falconer, I., Bartram, J., Chorus, I., Kuiper-Goodman, T., Utkilen, H., Burch, M. and Codd, G.A., 1999. Safe levels and safe practices. *In*: Chorus, I. and Bartram J., eds., *Toxic cyanobacteria in water: a guide to their public health consequences, monitoring and management*. London: E & FN Spon, 155-178.

Fausey, J.C., 2003. Controlling liverwort and moss now and in the future. *HortTechnology*, 13 (1), 35-38.

Fenchel, T., 1980. Suspension feeding in ciliated protozoa: feeding rates and their ecological significance. *Microbial Ecology*, 6 (1), 13-25.

Fenchel, T., 1982. Ecology of heterotrophic microflagellates. III. Adaptations to heterogeneous environments. *Marine Ecology Progress Series*, 9, 25-33.

Ferrier, M.D., Butler, B.R., Terlizzi, D.E. and Lacouture, R.V., 2005. The effects of barley straw (*Hordeum vulgare*) on the growth of freshwater algae. *Bioresource Technology*, 96 (16), 1788-1795.

Fink, P., Pflitsch, C. and Marin, K., 2011. Dietary essential amino acids affect the reproduction of the keystone herbivore *Daphnia pulex*. *PLoS One*, 6 (12), e28498.

Finlay, B.J. and Esteban, G.F., 1998. Freshwater protozoa: biodiversity and ecological function. *Biodiversity & Conservation*, 7 (9), 1163-1186.

Flemming, H.C. and Wingender, J., 2010. The biofilm matrix. *Nature Reviews Microbiology*, 8 (9), 623-633.

Food and Agriculture Organization of the United Nations (FAO), 2006. Guidelines on Efficacy Evaluation for the Registration of Plant Protection Products in *International Code of Conduct on the Distribution and Use of Pesticides,* Rome, Italy.

Francis, G., 1878. Poisonous Australian lake. *Nature*, 18, 11-12.

Franklin, D.J., Airs, R.L., Fernandes, M., Bell, T.G., Bongaerts, R.J., Berges, J.A. and Malin, G., 2012. Identification of senescence and death in *Emiliania huxleyi* and *Thalassiosira pseudonana*: cell staining, chlorophyll alterations, and dimethylsulfoniopropionate (DMSP) metabolism. *Limnology and Oceanography*, 57 (1), 305-317.

Franklin, D.J., Brussaard, C.P. and Berges, J.A., 2006. What is the role and nature of programmed cell death in phytoplankton ecology? *European Journal of Phycology*, 41 (1), 1-14.

Fulton III, R.S. and Paerl, H.W., 1988. Effects of the blue-green alga *Microcystis aeruginosa* on zooplankton competitive relations. *Oecologia*, 76 (3), 383-389.

Fuqua, W.C., Winans, S.C. and Greenberg, E.P., 1994. Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *Journal of Bacteriology*, 176 (2), 269.

Gaikowski, M.P., Rach, J.J. and Ramsay, R.T., 1999. Acute toxicity of hydrogen peroxide treatments to selected lifestages of cold-, cool-, and warmwater fish. *Aquaculture*, 178 (3), 191-207.

Garcia-Pichel, F., Belnap, J., Neuer, S. and Schanz, F., 2003. Estimates of global cyanobacterial biomass and its distribution. *Algological Studies*, 109 (1), 213-227.

Gasol, J.M. and Del Giorgio, P.A., 2000. Using flow cytometry for counting natural planktonic bacteria and understanding the structure of planktonic bacterial communities. *Scientia Marina*, 64 (2), 197-224.

Ger, K.A., Hansson, L.A. and Lürling, M., 2014. Understanding cyanobacteria-zooplankton interactions in a more eutrophic world. *Freshwater Biology*, 59 (9), 1783-1798.

Ger, K.A., Urrutia-Cordero, P., Frost, P.C., Hansson, L.A., Sarnelle, O., Wilson, A.E. and Lürling, M., 2016. The interaction between cyanobacteria and zooplankton in a more eutrophic world. *Harmful Algae*, 54, 28-144.

Gerten, D. and Adrian, R., 2000. Climate-driven changes in spring plankton dynamics and the sensitivity of shallow polymictic lakes to the North Atlantic Oscillation. *Limnology and Oceanography*, 45 (5), 1058-1066.

Getsinger, K.D., 1998. Appropriate use of aquatic herbicides. *Land and Water*, 42 (4), 44-48.

Giacomazzi, S. and Cochet, N., 2004. Environmental impact of diuron transformation: a review. *Chemosphere*, 56 (11), 1021-1032.

Giese, A.C., 1938. Cannibalism and gigantism in *Blepharisma*. *Transactions of the American Microscopical Society*, 57 (3), 245-255.

Giese, A.C., 1973. *Blepharisma*: the biology of a light-sensitive protozoan. California: Stanford University Press.

Gilbride, K.A., Lee, D.Y. and Beaudette, L.A., 2006. Molecular techniques in wastewater: understanding microbial communities, detecting pathogens, and real-time process control. *Journal of Microbiological Methods*, 66 (1), 1-20.

Gniazdowska, A. and Bogatek, R., 2005. Allelopathic interactions between plants. Multi-site action of allelochemicals. *Acta Physiologiae Plantarum*, 27 (3), 395-407.

Gorham, P.R., McLachlan, J.S., Hammer, U.T. and Kim, W.K., 1964. Isolation and culture of toxic strains of *Anabaena flos-aquae* (Lyngb*.). Internationale Vereinigung fuer Theoretische und Angewandte Limnologie*, 15, 796-804.

Görl, M., Sauer, J., Baier, T. and Forchhammer, K., 1998. Nitrogen-starvation-induced chlorosis in *Synechococcus* PCC 7942: adaptation to long-term survival. *Microbiology*, 144 (9), 2449-2458.

Gray, J.V., Petsko, G.A., Johnston, G.C., Ringe, D., Singer, R.A. and Werner-Washburne, M., 2004. “Sleeping beauty”: quiescence in *Saccharomyces cerevisiae*. *Microbiology and Molecular Biology Reviews*, 68 (2), 187-206.

Grover, J.P. and Chrzanowski, T.H., 2006. Seasonal dynamics of phytoplankton in two warm temperate reservoirs: association of taxonomic composition with temperature. *Journal of Plankton Research*, 28 (1), 1-17.

Guaragnella, N., Antonacci, L., Passarella, S., Marra, E. and Giannattasio, S., 2007. Hydrogen peroxide and superoxide anion production during acetic acid-induced yeast programmed cell death. *Folia Microbiologica*, 52 (3), 237-240.

Guo, L., 2007. Doing battle with the green monster of Taihu Lake. *Science*, 317 (5842), 1166-1166.

Hakkila, K., Antal, T., Rehman, A.U., Kurkela, J., Wada, H., Vass, I., Tyystjärvi, E. and Tyystjärvi, T., 2014. Oxidative stress and photoinhibition can be separated in the cyanobacterium *Synechocystis* sp. PCC 6803. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, 1837 (2), 217-225.

Halliwell, B., Clement, M.V. and Long, L.H., 2000. Hydrogen peroxide in the human body. *FEBS Letters*, 486 (1), 10-13.

Halvorson, R.A., Leng, W. and Vikesland, P.J., 2011. Differentiation of microcystin, nodularin, and their component amino acids by drop-coating deposition Raman spectroscopy. *Analytical Chemistry*, 83 (24), 9273-9280.

Hammes, F., Berney, M., Wang, Y., Vital, M., Köster, O. and Egli, T., 2008. Flow-cytometric total bacterial cell counts as a descriptive microbiological parameter for drinking water treatment processes. *Water Research*, 42 (1), 269-277.

Hannah, D.M. and Garner, G., 2015. River water temperature in the United Kingdom: changes over the 20th century and possible changes over the 21st century. *Progress in Physical Geography*, 39 (1), 68-92.

Hartley, B., 1996. *An atlas of British diatoms*. Stuttgart: Balogh Scientific Books.

Hartnell, D.M., Chapman, I.J., Esteban, G.F. and Franklin, D.J., 2016. Exploiting eco-physiological niche to facilitate the separation of the freshwater cyanobacteria *Microcystis* sp. and *Synechococcus* sp. *Journal of Microbiological Methods*, 122, 13-15.

Haughey, M.A., Anderson, M.A., Whitney, R.D., Taylor, W.D. and Losee, R.F., 2000. Forms and fate of Cu in a source drinking water reservoir following $CuSO_4$ treatment. *Water Research*, 34 (13), 3440-3452.

Helm, R.F., and Potts, M., 2012. *Extracellular Matrix (ECM)*. *In*: Whitton B.A., ed., *Ecology of cyanobacteria II: their diversity in space and time*. 2nd edition. Dordrecht: Springer, 461-480.

Helman, Y., Tchernov, D., Reinhold, L., Shibata, M., Ogawa, T., Schwarz, R., Ohad, I. and Kaplan, A., 2003. Genes encoding A-type flavoproteins are essential for photoreduction of O_2 in cyanobacteria. *Current Biology*, 13 (3), 230-235.

Hirschberg, J. and Chamovitz, D., 1994. Carotenoids in cyanobacteria. *In*: Bryant, D.A., ed., *The molecular biology of cyanobacteria*. Dordrecht: Kluwer Academic Publishers, 559-579.

Hitzfeld, B.C., Lampert, C.S., Späth, N., Mountfort, D., Kaspar, H. and Dietrich, D.R., 2000. Toxin production in cyanobacterial mats from ponds on the McMurdo Ice Shelf, Antarctica. *Toxicon*, 38 (12), 1731-1748.

Hoffman, R.A., 2009. Pulse width for particle sizing. *In*: Robinson J.P., ed., *Current protocols in cytometry*. Pennsylvania: John Wiley & Sons, Inc., 1.23.1-1.23.17.

Hoiczyk, E. and Hansel, A., 2000. Cyanobacterial cell walls: news from an unusual prokaryotic envelope. *Journal of Bacteriology*, 182 (5), 1191-1199.

Holm, N.P. and Shapiro, J., 1984. An examination of lipid reserves and the nutritional status of *Daphnia pulex* fed *Aphanizomenon flos-aquae*. *Limnology and Oceanography*, 29 (5), 1137-1140.

Huisman, J., van Oostveen, P. and Weissing, F.J., 1999. Species dynamics in phytoplankton blooms: incomplete mixing and competition for light. *The American Naturalist*, 154 (1), 46-68.

Huisman, J., Sharples, J., Stroom, J.M., Visser, P.M., Kardinaal, W.E.A., Verspagen, J.M. and Sommeijer, B., 2004. Changes in turbulent mixing shift competition for light between phytoplankton species. *Ecology*, 85 (11), 2960-2970.

Hulme, M., Jenkins, G.J., Lu, X., Turnpenny, J.R., Mitchell, T.D., Jones, R.G., Lowe, J., Murphy, J.M., Hassell, D., Boorman, P., McDonald, R. and Hill, S., 2002. *Climate change scenarios for the United Kingdom: the UKCIP02 scientific report*. Tyndall Centre for Climate Change Research, School of Environmental Sciences, University of East Anglia, Norwich, UK.

Huo, X., Chang, D.W., Tseng, J.H., Burch, M.D. and Lin, T.F., 2015. Exposure of *Microcystis aeruginosa* to hydrogen peroxide under light: kinetic modelling of cell rupture and simultaneous microcystin degradation. *Environmental Science & Technology*, 49 (9), 5502-5510.

Infante, A. and Abella, S.E., 1985. Inhibition of *Daphnia* by *Oscillatoria* in Lake Washington. *Limnology and Oceanography*, 30 (5), 1046-1052.

Jacobson, L. and Halmann, M., 1982. Polyphosphate metabolism in the blue-green alga *Microcystis aeruginosa*. *Journal of Plankton Research*, 4 (3), 481-488.

Jacoby, J.M., Collier, D.C., Welch, E.B., Hardy, F.J. and Crayton, M., 2000. Environmental factors associated with a toxic bloom of *Microcystis aeruginosa*. *Canadian Journal of Fisheries and Aquatic Sciences*, 57 (1), 231-240.

Jančula, D., Drábková, M., Černý, J., Karásková, M., Kořínková, R., Rakušan, J. and Maršálek, B., 2008. Algicidal activity of phthalocyanines - screening of 31 compounds. *Environmental Toxicology*, 23 (2), 218-223.

Jančula, D. and Maršálek, B., 2011. Critical review of actually available chemical compounds for prevention and management of cyanobacterial blooms. *Chemosphere*, 85 (9), 1415-1422.

Jančula, D. and Maršálek, B., 2012. Seven years from the first application of polyaluminium chloride in the Czech Republic - effects on phytoplankton communities in three water bodies. *Chemistry and Ecology*, 28 (6), 535-544.

Jeppesen, E., Søndergaard, M., Lauridsen, T.L., Davidson, T.A., Liu, Z., Mazzeo, N., Trochine, C., Özkan, K., Jensen, H.S., Trolle, D., Starling, F., Lazzaro, X., Johansson, L.S., Bjerring, R., Liboriussen, L., Larsen, S.E., Landkildehus, F., Egemose, S. and Meerhoff, M., 2012. Biomanipulation as a restoration tool to combat eutrophication: recent advances and future challenges. *Advances in Ecological Research*, 47, 411-488.

Jiang, H. and Qiu, B., 2005. Photosynthetic adaptation of a bloom-forming cyanobacterium *Microcystis aeruginosa* (cyanophyceae) to prolonged UV-b exposure. *Journal of Phycology*, 41 (5), 983-992.

Joung, S., Kim, C., Ahn, C., Jang, K., Boo, S.M. and Oh, H., 2006. Simple method for a cell count of the colonial cyanobacterium, *Microcystis* sp. *Journal of Microbiology-Seoul*, 44 (5), 562.

Jürgens, U.J., Martin, C. and Weckesser, J., 1989. Cell wall constituents of *Microcystis* sp. PCC 7806. *FEMS Microbiology Letters*, 65 (1-2), 47-51.

Kaneshiro, E.S., Wyder, M.A., Wu, Y.P. and Cushion, M.T., 1993. Reliability of calcein acetoxy methyl ester and ethidium homodimer or propidium iodide for viability assessment of microbes. *Journal of Microbiological Methods*, 17 (1), 1-16.

Karatan, E. and Watnick, P., 2009. Signals, regulatory networks, and materials that build and break bacterial biofilms. *Microbiology and Molecular Biology Reviews*, 73 (2), 310-347.

Kell, D.B., Kaprelyants, A.S., Weichart, D.H., Harwood, C.R. and Barer, M.R., 1998. Viability and activity in readily culturable bacteria: a review and discussion of the practical issues. *Antonie van Leeuwenhoek*, 73 (2), 169-187.

Klausmeier, C.A. and Litchman, E., 2001. Algal games: the vertical distribution of phytoplankton in poorly mixed water columns. *Limnology and Oceanography*, 46 (8), 1998-2007.

Knappe, D.R.U., Belk, R.C., Briley, D.S., Gandy, S.R., Rastogi, N. and Rike, A.H., 2004. *Algae detection and removal strategies for drinking water*. Colorado: AWWA Research Foundation.

Komárek, J., 2010. Recent changes (2008) in cyanobacteria taxonomy based on a combination of molecular background with phenotype and ecological consequences (genus and species concept). *Hydrobiologia*, 639 (1), 245-259.

Komárek, J., 2012. Nomenclatural changes in heterocytous cyanoprokaryotes (cyanobacteria, cyanophytes). *Fottea*, 12 (1), 141-148.

Komárek, J., 2016. Review of the cyanobacterial genera implying planktic species after recent taxonomic revisions according to polyphasic methods: state as of 2014. *Hydrobiologia*, 764 (1), 259-270.

Komárek, J., Kastovsky, J., Mares, J. and Johansen, J.R., 2014. Taxonomic classification of cyanoprokaryotes (cyanobacterial genera) 2014, using a polyphasic approach. *Preslia*, 86 (4), 295-335.

Komárek, J. and Komárková, J., 2002. Review of the European *Microcystis* - morphospecies (cyanoprokaryotes) from nature. *Czech Phycology, Olomouc*, 2, 1-24.

Kramer, J.F., 1997. Peracetic acid: A new biocide for industrial water applications. *Materials performance*, 36 (8).

Kuczynska, P., Jemiola-Rzeminska, M. and Strzalka, K., 2015. Photosynthetic pigments in diatoms. *Marine drugs*, 13 (9), 5847-5881.

Kuniyoshi, T.M., Sevilla, E., Bes, M.T., Fillat, M.F. and Peleato, M.L., 2013. Phosphate deficiency (N/P 40: 1) induces mcyD transcription and microcystin synthesis in *Microcystis aeruginosa* PCC 7806. *Plant Physiology and Biochemistry*, 65, 120-124.

Kurmayer, R., Christiansen, G. and Chorus, I., 2003. The abundance of microcystin-producing genotypes correlates positively with colony size in *Microcystis* sp. and determines its microcystin net production in Lake Wannsee. *Applied and Environmental Microbiology*, 69 (2), 787-795.

Lakeman, M.B., von Dassow, P. and Cattolico, R.A., 2009. The strain concept in phytoplankton ecology. *Harmful Algae*, 8 (5), 746-758.

Lam, A.K.Y., Prepas, E.E., Spink, D. and Hrudey, S.E., 1995. Chemical control of hepatotoxic phytoplankton blooms: implications for human health. *Water Research*, 29 (8), 1845-1854.

Lampert, W., 1987. Laboratory studies on zooplankton-cyanobacteria interactions. *New Zealand Journal of Marine and Freshwater Research*, 21 (3), 483-490.

Latour, D., Giraudet, H. and Berthon, J.L., 2004. Frequency of dividing cells and viability *of Microcystis aeruginosa* in sediment of a eutrophic reservoir. *Aquatic Microbial Ecology*, 36 (2), 117-122.

Latour, D., Salençon, M.J., Reyss, J.L. and Giraudet, H., 2007. Sedimentary imprint of *Microcystis aeruginosa* (cyanobacteria) blooms in Grangent reservoir (Loire, France). *Journal of Phycology*, 43 (3), 417-425.

Lawton, L.A., Edwards, C. and Codd, G.A., 1994. Extraction and high-performance liquid chromatographic method for the determination of microcystins in raw and treated waters. *Analyst*, 119 (7), 1525-1530.

Le Berre, C., Serp, P., Kalck, P. and Torrence, G.P., 2014. Acetic acid. *Ullmann's encyclopedia of industrial chemistry*, 1-34.

Leão, P.N., Vasconcelos, M.T.S. and Vasconcelos, V.M., 2009. Allelopathy in freshwater cyanobacteria. *Critical Reviews in Microbiology*, 35 (4), 271-282.

Lee, S.J., Jang, M.H., Kim, H.S., Yoon, B.D. and Oh, H.M., 2000. Variation of microcystin content of *Microcystis aeruginosa* relative to medium N: P ratio and growth stage. *Journal of Applied Microbiology*, 89 (2), 323-329.

Lee, T.J., Nakano, K. and Matsumura, M., 2000. A new method for the rapid evaluation of gas vacuoles regeneration and viability of cyanobacteria by flow cytometry. *Biotechnology Letters*, 22 (23), 183.

Lee, T.A., Rollwagen-Bollens, G. and Bollens, S.M., 2015. The influence of water quality variables on cyanobacterial blooms and phytoplankton community composition in a shallow temperate lake. *Environmental Monitoring and Assessment*, 187 (6), 1-19.

Lidstrom, M.E. and Konopka, M.C., 2010. The role of physiological heterogeneity in microbial population behaviour. *Nature Chemical Biology*, 6 (10), 705-712.

Litton, G. and Hendry, K., 1999. Development of operational and policy guidance on the use of hydrogen peroxide. R&D Technical Report P191. Bristol: Environment Agency.

Liu, Y., Tan, W., Wu, X., Wu, Z., Yu, G. and Li, R., 2011. First report of microcystin production in *Microcystis smithii* Komárek and Anagnostidis (cyanobacteria) from a water bloom in eastern China. *Journal of Environmental Sciences*, 23 (1),102-107.

Lloyd, D. and Hayes, A.J., 1995. Vigour, vitality and viability of microorganisms. *FEMS Microbiology Letters*, 133 (1-2), 1-7.

Ludovico, P., Sousa, M.J., Silva, M.T., Leão, C. and Côrte-Real, M., 2001. *Saccharomyces cerevisiae* commits to a programmed cell death process in response to acetic acid. *Microbiology*, 147 (9), 2409-2415.

Lürling, M., Meng, D. and Faassen, E.J., 2014. Effects of hydrogen peroxide and ultrasound on biomass reduction and toxin release in the cyanobacterium, *Microcystis aeruginosa*. *Toxins*, 6 (12), 3260-3280.

Lynch, M., 1980. *Aphanizomenon* blooms: alternate control and cultivation by *Daphnia pulex*. *In:* Kerfoot, W.D., ed*., Evolution and ecology of zooplankton communities*. Hanover: University Press of New England, 299-304.

Lynch, M., Gabriel, W. and Wood, A.M., 1991. Adaptive and demographic responses of plankton populations to environmental change. *Limnology and Oceanography*, 36 (7), 1301-1312.

Mackerras, A.H., de Chazal, N.M. and Smith, G.D., 1990. Transient accumulations of cyanophycin in *Anabaena cylindrica* and *Synechocystis* 6308. *Microbiology*, 136 (10), 2057-2065.

Madigan, M.T., Clark, D.P., Stahl, D. and Martinko, J.M., 2010. *Brock biology of microorganisms*. 13th edition. San Francisco: Benjamin Cummings.

Maecker, H.T. and Trotter, J., 2006. Flow cytometry controls, instrument setup, and the determination of positivity. *Cytometry Part A*, 69 (9), 1037-1042.

Maileht, K., Nõges, T., Nõges, P., Ott, I., Mischke, U., Carvalho, L. and Dudley, B., 2013. Water colour, phosphorus and alkalinity are the major determinants of the dominant phytoplankton species in European lakes. *Hydrobiologia*, 704 (1), 115-126.

Marie, D., Simon, N. and Vaulot, D., 2005. Phytoplankton cell counting by flow cytometry. *Algal Culturing Techniques*, 1, 253-267.

Martin, D. and Ridge, I., 1999. The relative sensitivity of algae to decomposing barley straw. *Journal of Applied Phycology*, 11 (3), 285-291.

Mason, C.F., 2002. *Biology of freshwater pollution*. 4th edition. Harlow: Pearson Education Limited.

Matthijs, H.C., Visser, P.M., Reeze, B., Meeuse, J., Slot, P.C., Wijn, G., Talens, R. and Huisman, J., 2012. Selective suppression of harmful cyanobacteria in an entire lake with hydrogen peroxide. *Water Research*, 46 (5), 1460-1472.

Mello, M.M., Soares, M.C.S., Roland, F. and Lürling, M., 2012. Growth inhibition and colony formation in the cyanobacterium *Microcystis aeruginosa* induced by the cyanobacterium *Cylindrospermopsis raciborskii*. *Journal of Plankton Research*, fbs056.

Metcalf, J.S., Banack, S.A., Lindsay, J., Morrison, L.F., Cox, P.A. and Codd, G.A., 2008. Co-occurrence of β-N-methylamino-l-alanine, a neurotoxic amino acid with other cyanobacterial toxins in British waterbodies, 1990–2004. *Environmental Microbiology*, 10 (3), 702-708.

Metcalf, J.S., Bell, S.G. and Codd, G.A., 2001. Colorimetric immuno-protein phosphatase inhibition assay for specific detection of microcystins and nodularins of cyanobacteria. *Applied and Environmental Microbiology*, 67 (2), 904-909.

Metcalf, J.S. and Codd, G.A., 2012. Cyanotoxins. *In*: Whitton B.A., ed., *Ecology of cyanobacteria II: their diversity in space and time*. 2nd edition. Dordrecht: Springer, 651–675.

Mikula, P., Zezulka, S., Jancula, D. and Marsalek, B., 2012. Metabolic activity and membrane integrity changes in *Microcystis aeruginosa* - new findings on hydrogen peroxide toxicity in cyanobacteria. *European Journal of Phycology*, 47 (3),195-206.

Mitrovic, S.M., Hardwick, L. and Dorani, F., 2011. Use of flow management to mitigate cyanobacterial blooms in the lower Darling River, Australia. *Journal of Plankton Research*, 33 (2), 229-241.

Moezelaar, R. and Stal, L.J., 1994. Fermentation in the unicellular cyanobacterium *Microcystis* PCC 7806. *Archives of Microbiology*, 162 (1-2), 63-69.

Mohamed, Z.A., 2001. Alum and lime-alum removal of toxic and nontoxic phytoplankton from the Nile River water: laboratory study. *Water Resources Management*, 15 (4), 213-221.

Molles, M.C., 2012. *Ecology: concepts and applications*. 6th edition. Boston: McGraw-Hill Higher Education.

Moran, P.J. and Greenberg, S.M., 2008. Winter cover crops and vinegar for early-season weed control in sustainable cotton. *Journal of Sustainable Agriculture*, 32 (3), 483-506.

Murray, D., Jefferson, B., Jarvis, P. and Parsons, S.A., 2010. Inhibition of three algae species using chemicals released from barley straw. *Environmental Technology*, 31 (4), 455-466.

