Factors affecting the Growth and Toxin Content of Key Cyanobacteria Species in a Changing World.

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Abstract

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Cyanobacteria are ubiquitous in marine, freshwater and terrestrial ecosystems. In freshwaters, under certain conditions cyanobacteria can form super abundant blooms. Many cyanobacteria taxa produce secondary metabolites, amongst these are many potent toxins (cyanotoxins), which can present substantial risks to human, animal and environmental health. Where risk mitigation strategies are implemented, they are usually based on cyanobacteria cell counts. However, not all cyanobacteria taxa produce toxins, and the drivers for cyanotoxin production and their eco-physiological role remain unknown. Thus, risk management strategies may over or under estimate risks. A predicted increase in the incidence of toxin producing cyanobacterial blooms has been proposed, linked to climate change driven water temperature increases and eutrophication. Consequently, better understanding of factors modulating cyanobacterial toxicity would assist in more proactive and effective water management.

In the first part of this study the effects of biotic and abiotic factors on the growth and toxicity a recently isolated strain of *Microcystis* sp. (CCAP1450/17) and *M. aeruginosa* reference strain (PCC7806) were examined. Strains responded similarly under controlled laboratory batch culture systems. Highest cell densities and growth rates were observed at medium light intensity (36 µmol of photons m^{-2} s^{-1}) compared to high (117 µmol of photons m^{-2} s^{-1}) and low (15 µmol of photons m^{-2} s^{-1}) light intensities. Nitrogen was an obligate requirement for microcystin production, but phosphorous was not, indicating that nitrogen eutrophication conditions (caused by agricultural run-off etc) would increase the risk of toxic blooms. Toxin (microcystin) concentrations were positively correlated with cell density, but microcystin synthesis was independent of growth rates under nitrogen replete conditions. Furthermore, smaller cells contained higher levels of toxins. Cellular microcystin content was significantly higher at 20°C compared to 25 & 30°C questioning the paradigm that increased water temperatures (caused by climate change) will favour toxic bloom formation. Although the role of microcystin is not clear, these data indicated that under nitrogen abundant conditions microcystins may perform an eco-physiological function, which is reduced under nitrogen deprivation and/or when cells are rapidly dividing.

In the second part of the study time series monitoring of cyanobacteria taxa, toxin concentrations and a range of environmental parameters was undertaken in two connected freshwater reservoirs over a twelve-month period. Cyanobacterial cell counts exceeding UK threshold levels of >20,000 cells mL^{-1} were recorded on four occasions. Toxins were detected in both reservoirs, concentrations were significantly higher in lake 2 (not stocked with fish) and did not correspond with highest levels of *Microcystis* cells, indicating that cyanobacterial species other than *Microcystis* were producing microcystins. Microcystin levels did not exceed the WHO medium health threshold of 20 µg L^{-1} although low threshold values (1.0 µg L^{-1}) were detected in 16% of samples. Monitoring data indicated complex bottom-up and top-down control mechanisms in the moderation of cyanobacterial taxa abundance and population structure, the latter potentially mediated by the presence of omnivorous fishes. Application of basic general linear modelling to the dataset indicated that approximately 60-65% of the variability could be explained by combined independent abiotic and biotic variables indicating the future applicability of this approach.

These results confirmed complex interactions between biotic and abiotic factors in both laboratory and field conditions but were broadly suggestive of additive effects with respect to microcystin production. Although the functional role of microcystins remains unclear it was considered possible that microcystins perform an eco-physiological function, perhaps conferring an advantage over non-toxic strains under nitrogen rich conditions. In environmental monitoring multiple species of cyanobacterial were implicated in toxin production, but cell numbers alone were not directly proportional to toxin levels supporting the proposition that management strategies based on cell counting alone may not be indicative of risk. Trophic relationships that influence cyanobacterial population dynamics and toxin production require further study to generate data to inform predictive models, but this series of studies suggested several avenues for further study which will provide water quality managers with improved tools to enable efficient, active management of water bodies.

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Publications and presentations

Publications

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. *J Microbiol. Methods,* 122, 13-15. (Appendix A).

Hartnell D.M., Esteban, G.F, Katsiadaki, I., Turner A.D. and Franklin, D.J. 2019. Long-term monitoring of cyanobacteria and cyanotoxins in two lowland freshwater reservoirs in southern Britain. *In preparation.*

Presentations

Hartnell, D.M. and Franklin, D.J., 2015. Autoinhibition of the growth in *Microcystis:* A process that could yield novel antimicrobials? Association for the Sciences of Limnology and Oceanography (ASLO) Aquatic Sciences Meeting, Granada, Spain. 22nd - 27th February 2015.

Hartnell, D.M. Franklin D.J., 2015 Autoinhibition of growth in Microcystis: A process that could control nuisance cyanobacteria blooms? Bournemouth University PGR Conference. 21st May 2015.

Hartnell, D.M., Chapman, I.J., Esteban, G.F. and Franklin, D.J., 2015. Using knowledge of ecological niche requirements to separate the freshwater cyanobacteria *Microcystis* sp. and *Synechococcus* sp. and create fresh culture lines. 6th European Phycological Congress, London 23rd – 28th August 2015.

Hartnell D.M., Esteban, G.F, Katsiadaki, I., Turner A.D. and Franklin, D.J. 2016. Predicting the future growth and toxin content of freshwater cyanobacteria in a changing world. Cefas Student Day, Lowestoft. 19th – 20th April 2016

Hartnell, D.M, Turner, A.D., Katsiadaki, I., Esteban, G.F. and Franklin, D.J. 2016. Understanding the environmental drivers of *Microcystis* blooms: A toxic cyanobacterium. Bournemouth University PGR Conference. 18st May 2016.

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Hartnell D.M., Esteban, G.F, Katsiadaki, I., Turner A.D. and Franklin, D.J. 2016. The Toxicity of Microcystin to the Greater Wax Moth larva *(Galleria mellonella):* A Simple and Cost-effective Cyanotoxin Bioassay? 10th International Conference on Toxic Cyanobacteria (ICTC10), Wuhan, China. 23rd – 28th October 2016.

Franklin, D.J., Foo, S., Chapman, I.J. and Hartnell, D.M. 2016 Lack of variability in the influence of hydrogen peroxide on growth, metabolic activity and membrane integrity in three strains of *Microcystis aeruginosa*. 10th International Conference on Toxic Cyanobacteria (ICTC10), Wuhan, China. 23rd – 28th October 2016.

Hartnell D.M., Esteban, G.F, Katsiadaki, I., Turner A.D. and Franklin, D.J. 2017. Monitoring the seasonal variation of cyanobacteria and toxins in a British lowland reservoir. Cefas Student Day, Weymouth. 9th – 10th May 2017.

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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.

1. Cyanobacteria and cyanotoxins: cell and population responses to climate change and eutrophication

1.1. Introduction

Cyanobacteria, also known as blue-green algae are a diverse phylum of oxygen evolving photosynthetic prokaryotes (Whitton 2011, Whitton and Potts 2012). The evolutionary history of cyanobacteria extends back ~3,500 million years (Schopf 2012) and in that time, they have had a major influence on the Earth system (see below and Canfield 2005). Globally cyanobacteria contribute significantly to primary production and are common in marine, freshwater and terrestrial ecosystems, both independently and as symbionts (Chisholm *et al.* 1988, Adams *et al.* 2012). Cyanobacteria demonstrate complex morphologies and life histories (Whitton 2011, Sciuto and Moro 2015) and it has been reported that freshwater taxa may have eco-physiological adaptions which offer competitive advantages over eukaryotic phytoplankton (Carey *et al.* 2012, Paerl and Paul 2012).

In fresh waters, especially lakes and reservoirs, cyanobacteria can bloom in super abundant events producing surface scums, adversely affecting water supplies by fouling treatment infrastructure, imparting bad odours and causing anoxic water conditions through decomposition (Sigee 2005, Paerl *et al.* 2011a). Furthermore, many cyanobacteria taxa produce potent toxins (cyanotoxins) which can act on a variety of biological pathways and are considered to present substantial risks to ecosystems and public health (Carmichael 1992, Codd *et al.* 2005, Metcalf and Codd 2012). A predicted increase in the incidence of cyanobacterial blooms has been proposed linked to anthropogenic climate change (Robarts and Zohary 1987 Paerl and Huisman 2009, O'Neil *et al.* 2012, Visser *et al.* 2016) causing water temperature increases and man-made eutrophication of water bodies by increased nutrient loading e.g. nitrogen and phosphorus (Paerl 1997, Codd 2000, Paerl *et al.* 2011b, O'Neil *et al.* 2012).

It is currently not clear if the impact of climate change, as characterised by increased water temperatures and prolonged warming events, and eutrophication, increasing availability of nitrogen and phosphorus, will be additive, synergistic or antagonistic with respect to bloom formation or frequency (Elliott 2012, O'Neil *et al.* 2012, Paerl and Otten 2013, Rigosi 2014). Although it is generally accepted that increased periods of warming together with higher eutrophication event frequency, will result in increased bloom occurrence (Davis *et al.* 2009, Wagner and Adrian 2009, Beaulieu *et al.* 2013, Visser *et al.* 2016).

However, it is important to note that it has been estimated that only approximately 40-70%, of cyanobacteria taxa produce toxins (Turner *et al*. 2018b) moreover the relative dominance of toxin producing strains in freshwater environments subject to climate change and eutrophication pressures is uncertain. Numerous studies have suggested that changes in biotic and abiotic conditions driven by climate change and eutrophication events increase the likelihood of toxic bloom formation (Paerl 1997, Johnk, *et al.* 2008, Davis *et al.* 2009, Paerl and Paul 2012 Liu *et al.* 2015.). Whereas, several reports, albeit fewer in number, have predicted that whilst cyanobacterial bloom formation may become more common, elevated toxicity during bloom events is not inevitable (Kardinaal *et al.* 2007, Van de Waal *et al.* 2011). Consequently, the public and animal health risks of climate and/or nutrient mediated cyanobacterial bloom formation are currently not fully understood.

1.2. The influence of cyanobacteria on the Earth-life system

Cyanobacteria have a long evolutionary history spanning around 3,500 million years, in that time they have influenced the Earth-life system in several significant ways. Stromatolites, trace fossils of layered carbonate rock formed by cyanobacterial mats dominate the fossil record of the Precambrian, from the formation of the planet 4,600 million years ago to the beginning of the Phanerozoic eon 542 million years ago. The precise dating of fossil evidence for the emergence of oxygenic photosynthesising and oxygenic respiring cyanobacteria, however, is disputed to between 3,800 and 3,200 million years ago (Schopf 2012).

Cyanobacteria are responsible for creating the dioxygen (O_2) rich atmosphere around 2,500 million years ago (Canfield 2005). The initial consequences of an O_2 rich atmosphere were negative with respect to the anaerobic prokaryotes which were the dominant life form but intolerant to oxidative stress (Sessions *et al.* 2009). In addition, O_2 probably destroyed, or contributed significantly to the destruction, of atmospheric methane (a greenhouse gas) causing a cascade of global glaciations in a 'Snowball Earth' event (Kopp *et al.* 2005). By approximately 1,000 million years ago, however, stabilisation of an O_2 rich atmosphere led to the formation of a layer of ozone (O_3). This O_3 layer shielded oceanic water molecules from cleavage and subsequent evaporation of hydrogen into space (Lane 2010) further protecting life from the detrimental effects of solar radiation (Sciuto and Moro 2015). Complex life is only considered possible under conditions of aerobic respiration, calculated to be at least an order of magnitude more efficient than anoxic processes (Catling *et al.* 2005) Thus, the importance of cyanobacteria in establishing an O_2 rich atmosphere and thereby establishing the conditions on Earth for the development of multicellular organisms cannot be overstated (Catling *et al.* 2005, Lane 2010, Beerling 2017).

Another influence of cyanobacteria on the Earth-life system is their direct contribution to the evolution of the eukaryotic cell. Chloroplasts, the photosynthetic organelles of algae and higher plants, are proposed to have evolved by the engulfment of cyanobacteria by eukaryotic cells (Archibald 2009b). There have been several 'rounds' of this symbiogenesis; primary engulfment occurred around 1,000 million years ago and through this process evolved the lineages of red algae and plants and green algae (Cavalier-Smith 2000). Subsequent secondary and tertiary engulfment has led to euglenophytes, chlorarachniophytes, cryptophytes, haptophytes and heterokontophytes (Archibald 2009b). It is proposed that symbiogenesis by engulfment of photosynthetic organisms is a continuing process (Archibald 2009a)

and there are many examples of cyanobacteria in symbiotic relationships with other organisms in freshwater, marine and terrestrial ecosystems (Shi and Hall 1988, Lane 2010, Adams *et al.* 2012). These include diatoms, coral, sponges, ascidians (sea squirt), fungi (as lichens), bryophytes, cycads (tree ferns) and ferns (aquatic and terrestrial) (Adams *et al.* 2012). Without cyanobacteria the Earth-life system would be very different, if not non-existent (Lane 2010).