Nagata, S., Tsutsumi, T., Hasegawa, A., Watanabe, M.F. and Ueno, Y., 1995. Determination of microcystin in environmental water by highly sensitive immunoassay. Proceedings of the 20th Symposium on Toxicology and Environmental Health, 21st - 22nd September 1994, Tokyo, Japan. *Japanese Journal of Toxicology and Environmental Health*, 41 (1), 10.

Nakai, S., Inoue, Y., Hosomi, M. and Murakami, A., 2000. *Myriophyllum spicatum*-released allelopathic polyphenols inhibiting growth of blue-green algae *Microcystis aeruginosa*. *Water Research*, 34 (11), 3026-3032.

Nakai, S., Inoue, Y. and Hosomi, M., 2001. Algal growth inhibition effects and inducement modes by plant-producing phenols. *Water Research*, 35 (7), 1855-1859.

Nealson, K.H., Platt, T. and Hastings, J.W., 1970. Cellular control of the synthesis and activity of the bacterial luminescent system. *Journal of Bacteriology*, 104 (1), 313-322.

Newman, J.R. and Barrett, P.R.F., 1993. Control of *Microcystis aeruginosa* by decomposing barley straw. *Journal of Aquatic Plant Management*, 31, 203-203

Newman, D.J. and Cragg, G.M., 2007. Natural products as sources of new drugs over the last 25 years. *Journal of Natural Products*, 70 (3), 461-477.

Nichol, B.E., Budd, K., Palmer, G.R. and MacArthur, J.D., 1987. The mechanisms of fluoride toxicity and fluoride resistance in *synechococcus leopoliensis* (cyanophyceae). *Journal of Phycology*, 23 (4), 535-541.

Nielsen, S.L., Enriquez, S., Duarte, C.M. and Sand-Jensen, K., 1996. Scaling maximum growth rates across photosynthetic organisms. *Functional Ecology*, 167-175.

Nixdorf, B. and Jander, J., 2003. Bacterial activities in shallow lakes - a comparison between extremely acidic and alkaline eutrophic hard water lakes. *Hydrobiologia*, 506 (1-3), 697-705.

Nõges, P., Adrian, R., Anneville, O., Arvola, L., Blenckner, T., George, G., Jankowski, T., Järvinen, M., Maberly, S., Padisák, J. and Straile, D., 2010. The impact of variations in the climate on seasonal dynamics of phytoplankton. *In*: George, G., ed., *The impact of climate change on European lakes*. Aquatic Ecology Series, 4. Dordrecht: Springer, 253-274.

Nohomovich, B., Nguyen, B.T., Quintanilla, M., Lee, L.H., Murray, S.R. and Chu, T.C., 2013. Physiological effects of nickel chloride on the freshwater cyanobacterium *Synechococcus* sp. IU 625. *Advances in Bioscience and Biotechnology*, 4 (7B), 10.

Nolan, J.P., 2015. Flow cytometry of extracellular vesicles: potential, pitfalls, and prospects. *Current Protocols in Cytometry*, 13-14.

National Sanitation Foundation (NSF), 2011. NSF/ANSI Standard 60: Drinking water treatment chemicals - health effects: acetic acid. Available from: http://info.nsf.org/Certified/PwsChemicals/Listings.asp?Company Name=&TradeName=acetic+ acid&ChemicalName=&ProductFunction=&PlantState=&PlantCountry=&PlantRegion= [Accessed 10th October 2015].

National Sanitation Foundation (NSF), 2011. NSF/ANSI Standard 60: Drinking water treatment chemicals - health effects: hydrogen peroxide Available from: http://info.nsf.org/Certified/PwsChemicals/Listings.asp?Chemical Name=Hydrogen+Peroxide& [Accessed 10th October 2015].

Nyström, B., Becker-Van Slooten, K., Bérard, A., Grandjean, D., Druart, J.C. and Leboulanger, C., 2002. Toxic effects of Irgarol 1051 on phytoplankton and macrophytes in Lake Geneva. *Water Research*, 36 (8), 2020-2028.

Ó hUallacháin, D. and Fenton, O., 2010. Barley (*Hordeum vulgare*)-induced growth inhibition of algae: a review. *Journal of Applied Phycology*, 22 (5), 651-658.

Oberholster, P.J., Botha, A.M. and Grobbelaar, J.U., 2004. *Microcystis aeruginosa*: source of toxic microcystins in drinking water. *African Journal of Biotechnology*, 3 (3), 159-168.

Ohko, Y., Nagao, Y., Okano, K., Sugiura, N., Fukuda, A., Yang, Y., Negishi, N., Takeuchi, M. and Hanada, S., 2009. Prevention of *Phormidium tenue* biofilm formation by TiO_2 photocatalysis. *Microbes and Environments*, 24 (3), 241-245.

Olenina, I., Hajdu, S., Edler, L., Andersson, A., Wasmund, N., Busch, S., Gobel, J., Gromisz, S., Huseby, S., Huttunen, M., ¨ Jaanus, A., Kokkonen, P., Ledaine, I., and Niemkiewicz, E. 2006. Biovolumes and size-classes of phytoplankton in the Baltic Sea. *HELCOM Baltic Sea Environment Proceedings,* 106, 1-144.

Olsson-Francis, K., de la Torre, R., Towner, M.C. and Cockell, C.S., 2009. Survival of akinetes (resting-state cells of cyanobacteria) in low Earth orbit and simulated extraterrestrial conditions. *Origins of Life and Evolution of Biospheres*, 39 (6), 565-579.

Otsuka, S., Suda, S., Li, R., Matsumoto, S. and Watanabe, M.M., 2000. Morphological variability of colonies of *Microcystis* morphospecies in culture. *The Journal of General and Applied Microbiology*, 46 (1), 39-50.

Otsuka, S., Suda, S., Li, R., Watanabe, M., Oyaizu, H., Hiroki, M., Mahakhant, A., Liu, Y., Matsumoto, S. and Watanabe, M.M., 1998. Phycoerythrin-containing *Microcystis* isolated from PR China and Thailand. *Phycological Research*, 46 (s2), 45-50.

Otsuka, S., Suda, S., Shibata, S., Oyaizu, H., Matsumoto, S. and Watanabe, M.M., 2001. A proposal for the unification of five species of the cyanobacterial genus *Microcystis* Kützing ex Lemmermann 1907 under the rules of the Bacteriological Code. *International Journal of Systematic and Evolutionary Microbiology*, 51 (3), 873-879.

Paerl, H.W. 2008. Nutrient and other environmental controls of harmful cyanobacterial blooms along the freshwater-marine continuum. *In*: Hudnell, H.K., ed., Cyanobacterial harmful algal

blooms: state of the science and research needs. *Advances in Experimental Medicine & Biology*, 619, 217-237.

Paerl, H.W., Fulton, R.S., Moisander, P.H. and Dyble, J., 2001. Harmful freshwater algal blooms, with an emphasis on cyanobacteria. *The Scientific World Journal*, 1, 76-113.

Paerl, H.W. and Huisman, J., 2009. Climate change: a catalyst for global expansion of harmful cyanobacterial blooms. *Environmental microbiology Reports*, 1 (1), 27-37.

Paerl, H.W. and Otten, T.G., 2013. Harmful cyanobacterial blooms: causes, consequences, and controls. *Microbial Ecology*, 65 (4), 995-1010.

Parker, D.L., Kumar, H.D., Rai, L.C. and Singh, J.B., 1997. Potassium salts inhibit growth of the cyanobacteria *Microcystis* spp. in pond water and defined media: implications for control of microcystin-producing aquatic blooms. *Applied and Environmental Microbiology*, 63 (6), 2324-2329.

Peperzak, L. and Brussaard, C.P., 2011. Flow cytometric applicability of fluorescent vitality probes on phytoplankton. *Journal of Phycology*, 47 (3), 692-702.

Perelman, A., Shaltiel, J., Sendersky, E. and Schwarz, R., 2004. Cyanobacterial spectrum. *BioTechniques*, 36 (6), 948-952.

Petz, W., Valbonesi, A., Schiftner, U., Quesada, A. and Ellis-Evans, J.C., 2007. Ciliate biogeography in Antarctic and Arctic freshwater ecosystems: endemism or global distribution of species? *FEMS Microbiology Ecology*, 59 (2), 396-408.

Pillinger, J.M., Cooper, J.A. and Ridge, I., 1994. Role of phenolic compounds in the antialgal activity of barley straw. *Journal of Chemical Ecology*, 20 (7), 1557-1569.

Porter, J., Diaper, J., Edwards, C. and Pickup, R., 1995. Direct measurements of natural planktonic bacterial community viability by flow cytometry. *Applied and Environmental Microbiology*, 61 (7), 2783-2786.

Potts, M., Slaughter, S.M., Hunneke, F.U., Garst, J.F. and Helm, R.F., 2005. Desiccation tolerance of prokaryotes: application of principles to human cells. *Integrative and Comparative Biology*, 45 (5), 800-809.

Poulton, N.J., 2016. FlowCam: quantification and classification of phytoplankton by imaging flow cytometry. *Imaging Flow Cytometry: Methods and Protocols*, 237-247.

Prepas, E.E., Pinel-Alloul, B., Chambers, P.A., Murphy, T.P., Reedyk, S., Sandland, G. and Serediak, M., 2001. Lime treatment and its effects on the chemistry and biota of hardwater eutrophic lakes. *Freshwater Biology*, 46 (8), 1049-1060.

Purcell, D., Parsons, S.A., Jefferson, B., Holden, S., Campbell, A., Wallen, A., Chipps, M., Holden, B. and Ellingham, A., 2013. Experiences of algal bloom control using green solutions barley straw and ultrasound, an industry perspective. *Water and Environment Journal*, 27 (2), 148-156.

Qian, H., Yu, S., Sun, Z., Xie, X., Liu, W. and Fu, Z., 2010. Effects of copper sulfate, hydrogen peroxide and N-phenyl-2-naphthylamine on oxidative stress and the expression of genes involved photosynthesis and microcystin disposition in *Microcystis aeruginosa*. *Aquatic Toxicology*, 99 (3), 405-412.

Qin, B., Xu, P., Wu, Q., Luo, L. and Zhang, Y., 2007. Environmental issues of Lake Taihu, China. *Hydrobiologia*, 581 (1), 3-14.

Rabouille, S., Thébault, J.M. and Salençon, M.J., 2003. Simulation of carbon reserve dynamics in *Microcystis* and its influence on vertical migration with Yoyo model. *Comptes Rendus Biologies*, 326 (4), 349-361.

Rach, J.J., Schreier, T.M., Howe, G.E. and Redman, S.D., 1997. Effect of species, life stage, and water temperature on the toxicity of hydrogen peroxide to fish. *The Progressive Fish-Culturist*, 59 (1), 41-46.

Rantala, A., Fewer, D.P., Hisbergues, M., Rouhiainen, L., Vaitomaa, J., Börner, T. and Sivonen, K., 2004. Phylogenetic evidence for the early evolution of microcystin synthesis. *Proceedings of the National Academy of Sciences of the United States of America*, 101 (2), 568-573.

Raps, S., Kycia, J.H., Ledbetter, M.C. and Siegelman, H.W., 1985. Light intensity adaptation and phycobilisome composition of *Microcystis aeruginosa*. *Plant Physiology*, 79 (4), 983-987.

Raspor, P. and Goranovič, D., 2008. Biotechnological applications of acetic acid bacteria. *Critical Reviews in Biotechnology*, 28 (2), 101-124.

Read, D.S., Bowes, M.J., Newbold, L.K. and Whiteley, A.S., 2014. Weekly flow cytometric analysis of riverine phytoplankton to determine seasonal bloom dynamics. *Environmental Science: Processes & Impacts*, 16 (3), 594-603.

Reynolds, C.S., 1973. Growth and buoyancy of *Microcystis aeruginosa* Kütz. emend. Elenkin in a shallow eutrophic lake. *Proceedings of the Royal Society of London B: Biological Sciences*, 184 (1074), 29-50.

Reynolds, C.S., 1984. Phytoplankton periodicity: the interactions of form, function and environmental variability. *Freshwater Biology*, 14 (2), 111-142.

Reynolds, C.S., 1987. Cyanobacterial water-blooms. *Advances in Botanical Research*, *13*, 67-143.

Reynolds, C.S., Irish, A.E. and Elliott, J.A., 2001. The ecological basis for simulating phytoplankton responses to environmental change (PROTECH). *Ecological Modelling*, 140 (3), 271-291.

Reynolds, C.S. and Jaworski, G.H.M., 1978. Enumeration of natural *Microcystis* populations. *British Phycological Journal*, 13 (3), 269-277.

Reynolds, C.S., Jaworski, G.H.M., Cmiech, H.A. and Leedale, G.F., 1981. On the annual cycle of the blue-green alga *Microcystis aeruginosa* Kütz. emend. Elenkin. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 293 (1068), 419-477.

Reynolds, C.S. and Rogers, D.A., 1976. Seasonal variations in the vertical distribution and buoyancy of *Microcystis aeruginosa* Kütz. Emend. Elenkin in Rostherne Mere, England. *Hydrobiologia*, 48 (1),17-23.

Ribeiro, G.F., Côrte-Real, M. and Johansson, B., 2006. Characterization of DNA damage in yeast apoptosis induced by hydrogen peroxide, acetic acid, and hyperosmotic shock. *Molecular Biology of the Cell*, 17 (10), 4584-4591.

Ridge, I. and Pillinger, J.M., 1996. Towards understanding the nature of algal inhibitors from barley straw. *Hydrobiologia*, 340, 301-305.

Roberts, E.C., Priscu, J.C. and Laybourn-Parry, J., 2004. Microplankton dynamics in a perennially ice-covered Antarctic lake - Lake Hoare. *Freshwater Biology*, 49 (7), 853-869.

Roegner, A.F., Brena, B., González-Sapienza, G. and Puschner, B., 2014. Microcystins in potable surface waters: toxic effects and removal strategies. *Journal of Applied Toxicology*, 34 (5), 441-457.

Russell, J.B., 1992. Another explanation for the toxicity of fermentation acids at low pH: anion accumulation versus uncoupling. *Journal of Applied Bacteriology*, 73 (5), 363-370.

Ryssel, H., Kloeters, O., Germann, G., Schäfer, T., Wiedemann, G. and Oehlbauer, M., 2009. The antimicrobial effect of acetic acid - an alternative to common local antiseptics? *Burns*, 35 (5), 695-700.

Rzymski, P., Poniedziałek, B., Kokociński, M., Jurczak, T., Lipski, D. and Wiktorowicz, K., 2014. Interspecific allelopathy in cyanobacteria: Cylindrospermopsin and *Cylindrospermopsis raciborskii* effect on the growth and metabolism of *Microcystis aeruginosa*. *Harmful Algae*, 35, 1-8.

Saha, S.K., Uma, L. and Subramanian, G., 2003. Nitrogen stress induced changes in the marine cyanobacterium *Oscillatoria willei* BDU 130511. *FEMS Microbiology Ecology*, 45 (3), 263-272.

Salmond, C.V., Kroll, R.G. and Booth, I.R., 1984. The effect of food preservatives on pH homeostasis in Escherichia coli. *Microbiology*, 130 (11), 2845-2850.

Sanseverino, I., Conduto, D., Pozzoli, L., Dobricic., S and Lettieri, T., 2016. *Algal bloom and its economic impact*. European Commission Joint Research Centre (JRC) Technical Reports. EUR 27905 EN. doi:10.2788/660478.

Sarnelle, O. and Wilson, A.E., 2005. Local adaptation of *Daphnia pulicaria* to toxic cyanobacteria. *Limnology and Oceanography*, 50 (5), 1565-1570.

Satoh, K., Hirai, M., Nishio, J., Yamaji, T., Kashino, Y. and Koike, H., 2002. Recovery of photosynthetic systems during rewetting is quite rapid in a terrestrial cyanobacterium, *Nostoc commune*. *Plant and Cell Physiology*, 43 (2), 170-176.

Sauer, J., Schreiber, U., Schmid, R., Völker, U. and Forchhammer, K., 2001. Nitrogen starvation-induced chlorosis in *Synechococcus* PCC 7942. low-level photosynthesis as a mechanism of long-term survival. *Plant Physiology*, 126 (1), 233-243.

Schatz, D., Nagar, E., Sendersky, E., Parnasa, R., Zilberman, S., Carmeli, S., Mastai, Y., Shimoni, E., Klein, E., Yeger, O. and Reich, Z., 2013. Self-suppression of biofilm formation in the cyanobacterium *Synechococcus elongatus*. *Environmental Microbiology*, 15 (6), 1786-1794.

Scheer, H., 2006. An overview of chlorophylls and bacteriochlorophylls: biochemistry, biophysics, functions and applications. *In*: Grimm, B., Porra, R.J., Rüdiger, W. and Scheer, H., eds., *Chlorophylls and bacteriochlorophylls: biochemistry, biophysics, functions and applications*. Dordrecht: Springer, 1-26.

Schindler, D.W., Hecky, R.E., Findlay, D.L., Stainton, M.P., Parker, B.R., Paterson, M.J., Beaty, K.G., Lyng, M. and Kasian, S.E.M., 2008. Eutrophication of lakes cannot be controlled by reducing nitrogen input: results of a 37-year whole-ecosystem experiment. *Proceedings of the National Academy of Sciences*, 105 (32), 11254-11258.

Schopf, J.W., 2000. The fossil record: tracing the roots of the cyanobacterial lineage. *In*: Whitton, B.A. and Potts, M., eds., *The ecology of cyanobacteria: their diversity in time and space.* Dordrecht: Kluwer Academic Publishers, 13-35.

Schopf, J.W., 2006. Fossil evidence of Archaean life. *Philosophical Transactions of the Royal Society of London B: Biological Sciences,* 361 (1470), 869-885.

Šejnohová, L., and Maršálek, B., 2012. *Microcystis*. *In*: Whitton B.A., ed., *Ecology of cyanobacteria II: their diversity in space and time*. 2nd edition. Dordrecht: Springer, 195-227.

Shapiro, J., 1990. Biomanipulation: the next phase - making it stable. *Hydrobiologia*, 200/201, 13-27.

Shapiro, J., Lamarra, V.A. and Lynch, M., 1975. Biomanipulation: an ecosystem approach to lake restoration. *In*: Brezonik, P.L. and Fox, J.L., eds. *Water quality management through biological control.* Report No. ENV-07–75–1. University of Florida, Gainsville, 85-96.

Shapiro, J.A., 1998. Thinking about bacterial populations as multicellular organisms. *Annual Reviews in Microbiology*, 52 (1), 81-104.

Shapiro, H.M., 1994. How flow cytometers work. *In*: Shapiro, H.M., eds, *Practical flow cytometry*. 3rd edition. New York: John Wiley & Sons, Inc., 101-223.

Shapiro, H.M., ed., 2003. *Practical flow cytometry*. 4th edition. New York: John Wiley & Sons, Inc.

Sharif, D.I., Gallon, J., Smith, C.J. and Dudley, E.D., 2008. Quorum sensing in cyanobacteria: N-octanoyl-homoserine lactone release and response, by the epilithic colonial cyanobacterium *Gloeothece* PCC 6909. *The ISME Journal*, 2 (12), 1171-1182.

Shao, J., Wu, Z., Yu, G., Peng, X. and Li, R., 2009. Allelopathic mechanism of pyrogallol to *Microcystis aeruginosa* PCC 7806 (cyanobacteria): from views of gene expression and antioxidant system. *Chemosphere*, 75 (7), 924-928.

Sidler, W.A., 1994. Phycobilisome and phycobiliprotein structures. *In*: Bryant, D.A., ed., *The molecular biology of cyanobacteria*. Dordrecht: Kluwer Academic Publishers, 139-216.

Sieracki, C.K., Sieracki, M.E. and Yentsch, C.S., 1998. An imaging-in-flow system for automated analysis of marine microplankton. *Marine Ecology Progress Series*, 168, 285-296.

Sigee, D.C., 2005. *Freshwater microbiology: biodiversity and dynamic interactions of microorganisms in the aquatic environment*. Chichester: John Wiley & Sons Ltd., Chichester.

Sigee, D.C., Glenn, R., Andrews, M.J., Bellinger, E.G., Butler, R.D., Epton, H.A.S. and Hendry, R.D., 1999. Biological control of cyanobacteria: principles and possibilities. *Hydrobiologia*, 395/396 (Dev. Hydrobiol. 136), 161-172.

Sigee, D.C., Selwyn, A., Gallois, P. and Dean, A.P., 2007. Patterns of cell death in freshwater colonial cyanobacteria during the late summer bloom. *Phycologia*, 46 (3), 284-292.

Simis, S.G. and Kauko, H.M., 2012. *In vivo* mass-specific absorption spectra of phycobilipigments through selective bleaching. *Limnology and Oceanography: Methods*, 10, 214-226.

Sivonen, K. and Jones, G., 1999. Cyanobacterial toxins. *In*: Chorus, I. and Bartram, J., eds., *Toxic cyanobacteria in water: a guide to natural toxins from cyanobacteria 19 public health significance, consequences, monitoring and management*. London: E & FN SPON, 41-111.

Sleytr, U.B., Schuster, B., Egelseer, E.M. and Pum, D., 2014. S-layers: principles and applications. *FEMS Microbiology Reviews*, 38 (5), 823-864.

Sommer, U., Adrian, R., de Senerpont Domis, L., Elser, J.J., Gaedke, U., Ibelings, B., Jeppesen, E., Lürling, M., Molinero, J.C., Mooij, W.M. and Van Donk, E., 2012. Beyond the Plankton Ecology Group (PEG) model: mechanisms driving plankton succession. *Annual Review of Ecology, Evolution, and Systematics*, 43, 429-448.

Spencer, D.F., Elmore, C.L., Ksander, G.G. and Roncoroni, J.A., 2003. Influence of dilute acetic acid treatments on American pondweed winter buds in the Nevada irrigation district, California. *Journal of Aquatic Plant Management*, 41, 65-68.

Stanier, R.Y., Kunisawa, R., Mandel, M. and Cohen-Bazire, G., 1971. Purification and properties of unicellular blue-green algae (order *Chroococcales*). *Bacteriological Reviews*, 35 (2), 171.

Stauber, J.L. and Jeffrey, S.W., 1988. Photosynthetic pigments in fifty-one species of marine diatoms. *Journal of Phycology*, 24 (2), 158-172.

Stevens Jr, S.E., Balkwill, D.L. and Paone, D.A.M., 1981. The effects of nitrogen limitation on the ultrastructure of the cyanobacterium *Agmenellum quadruplicatum*. *Archives of Microbiology*, 130 (3), 204-212.

Stuart, R.K., Mayali, X., Lee, J.Z., Everroad, R.C., Hwang, M., Bebout, B.M., Weber, P.K., Pett-Ridge, J. and Thelen, M.P., 2015. Cyanobacterial reuse of extracellular organic carbon in microbial mats. *The ISME journal*. 10 (5), 1240-1251.

Suzuki, S., 1951. Morphological study of *Blepharisma undulans* from Japan. *Bulletin of the Yamagata University. Natural science*, 1 (3), 275-280.

Talling, J.F., Spencer, H.J. and Morison, H.R., 2005. The ‘shock period’: dynamics of phytoplankton during the spring–summer transition of a stratifying English lake. *Hydrobiologia*, 533 (1-3), 15-28.

Taori, K., Paul, V.J. and Luesch, H., 2008. Structure and activity of largazole, a potent antiproliferative agent from the Floridian marine cyanobacterium *Symploca* sp.. *Journal of the American Chemical Society*, 130 (6), 1806-1807.

Taranu, Z.E., Gregory-Eaves, I., Leavitt, P.R., Bunting, L., Buchaca, T., Catalan, J., Domaizon, I., Guilizzoni, P., Lami, A., McGowan, S. and Moorhouse, H., 2015. Acceleration of cyanobacterial dominance in north temperate-subarctic lakes during the Anthropocene. *Ecology Letters*, 18 (4), 375-384.

Taranu, Z.E., Zurawell, R.W., Pick, F. and Gregory-Eaves, I., 2012. Predicting cyanobacterial dynamics in the face of global change: the importance of scale and environmental context. *Global Change Biology*, 18 (12), 3477-3490.

Thomas, R.H. and Walsby, A.E., 1985. Buoyancy regulation in a strain of *Microcystis*. *Microbiology*, 131 (4), 799-809.

Tillmann, U., 2004. Interactions between planktonic microalgae and protozoan grazers. *Journal of Eukaryotic Microbiology*, 51 (2), 156-168.

Toepel, J., Langner, U. and Wilhelm, C., 2005. Combination of flow cytometry and single-cell absorption spectroscopy to study the phytoplankton structure and to calculate the Chl *a* specific absorption coefficients at the taxon Level. *Journal of Phycology*, 41 (6), 1099-1109.

Triest, L., Stiers, I. and Van Onsem, S., 2015. Biomanipulation as a nature-based solution to reduce cyanobacterial blooms. *Aquatic Ecology*, 1-23.

Tweeddale, H., Notley-McRobb, L. and Ferenci, T., 1998. Effect of slow growth on metabolism of *Escherichia coli*, as revealed by global metabolite pool ("metabolome") analysis. *Journal of Bacteriology*, 180 (19), 5109-5116.

Tzur, A., Moore, J.K., Jorgensen, P., Shapiro, H.M. and Kirschner, M.W., 2011. Optimizing optical flow cytometry for cell volume-based sorting and analysis. *PloS One*, i6 (1), e16053.

UK Technical Advisory Group (TAG), 2008. *UK environmental standards and conditions (Phase 2)*. Final Report (SR1–2007). March 2008. UK Technical Advisory Group on the Water Framework Directive. Edinburgh: SNIFFER.

United Nations Educational, Scientific and Cultural Organization (UNESCO), 2006. *Water: a shared responsibility - the United Nations world water development report 2*. Paris: UNESCO.

United Nations General Assembly (UNGA), 2010. *The human right to water and sanitation*. A/RES/64/292 (28th July 2010). Available from: undocs.org/A/RES/64/292. [Accessed 10th July 2013].

U.S. Food and Drug Administration (FDA), 2015. 21 CFR Regulation: citation in Title 21 of the U.S. Code of Federal Regulations - acetic acid 184.1005 (1982). Available from: http://www.ecfr.gov/cgi-bin/retrieveECFR?gp=&SID=af825799ab6dec1b4b4704987d373298&mc=true&n=pt21.3.184&r=PART&ty=HTML#se21.3.184_11005 [Accessed 10th October 2015].

Van de Water, S.D. and Simon, R.D., 1982. Induction and differentiation of heterocysts in the filamentous cyanobacterium *Cylindrospermum licheniforme*. *Microbiology*, 128 (5), 917-925.

Van Oosterhout, F. and Lürling, M., 2011. Effects of the novel 'Flock & Lock' lake restoration technique on Daphnia in Lake Rauwbraken (The Netherlands). *Journal of Plankton Research*, 33 (2), 255-263.

Van Wichelen, J., Van Gremberghe, I., Vanormelingen, P., Debeer, A.E., Leporcq, B., Menzel, D., Codd, G.A., Descy, J.P. and Vyverman, W., 2010. Strong effects of amoebae grazing on the biomass and genetic structure of a *Microcystis bloom* (cyanobacteria). *Environmental Microbiology*, 12 (10), 2797-2813.

Veal, E.A., Day, A.M. and Morgan, B.A., 2007. Hydrogen peroxide sensing and signaling. *Molecular Cell*, 26 (1), 1-14.

Veldhuis, M.J. and Kraay, G.W., 2000. Application of flow cytometry in marine phytoplankton research: current applications and future perspectives. *Scientia Marina*, 64 (2), 121-134.

Verspagen, J.M., Snelder, E.O., Visser, P.M., Joehnk, K.D., Ibelings, B.W., Mur, L.R. and Huisman, J., 2005. Benthic–pelagic coupling in the population dynamics of the harmful cyanobacterium *Microcystis*. *Freshwater Biology*, 50 (5), 854-867.

Vidal, R., López-Maury, L., Guerrero, M.G. and Florencio, F.J., 2009. Characterization of an alcohol dehydrogenase from the cyanobacterium *Synechocystis* sp. strain PCC 6803 that responds to environmental stress conditions via the Hik34-Rre1 two-component system. *Journal of Bacteriology*, 191 (13), 4383-4391

Visser, P.M., Passarge, J. and Mur, L.R., 1997. Modelling vertical migration of the cyanobacterium *Microcystis*. *Hydrobiologia*, 349 (1-3), 99-109.

Wagner, C. and Adrian, R., 2009. Cyanobacteria dominance: quantifying the effects of climate change. *Limnology and Oceanography*, 54 (6, part 2), 2460-2468.

Walsby, A.E., 1969. The permeability of blue-green algal gas-vacuole membranes to gas. *Proceedings of the Royal Society of London B: Biological Sciences*, 173 (1031), 235-255.

Walsby, A.E., 1972. Structure and function of gas vacuoles. *Bacteriological Reviews*, 36 (1), 1-32.

Walsby, A.E., 1994. Gas vesicles. *Microbiological Reviews*, 58 (1), 94-144.

Wang, Z., Li, D., Qin, H. and Li, Y., 2012. An integrated method for removal of harmful cyanobacterial blooms in eutrophic lakes. *Environmental Pollution*, 160, 34-41.

Wang, B., Shao, Y. and Chen, F., 2015. Overview on mechanisms of acetic acid resistance in acetic acid bacteria. *World Journal of Microbiology and Biotechnology*, 31 (2), 255-263.

Wang, J. and Xie, P., 2007. Antioxidant enzyme activities of *Microcystis aeruginosa* in response to nonylphenols and degradation of nonylphenols by *M. aeruginosa*. *Environmental Geochemistry and Health*, 29 (5), 375-383.

Watanabe, M.F. and Oishi, S., 1985. Effects of environmental factors on toxicity of a cyanobacterium (*Microcystis aeruginosa*) under culture conditions. *Applied and Environmental Microbiology*, 49 (5), 1342-1344.

Watanabe, M.F., Tsuji, K., Watanabe, Y., Harada, K.I. and Suzuki, M., 1992. Release of heptapeptide toxin (microcystin) during the decomposition process of *Microcystis aeruginosa*. *Natural Toxins*, 1 (1), 48-53.

Water Framework Directive (WFD), 2000. *Directive 2000/60/EC of the European Parliament and of the Council establishing a framework for the Community action in the field of water policy*. Brussels, Belgium: The European Parliament and the Council of the European Union.

Waybright, T.J., Terlizzi, D.E. and Ferrier, M.D., 2009. Chemical characterization of the aqueous algistatic fraction of barley straw (*Hordeum vulgare*) inhibiting *Microcystis aeruginosa*. *Journal of Applied Phycology*, 21 (3), 333-340.

Wetzel, R.G., 2001. *Limnology: lake and river ecosystems*. 3rd edition. San Diego: Academic Press.

Whitton, B.A., 1992. Diversity, ecology, and taxonomy of the cyanobacteria. *In:* N.G. Carr, eds. *Photosynthetic prokaryotes*. Springer US, 1-51.

Whitton, B.A. and Potts, M., 2012. Introduction to the cyanobacteria. *In*: Whitton B.A., ed., *Ecology of cyanobacteria II: their diversity in space and time*. 2nd edition. Dordrecht: Springer, 1-13.

Wiedner, C., Visser, P.M., Fastner, J., Metcalf, J.S., Codd, G.A. and Mur, L.R., 2003. Effects of light on the microcystin content of *Microcystis* strain PCC 7806. *Applied and Environmental Microbiology*, 69 (3), 1475-1481.

Wilhelm, S.W., 1995. Ecology of iron-limited cyanobacteria: a review of physiological responses and implications for aquatic systems. *Aquatic Microbial Ecology*, 9 (3), 295-303.

Williams, J.G., 1988. Construction of specific mutations in photosystem II photosynthetic reaction center by genetic engineering methods in *Synechocystis* 6803. *Methods in Enzymology*, 167, 766-778.

Wilson, A.E., Sarnelle, O. and Tillmanns, A.R., 2006. Effects of cyanobacterial toxicity and morphology on the population growth of freshwater zooplankton: meta-analyses of laboratory experiments. *Limnology and Oceanography*, 51 (4),1915-1924.

World Health Organization (WHO), 1999. *Toxic cyanobacteria in water: A guide to their public health consequences, monitoring and management*. London: E & FN Spon.

World Health Organization (WHO), 2008. *Guidelines for drinking-water quality* (Vol. 3). Geneva: World Health Organization.

World Health Organization (WHO), 2011. *Guidelines for drinking-water quality* (Vol. 4). Geneva: World Health Organization.

Yang, Z., Kong, F., Shi, X., Zhang, M., Xing, P. and Cao, H., 2008. Changes in the morphology and polysaccharide content of *Microcystis aeruginosa* (cyanobacteria) during flagellate grazing. *Journal of Phycology*, 44 (3), 716-720.

Zhang, Y. and Prepas, E.E., 1996. Short-term effects of Ca(OH)2 additions on phytoplankton biomass: a comparison of laboratory and in situ experiments. *Water Research*, 30 (5), 1285-1294.

Zhou, Q., Chen, W., Zhang, H., Peng, L., Liu, L., Han, Z., Wan, N., Li, L. and Song, L., 2012. A flow cytometer based protocol for quantitative analysis of bloom-forming cyanobacteria (*Microcystis*) in lake sediments. *Journal of Environmental Sciences*, 24 (9), 1709-1716.

This page intentionally left blank

Appendix A

Physical control of cyanobacteria

Sediment removal is one method of reducing nutrients (nitrates and phosphates), with the aim to decrease cyanobacterial densities. Dredging sediments is effective at reducing *Microcystis* biomass in hypertrophic ponds (Lürling and Faassen 2012) but is expensive and requires storage of the extracted wet biomatter if carried out on an industrial scale. A more viable method of sediment manipulation in small water bodies is capping, whereby a benthic barrier is created from a sand based mixture preventing phosphorus mixing of the water column. However, the stability of the barrier is often comprised by bioturbation from benthic organisms (Klapper 2003).

If pelagic phosphorus levels are high, nutrient immobilisation can be obtained through coagulants, preventing planktonic algae accessing bioavailable phosphorus (Pęczuła 2012). Consequently this type of nutrient capping (e.g. Phoslock) will potentially destabilise the nutrient dynamics of an ecosystem. Cyanobacterial blooms can also be limited by diluting or flushing systems which reduces nutrient concentration leading to intensification of seston sedimentation (Cooke *et al.* 2005). Aeration / artificial destratification has also been used to reduce cyanobacterial densities in reservoirs. Although, destratification does not significantly reduce the total amount of community microbial biomass, it only shifts the resident domination of cyanobacteria to diatoms (Heo and Kim 2004).

A more modern technique like ultrasound has the capability of shearing *Microcystis* cells (Cameron *et al.* 2008), collapsing gas vesicles (Lee *et al.* 2001) and inducing the formation of external reactive oxygen species (ROS) such as HO·, O· and tert-butanol (Koda *et al.* 2009). In addition sonication can also degrade chlorophyll *a* and separate colonies (Wu *et al.* 2012). Despite ultrasound working on cyanobacteria it strongly depends on the acoustic parameters (frequency and intensity) with differing optimal exposures for species (Lee *et al.* 2001; Hao *et al.* 2004; Joyce *et al.* 2010) and as of yet no long term studies of cyanobacterial population persistence has been assessed. UV-radiation has also induced mortality of *Microcystis* cells but again like the above treatment processes it requires high energy consumption resulting in increased costs (Alam *et al.* 2001).

References

Alam, M.Z.B., Otaki, M., Furumai, H. and Ohgaki, S., 2001. Direct and indirect inactivation of *Microcystis aeruginosa* by UV-radiation. *Water Research*, 35 (4), 1008-1014.

Cameron, M., McMaster, L.D. and Britz, T.J., 2008. Electron microscopic analysis of dairy microbes inactivated by ultrasound. *Ultrasonics Sonochemistry*, 15 (6), 960-964.

Cooke, G.D., Welch, E.B., Peterson, S. and Nichols, S.A., 2005. Restoration and management of lakes and reservoirs. 3rd edition. Florida: CRC Press.

Heo, W.M. and Kim, B., 2004. The effect of artificial destratification on phytoplankton in a reservoir. *Hydrobiologia*, 524 (1), 229-239.

Hao, H., Wu, M., Chen, Y., Tang, J. and Wu, Q., 2004. Cavitation mechanism in cyanobacterial growth inhibition by ultrasonic irradiation. *Colloids and Surfaces B: Biointerfaces*, 33 (3), 151-156.

Joyce, E.M., Wu, X. and Mason, T.J., 2010. Effect of ultrasonic frequency and power on algae suspensions. *Journal of Environmental Science and Health Part A*, 45 (7), 863-866.

Klapper, H., 2003. Technologies for lake restoration. *Journal of Limnology*, 62 (1s), 73-90.

Koda, S., Miyamoto, M., Toma, M., Matsuoka, T. and Maebayashi, M., 2009. Inactivation of *Escherichia coli* and *Streptococcus* mutants by ultrasound at 500kHz. *Ultrasonics Sonochemistry*, 16 (5), 655-659.

Lee, T.J., Nakano, K. and Matsumara, M., 2001. Ultrasonic irradiation for blue-green algae bloom control. *Environmental Technology*, 22 (4), 383-390.

Lürling, M. and Faassen, E.J., 2012. Controlling toxic cyanobacteria: effects of dredging and phosphorus-binding clay on cyanobacteria and microcystins. *Water Research*, 46 (5), 1447-1459.

Pęczuła, W., 2012. Methods applied in cyanobacterial bloom control in shallow lakes and reservoirs. *Ecological Chemistry and Engineering. A*, 19 (7), 795-806.

Wu, X., Joyce, E.M. and Mason, T.J., 2012. Evaluation of the mechanisms of the effect of ultrasound on *Microcystis aeruginosa* at different ultrasonic frequencies. *Water Research*, 46 (9), 2851-2858.

Appendix B

Appendix B1: BD Accuri C6 flow cytometer specifications

Laser(s) excitation	488 nm 640 nm
Laser profile	10 x 75 µm
Laser power	14.7 mW 640 nm diode red laser 20 mW 488 nm solid state blue laser
Scatter detection	Forward (0 degrees, ± 13) Side (90 degrees, ± 13)
Emission detection	FL1 533 ± 15 nm FL2 585 ± 20 nm FL3 > 670 nm FL4 675 ± 12.5 nm
Flow cell	200 µm ID quartz capillary
Minimum detectable particle size	0.5 µm
Minimum sample volume	300 µL (in 12 x 75 mm tube)
Pre-set flow rates and core sizes	Slow: 14 µL/min, 10 µm core Medium: 35 µL/min, 16 µm core Fast: 66 µL/min, 22 µm core
Custom sample flow rates	10 - 100 µL/min
Custom core diameter	5 - 40 µm
Sheath fluid	0.22 µm filtered deionized water
Maximum events per sample	1 million events per sample
Fluorescence linearity	2 ± 0.05% for chick erythrocyte nuclei (CEN)
Fluorescence precision	≤ 3% CV for CEN
Data acquisition rates	10,000 event/second maximum

Appendix B2: FSC-W data (arbitrary units) for a six-point calibration curve from slow and custom settings

	Sizing Bead (µm)					
	2.0	3.4	5.1	7.4	10.3	14.5
Slow						
Mean FSC-W (AU)	39.41	47.55	52.72	62.48	74.84	91.38
S.D.	0.07	0.02	0.04	0.02	0.04	0.08
S.E.	0.04	0.01	0.03	0.01	0.02	0.05
Custom						
Mean FSC-W (AU)	16.15	22.10	23.52	28.76	34.14	42.04
S.D.	0.01	0.02	0.04	0.05	0.02	0.18
S.E.	0.01	0.01	0.02	0.03	0.01	0.10

Appendix B3: Publication

Hartnell, D.M., Chapman, I.J., Esteban, G.F. and Franklin, D.J., 2016. Exploiting eco-physiological niche to facilitate the separation of the freshwater cyanobacteria *Microcystis* sp. and *Synechococcus* sp. *Journal of Microbiological Methods*, 122, 13-15.

Journal of Microbiological Methods 122 (2016) 13–15

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Note

Exploiting eco-physiological niche to facilitate the separation of the freshwater cyanobacteria *Microcystis* sp. and *Synechococcus* sp.

David M. Hartnell [a,b,*], Ian J. Chapman [a], Genoveva F. Esteban [a], Daniel J. Franklin [a]

[a] Bournemouth University, Centre for Ecology, Environment and Sustainability, Department of Life and Environmental Sciences, Faculty of Science and Technology, Dorset BH12 5BB, UK
[b] Centre for Environment, Fisheries and Aquaculture Science (Cefas), Barrack Rd, The Nothe, Weymouth, Dorset DT4 8UB, UK

ARTICLE INFO

Article history:
Received 3 September 2015
Received in revised form 4 January 2016
Accepted 7 January 2016
Available online 9 January 2016

Keywords:
Cyanobacteria
Isolation
Microcystis
Purification
Synechococcus

ABSTRACT

In a novel approach to separate the co-occurring freshwater cyanobacteria *Microcystis* and *Synechococcus*, published ecological characteristics are used to manipulate temperature and nutrient concentrations to successfully establish a unialgal *Microcystis* strain. The simple protocol has implications for future cyanobacterial culturing approaches and the establishment of new cyanobacteria strains.

Dense blooms of cyanobacteria (blue-green algae) can adversely affect freshwater supplies and ecosystems by releasing toxins, fouling infrastructure and causing anoxic conditions (Paerl and Huisman, 2009). Bloom forming genera of cyanobacteria have an array of eco-physiological adaptations which can allow their domination of freshwater ecosystems under favourable environmental conditions (Carey et al., 2012). Experimental work into the ecology and physiology of these important organisms often relies on long-established culture strains which may have been maintained under laboratory conditions for years or even decades. The physiological representativeness of these laboratory strains can be questioned (Lakeman et al., 2009) providing impetus for the development of simple protocols to isolate fresh cultures from natural populations.

Cyanobacteria isolation and purification techniques fall into two broad categories; mechanical (e.g. micro-pipetting, centrifugal washing and cell sorting) and physiological separation (e.g. antibiotic resistance, or changes in environmental parameters; light, temperature, nutrients). The majority of these isolation techniques require only simple apparatus. In this work we tested several techniques, alone and in combination, in order to design a protocol which yielded a unialgal culture of *Microcystis* after separation from the co-occurring *Synechococcus*.

* Corresponding author at: Bournemouth University, Centre for Ecology, Environment and Sustainability, Department of Life and Environmental Sciences, Faculty of Science and Technology, Dorset BH12 5BB, UK.
E-mail address: dhartnell@bournemouth.ac.uk (D.M. Hartnell).

http://dx.doi.org/10.1016/j.mimet.2016.01.003
0167-7012/

1. Step one (picking and crude selection)

Water samples were collected using 1 L Duran bottles from Ivy Lake, a flooded gravel pit, now used for drinking water storage and as a nature reserve in Dorset, UK (50°52′7.86″N, 1°47′7.87″W). Using light microscopy (10x light magnification) *Microcystis* sp. colonies were transferred by micro-pipette into a 25 mL conical flask containing 10 mL of 0.1 μm filtered reservoir water (collected along with the sample) and maintained at room temperature (18–22 °C) in a North facing window for 7 days. 1 mL of this mixed culture was transferred by pipette into a 15 mL centrifuge tube and diluted 1 in 6 with ultra-pure water (Millipore, USA) and then centrifuged at 1000 × g for 20 min. The supernatant biomass was removed by pipetting and transferred to a separate sterile centrifuge tube. This process was repeated three times to select for buoyant cyanobacteria following the methods of Bloch and Blackburn (1995). Processed cells were placed in 150 mL conical flasks with 50 mL of 50% BG-11 medium (Sigma-Aldrich, C3061) diluted by adding 0.5 mL to 50 mL ultra-pure water. All samples were then placed into an incubator (Conviron, CMP6010) at 25 ± 1 °C, on 12 h light/dark. Light was provided by a single 58 watt fluorescent tube (Luminex, Cool white) at 25 μmol quanta $m^{-2} s^{-1}$ (Biospherical Instrument Inc., PAR Scalar Irradiance sensor) for 18 days. Colonies were homogenised by vortex and cell division monitored by flow cytometry (Accuri, C6) for 2 min using a 10 μL core size, 14 μL/min flow rate and threshold set at 20,000 on forward scatter light (FSC) signal.

After 10 days there was an increase in the biomass of *Microcystis* cells as cell density increased from 270,000 to 4,000,000 per millilitre. However, closer examination under light microscopy revealed cultures

Appendix B4: *Microcystis* colony fragmentation and FCM analysis

Introduction

As *Microcystis* can exist either as uni-cells or colonies, it may be necessary to use physical or chemical separation techniques to achieve more accurate flow cytometry (FCM) cell counts. Pre-treatment with potassium hydroxide, enzymes, vortexing, boiling and ultrasound have all been used to separate *Microcystis* colonies, with ultra sound exposure more commonly applied when establishing cell counts for *in-situ Microcystis* (Reynolds 1973; Reynolds and Jaworski 1978; Kurmayer *et al.* 2003; Joung *et al.* 2006; Karatan and Watnick 2009).

The fragmentation of colonies from various methods like ultrasound may also cause morphological changes in algal species, inducing gas vesicle (GV) collapse or changes in cell size (Lee *et al.* 2000) potentially shifting FCM outputs. A number of cyanobacteria such as *Microcystis* contain GVs which can act as a biomolecular sensor reporting cellular integrity (Shapiro *et al.* 2014) or help identify algal groups from environmental monitoring (Crosbie *et al.* 2003; Read *et al.* 2014).

Ultrasound has the ability to collapse *Microcystis* GVs depending on the acoustic parameters. Specific frequencies and power intensities can also cause cell mortality by cell shearing, free radicals formation (e.g. HO·, O·) and / or degradation of chlorophyll *a* (Lee *et al.* 2001; Cameron *et al.* 2008; Koda *et al.* 2009; Wu *et al.* 2012).

Although at low power ultrasound energy can fragment *Microcystis* colonies (Wu *et al.* 2012) the energy generated can also lead to damage in photosynthetic apparatus (Zhang *et al.* 2006). In order to obtain high resolution data from colony disruption precise ultrasound parameters need to be tested, as cell mortality (lysis) would interfere with accurate enumeration. As ultrasound has been observed to cause shifts in both SSC-H and FSC-H outputs, along with the potential to damage photosynthetic apparatus (Lee *et al.* 2000; Zhang *et al.* 2006) any potential shift in FCM outputs due to colony disruption techniques must be taken into consideration.

As the research aims to monitor water systems, mechanical instruments such as water inflow and extraction equipment commonly found in reservoirs can potentially cause shifts in FCM outputs (e.g. SSC-H from collapsed GVs). Therefore, a high and low impact colony fragmentation technique was tested to assess changes in *Microcystis* colony fragmentation, morphology and cell physiology (auto fluorescence) in both colonial forming and unicellular cultures.

Methodology

The unicellular *Microcystis aeruginosa* PCC 7806 (+mycB) (PCC, Institut Pasteur, France) and a colony forming sample identified as *Microcystis* sp. Longham which was freshly isolated from Longham reservoir 1 (Hartnell *et al.* 2016) were used in this experiment. Both cultures were incubated at 25±1^{o}C, under an irradiance range 15 – 19.5 µE m^{-2} s^{-1} (Luminex, 58 watt fluorescent cool white tube) on a 12 hour light/dark cycle (Conviron, CMP6010, Canada) in BG-11 media (Sigma-Aldrich, U.K.). Cell densities were analysed at approximately 3 - 4x10^{6} cells/mL, using custom FCM settings (as used for the environmental monitoring research, Chapter 3) and sampled for 2 minutes.

The *Microcystis* samples were subjected to two types of common separation techniques; a low impact vortex (fixed speed, 2800 rpm, IKEA-works, Germany) and a high impact ultrasonic bath (38kHz±10%, 75W, KC2, Kerry, USA). All samples were contained in a 3.5 mL round based haemolysis tube (12x75mm, polypropylene and borosilicate glass, Thermo Fisher Scientific, USA), which were used for all FCM analysis throughout this research at a total sample volume of 2 mL. The ultrasonic bath was filled with 5 cm of distilled water with haemolysis tubes held under 2 cm from the surface during testing. Experimental times were conducted over 0 (control), 20, 40, 50 and 60 seconds (n=10).

Microcystis sp. Longham spherical colonies were measured to observe colony fragmentation through post-treatment mean diameters under light microscopy (Olympus BX51, Japan). For both strains, cell numbers and mean wavelengths (arbitrary units) were measured for FSC-W, SSC-H, FL3-H and FL4-H. A one-way ANOVA (or a Kruskal-Wallis one-way ANOVA on ranks for non-parametric data) with *post hoc* analysis was tested to report significant difference (p < 0.05) in colony diameter, cell numbers and shifts from FCM outputs in both separation techniques over time.

Results

Colony dimensions

A one-way ANOVA revealed a statistically significant difference between *Microcystis* sp. Longham colony sizes over time using ultrasound ($p < 0.001$) but not vortexing ($p > 0.05$) (Table B4.1). A pairwise analysis (Tukey's test) for the ultrasound technique revealed two homogeneous subsets, with 0 and 20 seconds having a combined mean colony diameter of 148.7±19.0 µm (Chapter 2, Figure 2.9), which was 91.6% larger than the mean colony diameter of 12.5±1.6 µm for 40, 50 and 60 seconds (Figure B4.1). Vortexing produced a mean colony diameter of 145.8±26.7 µm, similar to 0 and 20 seconds of ultrasound exposure (Table B4.2).

Table B4.1. High and low impact separation techniques on *Microcystis* sp. Longham tested through a Kruskal-Wallis one-way ANOVA on ranks, with statistically significant difference in bold, *denotes the parametric alternative one-way ANOVA.