1.3. Cyanobacterial taxonomy

Taxonmically, cyanobacteria form a monophyletic group as the only photosynthetic prokaryotic algae (Whitton 2011). Classified in the domain of bacteria, cyanobacteria are closely related to purple sulphur bacteria and Gram-positive bacteria (Woese 1987). The phylum cyanobacterium is morphologically and physiologically diverse, and is divided into four orders (Whitton 2011):

- Chroococcales – unicellular or colonial, never forming true filaments with some cell motility.
  Example genera; *Coelosphaerium, Microcystis, Synechococcus.*
- Oscillatoriales – cells form filaments and undergo binary fission in one plane. Specialist heterocyst cells for the fixation of atmospheric nitrogen (N_2) and akinete dormancy cells are absent.
  Example genera; *Lyngbya, Oscillatoria Planktothrix, Pseudanabaena.*
- Nostocales - cells form filaments, cells undergo binary fission in one plane and all cells can develop into heterocysts. In some genera the heterocysts can further differentiate into akinetes.
  Example genera; *Anabaena, Aphanizomenon, Cylindrospermum, Nodularia,*
- Stigonematales – morphologically complex, binary fission occurs on more than one plane allowing branched filaments, cells can form into heterocysts and akinetes. The order is not as diverse as the others.
  Example genera; *Hapalosiphon, Stigonema*

The systematic taxonomy of cyanobacteria has been problematic (Stanier *et al.* 1978). This is in part because prokaryotes do not fall precisely into the biological species criterion and that historically, two codes of nomenclature have been used interchangeably by workers. The difficulties arise from the observation that cyanobacteria do not reproduce sexually, which is considered a requirement of the traditional biological species criterion (Palinska and Surosz 2014). Additionally, cyanobacteria have a very well conserved genetic core (Shi and Falkowski 2008) and possess the ability to express differing phenotypes according to environmental conditions (ecotypes) (Sejnohova and Marsalek 2013). Historically, cyanobacteria were named ‘blue-green algae’ and were categorised according to rules of the Botanical Code. The Botanical Code classifies species as the basic unit distinguished by morphology. Type-specimens are preserved, and new species can be widely published (Palinska and Surosz 2014). The similarities between cyanobacteria and

bacteria had however been recognised for over 100 years (Stanier *et al.* 1978). The phylogenetic basis of this morphological relationship has been confirmed subsequently by molecular analysis (Woese 1987). In 1978 cyanobacteria were re-classified by the Bacterial Code of nomenclature (Stanier *et al.* 1978), which is a system of categorisation based upon strain differentiation informed by genetic relatedness. Characterised cultured strains with numbered designations are the basic units and new strains can only be published in the International Journal of Systematic and Evolutionary Microbiology (Stanier *et al.* 1978). However, despite the advances in genomic characterisation techniques most cyanobacterial researchers agree that both systems can be valuable but would benefit from integration. Consequently, both codes are used as best fit specific applications.

The outcome of this dual approach to cyanobacteria taxonomy is that an excessive number of species are described by the Botanical Code (although the exact figure is often revised downwards) (Palinska and Surosz 2014) and arguably too few strains are recorded by the Bacteria Code which are therefore unrepresentative of the true diversity of this group (Palinska and Surosz 2014).

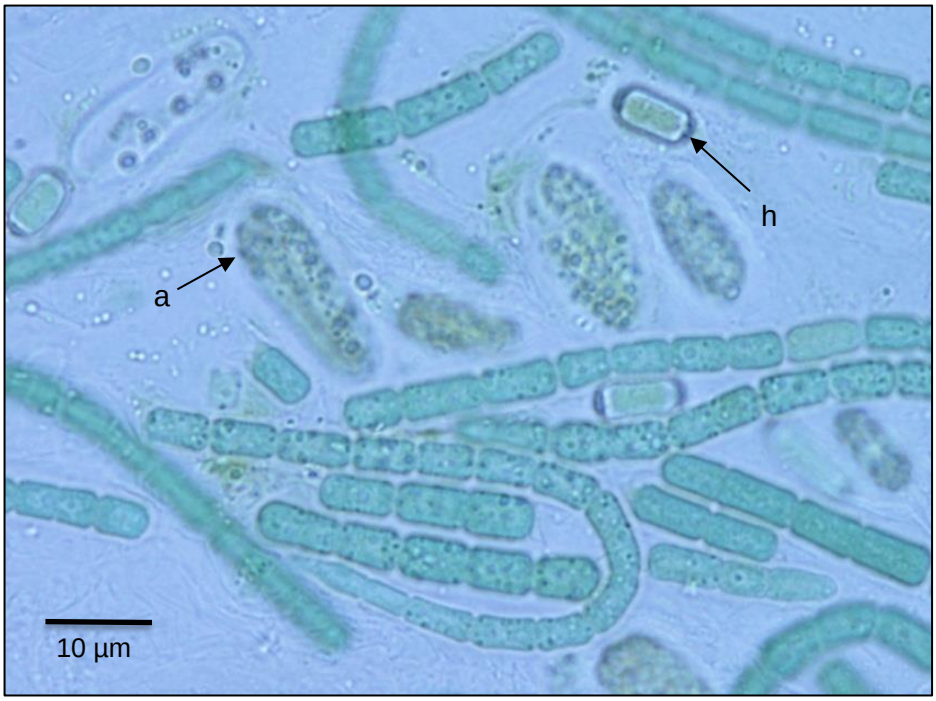


Figure 1.1. Micrograph of *Anabaena* (CCAP 1446/1A) cells at 800x magnification, a = akinetes and h = heterocyst (Micrograph by D.M. Hartnell).

1.4. Important bloom forming cyanobacteria

The bloom forming genera of cyanobacteria considered in this work are *Anabaena, Aphanizomenon, Microcystis* and *Oscillatoria.* These represent three of the four orders of cyanobacteria, the fourth and minor order being the Stigonematales. *Anabaena, Aphanizomenon, Microcystis* and *Oscillatoria* demonstrate a wide range of physical adaptations and produce an abundance of extracellular metabolites, generally termed secondary metabolites (Kurmayer *et al.* 2009) including some which are potent hepatotoxins (Carmichael 1992, Metcalf and Codd 2012). The biological functions of cyanobacterial metabolites are uncertain, but it has been reported that they may confer physiological advantages under fluctuating environmental or stress conditions thus facilitating differential survival of certain species and strains (Carey *et al.* 2012, O'Neil *et al.* 2012, Paerl and Paul 2012). Reference or type strains of *Anabaena, Aphanizomenon, Microcystis* and *Oscillatoria* are available from culture collections e.g. Culture Collection of Algae and Protozoa (CCAP, UK), Pasteur Culture Collection of Cyanobacteria (PCC, France), with accession supported by substantial characterisation data. These reference and type strains provide an invaluable resource for the robust and reproducible study of cyanobacteria; however, it has been suggested that laboratory-adapted reference strains may lose key physiological traits and not therefore be representative of cells in the environment (Lakeman *et al.* 2009). This subject will be further explored in the laboratory-based culture studies of this work, notwithstanding that point, the main characteristics for each of the genera considered are described below.

Anabaena (Figures 1.1. 1.2. & 1.3.) is a large genus of freshwater filamentous cyanobacteria in the order Nostocales, some species have been re-designated to the genus *Dolichospermum* (Komárek and Mareš 2012). Almost all members of the order reproduce via the germination of spore-like cells known as akinetes and fix atmospheric N_2 through heterocyst formation (Figure 1.1.) (Whitton 2011). *Anabaena* is globally distributed in freshwater lakes and reservoirs (Whitton 2011); some species are known to form symbiotic relationships with plants (Shi and Hal 1988). *Anabaena* sp. are reported to be one of the most prevalent bloom forming group (O'Neil *et al.* 2012) and is the most widespread toxic cyanobacterial genus worldwide (Islam and Beardall 2017). Certain species produce potent microcystins (MCs), anatoxin-a and anatoxin-a(S) or cylindrospermopsin (CYN), whilst others, generally strains of *A. circinalis*, produce a saxitoxin (STX) (Dittmann *et al.* 2012, Islam and Beardall 2017) and β-N-methylamino-L-alanine (BMAA) (Dittmann *et al.* 2012).

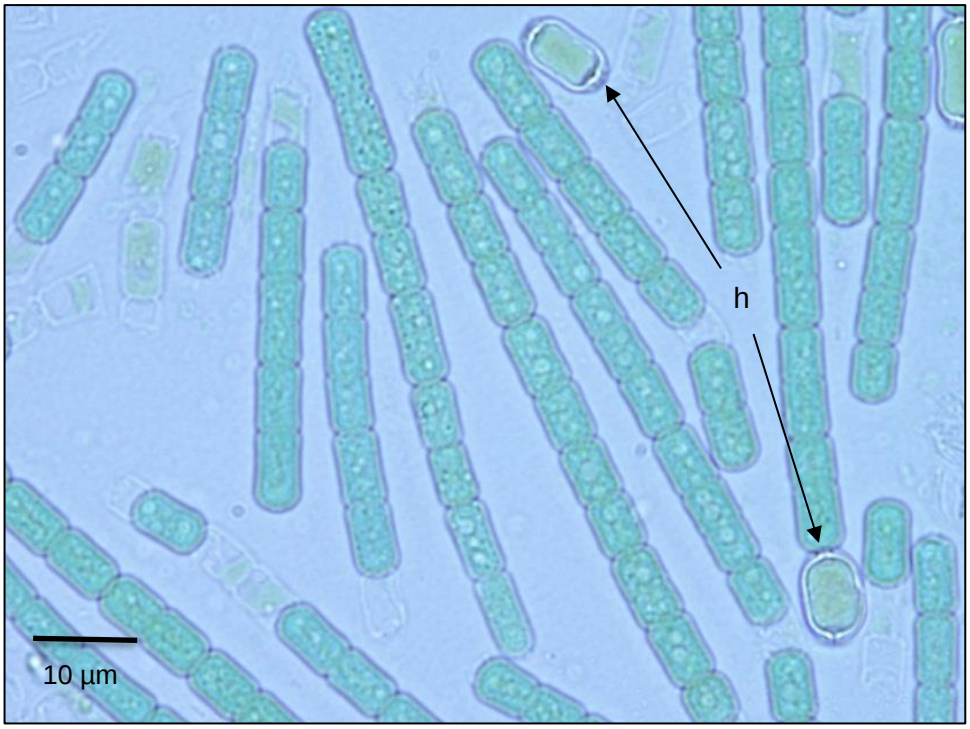


Figure 1.2. Micrograph of *Anabaena* (CCAP 1446/1A) cells at 800x magnification, h = heterocyst (Micrograph by D.M. Hartnell).

Aphanizomenon (Figure 1.3.) is a genus of motile filamentous cyanobacteria of the order Nostocales, found in predominantly in brackish, estuarine waters (O'Neil *et al.* 2012). *Aphanizomenon* can form large colonies and, similarly to *Anabaena*, generally reproduces via the formation of akinete cells (Whitton 2011). N_2 fixation is through the generation of heterocysts, and in addition many members of the genus *Aphanizomenon* modulate phosphate availability through the production of the hepatotoxic alkaloid, CYN. Whilst the biological role of CYN remains a subject of debate, it has been suggested that it functions as an allelopathic molecule inducing contiguous cyanobacteria and eukaryotic algae to increase phosphatase activity under phosphate limited conditions. This in turn increases soluble phosphate availability to *Aphanizomenon* (Bar-Yosef *et al.* 2010). *Aphanizomenon* has been described to be the dominant bloom forming genera in brackish waters where it outcompetes other cyanobacteria and phytoplankton (Bar-Yosef *et al.* 2010). Some species have also been associated with the production of anatoxin-a, STX, MCs and BMAA (Dittmann *et al.* 2012).

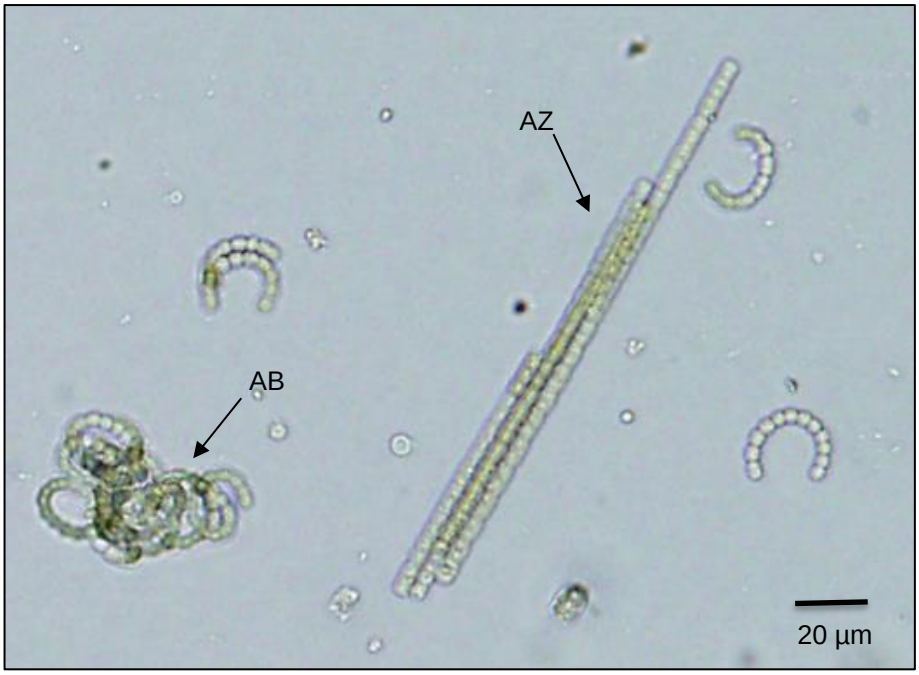


Figure 1.3. Micrograph of *Aphanizomenon* sp. (AZ) and *Anabaena* sp. (AB) from Longham Lakes, Dorset (200x magnification) (Micrograph by D.M. Hartnell).