Parameter	Ultrasound			Vortexing		
	X^2	d.f.	*p*	X^2	d.f.	*p*
Colony size (µm)	40.72	4	< 0.001	0.30	4	> 0.05
Cell count (cells/mL)	45.86	4	< 0.001	194.04*	4,45	< 0.001
Cell size (µm)	247.26*	4,45	< 0.001	5.17	4	> 0.05
SSC-H (AU)	23.77	4	< 0.001	1.58*	4,45	> 0.05
FL3-H (AU)	42.50	4	< 0.001	0.59*	4,45	> 0.05
FL4-H (AU)	45.73	4	< 0.001	1.26	4	> 0.05

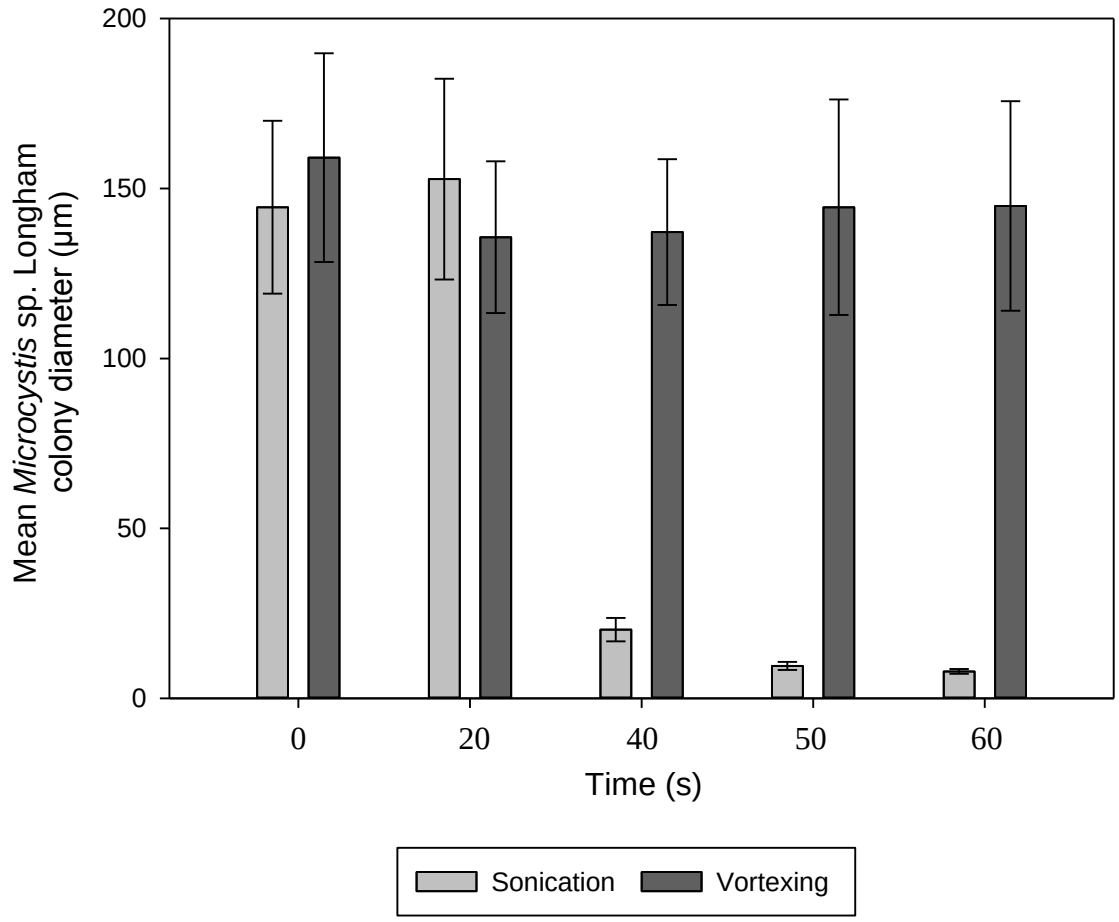


Figure B4.1. Mean diameter of *Microcystis* sp. Longham colonies after exposure to a low and high impact separation technique over 60s, including standard error bars (S.E.) n = 10.

Table B4.2. *Microcystis* sp. Longham colony mean diameter after exposure to ultrasound and vortexing between 0 – 60s, with standard error (S.E.).

Time (s)	Mean colony diameter (µm)	
	Ultrasound	Vortexing
0 (control)	144.5 ± 25.4	159.1 ± 30.7
20	152.8 ± 29.5	135.7 ± 22.3
40	20.2 ± 3.4	137.2 ± 21.4
50	9.5 ± 1.2	144.5 ± 31.7
60	7.9 ± 0.7	144.9 ± 30.8

Cell counts

Cell counts for *Microcystis* sp. Longham were significantly different over time when exposed to ultrasound (p < 0.001) (Table B4.3). Cell numbers recorded an increase through application time, with the control (0s) and 20s cultures reporting a combined mean of $6.9x10^5 \pm 5.1x10^4$ cells/mL, 5.9 times less than the mean of $4.1x10^6 \pm 4.3x10^4$

cells/mL for 50 and 60s (Figure B4.2). Vortexing in *Microcystis* sp. Longham also saw a significant difference in cell numbers analysed through FCM ($p < 0.001$) but much lower than ultrasound application. There was a 23.65% increase from the low impact technique between the first two application times (0 and 20s) and the last three (40, 50, and 60s), increasing from $4.8x10^5 \pm 4.2x10^3$ cells/mL to $6.0x10^5 \pm 1.9x10^3$ cells/mL.

The unicellular *M. aeruginosa* (PCC 7806) reported significant difference in cell numbers recorded when exposed to ultrasound ($p < 0.001$) (Table B4.4), which reported a decreased over time. Two homogeneous subsets from *M. aeruginosa* (PCC 7806) were reported i) between 0 - 50s with a combined mean of $3.1x10^6 \pm 1.0x10^4$ cells/mL and ii) 60 s which had 7.9% fewer cells ($2.9x10^6 \pm 2.6x10^4$ cells/mL). Vortexing had no significant impact on cell enumeration in *M. aeruginosa* (PCC 7806) recording a mean of $3.1x10^6 \pm 8.6x10^3$ cells/mL over all time periods tested ($p > 0.05$) (Table B4.4).

Table B4.3. Statistical analysis from a Kruskal-Wallis one-way ANOVA on ranks for all parameters tested over time when a high (ultrasound) and low (vortexing) technique was used on colony forming *Microcystis* sp. Longham. Statistically significant difference are in bold (*denotes the parametric alternative one-way ANOVA on ranks).

Parameter	Sonication			Vortexing		
	X^2	d.f.	*p*	X^2	d.f.	*p*
Colony size (µm)	40.72	4	**< 0.001**	0.30	4	> 0.05
Cell count (cells/mL)	45.86	4	**< 0.001**	194.04*	4,45	**< 0.001**
Cell size (µm)	247.26*	4,45	**< 0.001**	5.17	4	> 0.05
SSC-H (AU)	23.77	4	**< 0.001**	1.58*	4,45	> 0.05
FL3-H (AU)	42.50	4	**< 0.001**	0.59*	4,45	> 0.05
FL4-H (AU)	45.73	4	**< 0.001**	1.26	4	> 0.05

Table B4.4. Statistical analysis from a one-way ANOVA for all parameters tested over time when a high (ultrasound) and low (vortexing) technique was used on unicellular *Microcystis aeruginosa* (PCC 7806). Statistically significant difference are in bold (*denotes the non-parametric alternative Kruskal-Wallis one-way ANOVA on ranks).

Parameter	Sonication			Vortexing		
	F	d.f.	*p*	*F*	d.f.	*p*
Cell count (cells/mL)	25.45	4,45	**< 0.001**	1.03	4,45	> 0.05
Cell size (µm)	24.26*	4	**< 0.001**	0.33	4,45	> 0.05
SSC-H (AU)	57,815.55	4,45	**< 0.001**	0.90	4,45	> 0.05
FL3-H (AU)	38.23	4,45	**< 0.001**	0.39	4,45	> 0.05
FL4-H (AU)	750.89	4,45	**< 0.001**	2.37*	4	> 0.05

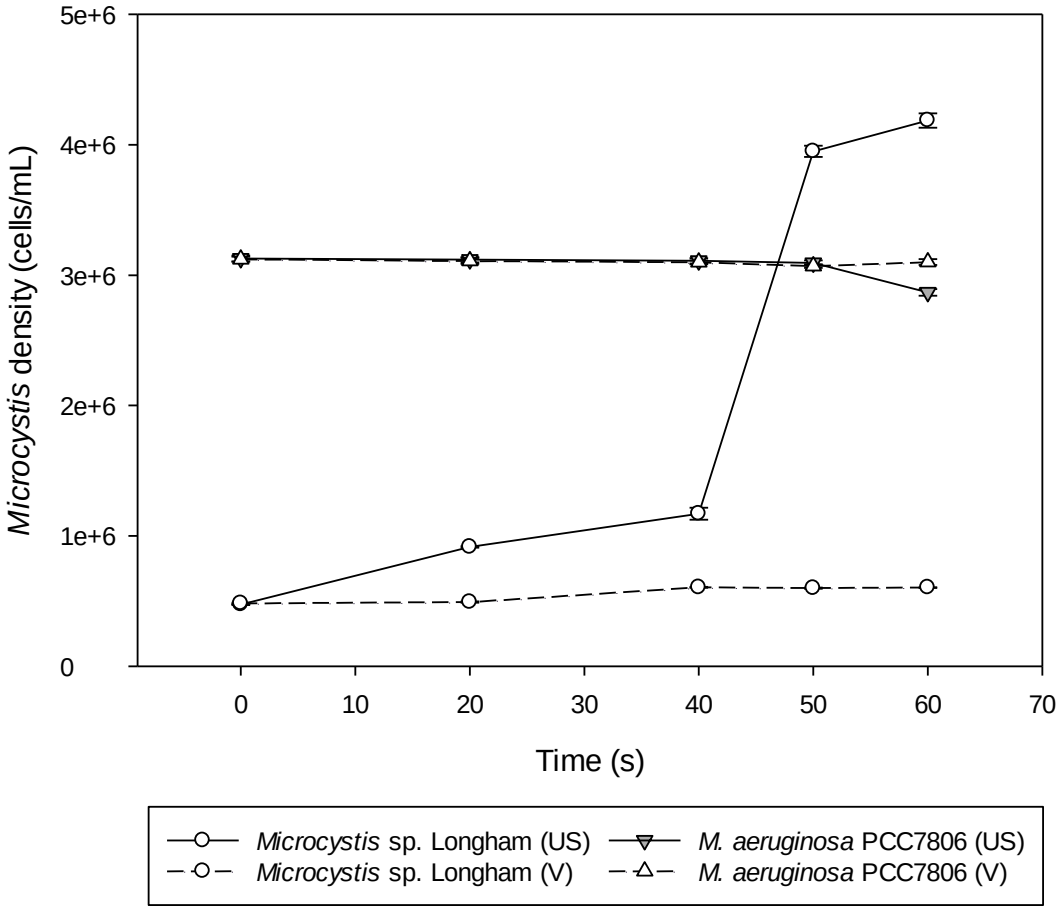


Figure B4.2. Cell densities (cells/mL) recorded through FCM for both the colony forming *Microcystis* sp. Longham and unicellular *Microcystis aeruginosa* (PCC 7806) cultures when exposed to ultra sound (US) and vortexing (V) over 60s.

Cell morphology and light scatter

The mean cell size (diameter) calculated from FSC-W in both *Microcystis* sp. Longham and *M. aeruginosa* (PCC 7806) had significantly increased over time when ultrasound was applied ($p < 0.001$) (Tables B4.3 and B4.4). *Microcystis* sp. Longham increased mean cell size (diameter) from 5.23$\pm$0.07 µm to 6.34$\pm$0.07 µm after 20s, which increased further to 7.51$\pm$0.04 µm at 60 s (Table B4.5). *M. aeruginosa* (PCC 7806) also recorded an increase after 20 s by 21.5% from 4.46$\pm$0.07 µm to 5.37$\pm$0.07 µm, where a final mean diameter of 5.46$\pm$0.06 µm was measured at 60s (Table B4.5). Vortexing had no significant effect on *Microcystis* sp. Longham or *M. aeruginosa* PCC 7806 sizes ($p > 0.05$).

Mean SSC-H mean peaks did significantly decrease over time for *Microcystis* sp. Longham and *M. aeruginosa* PCC 7806 when samples were subjected to ultrasound ($p < 0.001$). After *Microcystis* sp. Longham was exposed to ultra sound SSC-H shifted from a mean peak of 350,597±2,523 AU (0s) to 64,435±377 AU (20s). Analysis of *M. aeruginosa* (PCC 7806) SSC-H mean peaks revealed a shift decrease of approximately an order of magnitude between non-application (control 0s) and application of ultrasound (20 - 60s) (Figure B4.3 A). Ultrasound exposure in both *Microcystis* cultures produced a smaller %CV, recording a narrower histogram peak (Figure B4.3 B). SSC-H mean peaks did not significantly shift after vortexing in both *Microcystis* species ($p > 0.05$).

Ultrasound exposure on both *Microcystis* cultures did record a significant shift in mean FL3-H peaks ($p < 0.001$) (Table B4.4). Ultrasound caused an increase mean FL3-H peak shift from 82,062±800 AU at 0s to 169,778±401 AU at 60s in *Microcystis* sp. Longham and a smaller 4.8% increase in *M. aeruginosa* (PCC 7806) (Figure B4.3 C). Vortexing had no significant impact on mean FL3-H peaks for both *Microcystis* sp. Longham and *M. aeruginosa* (PCC 7806) ($p > 0.05$) (Table B4.6).

Both *Microcystis* samples subjected to ultrasound did show a significant shift in mean FL4-H peaks ($p < 0.001$). Sonication increased mean FL4-H peaks from 738,154±2,522 AU at 0s to 1,443,007±17,840 AU at 60 s in *Microcystis* sp. Longham and by 31.6% in *M. aeruginosa* (PCC 7806) (Figure B4.3 D). Vortexing again did not have a significant impact on mean FL4-H peaks in both *Microcystis* species ($p > 0.05$).

Table B4.5. FCM mean histogram peak data from *Microcystis* sp. Longham and *M. aeruginosa* (PCC 7806) after exposure to ultra sound.

Microcystis sp. Longham

	Colony size (µm)		Cell count (cells/mL)		Cell size (µm)		SSC-H (AU)		FL3-H (AU)		FL4-H (AU)	
Time (s)	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.
0	144.50	25.44	4.7E+05	3.7E+03	5.23	0.07	3.5.E+05	2.5.E+03	8.2.E+04	8.0.E+02	7.4.E+05	2.5.E+03
20	152.80	29.52	9.2E+05	5.6E+03	6.34	0.06	6.5.E+04	7.4.E+02	1.1.E+05	1.5.E+03	1.1.E+06	1.3.E+04
40	20.20	3.44	1.2E+06	4.6E+04	6.48	0.06	6.4.E+04	7.9.E+02	1.1.E+05	1.3.E+03	1.2.E+06	7.4.E+03
50	9.50	1.18	3.9E+06	4.3E+04	7.44	0.06	6.4.E+04	7.0.E+02	1.7.E+05	4.8.E+02	1.3.E+06	1.2.E+04
60	7.90	0.69	4.2E+06	5.5E+04	7.51	0.04	6.5.E+04	8.9.E+02	1.7.E+05	4.0.E+02	1.4.E+06	1.8.E+04

Microcystis aeruginosa (PCC 7806)

	Cell count (cells/mL)		Cell size (µm)		SSC-H (AU)		FL3-H (AU)		FL4-H (AU)	
Time (s)	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.
0	3.1E+06	2.3E+04	4.42	0.07	2.0.E+05	5.0.E+02	1.2.E+05	1.9.E+02	4.1.E+05	1.0.E+03
20	3.1E+06	2.0E+04	5.37	0.07	2.4.E+04	3.3.E+02	1.2.E+05	3.3.E+02	5.4.E+05	2.6.E+03
40	3.1E+06	2.0E+04	5.36	0.08	2.3.E+04	3.3.E+02	1.2.E+05	4.7.E+02	5.4.E+05	1.7.E+03
50	3.1E+06	1.9E+04	5.42	0.06	2.4.E+04	2.3.E+02	1.2.E+05	4.3.E+02	5.3.E+05	2.0.E+03
60	2.9E+06	2.6E+04	5.46	0.06	2.4.E+04	1.9.E+02	1.2.E+05	5.1.E+02	5.3.E+05	2.7.E+03

Table B4.6. FCM mean histogram peak data from *Microcystis* sp. Longham and *M. aeruginosa* (PCC 7806) after vortexing.

Microcystis sp. Longham

	Colony size (µm)		Cell count (cells/mL)		Cell size (µm)		SSC-H (AU)		FL3-H (AU)		FL4-H (AU)	
Time (s)	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.
0	159.10	30.71	4.8E+05	6.2E+03	5.19	0.08	3.5.E+05	1.9.E+03	8.1.E+04	7.8.E+02	7.5.E+05	4.1.E+03
20	135.70	22.31	4.9E+05	5.5E+03	5.26	0.08	3.5.E+05	2.6.E+03	8.3.E+04	7.4.E+02	7.4.E+05	4.2.E+03
40	137.20	21.44	6.1E+05	3.0E+03	5.06	0.08	3.6.E+05	2.0.E+03	8.2.E+04	6.4.E+02	7.4.E+05	5.4.E+03
50	144.50	31.70	6.0E+05	4.2E+03	5.07	0.06	3.5.E+05	2.6.E+03	8.2.E+04	7.7.E+02	7.4.E+05	5.3.E+03
60	144.90	30.82	6.0E+05	2.8E+03	5.19	0.08	3.6.E+05	2.0.E+03	8.2.E+04	5.2.E+02	7.4.E+05	5.3.E+03

Microcystis aeruginosa (PCC 7806)

	Cell count (cells/mL)		Cell size (µm)		SSC-H (AU)		FL3-H (AU)		FL4-H (AU)	
Time (s)	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.
0	3.1E+06	1.9E+04	4.41	0.06	2.0.E+05	5.3.E+02	1.2.E+05	1.4.E+02	4.1.E+05	9.5.E+02
20	3.1E+06	2.3E+04	4.49	0.07	2.0.E+05	5.7.E+02	1.2.E+05	2.1.E+02	4.0.E+05	1.1.E+03
40	3.1E+06	1.5E+04	4.50	0.07	2.0.E+05	3.9.E+02	1.2.E+05	1.6.E+02	4.1.E+05	1.1.E+03
50	3.1E+06	1.4E+04	4.44	0.04	2.0.E+05	3.9.E+02	1.2.E+05	1.8.E+02	4.1.E+05	1.2.E+03
60	3.1E+06	2.3E+04	4.44	0.08	2.0.E+05	5.1.E+02	1.2.E+05	1.8.E+02	4.1.E+05	1.0.E+03

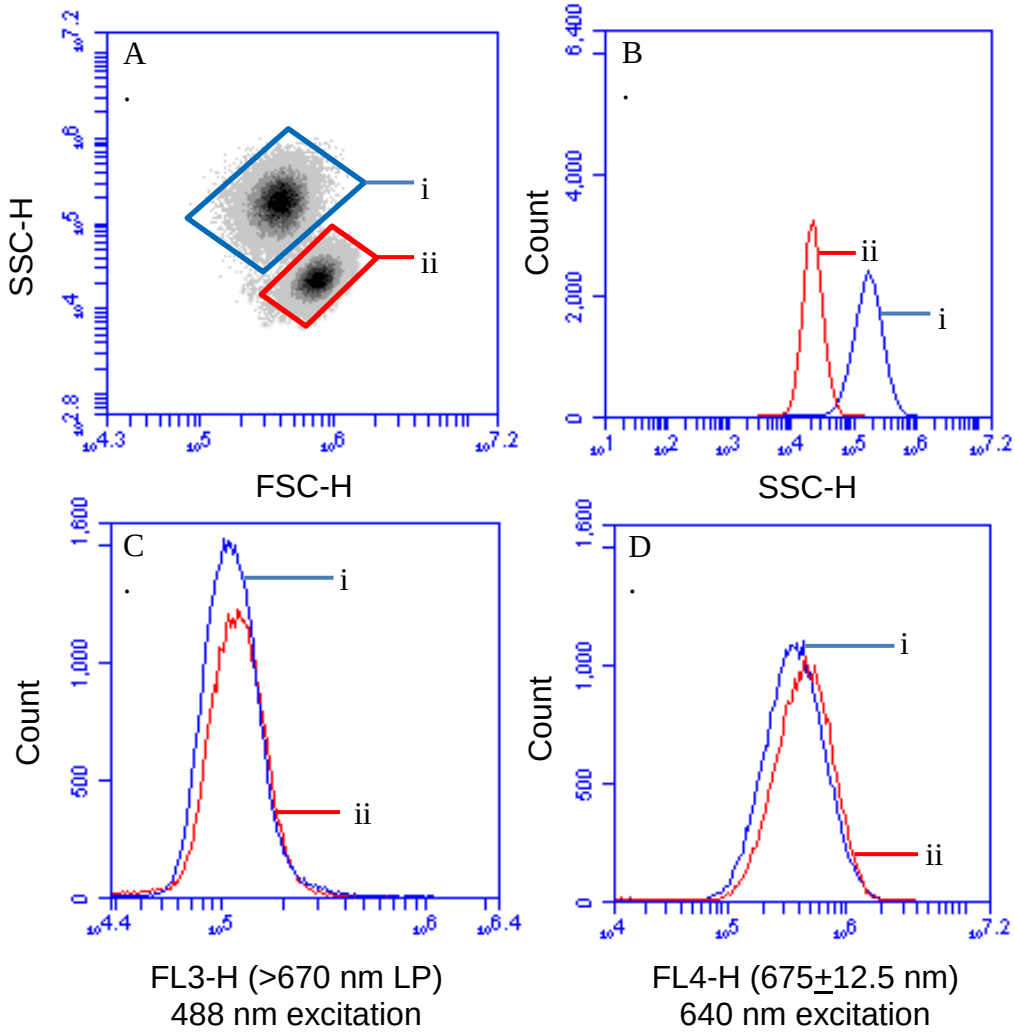


Figure B4.3. *Microcystis aeruginosa* (PCC 7806) i) pre and ii) post exposure to ultrasound. A) FCM density plot from forward and side light scatter recordings (for clarity in the monograph, FSC is reported as FSC-H instead of FSC-W). Mean peak histogram shifts were also observed in B) SSC-H, C) FL3-H and D) FL4-H.

Discussion

The use of separation techniques for *Microcystis* colonies produced a morphological and physiological response in single-cells, which affected the accuracy of cell counts. Colony separation through ultra sound was found to be more effective than the use of vortexing. Here *Microcystis* sp. Longham clearly showed good separation after 40s of sonication, as mean colony diameters reduced by an order of magnitude and many more individual cells were observed (Chapter 2, Figure 2.9). However, unicellular *Microcystis* reported a significant drop after 60s, indicating that prolonged exposure at those particular settings caused cell mortality, thereby reducing cell densities and ultimately enumeration accuracy. Microscope observations also confirmed cell membrane injury and a distinct green colour increase in culture suspension due to phytopigment release.

When using such a technique like ultrasound acoustic the power, frequency and time applied will need to be predetermined, along with the ultra sound equipment used. Ultrasonic baths provide a relatively even power distribution, whereas direct 'horn tip' immersion devices deliver more concentrated energy (Joyce *et al.* 2010). The contribution of vortexing is minimal as the energy required is higher for separating individual cells in colonial forms. Although *Microcystis* exists as both unicellular and colonial forms, natural and artificial process can cause GV collapse, so FCM gating will need to take into account shifts in SSC-H histogram peaks.

The mean cell size of individual *Microcystis* cells increased the longer they were exposed to ultra sound. Damage to DNA and the arrest of the cell cycle can lead to uncontrolled cell expansion, however, this would take longer than the 20 – 60s time period tested. Cavitation is one well recognised process of ultrasound, where high enough energy within a liquid-gas mixture forms bubbles that consequently collapse. To cause cavitation bubbles must be capable of expanding before violently collapsing and before the total pressure reaches its minimal value (Ensminger and Bond 2011), which may see internal cavitation of *Microcystis* expand before membrane rupture. Although there was a statistically significant increase of fluorescence peaks (FL3 and 4-H) the shifts were minimal (Figure B4.3) and would not change the gating range when identifying *Microcystis* for quantification.

The data recorded from colony separation does need to be understood to create the highest resolution for data, especially when monitoring in the environment. However, *in-situ* samples may only contain unicellular forms or colonies that could potentially be broken up by less invasive methods (Chapter 3). Therefore low energy techniques, like vortexing could be favourable when assessing physiological responses such as community viability, due to the reduced risk of altering cellular states e.g. induce mortality.

Homogeneity of samples must also be undertaken, as observations were made between 5 - 8 minutes from *Microcystis* sp. Longham after exposure to ultra sound (50s) seeing cells became re-buoyant, with the highest density of cells floating to the top. Here a sonication (ultrasound batch) recommendation of 50s will be implemented if colonial *Microcystis* counts are observed, followed by a pre-vortexing before FCM analysis.

References

Cameron, M., McMaster, L.D. and Britz, T.J., 2008. Electron microscopic analysis of dairy microbes inactivated by ultrasound. *Ultrasonics Sonochemistry*, 15 (6), 960-964.

Crosbie, N.D., Teubner, K. and Weisse, T., 2003. Flow-cytometric mapping provides novel insights into the seasonal and vertical distributions of freshwater autotrophic picoplankton. *Aquatic Microbial Ecology*, 33 (1), 53-66.

Ensminger, D. and Bond, L.J., 2011. *Ultrasonics: fundamentals, technologies, and applications*. 3rd edition. Boca Raton: CRC press, 27-101.

Hartnell, D.M., Chapman, I.J., Esteban, G.F. and Franklin, D.J., 2016. Exploiting eco-physiological niche to facilitate the separation of the freshwater cyanobacteria *Microcystis* sp. and *Synechococcus* sp. *Journal of microbiological methods*, 122, 13-15.

Joung, S., Kim, C., Ahn, C., Jang, K., Boo, S.M. and Oh, H., 2006. Simple method for a cell count of the colonial cyanobacterium, *Microcystis* sp. *Journal of Microbiology-Seoul*, 44 (5), 562.

Joyce, E.M., Wu, X. and Mason, T.J., 2010. Effect of ultrasonic frequency and power on algae suspensions. *Journal of Environmental Science and Health Part A*, 45 (7), 863-866.

Karatan, E. and Watnick, P., 2009. Signals, regulatory networks, and materials that build and break bacterial biofilms. *Microbiology and Molecular Biology Reviews*, 73 (2), 310-347.

Koda, S., Miyamoto, M., Toma, M., Matsuoka, T. and Maebayashi, M., 2009. Inactivation of *Escherichia coli* and *Streptococcus* mutants by ultrasound at 500kHz. *Ultrasonics sonochemistry*, 16 (5), 655-659.

Kurmayer, R., Christiansen, G. and Chorus, I., 2003. The abundance of microcystin-producing genotypes correlates positively with colony size in *Microcystis* sp. and determines its microcystin net production in Lake Wannsee. *Applied and Environmental Microbiology*, 69 (2), 787-795.

Lee, T.J., Nakano, K. and Matsumura, M., 2000. A new method for the rapid evaluation of gas vacuoles regeneration and viability of cyanobacteria by flow cytometry. *Biotechnology Letters*, 22 (23), 183.

Lee, T.J., Nakano, K. and Matsumara, M., 2001. Ultrasonic irradiation for blue-green algae bloom control. *Environmental technology*, 22 (4), 383-390.

Read, D.S., Bowes, M.J., Newbold, L.K. and Whiteley, A.S., 2014. Weekly flow cytometric analysis of riverine phytoplankton to determine seasonal bloom dynamics. *Environmental Science: Processes & Impacts*, 16 (3), 594-603.

Reynolds, C.S., 1973. Growth and buoyancy of *Microcystis aeruginosa* Kütz. emend. Elenkin in a shallow eutrophic lake. *Proceedings of the Royal Society of London B: Biological Sciences*, 184 (1074), 29-50.

Reynolds, C.S. and Jaworski, G.H.M., 1978. Enumeration of natural *Microcystis* populations. *British Phycological Journal*, 13 (3), 269-277.

Shapiro, M.G., Goodwill, P.W., Neogy, A., Yin, M., Foster, F.S., Schaffer, D.V. and Conolly, S.M., 2014. Biogenic gas nanostructures as ultrasonic molecular reporters. *Nature Nanotechnology*, 9 (4), 311.

Wu, X., Joyce, E.M. and Mason, T.J., 2012. Evaluation of the mechanisms of the effect of ultrasound on *Microcystis aeruginosa* at different ultrasonic frequencies. *Water Research*, 46 (9), 2851-2858.

Zhang, G., Zhang, P., Liu, H. and Wang, B., 2006. Ultrasonic damages on cyanobacterial photosynthesis. *Ultrasonics Sonochemistry*, 13 (6), 501-505.

Appendix B5: Publication

Chapman, I.J., Esteban, G.F. and Franklin, D.J., 2016. Molecular probe optimization to determine cell mortality in a photosynthetic organism (*Microcystis aeruginosa*) using flow cytometry. *JoVE (Journal of Visualized Experiments)*, 107, e53036-e53036.

jove Journal of Visualized Experiments www.jove.com

Video Article

Molecular Probe Optimization to Determine Cell Mortality in a Photosynthetic Organism (*Microcystis aeruginosa*) Using Flow Cytometry

Ian J. Chapman[1], Genoveva F. Esteban[1], Daniel J. Franklin[1]

[1]Department of Life and Environmental Sciences, Faculty of Science and Technology, Bournemouth University

Correspondence to: Ian J. Chapman at ichapman@bournemouth.ac.uk

URL:http://www.jove.com/video/5306
DOI: doi:10.3791/53036

Keywords: Medicine, Issue 107, Flow cytometry, molecular probe,

cyanobacteria, *Microcystis*, cell viability Date Published: 1/29/2016

Citation: Chapman, I.J., Esteban, G.F., Franklin, D.J. Molecular Probe Optimization to Determine Cell Mortality in a Photosynthetic Organism (*Microcystis aeruginosa*) Using Flow Cytometry. *J. Vis. Exp.* (107), e53036, doi:10.3791/53036 (2016).

Abstract

Microbial subpopulations in field and laboratory studies have been shown to display high heterogeneity in morphological and physiological parameters. Determining the real time state of a microbial cell goes beyond live or dead categories, as microbes can exist in a dormant state, whereby cell division and metabolic activities are reduced. Given the need for detection and quantification of microbes, flow cytometry (FCM) with molecular probes provides a rapid and accurate method to help determine overall population viability. By using SYTOX Green and SYTOX Orange in the model cyanobacteria *Microcystis aeruginosa* to detect membrane integrity, we develop a transferable method for rapid indication of single cell mortality. The molecular probes used within this journal will be referred to as green or orange nucleic acid probes respectively (although there are other products with similar excitation and emission wavelengths that have a comparable modes of action, we specifically refer to the fore mentioned probes). Protocols using molecular probes vary between species, differing principally in concentration and incubation times. Following this protocol set out on *M.aeruginosa* the green nucleic acid probe was optimized at concentrations of 0.5 µM after 30 min of incubation and the orange nucleic acid probe at 1 µM after 10 min. In both probes concentrations less than the stated optimal led to an under reporting of cells with membrane damage. Conversely, 5 µM concentrations and higher in both probes exhibited a type of non-specific staining, whereby 'live' cells produced a target fluorescence, leading to an over representation of 'non-viable' cell numbers. The positive controls (heat- killed) provided testable dead biomass, although the appropriateness of control generation remains subject to debate. By demonstrating a logical sequence of steps for optimizing the green and orange nucleic acid probes we demonstrate how to create a protocol that can be used to analyse cyanobacterial physiological state effectively.

Appendix B6: Mean percentage (%) of *Microcystis* cells that took up molecule probes during optimisation of CMFDA and CM-H_2DCFDA (standard error ± underlined)

CMFDA

Concentration	Time (min)									
(µM)	1		5		10		30		60	
0.5	1.9	0.1	28.5	3.8	65.9	7.0	97.5	0.6	99.1	0.1
1	7.2	1.1	61.1	1.7	96.3	0.2	99.6	0.0	99.7	0.0
5	36.8	13.8	97.7	1.4	99.9	0.3	100.0	0.5	100.0	0.5
10	60.0	12.3	98.5	0.7	100.0	0.2	99.8	0.1	100.0	0.5
20	42.6	4.4	99.1	0.3	99.6	0.2	99.8	0.1	99.7	0.2
50	23.3	6.0	99.0	0.6	99.4	0.3	99.4	0.3	99.6	0.2

CM-H_2DCFDA

Concentration	Time (min)							
(µM)	10		30		60		90	
5	0.7	0.1	17.6	1.7	66.6	1.5	83.4	1.2
10	1.6	0.2	46.4	1.5	87.6	0.4	93.2	0.5
25	2.9	0.1	63.1	1.6	97.1	0.4	95.3	0.3

Appendix B7: Chemicals and standards for cyanotoxin detection and quantification in *Microcystis*

Instrument solvents used for preparation of mobile phases were of LC-MS-grade (Fisher Optima, ThermoFisher, UK) and all chemicals were LC-MS reagent grade where possible. Sample preparation reagents were HPLC grade. Certified reference toxins (MC-RR, MC-LA, MC-LY, MC-LF, MC-LW, MC-YR, MC-WR, MC-HilR, MC-HtyR, MC-LR, Asp3-MC-LR and Nodularin) were all obtained from Enzo Life Sciences, Exeter, UK. A certified standard of [Dha^7]-MC-LR and a pre-certified freeze-dried matrix reference material of blue-green algae (RM-BGA, Lot 201301) containing a range of microcystins was obtained from the Institute of Biotoxin Metrology, National Research Council Canada (NRCC).

For preparation of a mixed stock solution, CRM ampoules were opened and aliquots accurately pipetted into a vial, giving a concentration of 327.7 ng/mL for each compound. A seven-level suite of working calibration standards was subsequently prepared through serial dilution of the mixed stock solution using 50% methanol as the diluent, resulting in a calibration range between 0.33 ng/mL to 327 ng/mL. 280 mg RM-BGA was extracted with 28.0 mL 50% aqueous MeOH + 0.1% acetic acid prior to centrifugation (4,500 rpm; 10 min) and the supernatant collected prior to analysis.

This page intentionally left blank

Appendix C

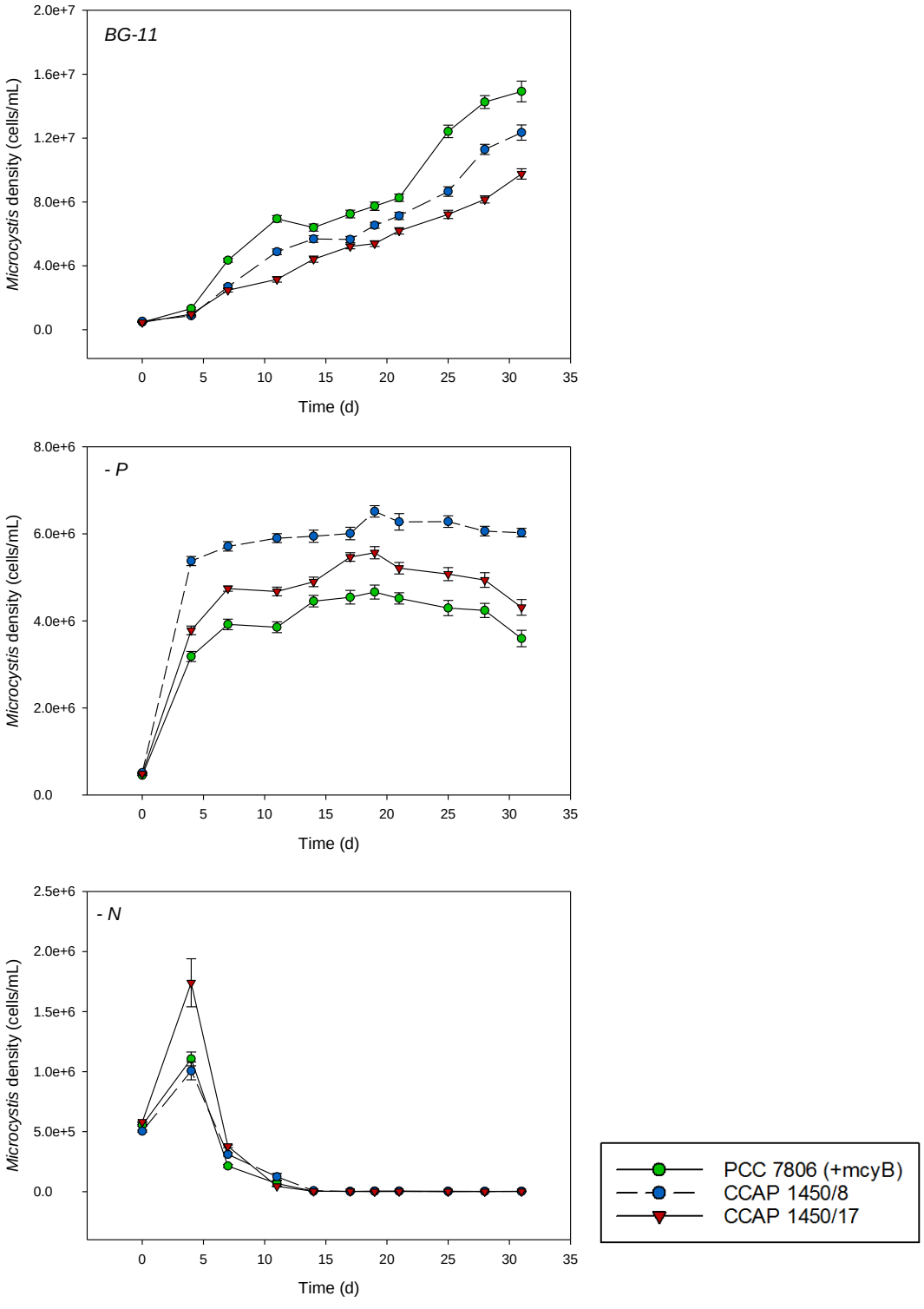
Appendix C1: Cell counts of three *Microcystis* strains cultured in three different media types over 31 days. Populations in BG-11 recorded continued growth, modified media without phosphates reached a stationary phase between 4-7 days and cells grown without nitrates exhibited a population crash after 4 days. Only three of the six *Microcystis* strains are represented here, standard error (S.E.) values underlined

	PCC 7806 (+mcyB)					
Day	BG-11		BG-11-N		BG-11-P	
0	4.7E+05	8.6E+03	5.5E+05	7.0E+03	4.5E+05	7.5E+03
4	1.3E+06	7.7E+04	1.1E+06	5.8E+04	3.2E+06	1.2E+05
7	4.3E+06	1.2E+05	2.1E+05	1.3E+04	3.9E+06	1.2E+05
11	6.9E+06	2.0E+05	6.8E+04	2.4E+04	3.9E+06	1.2E+05
14	6.0E+06	2.2E+05	3.9E+03	1.5E+03	4.5E+06	1.3E+05
17	7.2E+06	2.4E+05	1.9E+03	9.5E+02	4.5E+06	1.6E+05
19	7.7E+06	2.6E+05	4.8E+03	1.3E+03	4.7E+06	1.6E+05
21	8.3E+06	2.3E+05	4.4E+03	1.4E+03	4.5E+06	1.3E+05
25	1.2E+07	3.9E+05	4.3E+03	1.2E+03	4.3E+06	1.8E+05
28	1.4E+07	4.0E+05	2.4E+03	8.3E+02	4.2E+06	1.6E+05
31	1.0E+07	6.5E+05	2.9E+03	7.3E+02	3.6E+06	1.9E+05

	CCAP 1450/8					
Day	BG-11		BG-11-N		BG-11-P	
0	5.2E+05	9.5E+03	5.0E+05	8.5E+03	5.1E+05	9.0E+03
4	8.7E+05	7.1E+04	1.0E+06	7.5E+04	5.4E+06	1.1E+05
7	2.7E+06	1.2E+05	3.1E+05	1.5E+04	5.7E+06	1.1E+05
11	4.9E+06	1.8E+05	1.2E+05	2.9E+04	5.9E+06	1.0E+05
14	5.7E+06	2.1E+05	7.7E+03	1.1E+03	5.9E+06	1.4E+05
17	5.6E+06	2.0E+05	1.8E+03	8.8E+02	6.0E+06	1.4E+05
19	6.5E+06	1.8E+05	2.3E+03	1.4E+03	6.5E+06	1.3E+05
21	7.1E+06	2.2E+05	3.5E+03	1.1E+03	6.3E+06	1.9E+05
25	8.7E+06	3.0E+05	8.4E+02	9.6E+02	6.3E+06	1.3E+05
28	1.1E+07	3.2E+05	2.5E+03	6.5E+02	6.1E+06	1.1E+05
31	1.2E+07	4.8E+05	2.6E+03	3.2E+02	6.0E+06	9.9E+04

	CCAP 1450/17					
Day	BG-11		BG-11-N		BG-11-P	
0	4.6E+05	4.6E+03	5.8E+05	4.2E+03	4.9E+05	4.1E+03
4	9.7E+05	1.0E+05	1.7E+06	2.1E+05	3.8E+06	9.9E+04
7	2.5E+06	1.1E+05	3.8E+05	1.0E+04	4.7E+06	6.0E+04
11	3.1E+06	1.7E+05	4.4E+04	2.4E+03	4.7E+06	9.9E+04
14	4.4E+06	1.9E+05	2.8E+03	9.5E+02	4.9E+06	1.1E+05
17	5.2E+06	1.5E+05	2.1E+03	7.7E+02	5.5E+06	9.8E+04
19	5.4E+06	1.8E+05	8.2E+02	5.6E+02	5.6E+06	1.4E+05
21	6.2E+06	2.0E+05	1.8E+03	5.1E+02	5.2E+06	1.3E+05
25	7.2E+06	2.6E+05	8.5E+02	2.3E+02	5.1E+06	1.5E+05
28	8.2E+06	2.3E+05	8.5E+02	5.5E+02	4.9E+06	1.7E+05
31	9.8E+06	3.2E+05	1.4E+03	6.5E+02	4.3E+06	1.8E+05

Appendix C2: Graphical representation of three *Microcystis* strains cultured over 31 days in various media types. *Microcystis* in BG-11 continued to grow, cells in BG-11 minus P reached a stationary phase between days 4-7 and populations in BG-11 minus N crashed after 4 days. Only three of the six *Microcystis* strains are shown here for clarity (PCC 7806 (+mcyB), CCAP 1450/8 and 1450/17)



Appendix C3: Mean cell sizes (diameter) of cultured *Microcystis* strains in batch cultures. All densities were grown from relatively low lag phase numbers (≈5x10^5 cells/mL) over 31 days in different media types

	PCC 7806 (+mcyB)	PCC 7806 (-mcyB)	CCAP 1450 / 8	CCAP 1450 / 13	CCAP 1450 / 17	Longham
BG-11						
Highest (µm)	4.61	5.16	4.59	4.68	5.29	6.80
Lowest (µm)	4.10	3.59	3.05	3.60	3.89	4.60
Mean (µm)	4.29 ± 0.06	4.02 ± 0.15	3.34 ± 0.14	3.96 ± 0.13	4.45 ± 0.15	5.74 ± 0.24
BG-11 (-P)						
Highest (µm)	5.57	5.35	4.73	5.66	5.51	-
Lowest (µm)	5.15	5.21	4.08	4.97	4.92	-
Mean (µm)	5.45 ± 0.04	5.28 ± 0.02	4.29 ± 0.08	5.40 ± 0.08	5.33 ± 0.05	-
BG-11 (-N)						
Highest (µm)	5.62	5.47	4.46	5.49	5.58	-
Lowest (µm)	5.13	5.14	3.51	4.24	4.72	-
Mean (µm)	5.30 ± 0.06	5.24 ± 0.03	3.93 ± 0.13	4.94 ± 0.13	5.11 ± 0.10	-

Appendix C4: Side light scatter (SSC-H) mean peak wavelengths (AU) from *Microcystis* calibrations in different media types. The mean SSC-H records were taken approximately every 3 days over a 31 day period. Only *Microcystis* sp. Longham was subjected to ultrasound for breaking colonial formation in this table

	PCC 7806 (+mycB)	PCC 7806 (-mycB)	CCAP 1450 / 8	CCAP 1450 / 13	CCAP 1450 / 17	Longham
BG-11						
Highest (AU)	1.49E+5	2.77E+5	3.60E+5	3.49E+5	1.60E+5	6.82E+4
Lowest (AU)	7.73E+4	1.71E+5	1.97E+5	1.74E+5	8.24E+4	5.98E+4
Mean (AU)	1.06E+5 ± 7.2E+3	2.04E+5 ± 10.4E+4	2.28E+5 ± 15.0E+4	2.53E+5 ± 2.0E+4	1.24E+5 ± 9.5E+3	6.46E+4 ± 9.5E+2
BG-11 (-P)						
Highest (AU)	1.53E+5	2.69E+5	3.54E+5	4.44E+5	1.72E+5	-
Lowest (AU)	1.18E+5	9.35E+4	2.21E+5	2.19E+5	6.83E+4	-
Mean (AU)	1.30E+5 ± 4.4E+3	1.61E+5 ± 1.77E+4	2.62E+5 ± 1.42E+4	2.68E+5 ± 2.12E+4	1.38E+5 ± 9.34E+3	-
BG-11 (-N)						
Highest (AU)	1.66E+5	3.17E+5	3.61E+5	5.37E+5	2.01E+5	-
Lowest (AU)	1.18E+5	8.93E+4	2.37E+5	2.69E+5	6.52E+4	-
Mean (AU)	1.30E+5 ± 5.4E+3	1.36E+5 ± 2.2E+4	2.92E+5 ± 1.5E+4	4.18E+5 ± 2.5E+4	1.33E+5 ± 1.3E+4	-

Appendix C5: FL2-H mean peak wavelengths (AU) from *Microcystis* calibrations over 31 days in three media types, with the highest, lowest and overall mean FL2-H wavelengths recorded

	PCC 7806 (+mcyB)	PCC 7806 (-mcyB)	CCAP 1450 / 8	CCAP 1450 / 13	CCAP 1450 / 17	Longham
BG-11						
Highest (AU)	2.80E+2	4.51E+2	5.78E+2	5.67E+2	3.72E+2	4.70E+2
Lowest (AU)	2.01E+2	2.37E+2	2.66E+2	2.48E+2	2.13E+2	3.59E+2
Mean (AU)	2.34E+2	3.14E+2	4.20E+2	3.91E+2	2.68E+2	4.22E+2
BG-11 (-P)						
Highest (AU)	3.88E+2	4.38E+2	4.65E+2	4.29E+2	4.11E+2	-
Lowest (AU)	2.22E+2	2.57E+2	2.12E+2	2.62E+2	2.45E+2	-
Mean (AU)	2.68E+2	3.22E+2	2.66E+2	3.48E+2	3.10E+2	-
BG-11 (-N)						
Highest (AU)	4.52E+2	6.27E+2	5.07E+2	4.66E+2	4.98E+2	-
Lowest (AU)	2.92E+2	3.59E+2	2.23E+2	2.61E+2	3.14E+2	-
Mean (AU)	3.27E+2	4.75E+2	3.07E+2	3.37E+2	3.86E+2	-

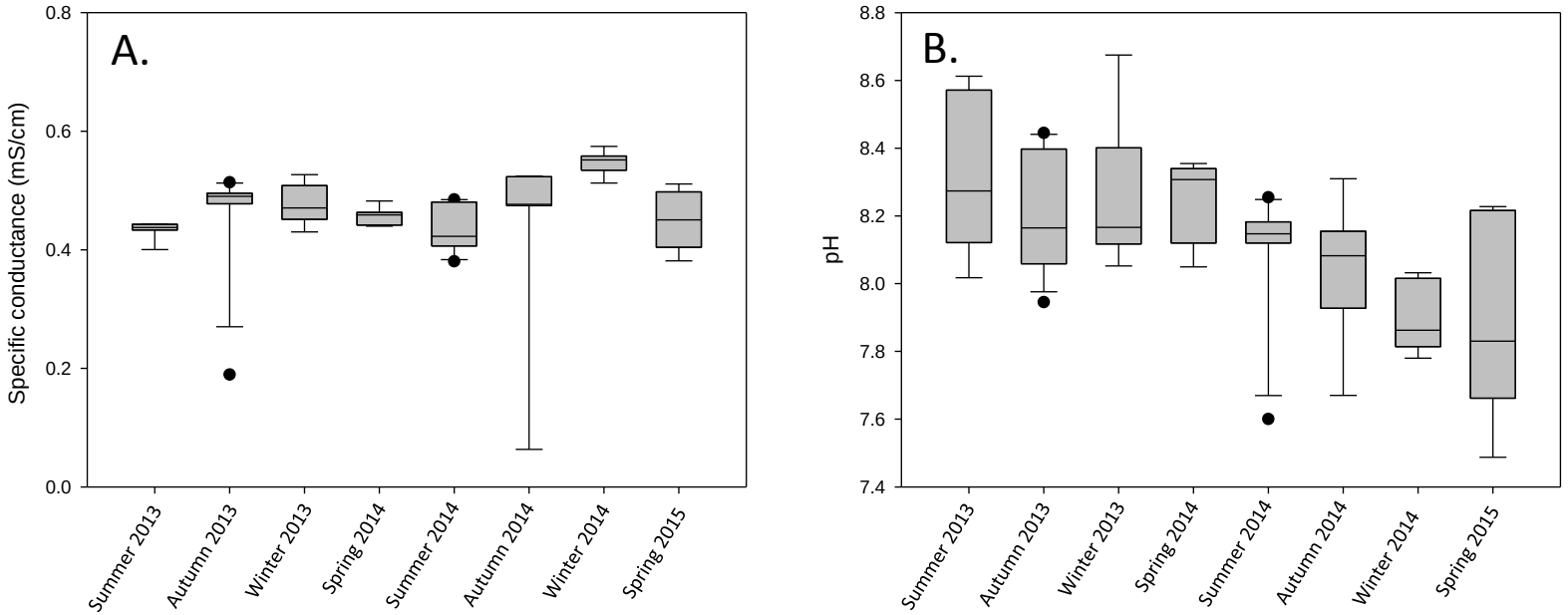
Appendix C6: Mean peak wavelengths (AU) from the FL3-H detection channel through the six *Microcystis* calibrations. The highest, lowest and mean FL3-H wavelengths are represented from each growth media through a 31 day batch culture cycle

	PCC 7806 (+mcyB)	PCC 7806 (-mcyB)	CCAP 1450 / 8	CCAP 1450 / 13	CCAP 1450 / 17	Longham
BG-11						
Highest (AU)	7.46E+4	1.16E+5	9.04E+4	1.12E+5	9.76E+4	1.79E+5
Lowest (AU)	5.45E+4	6.53E+4	5.19E+4	5.17E+4	5.90E+4	8.93E+4
Mean (AU)	6.26E+4	7.84E+4	6.73E+4	7.37E+4	6.80E+4	1.36E+5
BG-11 (-P)						
Highest (AU)	8.19E+4	9.02E+4	7.50E+4	8.36E+4	7.90E+4	-
Lowest (AU)	5.24E+4	6.53E+4	2.42E+4	5.20E+4	5.93E+4	-
Mean (AU)	6.26E+4	7.53E+4	3.86E+4	7.43E+4	6.83E+4	-
BG-11 (-N)						
Highest (AU)	9.45E+4	1.22E+5	8.71E+4	8.53E+4	9.87E+4	-
Lowest (AU)	5.85E+4	5.13E+4	2.40E+4	3.98E+4	5.74E+4	-
Mean (AU)	6.90E+4	8.28E+4	4.65E+4	5.25E+4	7.21E+4	-