Oscillatoria (Figure 1.4.) is a genus of motile filamentous, non-nitrogen fixing cyanobacteria of the order Oscillatoriales. Unlike *Anabaena* and *Aphanizomenon* reproduction is by fragmentation into hormogonia cells that disperse and grow into new filaments (Whitton 2011). CYN, anatoxin-*a* and MCYs production have been recorded (Dittmann *et al.* 2012). Furthermore, *Oscillatoria* spp. are also associated with the production of 2-methylisoborneol, the cause of odour and taste issues, and subsequent economic losses, in channel catfish aquaculture (Schrader *et al.* 1998). *Oscillatoria* have been reported as major components in persistent blooms in a number of countries, and are considered particularly problematic in some European, shallow freshwater lakes (Edwards *et al.* 1992, Hosper 1998).

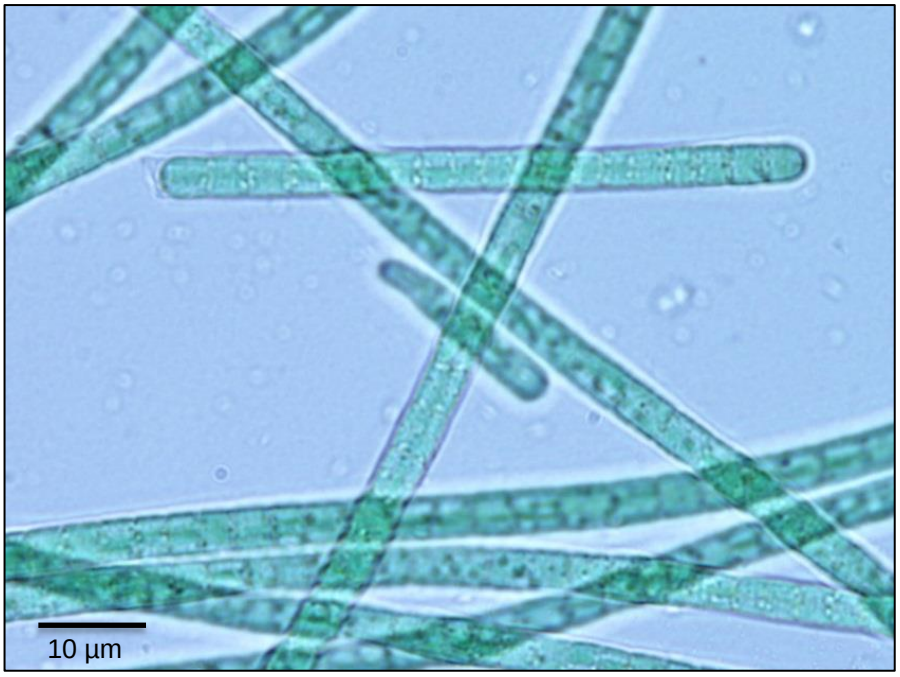


Figure 1.4. Micrograph of *Oscillatoria* (CCAP 1459/6) cells at 800x magnification (Micrograph by D.M. Hartnell).

The genus *Microcystis* (Figure 1.5.) is a colony forming freshwater cyanobacterium in the order Chroococcales and is ubiquitous in temperate and tropical regions. The genus is frequently considered problematic due predominantly to the production of a range of toxins, most notably microcystins, and its reported ability to exploit climate and nutrient perturbation (O'Neil *et al.* 2012, Harke *et al.* 2016). *Microcystis* species possess the ability to take advantage of, and proliferate under, changeable environmental conditions including wide (often higher) temperature ranges (20-30°C) and high pH (up to pH 12.0) conditions relative to eukaryotic phytoplankton (Sejnohova and Marsalek 2013). They also commonly exhibit the ability to control intracellular nutrient storage, meaning that they can favourably maximise utilisation of high phosphorous (P) to nitrogen (N) ratios. Furthermore, by regulating their buoyancy through the use of gas vesicles (and as a product of nutrient storage) they exhibit physiological advantages in conditions of higher irradiance (light) (Sejnohova and Marsalek 2013).

Certain strains of *Microcystis* can produce the hepatotoxins microcystins and the neurotoxins anatoxin-*a* and BMAA, however toxin production is not ubiquitous across the genera, and many strains isolated in the environment are reported to be non-toxic (Harke *et al.* 2016). Microcystin production is regulated by the expression of the *Mcy* gene cluster (Neilan *et al.* 2013). Reference microcystin *Mcy* gene complex 'knock out' strains are available removing toxicity (Dittmann *et al.* 1997) and which have

enabled a number of comparative studies on the relative, biological functionality of microcystins (Phelan and Downing 2011, Van de Waal *et al.* 2011, Zilliges *et al.* 2011) although to date no consensus on that issue has been reached.

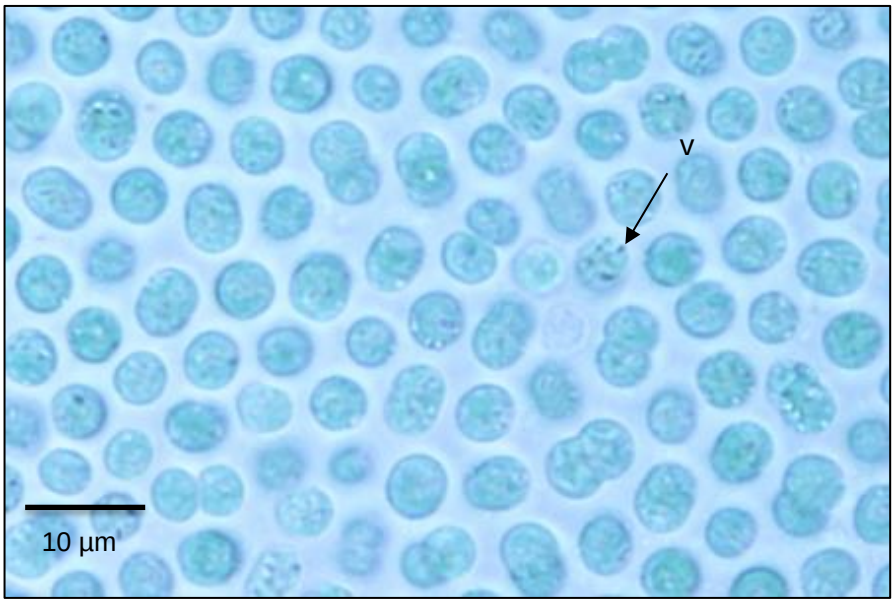


Figure 1.5. Micrograph of *Microcystis* cells (PCC 7806) at 800x magnification, vesicles are visible as dark areas on cell surface (v) (Micrograph by D.M. Hartnell).

All species attributed to *Microcystis* share several traits and it is considered a robust grouping, as defined by genetic and morphological criteria (Harke *et al.* 2016). Botanical nomenclature has described over 15 species of *Microcystis* according to cell diameter, pigment ratio, life cycles and the cell density, shape and mucilage content of colonies. Molecular sequencing of 16S rRNA has highlighted the close relatedness between *Microcystis* spp. and led to calls for several species to be unified within the rules of Bacterial nomenclature (Otsuka *et al.* 2001). However, by combining morphological and genetic techniques it is proposed *Microcystis* can divided into three clusters of 'morphospecies' based on cell size, mucilage, colony formation and cell arrangement (Sejnohova and Marsalek 2013).

Microcystis sp. are commonly encountered as macro-colonies forming surface blooms in freshwater bodies during the summer months. This is however just one phase of an annual cycle (Figure 1.6.). A proportion of *Microcystis* cells overwinter by sinking down to the upper layers of sediment. As conditions warm there is a reinvasion of the water column in spring, for summer blooms. The cycle is closed in autumn as a proportion sink back to the sediment to overwinter (Sejnohova and Marsalek 2013). Gas vesicles are present at least once in the annual growth cycle, these hollow protein structures regulate buoyancy in the water column allowing the

most advantageous exploitation of light and nutrients (Whitton 2011). Gas vesicles are refractile and under light microscopy appear as dark granulations on the *Microcystis* cells (Figure 1.5.). Colony formation is a ubiquitous survival strategy under favourable environmental conditions; however how and why colonies form exactly is unclear (Ma *et al.* 2014). Explanations for colony formation include, protection from zooplankton grazing, protection from viral or bacterial attack and enhanced buoyancy control. Notably *Microcystis* cells subject to serial subculture and considered laboratory adapted generally lose their colony forming characteristics (Ma *et al.* 2014, Hartnell *et al.* 2016).

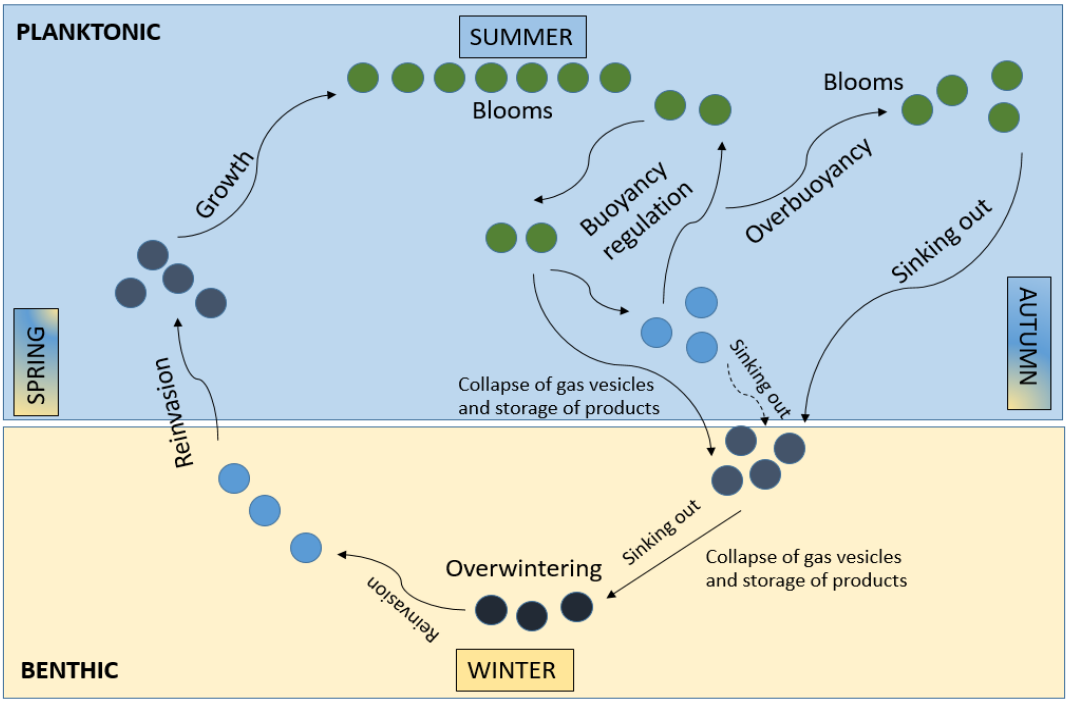


Figure 1.6. Annual cycle of *Microcystis* in a temperate regional lake (Reproduced with modifications from Sejnohova and Marsalek, 2012).