Appendix C7: FL4-H mean peak wavelengths (AU) from the six *Microcystis* calibrations in different growth media over a 31 day batch cycle incubation period, showing the highest, lowest and mean wavelengths

	PCC 7806 (+mcyB)	PCC 7806 (-mcyB)	CCAP 1450 / 8	CCAP 1450 / 13	CCAP 1450 / 17	Longham
BG-11						
Highest (AU)	3.28E+5	5.52E+5	3.53E+5	5.06E+5	4.36E+5	1.45E+6
Lowest (AU)	1.77E+5	1.62E+5	1.06E+5	1.35E+5	1.61E+5	5.93E+5
Mean (AU)	2.43E+5	2.98E+5	2.06E+5	2.80E+5	2.85E+5	1.02E+6
BG-11 (-P)						
Highest (AU)	3.87E+5	5.10E+5	3.16E+5	6.29E+5	4.86E+5	-
Lowest (AU)	2.36E+5	2.81E+5	1.18E+5	1.67E+5	2.59E+5	-
Mean (AU)	3.17E+5	3.85E+5	1.61E+5	4.18E+5	3.52E+5	-
BG-11 (-N)						
Highest (AU)	4.19E+5	5.64E+5	3.26E+5	3.63E+5	4.51E+5	-
Lowest (AU)	3.06E+5	3.46E+5	1.07E+5	1.64E+5	3.15E+5	-
Mean (AU)	3.50E+5	4.44E+5	2.09E+5	2.40E+5	3.82E+5	-

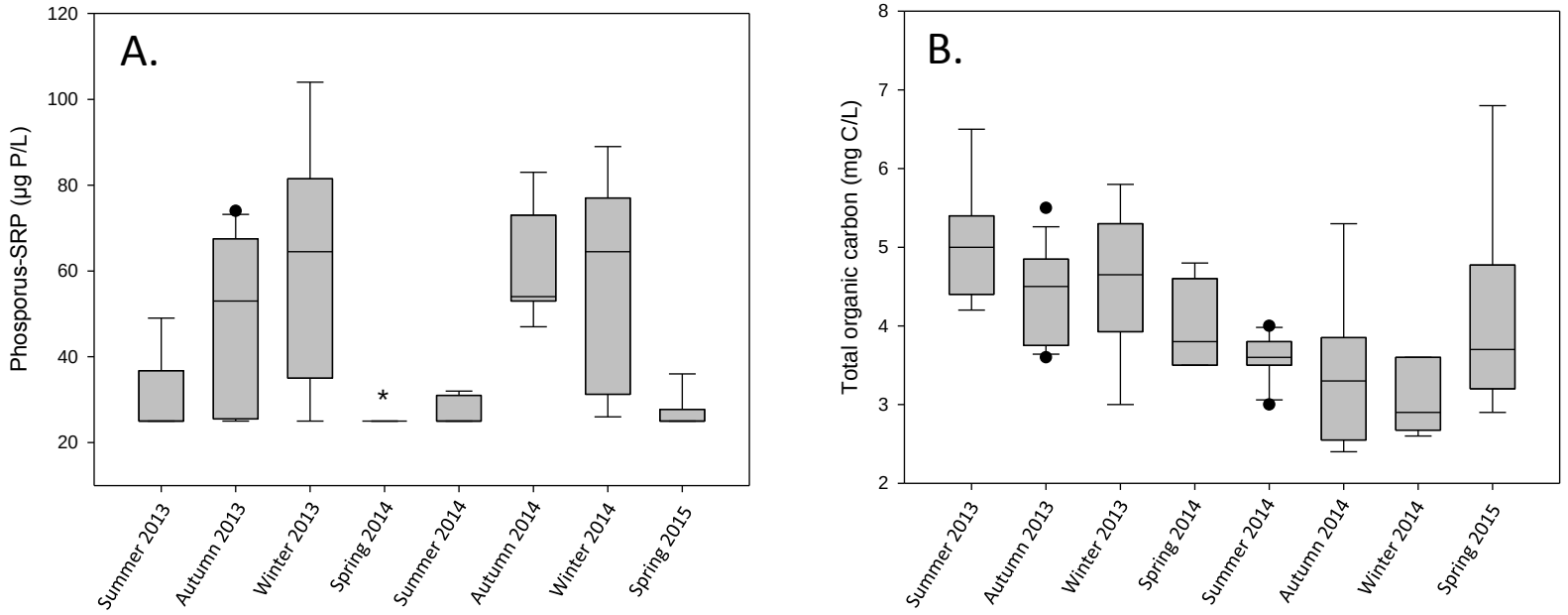
Appendix C8: Water chemistry data record over the monitoring period at Longham reserve for A) specific conductance B) pH C) turbidity and D) optical dissolved oxygen



C.
Turbidity (NTU)
210
10
5
0
Summer 2013
Autumn 2013
Winter 2013
Spring 2014
Summer 2014
Autumn 2014
Winter 2014
Spring 2015

D.
ODO (%)
220
200
180
160
140
120
100
80
60
Summer 2013
Autumn 2013
Winter 2013
Spring 2014
Summer 2014
Autumn 2014
Winter 2014
Spring 2015

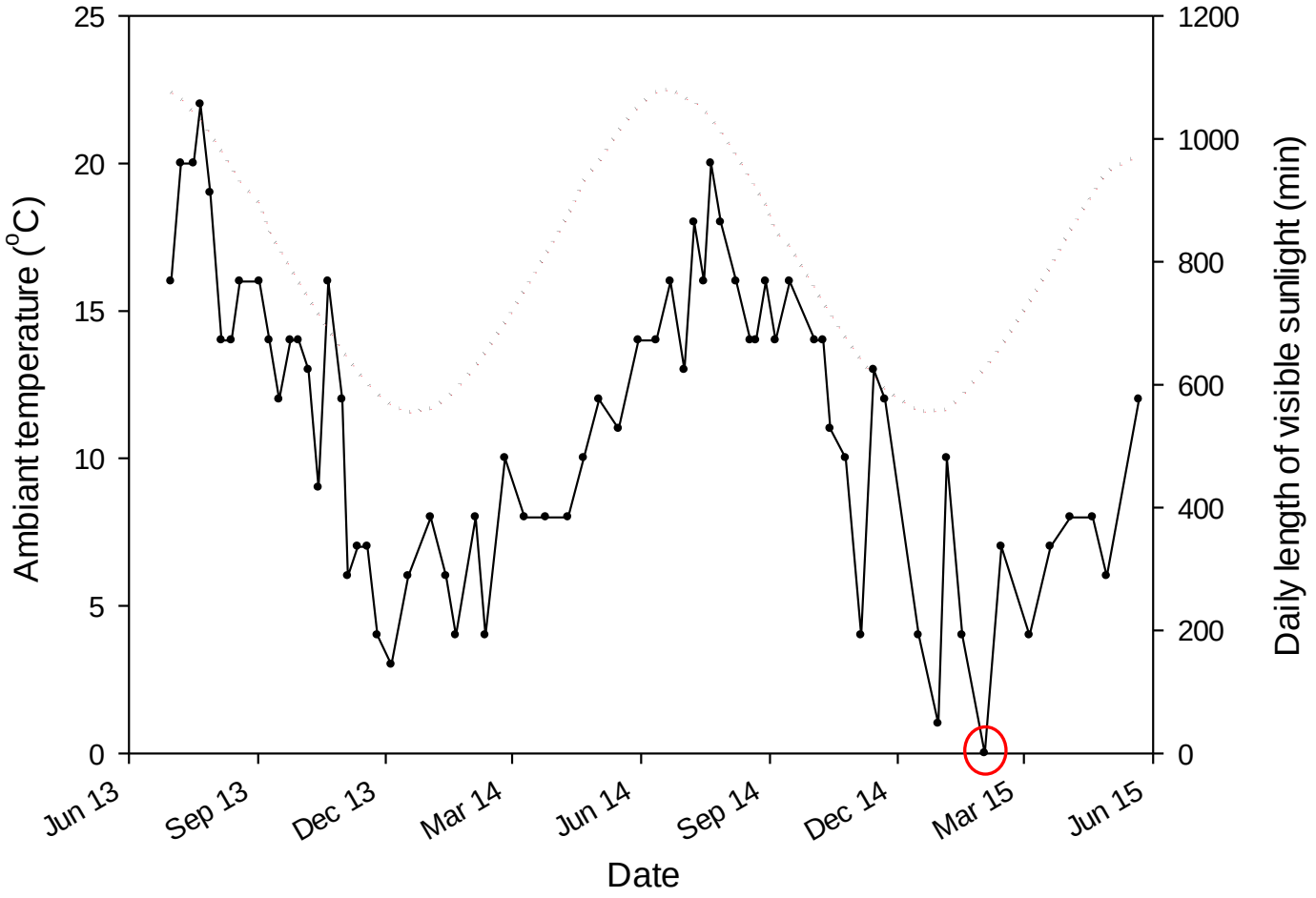
Appendix C9: Hydrological and water chemistry parameters recorded over 22 months at Longham reservoir for A) phosphorus B) organic carbon C) suspended solids and D) rain fall (*phosphorus had a constant 25 µg P/L in spring 2014 and no † precipitation was recorded in summer 2013)



C.
Suspended solids (mg/L)
0
5
10
15
20
25
Summer 2013
Autumn 2013
Winter 2013
Spring 2014
Summer 2014
Autumn 2014
Winter 2014
Spring 2015

D.
Precipitation (mm)
0
2
4
6
8
10
12
14
†
Summer 2013
Autumn 2013
Winter 2013
Spring 2014
Summer 2014
Autumn 2014
Winter 2014
Spring 2015

Appendix C10: Ambient air temperature (solid line) and the daily length of visible sunlight (dotted line) measured from Hurn airport (Dorset, UK) on days environmental monitoring was carried out. Red circle represents a 0^{O}C recording from February 2014, which saw a partial frozen reservoir



Appendix C11: i) Seasonal means for ambient environmental parameters at Longham reservoirs with standard error (+S.E.). ii) Ambient mean parameters between single seasons (2013 – 2015) compared through a student's T-test (* Mann-Whitney *U* test for non-parametric data)

i)

Date (Season/yyyy)	Mean daily temperature (^{O}C)	Precipitation (mm)	Length of visible sunlight (min)
Summer 2013	17.63 ± 1.07	0.00 ± 0.00	1,010 ± 19
Autumn 2013	11.08 ± 1.11	0.75 ± 0.35	721 ± 28
Winter 2013	6.13 ± 0.85	1.18 ± 0.52	606 ± 19
Spring 2014	10.14 ± 0.88	0.29 ± 0.29	917 ± 41
Summer 2014	16.11 ± 0.68	0.58 ± 0.50	1,032 ± 15
Autumn 2014	12.00 ± 1.17	2.07 ± 1.32	711 ± 30
Winter 2014	4.33 ± 1.52	2.00 ± 0.99	592 ± 18
Spring 2015	7.50 ± 1.09	0.20 ± 0.16	870 ± 38

ii)

Season	Ambient Temperature (^{O}C)		Precipitation (mm)		Length of daily visible sunlight (min)	
	t (d.f.)	*p*	*t* (d.f.)	*p*	*t* (d.f.)	*p*
Summer	1.46 (17)	> 0.05	32*	> 0.05	0.04 (17)	> 0.05
Autumn	-0.56 (20)	> 0.05	58*	> 0.05	0.23 (20)	> 0.05
Winter	1.10 (12)	> 0.05	20.5*	> 0.05	19*	> 0.05
Spring	1.91 (11)	> 0.05	18*	> 0.05	0.83 (11)	> 0.05

Appendix C12: Mann-Whitney *U* test for *Microcystis*-like cell densities and morphological parameters between single seasons over the monitoring period (* students T-test test for parametric data with degrees of freedom in brackets)

Season	Cell density (cells/mL)		Cell size (µm)		Cell biovolume (µm^3)		Total biovolume (µm^3/mL)	
	U	*p*	*U*	*p*	*U*	*p*	*U*	*p*
Summer	42	> 0.05	-2.78 (17)*	< 0.05	13	< 0.05	30	> 0.05
Autumn	34.5	> 0.05	28	> 0.05	28	> 0.05	28	> 0.05
Winter	0	< 0.001	2.23 (10)*	< 0.05	2.26 (10)*	< 0.05	0	< 0.001
Spring	13	> 0.05	16	> 0.05	16	> 0.05	14	> 0.05

Appendix C13: Statistical data from environmental parameters and *Microcystis*-like cell counts at Longham reservoir in autumn 2013 i) within the middle sampling points water column (Kruskal-Wallis one-way ANOVA on ranks) and ii) parameters between the middle and the original sampling point (Mann-Whitney *U* test)

i)

Parameter	X^2	*d.f.*	*p*
Temperature (^{O}C)	0.09	3	> 0.05
SpCond (mS/cm)	1.31	3	> 0.05
pH	0.04	3	> 0.05
Turbidity (NTU)	0.02	3	> 0.05
ODO (%)	0.03	3	> 0.05
Cell Count (cells/mL)	0.83	3	> 0.05

ii)

Parameter	*U*	*p*
Temperature (^{O}C)	417.0	> 0.05
SpCond (mS/cm)	419.5	> 0.05
pH	481.5	> 0.05
Turbidity (NTU)	408.0	> 0.05
ODO (%)	399.5	> 0.05
Cell Count (cells/mL)	424.5	> 0.05

This page intentionally left blank

Appendix D

Appendix D1: Cell counts, sizes (diameter) and total biovolumes of toxic *Microcystis aeruginosa* incubated with and without *Blepharisma americanum* at initial densities of ≈1.5x10^6 cells/mL (high), standard error (S.E.) included

Control

Day	Cell count		Cell size		Total biovolume	
	(cell/mL)	S.E.	(µm)	S.E.	(µm^3)	S.E.
0	1.5E+6	4.3E+4	5.25	0.02	1.2E+8	2.7E+6
2	2.1E+6	2.1E+4	4.97	0.24	1.4E+8	4.1E+6
4	2.7E+6	9.1E+4	5.00	0.10	1.7E+8	7.7E+6
6	4.0E+6	4.0E+4	4.99	0.02	2.6E+8	1.9E+6
9	5.2E+6	1.2E+5	4.99	0.07	3.4E+8	8.7E+6
11	6.8E+6	1.2E+5	4.81	0.73	4.0E+8	4.9E+7
13	7.5E+6	4.4E+4	4.90	0.19	4.6E+8	1.5E+7

Test

Day	Cell count		Cell size		Total biovolume	
	(cell/mL)	S.E.	(µm)	S.E.	(µm^3)	S.E.
0	1.5E+6	4.3E+4	5.25	0.02	1.2E+8	2.7E+6
2	2.1E+6	5.2E+4	5.29	0.01	1.6E+8	4.0E+6
4	2.6E+6	2.0E+4	4.99	0.01	1.7E+8	1.1E+6
6	3.9E+6	4.2E+4	5.02	0.01	2.6E+8	4.2E+6
9	5.1E+6	9.8E+4	4.85	0.01	3.1E+8	7.7E+6
11	5.8E+6	2.8E+5	4.60	0.02	3.0E+8	1.2E+7
13	6.9E+6	3.7E+5	4.84	0.02	4.1E+8	1.3E+7

Appendix D2: Cell counts, sizes (diameter) and total biovolumes of toxic *Microcystis aeruginosa* incubated with and without *Blepharisma americanum* at initial densities of ≈2.8x10^5 cells/mL (low), standard error (S.E.) included

Control

Day	Cell count		Cell size		Total biovolume	
	(cell/mL)	S.E.	(µm)	S.E.	(µm^3)	S.E.
0	2.8E+5	1.5E+4	5.64	0.02	2.6E+7	1.8E+6
2	3.8E+5	7.5E+3	5.34	0.13	3.1E+7	8.0E+5
4	6.4E+5	1.4E+4	5.16	0.08	4.6E+7	5.4E+5
6	9.3E+5	8.5E+3	5.09	0.03	6.4E+7	4.6E+5
9	1.4E+6	5.4E+4	5.10	0.38	9.8E+7	5.2E+6
11	1.9E+6	2.4E+4	4.85	0.19	1.1E+8	3.7E+6
13	2.4E+6	2.8E+4	4.84	0.04	1.4E+8	2.5E+6

Test

Day	Cell count		Cell size		Total biovolume	
	(cell/mL)	S.E.	(µm)	S.E.	(µm^3)	S.E.
0	2.7E+5	5.9E+3	5.64	0.02	2.5E+7	2.3E+5
2	4.0E+5	1.4E+4	5.30	0.01	3.1E+7	1.1E+6
4	7.5E+5	2.4E+4	5.69	0.00	7.3E+7	1.5E+6
6	1.1E+6	2.3E+4	5.44	0.01	9.0E+7	2.8E+6
9	1.4E+6	3.7E+3	4.89	0.01	8.5E+7	1.1E+6
11	1.6E+6	1.3E+5	4.53	0.04	7.8E+7	5.7E+6
13	2.6E+6	2.3E+4	4.76	0.04	1.5E+8	3.2E+6

Appendix D3: Cell counts, sizes (diameter) and total biovolumes of non-toxic *Microcystis aeruginosa* incubated with and without *Blepharisma americanum* at initial densities of ≈1.5x10^6 cells/mL (high), standard error (S.E.) included

Control

Day	Cell count		Cell size		Total biovolume	
	(cell/mL)	S.E.	(µm)	S.E.	(µm^3)	S.E.
0	1.4E+6	2.9E+4	4.25	0.02	5.8E+7	5.3E+5
2	1.6E+6	1.4E+4	4.30	0.01	6.5E+7	3.6E+6
4	2.2E+6	3.2E+4	3.70	0.01	5.9E+7	9.5E+5
6	2.9E+6	1.2E+5	3.63	0.01	7.2E+7	2.9E+6
9	4.3E+6	1.1E+5	3.50	0.02	9.6E+7	4.9E+5
11	4.9E+6	3.3E+4	3.56	0.02	1.2E+8	1.3E+6
13	6.2E+6	2.1E+5	4.01	0.02	2.1E+8	7.0E+6
16	8.1E+6	3.0E+5	3.76	0.01	2.3E+8	1.6E+7
18	1.0E+7	2.7E+5	3.05	0.01	1.5E+8	3.2E+6
21	1.1E+7	4.0E+5	3.24	0.00	2.0E+8	3.0E+6
25	1.6E+7	7.4E+5	3.70	0.00	4.4E+8	1.9E+7
29	1.9E+7	4.3E+5	3.62	0.01	4.7E+8	2.1E+7
32	1.9E+7	7.5E+5	3.48	0.01	4.3E+8	2.7E+7

Test

Day	Cell count		Cell size		Total biovolume	
	(cell/mL)	S.E.	(µm)	S.E.	(µm^3)	S.E.
0	1.5E+6	1.9E+4	4.25	0.02	6.2E+7	6.1E+5
2	1.8E+6	9.6E+4	4.47	0.03	8.5E+7	4.5E+6
4	2.0E+6	9.1E+4	4.50	0.02	9.4E+7	5.7E+6
6	2.3E+6	9.9E+4	4.49	0.01	1.1E+8	6.9E+6
9	2.7E+6	7.3E+4	4.21	0.01	1.1E+8	7.8E+6
11	3.4E+6	2.9E+5	3.91	0.04	1.1E+8	1.2E+7
13	3.8E+6	1.9E+5	3.55	0.05	8.8E+7	2.3E+6
16	4.1E+6	1.2E+5	3.33	0.01	8.0E+7	1.7E+6
18	5.8E+6	1.7E+5	3.32	0.01	1.1E+8	5.6E+6
21	5.4E+6	8.6E+4	3.25	0.02	9.7E+7	9.3E+6
25	5.4E+6	3.7E+5	3.33	0.02	1.1E+8	7.1E+6
29	5.0E+6	5.7E+5	3.55	0.01	1.2E+8	1.2E+7
32	5.2E+6	5.7E+5	3.43	0.07	1.1E+8	1.6E+7

Appendix D4: Cell counts, sizes (diameter) and total biovolumes of non-toxic *Microcystis aeruginosa* incubated with and without *Blepharisma americanum* at initial densities of ≈2.8x10^5 cells/mL (low), including the standard error (S.E.)

Control

Day	Cell count		Cell size		Total biovolume	
	(cell/mL)	S.E.	(µm)	S.E.	(µm^3)	S.E.
0	2.8E+5	3.7E+3	4.66	0.02	1.5E+7	2.9E+5
2	3.7E+5	4.7E+3	4.35	0.01	1.6E+7	4.1E+4
4	5.8E+5	4.0E+3	3.74	0.01	1.6E+7	1.4E+5
6	7.3E+5	6.5E+3	3.62	0.01	1.8E+7	4.6E+5
9	1.1E+6	6.8E+4	3.46	0.02	2.3E+7	8.7E+5
11	1.3E+6	9.1E+4	3.37	0.00	2.7E+7	4.0E+6
13	1.2E+6	2.6E+5	3.63	0.08	3.2E+7	1.4E+7
16	1.7E+6	1.6E+5	3.47	0.09	3.9E+7	1.2E+7
18	2.5E+6	2.4E+5	2.81	0.05	3.0E+7	6.4E+6
21	3.0E+6	2.7E+5	2.83	0.02	3.7E+7	7.9E+6
25	4.6E+6	5.2E+5	3.22	0.01	8.5E+7	2.3E+7
29	5.5E+6	3.9E+5	3.13	0.01	9.2E+7	1.9E+7
32	6.2E+6	5.0E+5	2.92	0.01	8.5E+7	1.9E+7

Test

Day	Cell count		Cell size		Total biovolume	
	(cell/mL)	S.E.	(µm)	S.E.	(µm^3)	S.E.
0	2.8E+5	3.7E+3	4.66	0.02	1.5E+7	2.9E+5
2	5.8E+5	2.0E+4	4.52	0.02	2.8E+7	9.7E+5
4	3.3E+5	5.0E+2	4.43	0.02	1.5E+7	1.5E+5
6	3.7E+5	3.3E+3	4.46	0.00	1.7E+7	1.8E+5
9	5.1E+5	2.9E+4	3.78	0.02	1.4E+7	8.6E+5
11	6.2E+5	2.8E+4	3.58	0.04	1.5E+7	8.5E+5
13	6.3E+5	5.2E+4	3.48	0.02	1.4E+7	1.8E+5
16	6.3E+5	9.3E+4	3.34	0.05	1.2E+7	1.8E+6
18	8.8E+5	7.8E+4	3.35	0.07	1.7E+7	3.2E+5
21	1.1E+6	8.4E+4	3.24	0.01	1.9E+7	5.1E+5
25	1.5E+6	8.6E+4	3.23	0.01	2.6E+7	5.8E+5
29	1.7E+6	1.2E+5	3.39	0.01	3.4E+7	2.8E+6
32	1.5E+6	6.5E+4	3.27	0.01	2.8E+7	7.0E+5

Appendix D5: *Blepharisma americanum* cell and cyst counts along with morphological dimensions in both control cultures, standard error (S.E.) included

Control

Day	Ciliate density		Ciliate length		Ciliate width		Ciliate biovolume		Cyst abundance	
	(cells/mL)	S.E.	(µm)	S.E.	(µm)	S.E.	(μm^3)	S.E.	(cysts/mL)	S.E.
0	100.0	0.0	118.3	5.6	39.9	2.3	110,984	19,784	5.6	1.2
2	13.3	0.7	124.5	8.8	40.4	2.2	113,840	15,488	4.7	2.9
4	18.7	1.3	108.6	8.6	37.7	2.2	83,136	9,796	17.3	0.7
6	2.0	0.0	132.1	10.6	30.2	1.3	63,849	9,518	6.7	2.9
9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Control + chloramphenicol

Day	Ciliate density		Ciliate length		Ciliate width		Ciliate biovolume		Cyst abundance	
	(cells/mL)	S.E.	(µm)	S.E.	(µm)	S.E.	(μm^3)	S.E.	(cysts/mL)	S.E.
0	100.0	0.0	122.3	9.7	42.8	2.8	114,156	18,654	5.6	1.2
2	18.7	4.4	111.6	8.1	41.5	1.7	105,847	13,696	0.7	0.7
4	8.7	2.4	118.0	8.2	36.4	1.9	86,687	11,561	16.0	2.3
6	10.7	3.3	130.9	6.9	27.1	2.7	51,131	10,733	8.7	4.7
9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Appendix D6: *Blepharisma americanum* cell and cyst counts along with morphological dimensions in both toxic *Microcystis aeruginosa* test cultures, standard error (S.E.) included