1.5. Cyanobacterial secondary metabolites and toxins

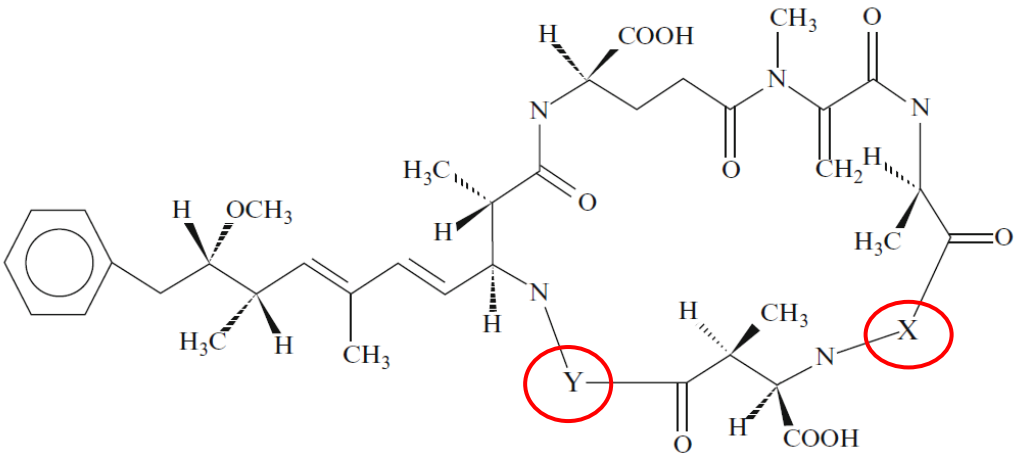
Cyanobacteria produce a wide range of chemically unique compounds, which it has been widely accepted are secondary metabolites; biological compounds which are not used by cells for primary metabolism e.g. for imminent survival, growth or reproduction (Carmichael 1992, Schatz *et al.* 2007). Examples of secondary metabolites produced by organisms include compounds that act as hormones, allelochemicals, antibiotics and toxins (Wiegand and Pflugmacher 2005, Holland and Kinnear, 2013). The role of secondary metabolites in the ecophysiology of cyanobacteria is unclear (Kaplan *et al.* 2012, Nielan *et al*. 2012). Recently reports indicating a core physiological role have been published suggesting that the maintenance of such metabolically expensive compounds over an extended evolutionary period is indicative of functionality within primary cellular metabolism (Nielan *et al*. 2012). Notwithstanding these considerations many metabolites of cyanobacteria are of special scientific interest both as potentially novel beneficial compounds (anti-microbial, cancer treatment, dietary supplements, etc.) and as potent toxins affecting human and ecosystem health (cyanotoxins).

Cyanotoxins are of concern to water quality managers and scientists of fresh and estuarine aquatic ecosystems, particularly those used for drinking water or recreational purposes (Carmichael 1992, Holland and Kinnear, 2013). Cyanotoxins include several classes of potent toxins (see section 1.4) (Dittmann *et al.* 2012). Structurally, the major cyanotoxins are varied, have a range of triggers for production and differ with respect to their modes of toxicity (Holland and Kinnear, 2013). They are classed into groups, characterised by their mode of toxicity on mammalian cells (Carmichael 1992, Codd 2000) and are named for the organism with which they were first associated. The chemical structures of the major cyanotoxins are illustrated in Figures 1.7. to 1.10. adapted from Metcalf and Codd (2012).

The following is a brief overview of the predominant groups with the common occurring and characterised cyanotoxins.

- Hepatotoxins (Figure 1.7. a. & b.) – inhibit enzyme activity in mammalian liver cells leading to loss of sinusoidal structure, haemorrhage, hemodynamic shock, and finally heart failure.
  Toxins include; microcystins, nodularins, cylindrospermopsin.

a



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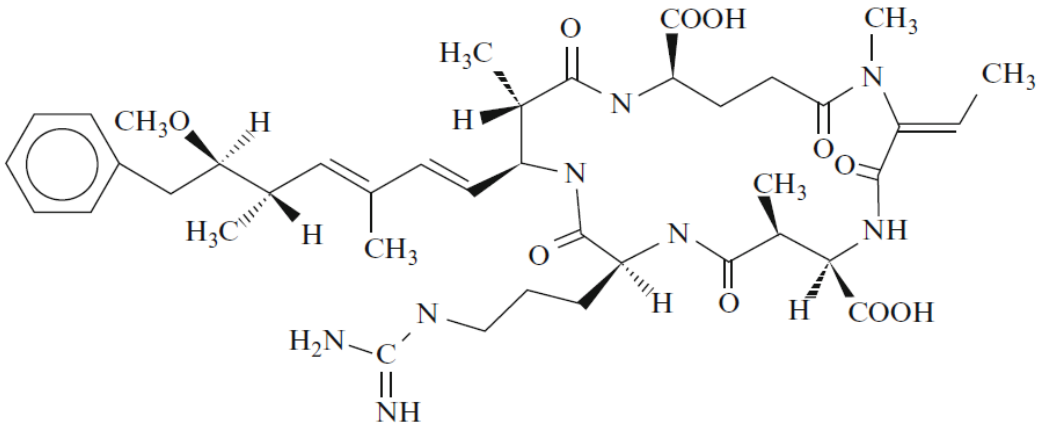


Figure 1.7. (a) Molecular structure of microcystin, X and Y (circled in red) are the sites of amino acid substitutions determining microcystin varients. (b) Molecular structure of nodularin, nodularins show less structual varibility than microcystins (Metcalf and Codd 2012).

- Neurotoxins (Figure 1.8.) – impede neuronal signals to avian and mammalian muscles, leading to respiratory arrest and death.
  Toxins include; anatoxin-*a*, saxitoxin β-N-methylamino-L-alanine (BMAA (an amino acid)).

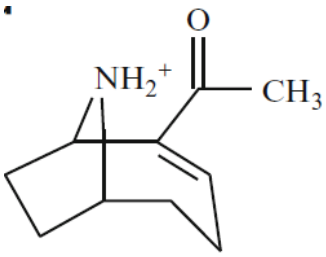


Figure 1.8. Molecular structure of anatoxin-*a* (Metcalf and Codd 2012).

- Cytotoxin (Figure 1.9.) – inhibits the synthesis of glutathione and proteins causing cytotoxic, hepatotoxic and neurotoxic effects in mammals. Toxin; cylindrospermopsin

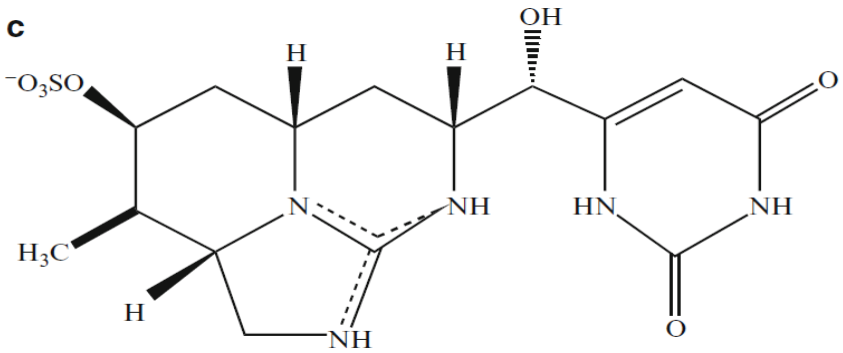


Figure 1.9. Molecular structure of cylindrospermopsin (Metcalf and Codd 2012).

- Dermatotoxins (Figure 1.10.) – causes blistering and inflammation of the skin in human swimmers.
  Toxins include; lynbyatoxin, aplysiatoxin

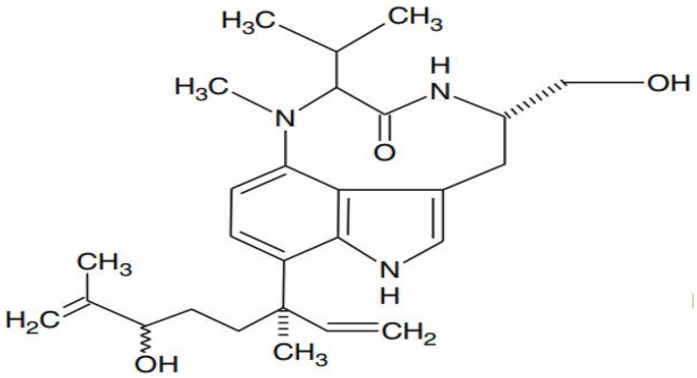


Figure 1.10. Molecular structure of lynbyatoxin (Metcalf and Codd 2012).

1.6. Existing control and mitigation measures for cyanobacterial blooms and toxins

The control and mitigation of potentially harmful cyanobacterial blooms varies considerably both between countries and between different types of water bodies based upon their intended use and management. In many countries, particularly, the More Economically Developed Countries (MECDs) mandatory or guideline standards for drinking waters and recreational waters following WHO recommendations have been developed. WHO 2 and 3 Alert Level Frameworks (ALFs), informed by cyanobacterial cell and/or chlorophyll concentrations have been widely adopted (Edition 2011, reviewed in Ibelings *et al*. 2016). These ALFs often trigger tiered mitigation plans allowing for proactive management of water bodies (Ibelings *et al.* 2016). Whilst these preventative strategies limit exposure to potentially toxic events, the presence of high levels of cyanobacterial cells does not automatically indicate harm, and the reliability with respect to toxicity has been questioned (Lawton and Codd 1991, WHO 2017). In a study on levels of microcystins in selected freshwater reservoirs in England, Turner and co-workers (2018b) showed that only 18% of samples exceeding alert framework levels contained microcystins above the WHO medium health criterion of 20µg L^{-1}.

A smaller number of countries have adopted the provisional WHO Guideline value for microcystin-LR of 1 µg L^{-1} in their national management plans for cyanotoxins in drinking waters (Ibelings *et al*. 2016), usually implementing restrictions following exceedances. However, there are a range of approaches in place with some countries using different advisory µg L^{-1} thresholds reflecting differing conditions or risk appetite, and others including microcystins other than microcystin-LR, reflective of the variability in toxin profiles. This lack of standardisation has the potential to create differential levels of public health protection.

In recent years the concept of risk assessment has been applied to the hazard of cyanobacteria in water courses. This approach is used increasingly in microbiological assessment of recreational water quality and is a requirement under the EU Bathing Water Directive, as amended (Directive 2006) where it facilitates prioritisation of resources and targeted action. Similarly, WHO Safe Water Plans for drinking waters advocate a comprehensive catchment level understanding to management (and prevent) exposure to risks (WHO and IWA 2010). Targeting regulatory activities towards mitigating the problem at source is gaining acceptance and likely to be the focus of future control measures but understanding of cyanobacteria toxicity remains

important for setting health-based targets and for safety verification (Ibelings *et al.* 2016).

1.7. Anthropogenic climate change

There is substantial evidence that the release of greenhouse gases from the burning of fossil fuels is the principal cause of current climate warming trends (Solomon *et al.* 2009, Moss *et al.* 2010). Since 1850, global estimates indicate that mean annual air temperatures have increased by 1ºC from 13.5ºC to 14.5ºC in 2010 (Moss *et al.* 2010, IPCC 2014). Over the same timeframe, atmospheric CO_2, a major greenhouse gas, has increased from ≈290ppt to ≈390ppt (Moss *et al.* 2010, IPCC 2014). There are many consequences of this temperature increase, in 2007 the International Panel on Climate Change (IPCC 2007) reported on anticipated impacts, including the following:

- A change in the distribution of rainfall, with more falling in wet seasons and less in dry seasons.
- An increase in the frequency of extreme weather events; storms, flooding and droughts.
- An increase in mean sea levels of ≈20cm from 1850, this is predominantly due to the thermal expansion of oceanic water mass (melting ice sheets are now contributing).
- A change in ecosystem community structure in marine, terrestrial and freshwaters, as species move poleward.

Anthropogenic climate change will potentially affect freshwater ecosystems (rivers, lakes etc.) more quickly as they are more susceptible to changes in temperature, precipitation and wind regimes than oceanic systems (Nickus *et al.* 2010). Climate change will also interact with other stressors, such as eutrophication, hydrological modifications, acidification and chemical pollution (Nickus *et al.* 2010). Consequently, an increase in cyanobacteria blooms has been widely reported to be linked to anthropogenic climate change (Pearl and Huisman 2008, Johnk, *et al.* 2008, Wagner and Adrian 2009, Sinden and Sinang 2016). Predicted effects of anthropogenic climate change on cyanobacteria are reported to be threefold (IPCC 2007). Firstly, rising water temperatures may provide favourable conditions for the proliferation of several genera of cyanobacterial over eukaryotic algae (Robarts and Zohary 1987). Secondly, the net effect of increased energy in the Earth's atmosphere will increase

the incidence of extreme weather events, such as extended droughts punctuated with increased flooding causing hydrological perturbations and nutrient flushing. Although drought conditions might be expected to increase residence times, Paerl and Paul (2012) contend that periodic flood events will also transport nutrients further downstream throughout watersheds, potentially also extending the size and range of the blooms. Finally, CO_2 is essential for the production of sugars via photosynthesis and enters the aquatic environment by direct absorption from the atmosphere and via dissolution in precipitation and is thus increasingly available to aquatic life.

How increased CO_2 levels will affect aquatic microbial community structure, particularly in relation to cyanobacterial toxin content, is poorly understood (Banares-Espana *et al.* 2006, Sandrini *et al.* 2014). Kardinaal and co-workers (2007) monitored the seasonal dynamics of toxic and non-toxic strains of *Microcystis* in three lakes in The Netherlands between April and November 2001. The authors reported succession of non-toxic strains in a deep stratified lake and toxic strain dominance in two shallow un-stratified lakes. The authors concluded that it was not clear how multi-variate factors affected the succession of toxic and non-toxic interaction and feedback. Paerl and Otten (2013) concluded that cyanobacteria can alter the phytoplankton community of a water body, this will cause a positive feedback with climate change selecting for toxic strains in blooms. Conversely, other workers have predicted increased atmospheric CO_2 will favour the dominance by non-toxic strains (Van de Waal *et al.* 2011, Zilliges *et al.* 2011).