High initial density

Day	Ciliate density		Ciliate length		Ciliate width		Ciliate biovolume		Cyst abundance	
	(cells/mL)	S.E.	(µm)	S.E.	(µm)	S.E.	(μm^3)	S.E.	(cysts/mL)	S.E.
0	100.0	0.0	121.1	9.8	41.8	6.9	172,513	80,123	5.6	1.2
2	18.0	5.0	138.9	12.9	42.5	5.9	189,361	95,722	1.3	0.7
4	18.7	2.4	121.4	6.6	35.8	2.4	84,062	10,231	7.3	3.3
6	16.7	0.7	118.4	6.0	30.2	1.3	58,206	5,725	6.7	1.3
9	5.3	1.8	127.8	19.5	27.0	2.6	49,749	10,497	0.7	0.7
11	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.3	1.3

Low initial density

Day	Ciliate density		Ciliate length		Ciliate width		Ciliate biovolume		Cyst abundance	
	(cells/mL)	S.E.	(µm)	S.E.	(µm)	S.E.	(μm^3)	S.E.	(cysts/mL)	S.E.
0	100.0	0.0	134.2	15.2	41.2	6.6	173,466	55,647	5.6	1.2
2	26.7	1.8	147.7	14.6	42.8	5.3	188,795	69,732	1.3	1.3
4	15.3	0.7	125.4	10.2	34.4	2.3	82,741	13,691	7.3	0.7
6	4.0	0.0	110.1	4.4	27.6	3.1	45,728	10,521	7.3	4.4
9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.7	0.7

Appendix D7: *Blepharisma americanum* cell and cyst counts along with morphological dimensions in both non-toxic *Microcystis aeruginosa* test cultures, standard error (S.E.) included

High initial density

Day	Ciliate density (cells/mL)	S.E.	Ciliate length (µm)	S.E.	Ciliate width (µm)	S.E.	Ciliate biovolume ($µm^3$)	S.E.	Cyst abundance (cysts/mL)	S.E.
0	100.0	0.0	128.4	9.4	41.8	3.8	118,323	11,326	5.7	1.2
2	22.0	2.0	131.3	11.6	37.1	1.8	100,455	15,993	0.0	0.0
4	30.7	1.8	127.1	9.6	38.9	4.6	129,028	48,118	2.0	2.0
6	31.3	1.8	117.6	4.1	42.4	1.4	113,695	10,664	4.0	1.2
9	17.3	3.7	113.1	5.1	41.0	1.8	104,429	12,425	0.7	0.7
11	14.0	3.1	131.2	4.8	30.1	1.7	65,208	8,496	2.7	0.7
13	14.0	2.3	130.1	5.0	29.8	1.2	62,052	6,349	8.7	1.8
16	13.3	1.3	125.5	5.4	28.8	1.1	55,629	5,209	8.7	0.7
18	15.3	1.8	118.3	6.8	29.3	1.2	55,305	6,384	0.0	0.0
21	13.3	2.4	114.3	5.7	28.6	1.3	50,946	6,944	2.7	2.7
25	22.0	1.2	115.5	8.7	27.7	2.5	55,070	14,739	0.0	0.0
29	22.7	2.4	117.2	10.0	26.5	1.8	43,971	5,932	0.0	0.0
32	33.3	2.4	121.9	11.4	27.6	2.0	50,565	6,951	0.0	0.0

Low initial density

Day	Ciliate density (cells/mL)	S.E.	Ciliate length (µm)	S.E.	Ciliate width (µm)	S.E.	Ciliate biovolume ($µm^3$)	S.E.	Cyst abundance (cysts/mL)	S.E.
0	100.0	0.0	132.7	12.2	42.1	3.2	124,561	20,165	5.7	1.2
2	21.3	2.9	137.0	9.7	36.1	1.5	97,662	12,011	0.7	0.7
4	28.7	3.7	129.4	11.3	40.1	4.3	135,450	38,407	1.3	1.3
6	10.7	4.1	119.1	5.9	41.3	1.9	111,944	15,296	6.7	2.4
9	16.0	3.1	113.8	6.8	39.2	2.3	97,622	14,827	2.7	1.3
11	14.7	2.4	129.0	7.2	29.8	2.4	67,724	13,854	4.7	1.3
13	13.3	2.4	118.5	6.9	27.6	1.8	51,801	9,390	2.7	0.7
16	12.7	1.8	122.9	6.7	27.0	1.1	48,446	5,776	0.7	0.7
18	18.7	0.7	113.4	8.8	27.1	1.0	45,900	6,052	0.7	0.7
21	24.0	4.2	113.0	6.9	27.4	0.7	45,418	4,302	0.7	0.7
25	26.0	1.2	115.6	8.6	27.1	1.5	47,815	7,162	1.0	0.0
29	42.0	7.2	107.2	8.3	25.0	1.3	35,171	3,612	1.0	0.0
32	95.0	15.9	127.3	9.6	29.2	0.7	57,780	5,738	1.0	0.0

Appendix D8: Total intracellular MC-LR (fg/cell) results from all toxic *M. aeruginosa* control and test cultures at both initial cyanobacterium densities.

High initial *M. aeruginosa* density

Day	Control		Test	
	Mean (fg/cell)	S.E.	Mean (fg/cell)	S.E.
0	2.64	0.71	1.90	0.07
2	2.42	0.06	2.12	0.03
6	2.86	0.07	1.77	0.15
11	2.60	0.07	2.06	0.04
16	1.72	0.36	1.31	0.20
25	2.51	0.08	1.69	0.10
29	3.60	0.30	3.04	0.20

Low initial *M. aeruginosa* density

Day	Control		Test	
	Mean (fg/cell)	S.E.	Mean (fg/cell)	S.E.
0	3.63	0.24	1.16	0.18
2	2.24	0.11	1.21	0.03
6	2.25	0.11	1.38	0.07
11	2.38	0.24	1.01	0.11
16	2.09	0.06	1.26	0.10
25	2.46	0.11	2.52	0.33
29	3.47	0.07	1.52	0.27

Appendix E

Appendix E1: Cell counts and % SYTOX® / CM-H_2DCFDA labelled cells of *Microcystis* exposed to H_2O_2 under light conditions with standard error (S.E.)

Cell count (cells/mL)	Time	Concentration (mg/L)									
	(h)	0	S.E.	1	S.E.	4	S.E.	10	S.E.	40	S.E.
	0	2,071,385	97,060	2,075,585	97,060	2,161,325	97,060	2,062,400	97,060	2,039,885	97,060
	2	2,363,590	34,545	2,468,538	49,576	2,331,179	11,080	2,334,872	10,804	2,219,974	19,311
	8	2,382,077	20,084	2,570,949	128,193	1,898,564	238,176	1,900,436	238,962	1,946,615	84,698
	24	3,330,128	47,846	3,315,205	22,959	814,436	89,586	811,872	90,392	135,179	15,712
	48	4,160,821	110,036	4,308,000	75,639	656,641	112,340	652,487	112,814	34,513	8,610
	72	5,758,103	259,187	5,635,974	718,870	518,846	50,194	511,923	49,386	19,564	6,619

CM-H_2DCFDA-labelled cells (% population)	Time	Concentration (mg/L)									
	(h)	0	S.E.	1	S.E.	4	S.E.	10	S.E.	40	S.E.
	0	0.24	0.05	0.23	0.04	0.24	0.07	0.26	0.06	0.23	0.05
	2	0.46	0.11	0.18	0.02	0.12	0.01	0.67	0.08	32.13	4.46
	8	0.48	0.07	0.38	0.05	2.63	0.19	13.65	1.02	15.04	0.83
	24	0.14	0.01	0.11	0.03	0.95	0.09	8.56	1.00	1.40	0.19
	48	0.05	0.01	0.09	0.01	1.57	0.35	10.11	1.39	0.48	0.11
	72	0.20	0.03	0.17	0.03	6.26	0.63	22.67	1.23	0.35	0.28

SYTOX®-labelled cells (% population)	Time	Concentration (mg/L)									
	(h)	0	S.E.	1	S.E.	4	S.E.	10	S.E.	40	S.E.
	0	0.14	0.03	0.13	0.05	0.15	0.04	0.14	0.04	0.16	0.03
	2	0.25	0.03	0.30	0.08	0.26	0.03	0.62	0.05	36.80	6.28
	8	0.18	0.01	0.24	0.04	0.51	0.09	9.98	2.47	89.48	1.40
	24	1.73	0.75	0.34	0.06	1.70	0.27	4.64	0.29	41.03	6.04
	48	0.33	0.06	0.45	0.11	1.46	0.06	7.50	1.48	20.50	4.42
	72	0.19	0.01	0.24	0.05	0.96	0.10	24.56	1.43	14.02	3.02

Appendix E2: Cell counts and % SYTOX® / CM-H_2DCFDA labelled cells of *Microcystis* exposed to H_2O_2 under dark conditions with standard error (S.E.)

Cell count (cells/mL)	Time	Concentration (mg/L)									
	(h)	0	S.E.	10	S.E.	40	S.E.	100	S.E.	400	S.E.
	0	2,132,103	101,061	2,222,103	101,061	2,090,103	101,061	2,107,703	101,061	2,200,013	101,061
	2	2,136,974	69,837	2,322,231	47,581	1,882,564	36,835	2,277,430	37,151	2,303,487	50,380
	8	2,018,844	53,933	2,260,692	40,499	1,135,462	66,823	2,056,769	143,103	2,138,128	13,168
	24	2,428,821	126,180	2,422,256	25,413	571,974	29,786	349,590	21,088	555,436	90,625
	48	2,144,564	15,852	2,137,590	32,219	466,795	18,421	117,692	13,577	113,538	13,190
	72	2,066,128	122,886	2,131,641	16,046	422,103	42,096	75,000	6,290	47,897	2,099

CM-H_2DCFDA-labelled (% population)	Time	Concentration (mg/L)									
	(h)	0	S.E.	10	S.E.	40	S.E.	100	S.E.	400	S.E.
	0	0.87	0.08	0.93	0.09	0.83	0.07	0.79	0.08	0.82	0.06
	2	0.41	0.07	0.13	0.01	1.06	0.16	2.61	0.24	18.03	0.26
	8	0.15	0.02	0.13	0.00	4.14	1.75	6.39	0.50	7.13	0.60
	24	0.09	0.02	0.09	0.01	6.68	1.45	0.27	0.07	0.68	0.05
	48	0.07	0.01	0.07	0.01	2.05	0.49	0.00	0.00	0.17	0.05
	72	0.18	0.02	0.30	0.03	1.67	0.59	0.01	0.01	0.13	0.07

SYTOX®-labelled (% population)	Time	Concentration (mg/L)									
	(h)	0	S.E.	10	S.E.	40	S.E.	100	S.E.	400	S.E.
	0	0.16	0.01	0.13	0.01	0.16	0.03	0.17	0.02	0.16	0.01
	2	0.36	0.14	0.25	0.02	0.46	0.05	2.87	1.05	77.63	0.35
	8	0.18	0.01	0.24	0.02	3.18	1.03	77.13	0.54	62.74	0.63
	24	0.28	0.15	1.67	1.14	66.52	3.08	19.09	0.52	29.70	0.21
	48	0.12	0.01	0.01	0.01	31.99	1.86	8.11	0.43	25.12	0.41
	72	0.40	0.24	0.24	0.02	18.23	1.05	5.60	0.27	20.29	0.40

Appendix E3: Cell counts and % SYTOX® / CM-H_2DCFDA labelled cells of *Microcystis* exposed to acetic acid under light conditions with standard error (S.E.)

Cell count (cells/mL)	Time	Concentration (mg/L)									
	(h)	0	S.E.	1	S.E.	4	S.E.	10	S.E.	40	S.E.
	0	2,230,282	44,576	2,213,382	44,576	2,129,213	44,576	2,198,861	44,576	2,230,987	44,576
	2	2,408,923	96,333	2,408,410	155,812	2,450,282	26,835	2,391,769	33,814	2,364,154	33,089
	8	2,615,872	82,583	2,649,795	137,054	2,730,436	59,373	2,711,667	79,325	2,368,897	94,818
	24	2,891,795	114,259	3,030,487	43,433	3,246,205	43,349	3,128,128	53,767	2,134,923	111,548
	48	3,866,667	231,338	4,282,026	31,738	4,241,897	20,401	4,265,538	217,916	1,317,795	127,628
	72	5,328,641	211,098	6,114,128	182,046	6,222,077	187,139	6,064,128	133,859	278,872	70,465

CM-H_2DCFDA-labelled (% population)	Time	Concentration (mg/L)									
	(h)	0	S.E.	1	S.E.	4	S.E.	10	S.E.	40	S.E.
	0	1.69	0.23	1.45	0.24	1.78	0.27	1.62	0.18	1.83	0.23
	2	1.16	0.24	0.94	0.05	0.83	0.17	11.20	3.09	71.79	2.75
	8	0.82	0.02	0.97	0.10	0.39	0.05	2.67	0.03	31.24	0.15
	24	0.95	0.06	0.89	0.08	0.89	0.26	19.63	5.76	8.58	0.55
	48	0.74	0.17	0.81	0.10	0.64	0.06	3.61	0.75	3.56	0.75
	72	0.94	0.05	0.75	0.10	0.77	0.08	4.36	0.66	2.69	0.28

SYTOX®-labelled (% population)	Time	Concentration (mg/L)									
	(h)	0	S.E.	1	S.E.	40	S.E.	10	S.E.	40	S.E.
	0	3.31	0.03	2.31	0.06	2.51	0.09	2.05	0.03	3.05	0.16
	2	8.09	1.33	9.94	1.94	19.87	2.17	39.83	5.16	66.56	2.92
	8	4.43	0.20	5.25	0.63	5.17	0.21	6.23	1.38	31.15	5.65
	24	11.44	1.99	15.09	4.37	19.64	8.31	19.42	3.46	56.61	5.09
	48	15.26	5.80	14.79	1.48	10.89	3.21	22.01	6.45	65.39	7.33
	72	2.90	0.51	3.39	0.26	5.67	0.34	26.46	3.55	87.75	10.80

Appendix E4: Cell counts and % SYTOX® / CM-H_2DCFDA labelled cells of *Microcystis* exposed to acetic acid under dark conditions with standard error (S.E.)

Cell count (cells/mL)	Time	Concentration (mg/L)									
	(h)	0	S.E.	10	S.E.	40	S.E.	100	S.E.	400	S.E.
	0	2,254,744	17,624	2,246,876	17,624	2,234,842	17,624	2,249,728	17,624	2,186,467	17,624
	2	2,462,205	36,799	2,457,385	50,951	2,224,538	31,488	2,291,026	45,377	958,385	93,051
	8	2,543,744	20,883	2,390,744	38,141	2,181,000	16,505	2,277,897	43,998	507,077	78,550
	24	2,402,974	75,862	2,255,051	30,790	1,923,256	19,187	2,170,385	18,580	527,718	121,901
	48	2,249,436	30,775	2,183,795	22,387	1,810,308	127,840	1,805,564	30,456	230,359	109,235
	72	2,358,974	79,196	2,189,641	103,792	1,233,897	170,384	759,462	40,839	164,615	44,228

CM-H_2DCFDA-labelled (% population)	Time	Concentration (mg/L)									
	(h)	0	S.E.	100	S.E.	40	S.E.	100	S.E.	400	S.E.
	0	1.16	0.09	1.14	0.08	1.52	0.12	1.32	0.19	1.01	0.09
	2	0.65	0.05	0.68	0.29	64.04	0.90	12.64	0.22	3.74	0.53
	8	0.11	0.04	0.06	0.01	37.11	5.68	4.04	0.19	1.84	0.10
	24	0.18	0.13	0.08	0.00	37.76	4.24	0.71	0.48	4.76	1.04
	48	0.09	0.03	0.08	0.01	1.38	0.18	0.09	0.03	7.14	1.19
	72	0.21	0.02	0.24	0.03	2.74	0.68	0.11	0.05	6.28	0.25

SYTOX-labelled (% population)	Time	Concentration (mg/L)									
	(h)	0	S.E.	10	S.E.	40	S.E.	100	S.E.	400	S.E.
	0	16.93	1.05	15.43	1.08	14.60	0.90	12.66	1.34	14.21	1.38
	2	5.49	1.88	8.22	3.06	30.32	6.13	52.86	8.27	95.80	0.51
	8	4.07	2.29	7.78	3.32	31.18	14.43	16.92	1.19	97.79	0.23
	24	14.38	4.40	13.60	3.18	51.40	10.63	90.82	2.28	92.82	0.37
	48	6.77	2.37	5.61	0.81	66.75	6.42	98.39	0.04	88.20	0.51
	72	1.61	0.07	0.24	0.03	75.02	3.66	98.09	0.12	94.18	0.32

Appendix E5: pH levels during the experiment when *Microcystis* cultures were exposed to H_2O_2 during i) light and ii) dark conditions and acetic acid, again during iii) light and iv) dark conditions, all with standard error (S.E.)

i) H_2O_2 light

Time	Concentration (mg/L)									
(h)	0	S.E.	1	S.E.	4	S.E.	10	S.E.	40	S.E.
0	8.5	0.1	8.5	0.3	8.4	0.1	8.4	0.2	8.5	0.3
2	8.9	0.1	8.7	0.0	8.8	0.1	8.7	0.0	8.7	0.1
8	9.0	0.1	9.4	0.0	8.6	0.1	8.6	0.1	8.5	0.0
24	9.0	0.1	9.1	0.1	8.8	0.1	8.4	0.0	8.4	0.0
48	9.7	0.1	10.0	0.1	9.5	0.1	8.7	0.1	8.5	0.1
72	10.3	0.2	10.2	0.2	10.2	0.1	8.6	0.1	8.3	0.0

ii) H_2O_2 dark

Time	Concentration (mg/L)									
(h)	0	S.E.	10	S.E.	40	S.E.	100	S.E.	400	S.E.
0	8.6	0.0	8.5	0.1	8.6	0.0	8.6	0.0	8.6	0.0
2	7.7	0.0	7.7	0.0	7.7	0.0	7.5	0.0	7.4	0.1
8	7.6	0.0	7.6	0.0	7.6	0.0	7.5	0.0	7.6	0.0
24	7.3	0.0	7.3	0.0	7.3	0.0	7.4	0.0	7.3	0.0
48	7.4	0.1	7.5	0.0	7.3	0.0	7.2	0.0	7.1	0.0
72	7.0	0.1	7.3	0.0	7.3	0.0	7.2	0.0	7.2	0.0

iii) Acetic acid Light

Time	Concentration (mg/L)									
(h)	0	S.E.	1	S.E.	4	S.E.	10	S.E.	40	S.E.
0	8.4	0.1	8.0	0.1	8.4	0.1	8.4	0.1	8.4	0.1
2	9.1	0.0	9.0	0.2	8.4	0.1	7.2	0.1	5.6	0.3
8	9.6	0.2	9.6	0.3	9.7	0.1	9.1	0.1	5.9	0.8
24	9.8	0.2	10.0	0.1	10.1	0.1	10.2	0.0	5.5	0.3
48	10.2	0.2	10.5	0.1	10.6	0.1	10.6	0.0	4.6	0.1
72	10.5	0.0	10.8	0.1	10.8	0.0	10.8	0.1	4.8	0.2

iv) Acetic acid dark

Time	Concentration (mg/L)									
(h)	0	S.E.	10	S.E.	40	S.E.	100	S.E.	400	S.E.
0	8.6	0.1	8.6	0.1	8.4	0.1	8.3	0.1	8.4	0.1
2	8.7	0.1	6.7	0.0	4.9	0.2	3.5	0.0	2.9	0.0
8	8.0	0.2	6.9	0.1	4.8	0.2	3.8	0.1	3.0	0.0
24	7.8	0.2	6.9	0.2	4.3	0.1	3.6	0.0	2.9	0.0
48	7.1	0.1	7.4	0.0	4.3	0.1	3.4	0.0	2.8	0.0
72	7.0	0.1	7.5	0.1	4.4	0.0	3.5	0.0	2.9	0.0

Appendix F

Appendix F1: Results from the exponential phase culutres in different CM v/v% concentrations under light conditions for cell counts, the % population density change and %-molecular probe labelled cells

75% CM

Day	Cells/mL	S.E.	Δ Density (%)	S.E.	SYTOX®%	S.E.	CMFDA%	S.E.
0	406,741	16,810	-	-	5.3	1.1	94.3	1.3
1	172,111	8,010	-57.4	3.6	46.8	3.1	49.3	3.3
2	147,086	4,267	-14.4	2.1	63.0	3.9	31.0	4.2
3	96,556	2,899	-34.2	3.4	71.7	4.6	26.3	4.6
4	57,568	3,126	-40.4	1.8	72.1	5.1	24.1	4.2
7	89,012	19,563	53.4	31.2	40.5	1.1	49.2	2.1

50% CM

Day	Cells/mL	S.E.	Δ Density (%)	S.E.	SYTOX®%	S.E.	CMFDA%	S.E.
0	853,346	33,797	-	-	4.7	0.9	93.7	0.7
1	417,123	5,303	-50.9	2.2	18.3	0.7	63.1	2.0
2	370,975	41,342	-10.8	10.9	36.1	2.0	51.2	1.7
3	321,309	38,237	-13.4	3.1	35.7	2.6	59.1	1.7
4	242,136	23,495	-21.0	15.1	26.8	2.5	70.0	1.6
7	301,901	35,176	29.5	26.8	23.6	0.7	72.8	1.4

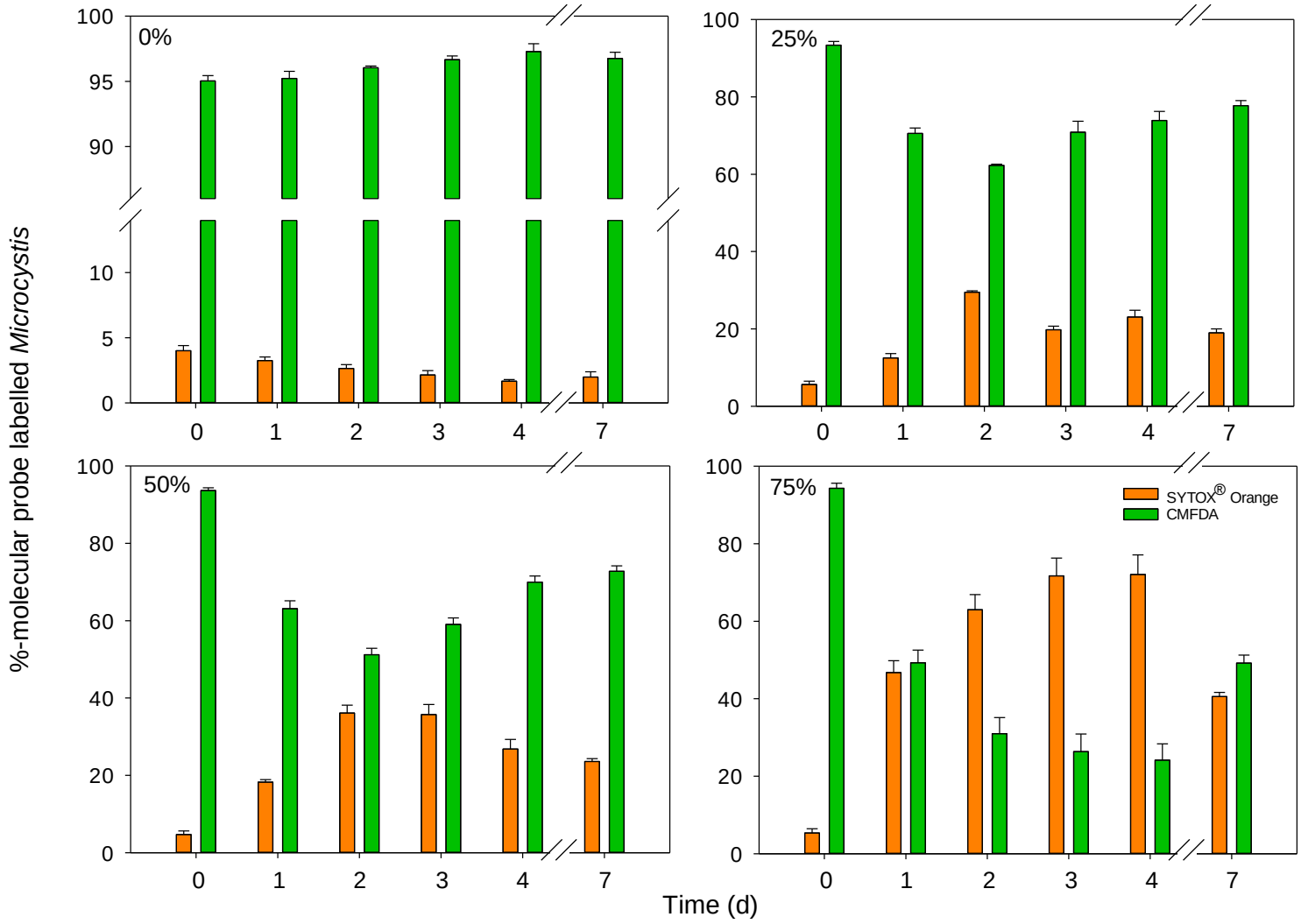
25% CM

Day	Cells/mL	S.E.	Δ Density (%)	S.E.	SYTOX®%	S.E.	CMFDA%	S.E.
0	1,111,309	34,321	-	-	5.6	0.9	93.4	1.0
1	676,728	6,311	-39.0	2.3	12.5	1.1	70.5	1.4
2	443,481	43,813	-34.5	6.2	29.5	0.4	62.3	0.3
3	446,136	40,339	1.7	8.9	19.8	0.9	70.9	2.8
4	532,037	56,479	20.0	12.9	23.1	1.8	73.9	2.4
7	784,469	93,215	48.7	14.2	19.0	1.0	77.7	1.3