Ultimately, the Earth-life system is driven and defined by climate. Climate determines the overall structure of ecosystems, drives the evolution of species and their life-histories. It also determines ocean circulation and subsequent nutrient cycling. From an anthropogenic point of view, it underlies many of our economic activities from agriculture to tourism. Therefore, a change in climate, such as we are currently experiencing, is a cause of concern for all.

1.8. Anthropogenic (cultural) eutrophication

The natural increase of inorganic plant nutrients in aquatic systems can cause the trophic state to shift towards increased microbial primary production in a process called eutrophication (Mason 2002). The release of nutrients e.g. nitrogen, phosphorous and carbon into aquatic systems from human activities e.g. sewage, agriculture and industry is termed cultural eutrophication (Smith *et al.* 1999). As some cyanobacteria can fix atmospheric N_2 (e.g. *Anabaena*) and others store P for later use (e.g. *Microcystis*) these taxa have a competitive advantage over P limited cyanobacteria and eukaryotic algae. Subsequently, the ratio of N to P has been viewed to be more important in the control of cyanobacterial blooms (Smith 1983, Mur *et al.* 1999). However, more recent research has challenged this dogma and has identified the importance of both N and P to algal community structure (Codd 2000, Wagner and Adrian 2009, Paerl *et al.* 2011a).

Modifications to the hydrology of water systems are twofold, either directly from human development or indirectly from global warming as described above. Freshwater is a valuable commodity and considerable infrastructure is dedicated for its supply and storage. In addition, substantial investment in infrastructure and remediation work is directed towards measures to counteract flood events. Human impacts, including water extraction, reservoir construction and sluices may increase residence times which favour cyanobacteria growth (Paerl and Huisman 2009). Wagner and Adrian (2009) concluded that periods of lake stratification over a three-week duration were the most significant factors in determining cyanobacteria dominance. Furthermore, the authors noted that absolute nutrient loading was a more important contributing factor favouring bloom formation than increased temperatures. In other metadata analyses researchers have established that nutrients were more significant than increased water temperatures (Paerl *et al.* 2011b, Rigosi *et al.* 2014). Consequently, it is generally considered that the impact of anthropogenic eutrophication was additive rather than synergistic in cyanobacterial bloom formation.

1.9. Aims and objectives

The aim of this study was to understand the relative importance and potential synergistic effects of factors associated with climate change (increased water temperature) and eutrophication (nutrient loading) which were examined by manipulating light, temperature and nutrients in batch cultures of strains of *Microcystis* spp. Total toxin concentrations and estimated toxin production per cell were measured using, IEC/ISO 17025 accredited ultra-performance liquid chromatography (UPLC) coupled to tandem mass spectrometry (MS/MS). Flow cytometry was used to monitor cyanobacteria cells during growth cycles, providing cell density, growth rate and cell size data.

Further to this, both UPLC MS/MS and flowcytometry will be applied to the environmental monitoring of two freshwater lakes in the South of England over at least a twelve-month period. Additionally, abiotic parameters (temperature, pH, turbidity, salinity) and biotic factors (chlorophyll-a and phycocyanin) were taken throughout the duration of the monitoring. The resultant data was examined for any causal relationships (additive, synergistic or antagonistic) between abiotic and biotic factors, and both total cyanobacteria and total and cellular toxin concentrations.

2. Methodological development: cell isolation, assessment of cell growth and toxin content

2.1. Introduction

To achieve the aims and objectives of this PhD project a series of laboratory and field techniques were developed. This chapter summarizes the research and development of the techniques and methods used:

- The isolation of cyanobacterial cells from the environment
- Batch culturing
- Cell abundance and size measured by flow cytometry
- Toxins qualified and quantified by liquid chromatography coupled to tandem mass spectrometry

Toxin analysis was completed in cooperation with the Centre for Environment, Fisheries and Aquaculture Science (Cefas), Weymouth, UK.

2.2 Isolating *Microcystis* cells from the natural environment

Up until the 1970's the isolation of cyanobacteria from their natural environment whether freshwater, marine or terrestrial was problematic, if not impossible (Rippka 1988). This was due in part to the apparent inability of unicellular cyanobacteria to grow on solid agar (Allen 1968) and the necessity to develop suitable growth media (Stainier 1971). However, there are now numerous cyanobacteria strains available from culture collections, with a considerable amount of published supporting data. Certain strains e.g. PCC 7806 (Pasteur Culture Collection of Cyanobacteria), PCC 7813 or PCC 7120 have been in culture for years or even decades, leading their ecological and physiological representativeness to be questioned (Lakeman *et al.* 2009, Alexova *et al.* 2011). For example, it has been observed that once maintained in culture *Microcystis* spp. will readily stop forming colonies (Ma *et al.* 2014, John Day (CCAP) pers. comm.).

Isolation and purification techniques fall into two broad categories; mechanical and physiological. Mechanical techniques include; micro-pipetting (Andersen and Kawachi 2005), serial dilutions (Yang *et al.* 2007), agar plating (Ferris *et al.* 1991) and cell sorting (Sieracki *et al.* 2005). Techniques that utilise the physiology of cyanobacteria include; differentiating buoyancy by centrifuge (Bloch and Blackburn 1996), nutrient gradients (Guillard 2005) and antibiotic resistance (Shirai *et al.* 1989). Most protocols are based on a combination of techniques and are generally ordered as; sampling, isolation, growth and (if necessary) purification. By the late 1980's over 500 cyanobacteria strains were successfully maintained as mono-cultures held by several collections globally (Rippka 1988).

The aim of this series of techniques in this research is to isolate freshwater cyanobacteria, in particular *Microcystis* spp., to provide non-laboratory adapted culture lines for ecological, toxicological and physiological experiments. Initially established techniques (as above) were used to create standardised protocols, but in addition a novel approach has been developed and published (Hartnell *et al.* 2016).

2.2.1. Isolation materials and methods

Four sites were sampled between 2014 and 2016 (Table 2.1.); where cyanobacteria blooms were visible as surface scums (Figure 2.1.) cells were collected in 1 L sterile Duran bottles. Otherwise, cells were collected by using a plankton net with a 50 µm mesh on a 5 m line. Netted phytoplankton cells were washed into a 30 mL polystyrene container. In the laboratory cyanobacteria were identified to the genus level by light microscopy. Samples containing *Microcystis* were placed in a north facing window at approximately 20°C for 7 days. Cells from samples containing *Microcystis* spp. were transferred to a 15 mL centrifuge tube and made up to 10 mL with ultra-pure water (Millipore, USA) and then centrifuged at 1000 x *g* for 20 minutes. The buoyant *Microcystis* cells on the surface of the centrifuge tube were removed by pipette to a new centrifuge tube, the process was repeated a further two times. The centrifugally washed cells (250 µL) were transferred to a 24 well plate prepared with four replicated 2 mL volumes of BG-11 (Stainier 1971) or Jaworski's Medium of media at 25, 50 & 75% concentrations (Figure 2.2.). The plates were placed in an incubator (Conviron, CMP6010) at 25 +/- 1°C, on 12-hour light/dark. Light was provided by a single 58-watt fluorescent tube (Luminex, cool white) at 9-16 µmol of photons m^{-2} s^{-1}. Isolate growth was assessed by eye. Wells in which bio-mass was seen to increase were transferred to 150 mL conical flask with 50 mL of BG-11 or Jaworski's Medium and stoppered, capped and returned to the incubator as previous light and temperature.

Figure 2.1. Surface scum from Microcystis bloom at Longham Lakes, Dorset, September 2015 (Photograph by D.M. Hartnell).

Table 2.1. Freshwater sites and cyanobacteria sampled.

Location	Grid reference	Surface Area	Use	Cyanobacteria sampled	Toxic (microcystins)
Blashford Lakes, Dorset, UK	SU 15247 07839	0.35 km²	Reservoir & nature reserve	*Anabaena* & *Microcystis*	Yes
Longham Lakes, Dorset, UK	SZ 06237 98079	0.86 km²	Emergency reservoir & angling	*Microcystis*	Yes
Roadford Lake, Devon, UK	SX 42747 92639	2.95 km²	Reservoir & nature reserve	*Anabaena* & *Woronichinia*	No
Cheshire pond (provided by Dr. A. Turner, Cefas)	Location withheld	Unknown	Ornamental pond	*Microcystis*	Yes

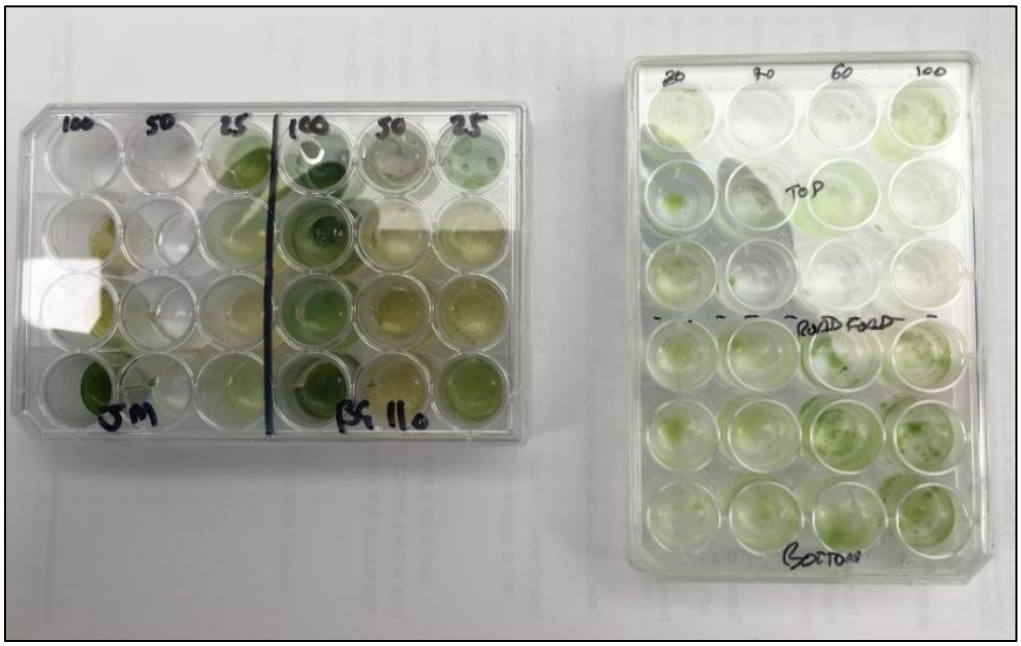


Figure 2.2. Two 24 well plates with dilution series of liquid media (BG-11 and Jaworski's), right samples of *Microcystis* from Longham Lakes and left *Anabaena* from Roadford Lake (Photograph by D.M. Hartnell).

2.2.2. Isolation results

An increase in biomass was observed visually and by light microscopy in the 24 well plates from the samples collected from Longham Lakes and Roadford Lake (Figure 2.2). The cells from Longham were identified as microcystin producing *Microcystis* (Toxins analysed as 2.5.), cells from Roadford were identified as *Anabaena* & *Woronichinia,* the *Anabaena* cells overgrew the *Woronichinia* cells, both proved negative for microcystin and nodularin toxins (Table 2.1.). Cells from Longham Lakes and a Cheshire pond (provided by A. Turner, Cefas) grew in BG-11 media and were transferred to 150 mL conical flasks.

Cells from a *Microcystis* sp. bloom collected from Blashford Lakes in September 2014, were found to be contaminated with *Synechococcus* sp. The co-culture was subjected to five additional purification techniques; spreading on agar plates, differential resistance/susceptibility to antibiotics, centrifuging in a sucrose gradient, susceptibility of *Synechococcus* cells to microcystin-LR and the use of an automated cell sorter (BD, FACSAria). None of the techniques applied was successful, with *Synechococcus* cells overgrowing the *Microcystis* cells in the cultures.

The solution was found in published eco-physiological data and the niche requirements of *Microcystis* and *Synechococcus.* Data from sampling >1000 lakes concluded *Microcystis* dominance is primarily driven by increased temperature, whereas *Synechococcus* dominance is driven by increased nutrients (Rigosi *et al.* 2014). Simple protocol manipulation of the temperature and nutrient regime allowed the establishment a uni-cellular culture of *Microcystis* (Hartnell *et al.* 2016) (Appendix I).

2.3. Batch culturing techniques

Batch cultures or limited volume cultures of microalgae are defined by a factor which finally limits cell growth. These factors can be: exhaustion of nutrient, gas supply, non-optimal pH, light via self-shading and/or autoinhibition (Fogg and Thake 1987). Batch cultures are characterised by a succession of five growth phases (Figure 2.3.). 1. Lag phase is a period after inoculation where no cell growth is recorded and is dependent on age of inoculation culture. Lag phase can be apparent where not all inoculation cells were viable or real where not all inoculation cells were in the correct condition to reproduce. 2. Exponential growth phase occurs when the conditions are ideal and maximum growth rate is reached.

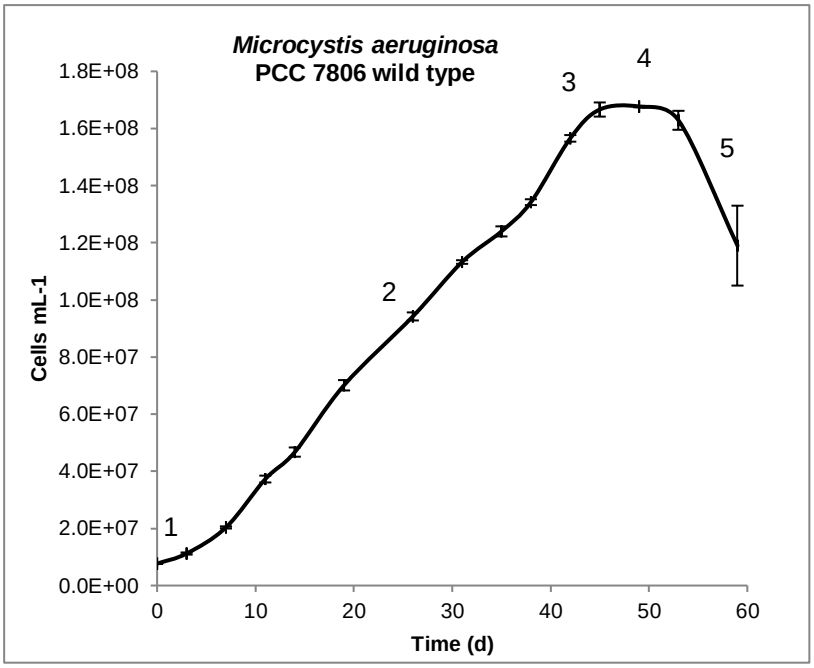


Figure 2.3. Characteristic pattern of growth phases in batch cultures of the cyanobacteria *Microcystis* (PCC 7806) in BG-11 media at 25°C. 1. Lag 2. Exponential 3. Declining 4. Stationary 5. Death. (error bars=SD, n=3).

3. Growth cannot be maintained indefinitely and will decline into a phase of declining growth. 4. Stationary growth phase where no growth is recorded, and cell density remains constant. 5. In death phase cell density decreases as cells are not replaced by reproduction. As there are many factors interacting in batch cultures some growth phases may be diminished and may be unapparent in some growth curves.

2.3.1. Batch culturing methods

Five strains from three genera of cyanobacteria were grown in batch cultures, three *Microcystis* strains PCC 7806 (wildtype and a *mcyB* gene-deficient strain), CCAP 1450/17 (Culture Collection of Algae and Protozoa), isolated at Bournemouth University (see Hartnell *et al.* 2016), *Anabaena inaequalis* (CCAP 1446/1A) and *Oscillatoria animalis* (CCAP 1459/6). Each strain was cultured in commercial BG-11 medium (Sigma-Aldrich C3061) prepared as per instructions with ultra-pure water (Millipore, USA) in 500 mL conical flasks with 200 mL of medium. The *mcyB* gene-deficient strain was produced by insertional mutagenesis of a chloramphenicol resistance gene cassette and maintained at 5 µg/ml chloramphenicol (Dittmann *et al.* 1997). All glassware was intensively washed and sealed with a foam bung and aluminium foil cap. The complete vessels and media were autoclaved to 121° C for 15 mins (Astell, AMP 430BT) prior to inoculation. All strain inoculations were standardised by measuring the chlorophyll *a* content of the inoculating culture (described below) and diluting all new cultures to approximately 0.4 mg/ml of chlorophyll *a,* this was supported by FCM counts for the *Microcystis* culture strains. Sampling and inoculation procedures were carried out in aseptic conditions in a laminar flow cabinet (Microflow, HLFWS). The culture vessels were placed in an incubator (Conviron CMP6010) at 25±1°C, on 12/12 light/dark with PAR at 16-20 µmol of photons m^{-2} s^{-1} (Figure 2.4.).

Figure 2.4. *Microcystis* cultures in 500 mL conical flasks with foam bungs and foil caps (Photograph by D.M. Hartnell).

Section 3 used the strains *Microcystis* strains PCC 7806 wild type and CCAP 1450/17, which were grown in triplicate batch cultures of 250 mL in 500 mL conical flasks. Culture conditions were as above, except that cultures were grown in an AlgaeTron AG230 incubator at three light levels. high, medium and low set on a 12/12 Light/Dark sine protocol on the light levels in Figure 2.5 and Table 2.2. in addition, BG-11 media was made up from stock solutions (Table 2.3.).

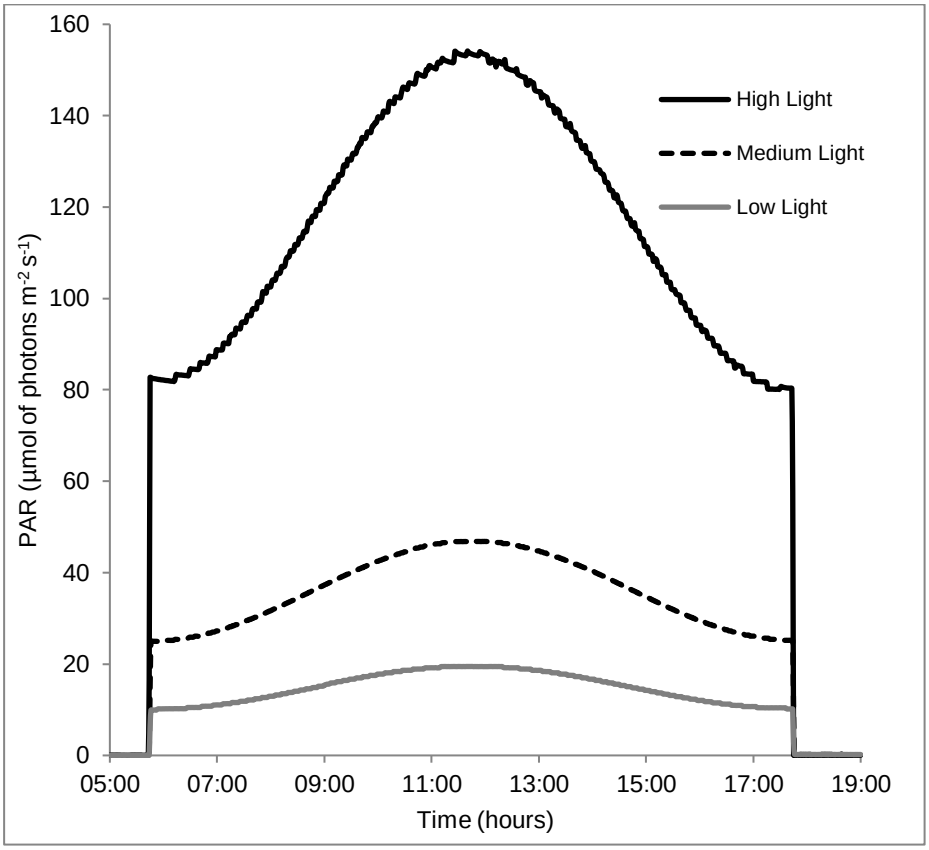


Figure 2.5. Three light levels in Algaetron AG230, high,medium & low set on a sine curve protocol over a 12 hour light period.

Table 2.2. Three light levels in Algaetron AG230, mean and range of Photosythetic Active Radiation measured in µmol of photons m^{-2} s^{-1} (PAR Scalar Irradiance sensor, Biospherical Instrument Inc.).

	Light Intensities		
	Average	High	Low
High Light	116.69	154	82
Medium Light	35.95	46	25
Low Light	14.81	20	10

Table 2.3. Recipe for BG-11 media from CCAP adapted from Stainier (1971).

Stock	Compound	Formula	Grams per Litre	Stock mL in 1 L	Final Concentration (mMol)
1	Sodium nitrate	$NaNO_3$	15	100	17.67
2	Di-potassium phosphate	K_2HPO_4	4	10	0.18
3	Magnesium sulphate heptahydrate	$MgSO_4.7H_2O$	7.5	10	0.3
4	Calcium chloride dihydrate	$CaCl_2.2H_2O$	3.6	10	0.25
5	Citric acid	$C_6H_8O_7$	0.6	10	0.029
6	Ammonium ferric citrate green	$C_6H_9FeNO_7$	0.6	10	0.03
7	Ethylenediaminetetraacetic acid	$C_{10}H_{16}N_2O_8$	0.1	10	0.0024
8	Sodium carbonate	Na_2CO_3	2	10	0.38
9	Boric acid	H_3BO_3	2.86	1	-
	Manganese (II) chloride tetrahydra	$MnCl_2.4H_2O$	1.81		-
	Zinc sulphate heptahydrate	$ZnSO_4.7H_2O$	0.22		-
	Sodium molybdate dihydrate	$Na2MoO_4.2H_2O$	0.39		-
	Copper (II) sulphate pentahydrate	$CuSO_4.5H_2O$	0.08		-
	Cobalt (II) nitrate hexahydrate	$Co(NO_3)_2.6H_2O$	0.05		-

2.3.2. Non Microcystis culturing and light intensities

All five strains, *Microcystis* PCC 7806, wild type and mutant, *Microcystis* CCAP 1450/17, *Anabaena* CCAP 1446/1A and *Oscillatoria* CCAP 1459/6 grew under culture condition as monitored by photosynthetic pigment extraction (chlorophyll *a*) (Figure 2.6). Flow cytometry data (Section 2.4.) for the *Microcystis* strains demonstrated all batch culture growth phases, however, data from filamentous strains (i.e. *Anabaena* and *Oscillatoria*) were inconclusive as counts were irregular (data not shown).

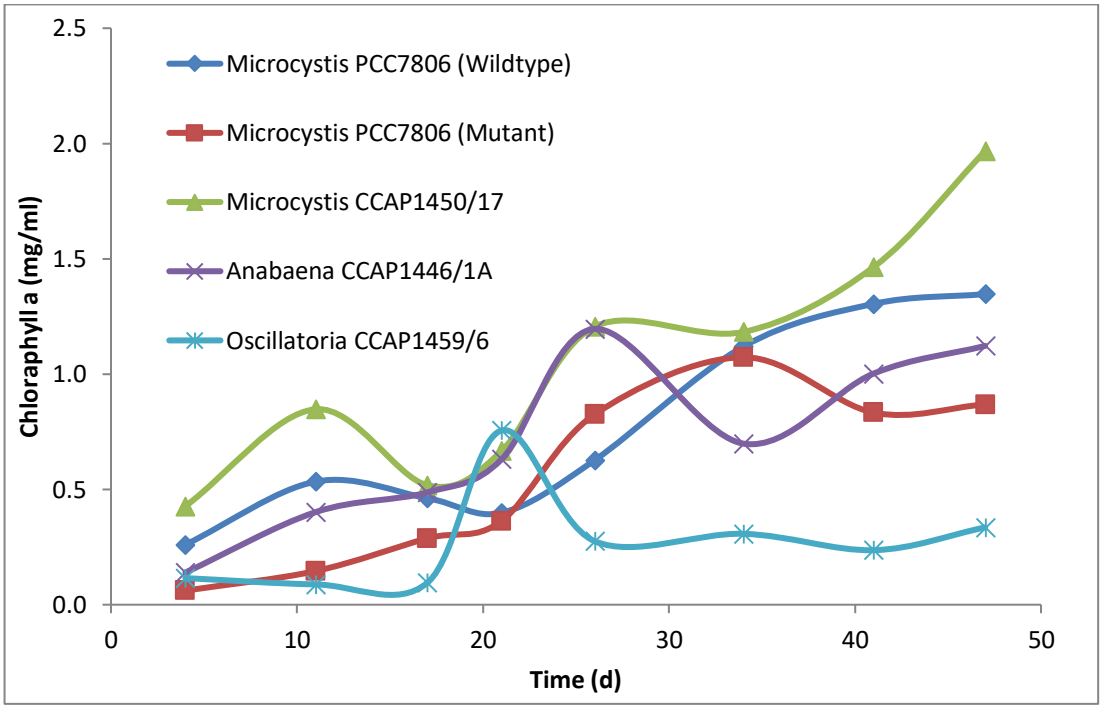


Figure 2.6. Chlorophyll a concentrations from batch cultures of Microcystis PCC 7806, wild type and mcyB gene-deficiant, CCAP 1450/17, Anabaena inaequalis (CCAP 1446/1A) and Oscillatoria animalis (CCAP 1459/6), grown at 25°C in 16-20 µmol of photons m-2 s-1 of light over a 50 day period.