0% CM

Day	Cells/mL	S.E.	Δ Density (%)	S.E.	SYTOX®%	S.E.	CMFDA%	S.E.
0	1,840,686	37,435	-	-	4.0	0.4	95.0	0.4
1	2,482,234	57,172	34.9	1.3	3.2	0.3	95.2	0.5
2	3,788,634	121,881	52.6	2.0	2.6	0.3	96.0	0.1
3	5,550,442	133,761	46.6	1.3	2.1	0.3	96.7	0.3
4	7,007,371	33,896	26.4	2.8	1.7	0.1	97.3	0.6
7	9,552,963	733,084	36.3	10.1	2.0	0.4	96.8	0.5

Appendix F2: Molecular probe data from SYTOX® Orange (membrane injured cells) and CMFDA (hydrolytic enzyme activity) for the population % of labelled cells. Results include all concentrations for exponential phase experiments over 7 days under light conditions



Appendix F3: Results from the exponential phase culutres in different CM v/v% concentrations under dark conditions for cell counts, the % population density change and %-molecular probe labelled cells

75% CM

Day	Cells/mL	S.E.	Δ Density (%)	S.E.	SYTOX®%	S.E.	CMFDA%	S.E.
0	412,000	12,945	-	-	3.5	0.8	93.7	0.5
1	430,383	6,596	4.1	4.1	4.9	1.0	88.0	0.2
2	432,210	9,497	-6.9	8.0	6.2	0.3	92.1	0.3
3	336,247	9,127	-14.4	7.6	4.6	0.5	93.8	0.7
4	331,798	8,748	-8.3	6.6	4.9	0.6	92.0	0.9
7	256,395	22,285	-23.3	11.6	7.3	3.4	84.4	4.6

50% CM

Day	Cells/mL	S.E.	Δ Density (%)	S.E.	SYTOX®%	S.E.	CMFDA%	S.E.
0	828,852	46,252	-	-	4.0	1.5	93.7	0.5
1	845,864	40,437	2.2	3.4	8.3	0.7	85.8	2.3
2	820,321	10,662	-2.4	6.1	9.6	0.3	88.9	0.5
3	856,160	114,535	4.4	14.0	13.7	1.7	84.8	1.7
4	828,568	28,477	0.5	14.1	17.8	0.3	77.6	0.3
7	662,247	22,375	-19.7	5.1	10.8	0.1	78.7	0.5

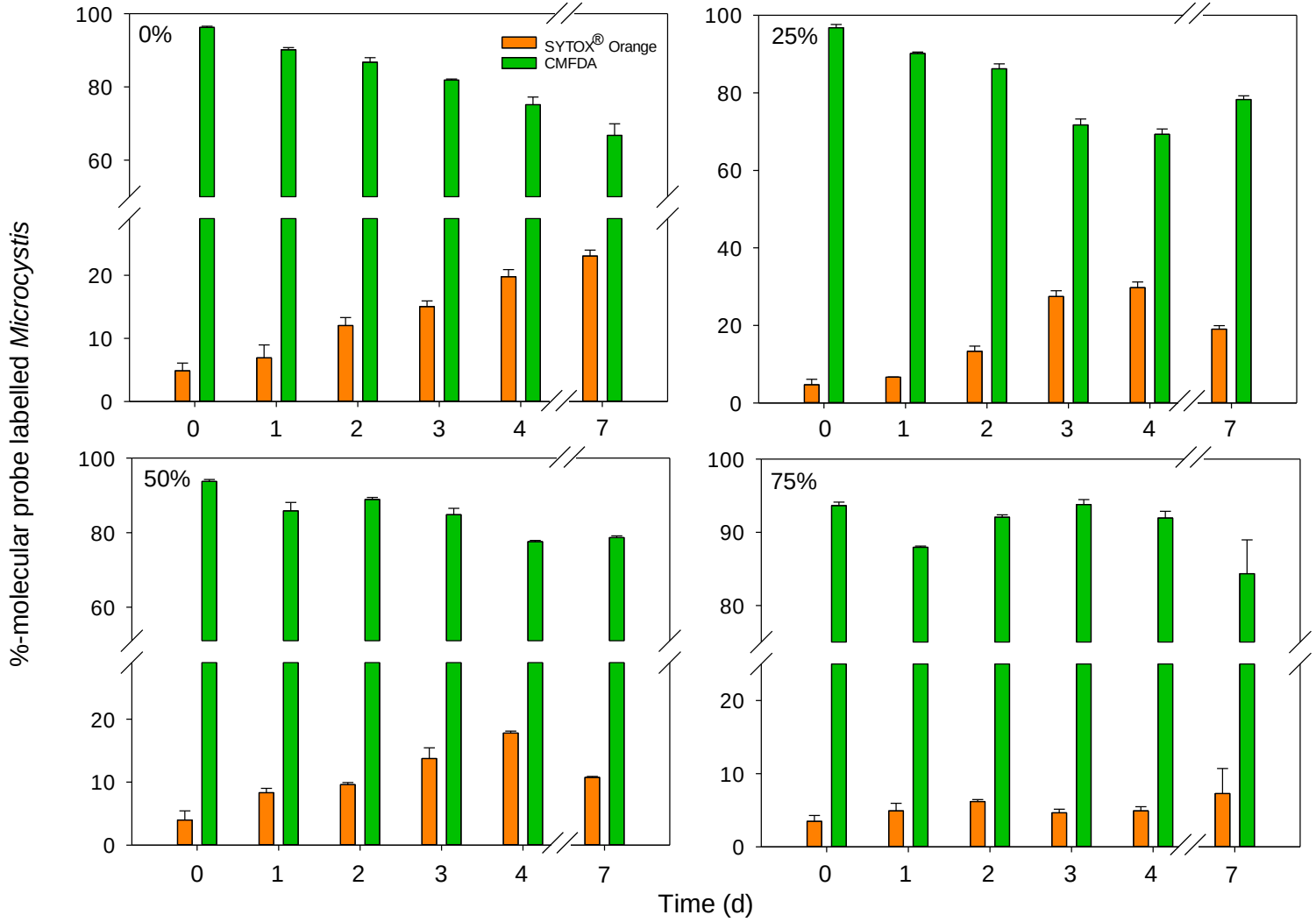
25% CM

Day	Cells/mL	S.E.	Δ Density (%)	S.E.	SYTOX®%	S.E.	CMFDA%	S.E.
0	1,101,420	23,905	-	-	4.7	1.4	96.8	0.8
1	1,192,975	10,796	8.5	3.4	6.6	0.1	90.2	0.4
2	1,216,370	14,609	2.0	0.4	13.4	1.3	86.2	1.3
3	1,175,123	9,035	-3.4	0.4	27.4	1.5	71.7	1.5
4	1,142,988	7,268	-2.7	1.2	29.7	1.5	69.3	1.4
7	749,222	39,857	-34.4	3.5	19.0	0.9	78.3	1.0

0% CM

Day	Cells/mL	S.E.	Δ Density (%)	S.E.	SYTOX®%	S.E.	CMFDA%	S.E.
0	1,833,704	15,065	-	-	4.9	1.2	96.2	0.3
1	1,811,531	30,495	-1.2	1.4	6.9	2.0	90.1	0.6
2	1,806,025	30,766	-0.3	0.1	12.0	1.3	86.8	1.2
3	1,781,617	25,793	-1.3	0.6	15.0	0.9	81.9	0.3
4	1,775,086	23,368	-0.4	0.2	19.8	1.1	75.1	2.1
7	1,347,642	84,977	-24.1	4.8	23.1	0.9	66.8	3.2

Appendix F4: Molecular probe data from SYTOX® Orange (membrane injured cells) and CMFDA (hydrolytic enzyme activity) for the population % of labelled cells. Results include all concentrations for exponential phase experiments over 7 days under dark conditions



Appendix F5: Results from the stationary phase culutres in different CM v/v% concentrations under light conditions for cell counts, the % population density change and %-molecular probe labelled cells

75% CM

Day	Cells/mL	S.E.	Δ Density (%)	S.E.	SYTOX®%	S.E.	CMFDA%	S.E.
0	4,090,444	75,469	-	-	4.9	1.5	94.0	1.5
1	4,822,436	36,263	17.9	1.4	25.4	0.3	67.8	0.4
2	5,717,444	118,060	18.6	2.4	20.8	0.8	72.6	0.5
3	7,038,679	314,891	23.0	3.0	13.9	0.5	77.1	0.5
4	7,735,247	493,101	9.8	2.9	9.9	0.5	79.5	1.2
7	8,943,370	378,909	15.9	2.5	5.4	0.3	91.3	0.5

50% CM

Day	Cells/mL	S.E.	Δ Density (%)	S.E.	SYTOX®%	S.E.	CMFDA%	S.E.
0	8,298,679	306,128	-	-	5.7	0.8	93.0	1.2
1	9,792,704	173,303	18.5	6.2	27.8	0.2	64.1	0.1
2	11,028,420	316,855	12.6	1.3	23.4	0.9	67.5	1.3
3	12,109,654	289,730	10.0	3.7	20.0	0.7	71.4	1.0
4	14,329,000	652,836	18.3	4.4	16.9	1.0	73.0	0.7
7	13,762,469	1,761,224	-4.4	9.7	11.1	0.7	86.9	0.7

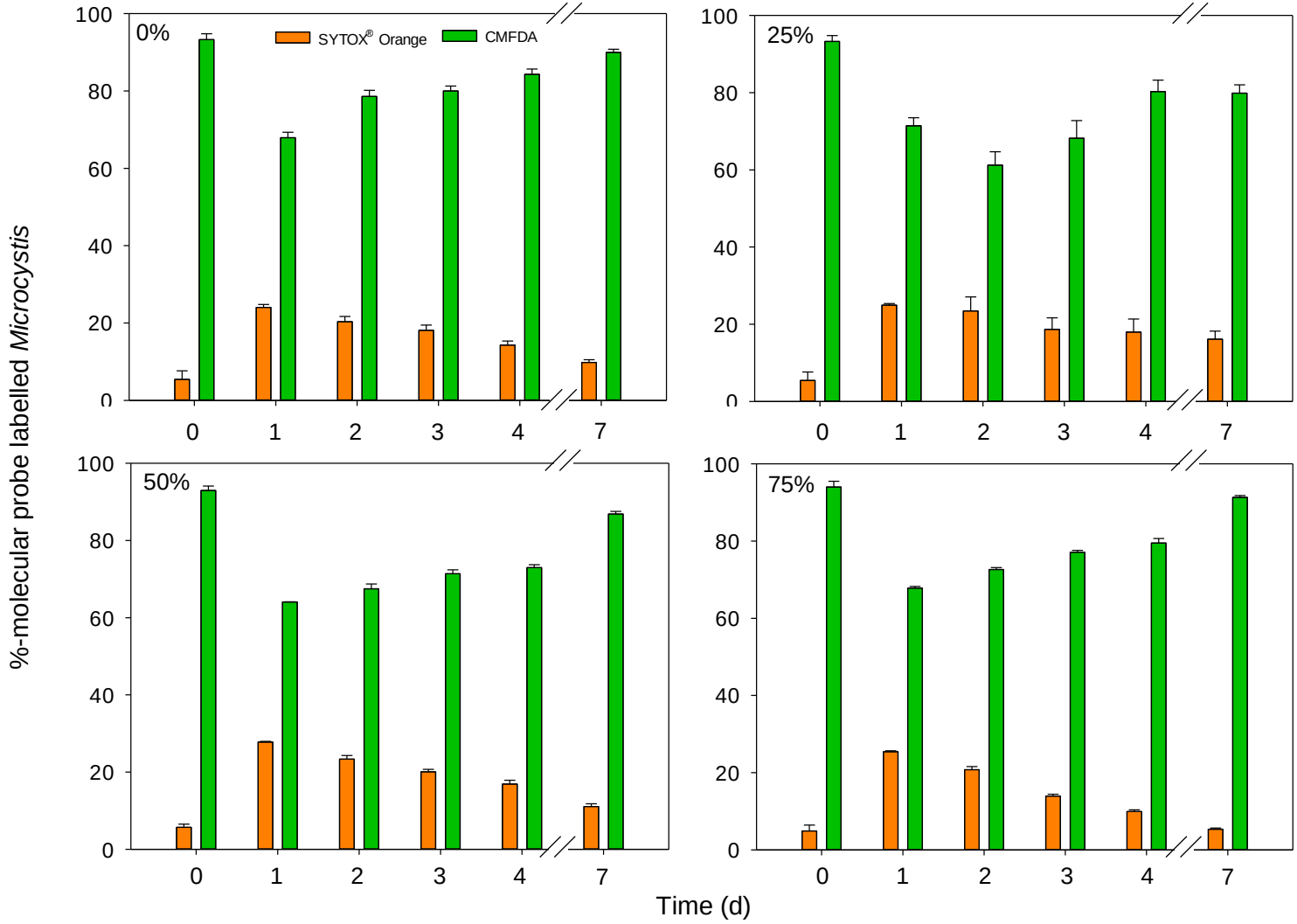
25% CM

Day	Cells/mL	S.E.	Δ Density (%)	S.E.	SYTOX®%	S.E.	CMFDA%	S.E.
0	12,114,741	208,443	-	-	5.4	2.2	93.3	1.5
1	12,381,136	302,335	2.2	0.9	24.9	0.4	71.4	2.1
2	12,811,630	406,823	3.4	1.3	23.4	3.6	61.2	3.5
3	13,468,951	835,068	5.1	5.0	18.7	3.0	68.2	4.6
4	15,378,309	987,091	14.2	3.9	18.0	3.4	80.3	3.0
7	16,672,630	3,150,500	10.2	22.8	16.1	2.1	79.9	2.1

0% CM

Day	Cells/mL	S.E.	Δ Density (%)	S.E.	SYTOX®%	S.E.	CMFDA%	S.E.
0	14,677,568	188,423	-	-	5.4	2.2	93.3	1.5
1	15,166,926	165,637	3.3	0.4	24.0	0.8	67.9	1.4
2	15,848,728	206,264	4.5	0.3	20.3	1.4	78.6	1.6
3	16,624,099	142,783	4.9	0.5	18.1	1.4	80.0	1.3
4	17,469,247	66,761	5.1	0.8	14.3	1.1	84.3	1.4
7	19,167,580	574,881	9.7	3.0	9.8	0.7	90.0	0.8

Appendix F6: Molecular probe data from SYTOX® Orange (membrane injured cells) and CMFDA (hydrolytic enzyme activity) for the population % of labelled cells. Results include all concentrations for stationary phase experiments over 7 days under light conditions



Appendix F7: Results from the stationary phase culutres in different CM v/v% concentrations under dark conditions for cell counts, the % population density change and %-molecular probe labelled cells

75% CM

Day	Cells/mL	S.E.	Δ Density (%)	S.E.	SYTOX®%	S.E.	CMFDA%	S.E.
0	3,904,259	46,013	-	-	5.4	2.2	93.3	1.5
1	3,437,679	53,986	-10.3	3.2	25.2	0.3	72.7	0.1
2	3,434,765	37,428	3.0	2.1	26.9	0.1	72.3	0.4
3	3,490,716	30,838	-0.8	0.9	25.7	0.1	73.8	0.2
4	3,416,667	43,083	-21.0	19.1	21.6	0.2	76.8	0.2
7	1,578,321	201,592	-51.3	7.0	19.8	0.4	79.2	0.6

50% CM

Day	Cells/mL	S.E.	Δ Density (%)	S.E.	SYTOX®%	S.E.	CMFDA%	S.E.
0	8,010,864	78,753	-	-	5.7	0.8	93.0	1.2
1	7,072,296	136,860	-11.7	2.2	27.7	0.1	69.1	0.0
2	6,827,605	139,658	-3.5	1.1	28.7	0.4	70.5	0.3
3	7,082,877	64,700	3.8	1.2	27.2	0.2	71.7	0.1
4	3,631,123	64,939	-48.7	0.6	21.4	0.2	77.5	0.2
7	2,881,988	136,418	-20.6	4.1	15.2	2.9	81.6	3.4

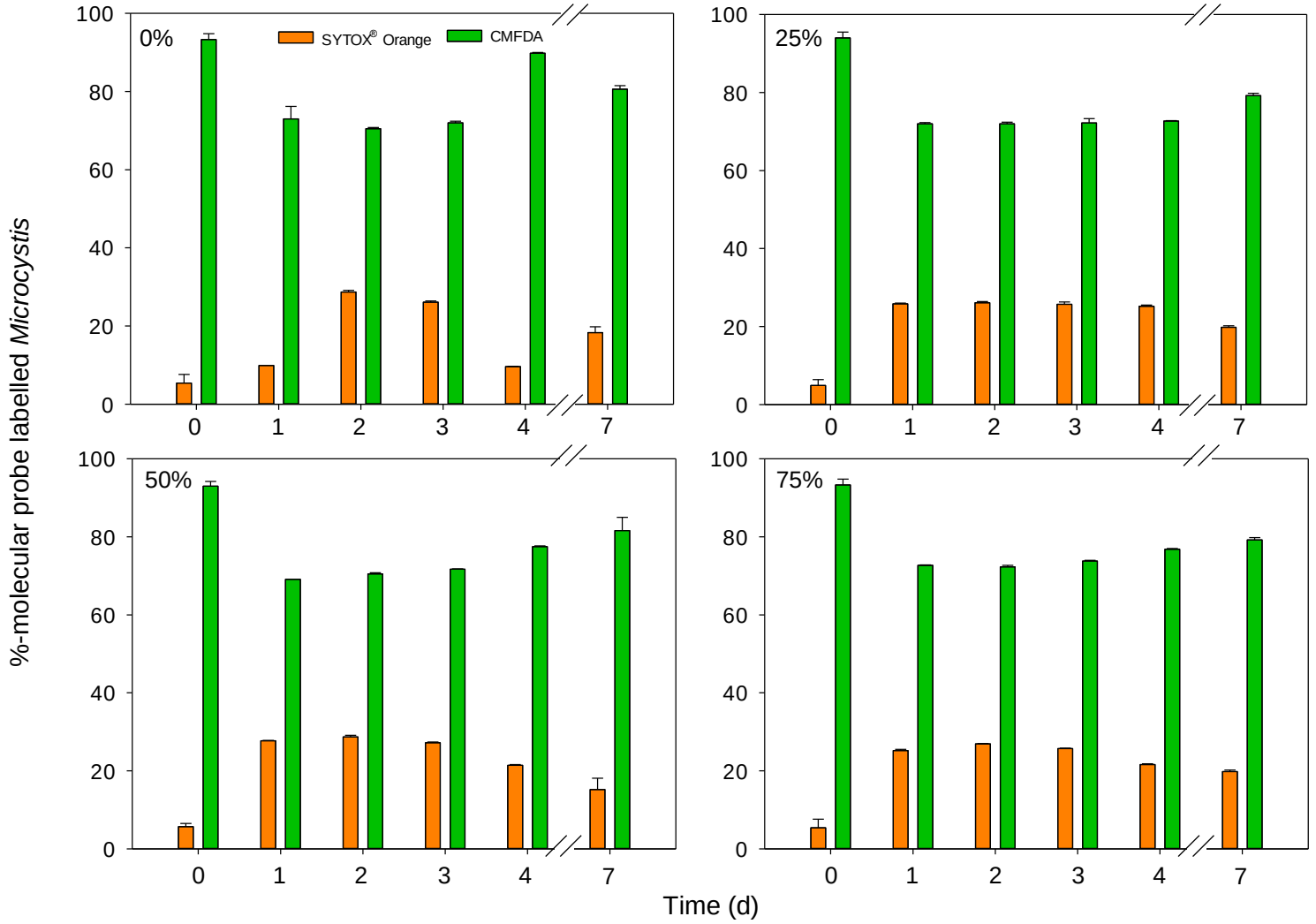
25% CM

Day	Cells/mL	S.E.	Δ Density (%)	S.E.	SYTOX®%	S.E.	CMFDA%	S.E.
0	11,489,605	169,226	-	-	4.9	1.5	94.0	1.5
1	10,351,963	262,468	-9.8	3.3	25.8	0.2	72.0	0.3
2	9,856,815	233,432	-4.7	2.1	26.1	0.3	72.0	0.4
3	10,415,185	149,278	5.7	2.0	25.7	0.6	72.2	1.1
4	4,966,173	102,679	-52.3	1.6	25.2	0.3	72.7	0.1
7	4,033,716	128,441	-18.6	4.0	19.8	0.4	79.2	0.6

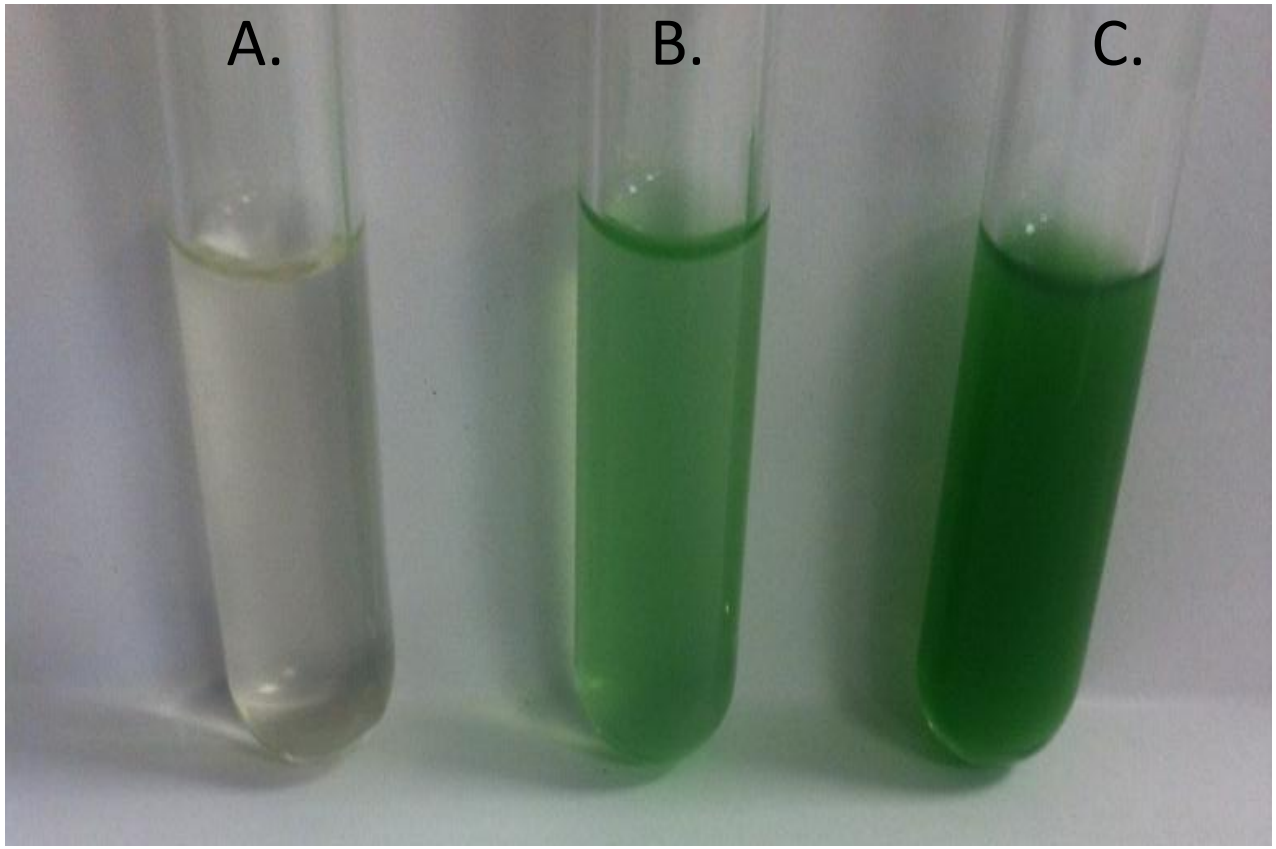
0% CM

Day	Cells/mL	S.E.	Δ Density (%)	S.E.	SYTOX®%	S.E.	CMFDA%	S.E.
0	14,835,914	1,080,617	-	-	5.4	2.2	93.3	1.5
1	12,881,198	39,678	-12.2	6.6	9.9	0.0	73.0	3.2
2	12,524,996	165,054	-2.8	1.5	28.7	0.4	70.5	0.3
3	12,234,864	116,141	-2.3	0.7	26.1	0.3	72.0	0.4
4	13,029,475	107,271	6.5	1.6	9.6	0.1	89.8	0.2
7	5,920,667	1,063,177	-54.4	8.5	18.3	1.5	80.6	0.9

Appendix F8: Molecular probe data from SYTOX® Orange (membrane injured cells) and CMFDA (hydrolytic enzyme activity) for the population % of labelled cells. Results include all concentrations for stationary phase experiments over 7 days under dark conditions



Appendix F9: Comparisons of cultures from A) 75% CM (lag), B) 25% CM (exponential) and C) 0% CM (stationary) after 7 days, under light conditions



Appendix F11: The inverse correlation of %-SYTOX® Orange and %-CMFDA for all cultures under A) light (including CM generation) and B) dark conditions, with Pearson's correlation results included. Physiological cell states indicates high cell viability in *M. aeruginosa* populations when %-SYTOX® labelled cells are low and %-CMFDA labelled cells are high