Growth of *Microcystis* PCC 7806 wildtype growth under three light levels at 25°C, were successfully monitored by flow cytometry of all replicates (Section 2.4.). Selected light levels demonstrated different growth characteristics on the cultures (Figure 2.7). High light caused rapid growth followed by an extended stationary phase. Initial growth in medium light was similar to high light, however, growth continued to a higher cell density indicating optimum light intensity. The cell densities of low light intensity were the lowest, indicating light limited growth.

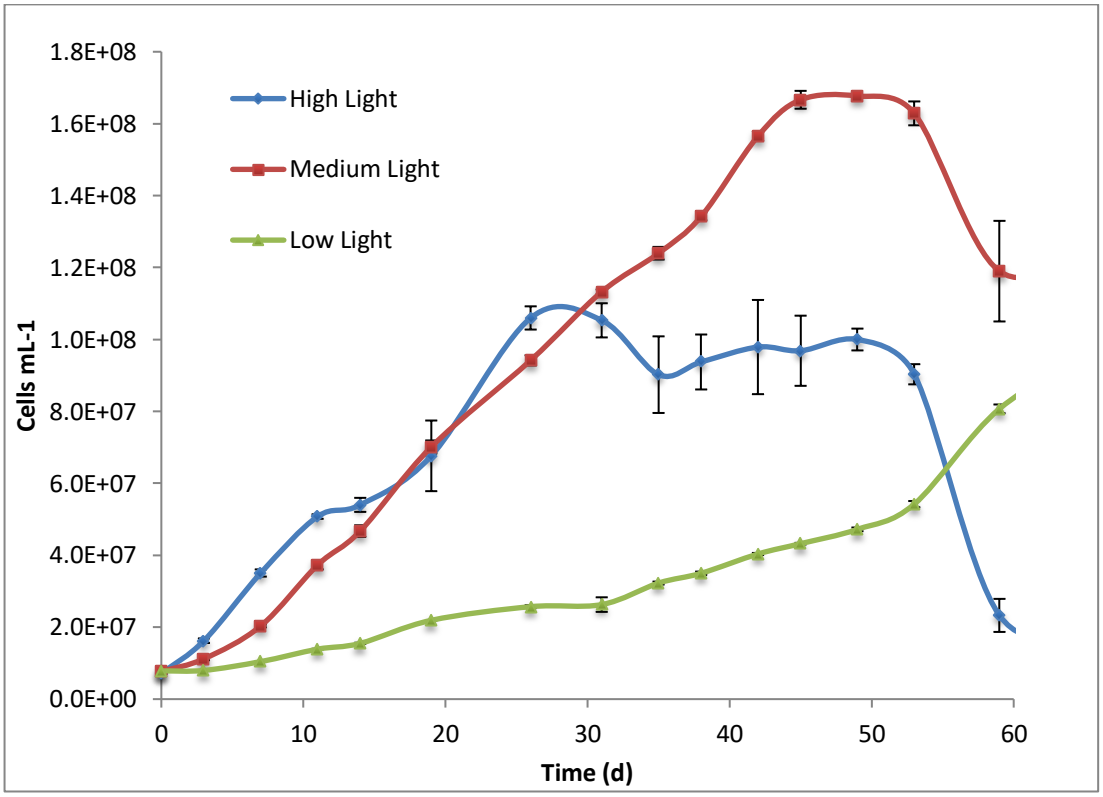


Figure 2.7. Cell density counts from flow cytometry (Section 2.4.) of *Microcystis* PCC 7806 wild type grown at three light intensities on sine 12/12/light/dark protocol at 25ºC (error bars=SD, n=3).

2.4. Cell density and cell size measured by flow cytometry and photosynthetic pigment extraction

2.4.1. The Principles and applications of flow cytometry

Flow cytometry (FCM) allows rapid counting and assessment of single cells in microbial populations. FCM was developed 50 years ago for research in immunology and cell biology, helping our understanding of diseases such as AIDS and some cancers (Robinson and Roederer 2016). However, the application of FCM to aquatic microbiology including phytoplankton research is a relatively recent application for this technology but a rapidly growing field (Wang *et al.* 2010).

In FCM particles and cells in suspension fluid are hydro-dynamically aligned in a narrow stream. The particles are intercepted by beam of light from a laser or lasers. The intensity of light scatter is dependent on the refractive index, size and shape of the particle. Additionally, cell pigments with corresponding absorption wavelengths to the light source will auto-fluorescence at a higher wavelength. In the case of phytoplankton, green chlorophyll is excited by a blue laser and emits red auto-fluorescence light. Forward scatter (FSC) light detectors are positioned at 180° and are related to size, side scatter (SSC) light detectors are positioned at 90°. SSC light is also related to size but indicates something of the cells surface and internal structure, often referred to as 'granularity'. Auto-fluorescence is collected by a series of lenses and filters (photomultipliers) to remove the excitation light and allow the recording of multiple emission wavelengths. With additional lasers at differing wavelengths the number of fluorescence wavelengths detected can be increased. The analogue signals from the photo-detectors are converted to digital data, to avoid saturated signals thresholds can be set on the detectors to remove particles that are not of interest. The data is processed in a computer and assigned arbitrary units (au), then presented as mono-parametric histograms and/or bi-parametric cytograms (Figure 2.8. & 2.10.). Data can be further processed by gating the signal to discriminate specific cell populations in the sample analysed. Measurements can be made at between 10 and 10,000 events per second; the event rate can be manipulated by adjusting the flow rate or core size (fluidics) and/or diluting or concentrating the sample. In recent years the technology of FCM has been applied to the field of phycology, both in culture and the natural environment (Marie *et al.* 2005).

2.4.2. Laboratory methods for flow cytometry

Cell density, growth rate, cell size and auto-fluorescence of laboratory batch cultures were monitored by FCM (Accuri, C6) at 3 and 4 day intervals. Subsamples of cultures (100-250 µL) were taken and diluted with ultra-pure water to a factor of 1 in 2 or 1 in 5, depending on growth phase. *Microcystis* strains were counted on the default slow fluidics (10 µm core and 14 µL/min flow rate) for 2 minutes, with a Forward scatter (FSC) threshold signal was set at 80,000 arbitrary units (au). The signal was gated with a first gate on far-red auto-fluorescence (675nm +/- 12nm (FL4)) between 55,000 and 950,000 au to distinguish cells containing phycocyanin. A second gate on red auto-fluorescence (670nm LP (FL3)) was set between 60,000 and 400,000 au to distinguish cells containing chlorophyll (Figure 2.7. & 2.9.). *Anabaena* and *Oscillatoria* cultures were counted on fast fluidics, 22 µm core and 660 µL/min flow rate, for 2 mins with a FSC threshold set at 40,000 au, no gate was applied.

The cell densities per millilitre of culture were calculated using the formula:

$$\text{cells per ml (C)} = \frac{\text{count}}{\text{volume (µL)}} \text{ x dilution factor x } 1000$$

Relative growth rate (µ) was calculated using the formulae (Fogg and Thake 1987):

$$\mu = \frac{\ln C1 - \ln C2}{t\,(d)}$$

Where ln = Natural log, C = Cell count at time intervals 1 & 2 and t (d) = Time in days.

Cell size was determined using calibration beads (Sphero, PPS-6K) in sizes 2, 3.4, 5.1, 7.4, 10.5 & 14.5 µm. The individual sizes were run through the flow cytometer set on slow fluidics for 2 minutes and the FSC signal recorded. All calibration bead sizes were combined in equal volumes and run through the flow cytometer on the same settings. Mean FSC au were used to construct a calibration curve to convert to cell size (µm).

2.4.3. Environmental methods for flow cytometry

Environmental samples from freshwater reservoirs were aliquoted (≈0.5 mL) into a sample tube and homogenised by vortex. A custom fluidic setting was selected of 25 µm core and 100 µL/min. Following Chapman (2016) unicellular *Microcystis*-like cells were resolved by size and three auto-florescence channels. The side scatter (SSC) signal was gated between 75,000 to 700,000 au, then yellow auto-florescence (FL2) gated at 40 to 2,000 au. Red auto-florescence (FL3) was used as an indicator of chlorophyll and gated between 180,000 to 2,200,000 au and finally far-red auto-florescence was used as an indicator of phycocyanin and gated between 11,000 to 500,000 au.

2.4.4. Photosynthetic pigment extraction materials and methods

For batch cultures triplicate 250 µL aliquots were taken for chlorophyll *a* extraction and filtered using a ø25mm 1.2 µm glass microfiber filters (Whatman, GF/C). Individual filter papers were placed in 15 mL plastic universal container and frozen at -80°C, on analysis samples were defrosted and dissolved in 10mL of analytical grade methanol (100% v/v) (ThermoFisher, UK) for 24±2 hours to facilitate extraction of chlorophyll *a*. Samples were analysed using an absorption spectrometer (Varian Cary 50 probe) at wavelengths of 652.4 and 665.2nm. Chlorophyll *a* (C_a) concentrations (µg/mL) were calculated according to the following equation (Wellburn 1994):

$$C_a = 16.72A_{665.2} - 9.16A_{652.4}$$

The mass of chlorophyll *a* per cell was calculated by dividing chlorophyll *a* concentration by cell density and multiplying by 10^6 to calculate the mass per cell in 1×10^{-15} gram (femtograms (fg)).

In later temperature and nutrient experiments (Section 3.), triplicate 250 µL samples were processed and preserved as above. However, samples were extracted in both 10mL of 100% methanol and 80% methanol with ultra-pure 20% H_2O (80/20 v/v). The results from 100% methanol and 80% methanol extractions were used to construct a calibration curve to correct chlorophyll *a* concentration in µg/mL.

Environmental samples of various volumes were filtered and preserved at -80°C, photosynthetic pigments were extracted using 10mL of 80% methanol and ultra-pure 20% H_2O (80/20 v/v) over 24±2 hours. Light absorption Samples were analysed at wavelengths of 470, 652.4 and 665.2nm, chlorophyll *a* (C_a) concentrations calculated as above. Chlorophyll *b* (C_b) concentrations were calculated according to the following equation (Wellburn 1994):

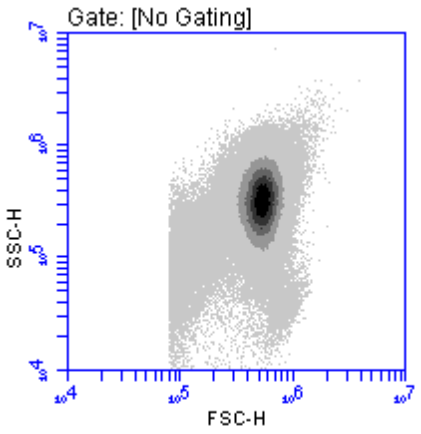
$$C_b = 34.09A_{662.4} - 15.28A_{665.2}$$

Total carotenoids (C_{x+c}) were calculated using the equation (Wellburn 1994):

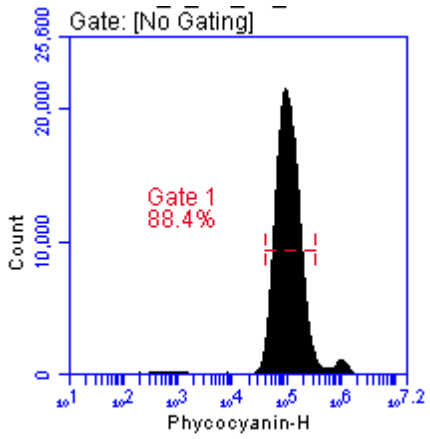
$$C_{x+c} = \frac{1000A_{470} - 1.63C_a - 104.96C_b}{221}$$

2.4.5. Flow cytometry results

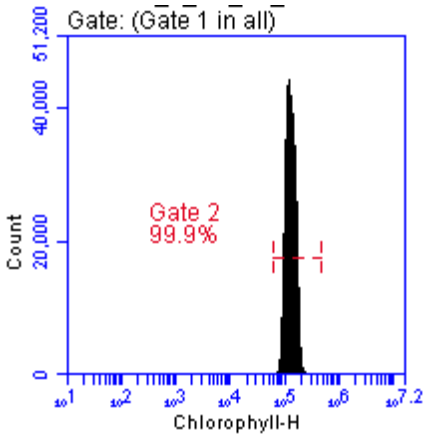
In the laboratory, batch cultured cells with high photosynthetic pigment content were distinguished using far-red and red signals as indicators of phycocyanin and chlorophyll respectively. Total flow cytometric count data were reduced from 13.5 million to 11.9 million per mL in batch cultures in an exponential growth phase (Figure 2.8.).



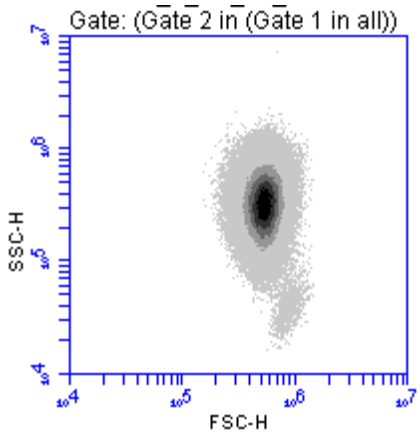
A. Un-gated cytogram of batch cultured *Microcystis* PCC7806 wildtype cells from side scatter (SSC) and forward scatter (FSC) signals. Count 13,500,000 mL-1



B. Histogram of Phycocyanin auto-florescence (far-red, FL4) signal to discriminate pigment productive *Microcystis* cells. Size count 12,000,000 mL-1



C. Histogram of Chlorophyll auto-florescence (red, FL3) signal to discriminate pigment productive *Microcystis* cells. Size count 11,900,000 mL-1



D. Phycocyanin & chlorophyll gated cytogram of batch cultured *Microcystis* PCC7806 wildtype cells from side scatter (SSC) and forward scatter (FSC) signals. Cell count 11,900,000 mL-1

Figure 2.8. Series of cytograms and histograms from flow cytometry demostrating the discrimination and counting of unicellullar *Microcystis* cells from batch cultures of PCC7806 wildtype.

The mean forward scatter (FSC) from six sizes of calibration beads (2, 3.4, 5.1, 7.4, 10.5 & 14.5 µm) were used to construct a calibration curve to calculate cell size on slow fluidics (Figure 2.9.) The trendline equations were $y = 195473x - 152330$, $R^2 = 0.9834$, therefore the mean FSC from Figure 2.8.D of 543,787 equals a mean spherical cell size of 3.56 µm.

$$3.56 = \frac{543{,}787 + 152{,}330}{195{,}473}$$

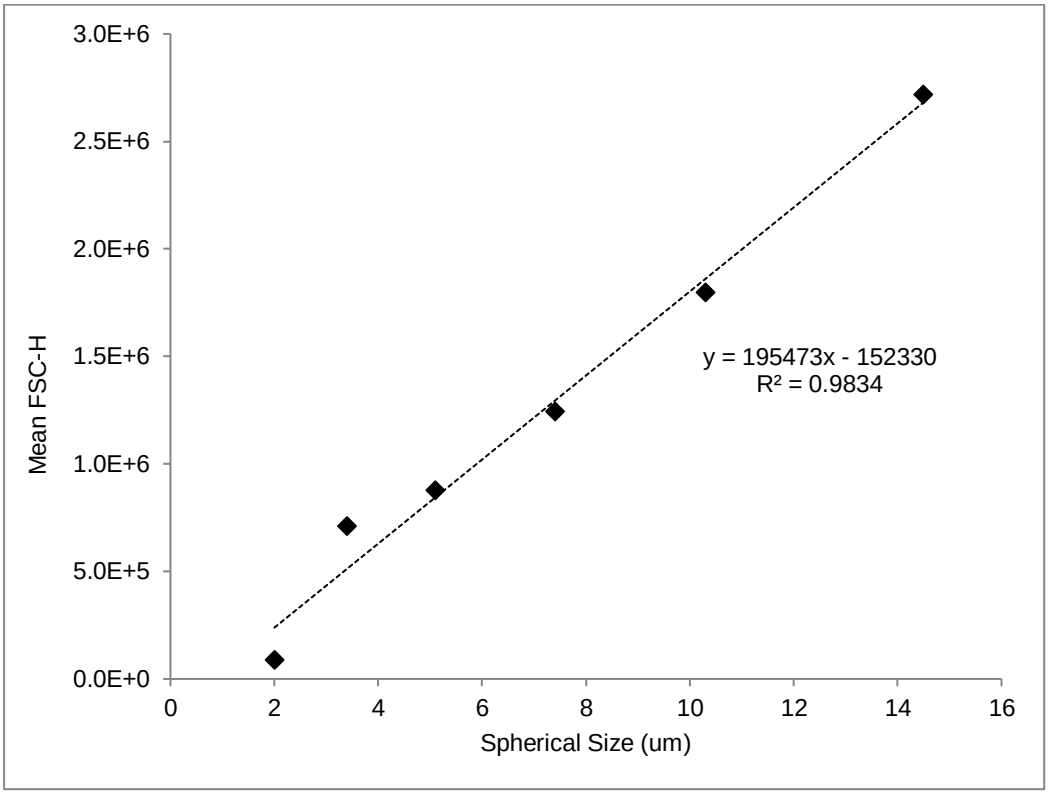
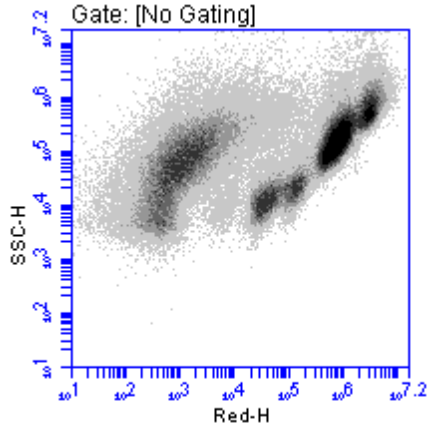
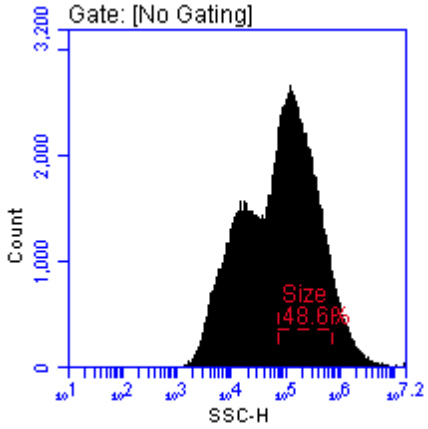


Figure 2.9. Calibration curve constructed from mean forward scatter (FSC) signal from spherical calibration beads. Trendline equations $y = 195473x - 152330$, $R^2 = 0.9834$.

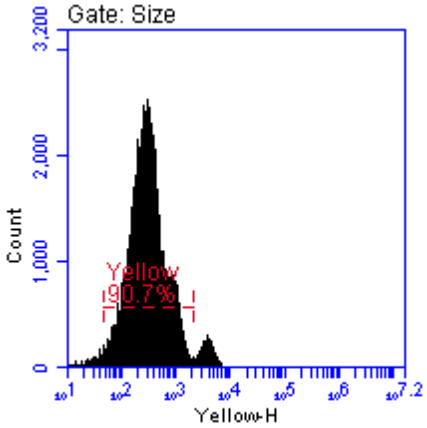
In environmental freshwater samples *Microcystis* like cells were distinguished using size (SSC), yellow, red and far-red auto-florescence. Total flow cytometric count data were reduced from 230,000 to 51,250 per mL in samples from a reservoir collected in mid-July 2016 (Figure 2.10.).



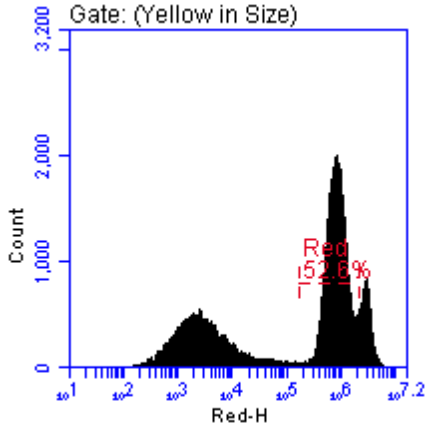
A. Un-gated cytogram of freshwater sample from side scatter (SSC) and red (FL3) auto-florescence signals. Count 230,000 mL-1



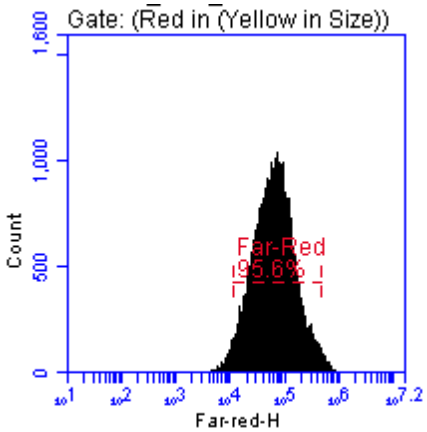
B. Histogram of side scatter (SSC) signal to discriminate size of *Microcystis* like cells. Size count 112,000 mL-1



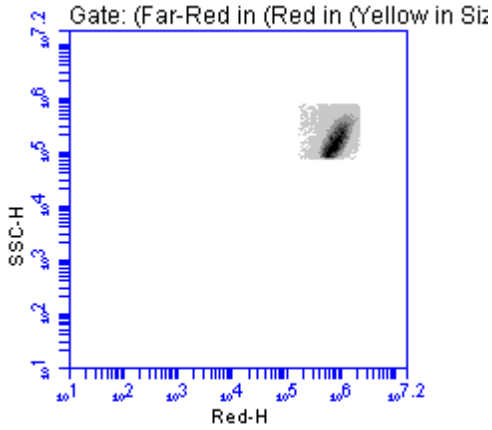
C. Histogram of size gated yellow (FL2) auto-florescence signal to discriminate *Microcystis* like cells. Yellow count 101,000 mL-1



D. Histogram of size & yellow gated red (FL3) auto-florescence signal to discriminate chlorophyll in *Microcystis* like cells. Red count 53,250 mL-1



E. Histogram of size, yellow & red gated far-red (FL4) auto-florescence signal to discriminate phycocyanin in *Microcystis* like cells. Far-red count 51,250 mL-1



F. Size, yellow, red & far-red gated cytogram of freshwater sample from side scatter (SSC) and red (FL3) auto-florescence signals. *Microcystis* like cell count 51,250 mL-1

Figure 2.10. Series of cytograms and histograms from flow cytometry demostrating the discrimination of unicellular *Microcystis* like cells from environmental sample collected from a freshwater reservoir.

2.4.6. Photosynthetic pigment extraction results

Chlorophyll *a* extracted from batch cultures of *Microcystis* PCC 7806 grown at three light levels generally displayed trends of growth phases, however, there was a drop in chlorophyll *a* extracted on day 45 across all light intensities (Figure 2.11.).

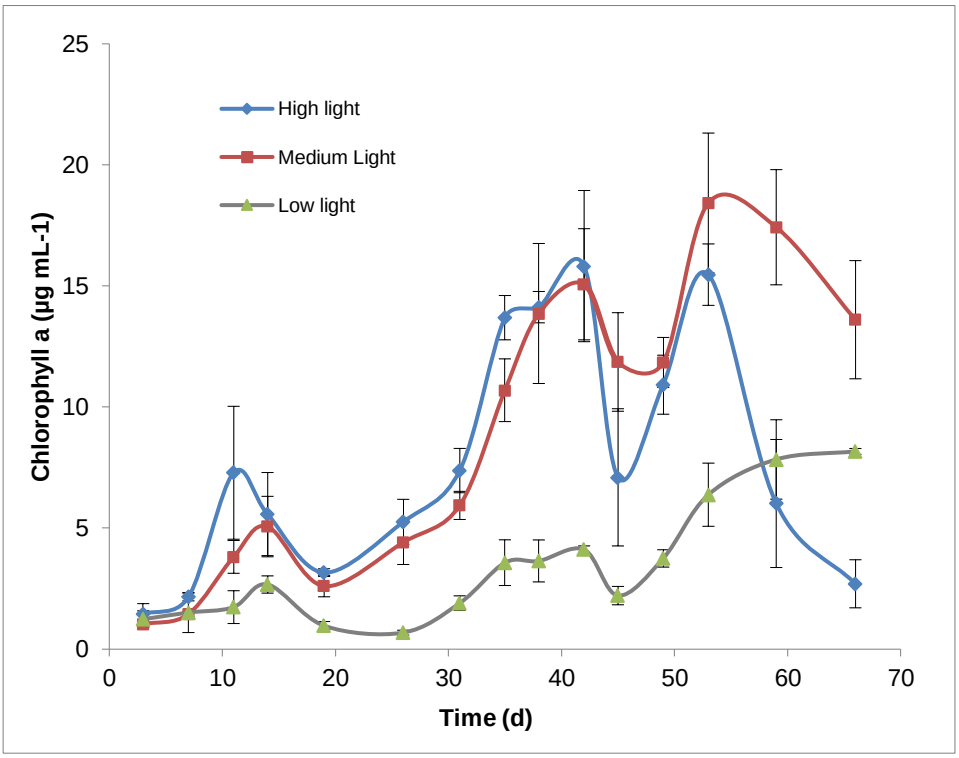


Figure 2.11. Concentration of chloropyll *a* from cultures of *Microcystis* PCC7806 wildtype grown under three light intensities sine wave 12/12/light/dark protocol at 25ºC (error bars=SD, n=3).

The data from calculated chlorophyll *a* concentrations determined from 100% and 80% methanol extractions were explored and found to be non-parametric. The two extraction methods were found to be significantly different using a Mann-Whitney test (*U=44763, p = .001*), results are presented on a box and whisker plot (Figure 2.12.).

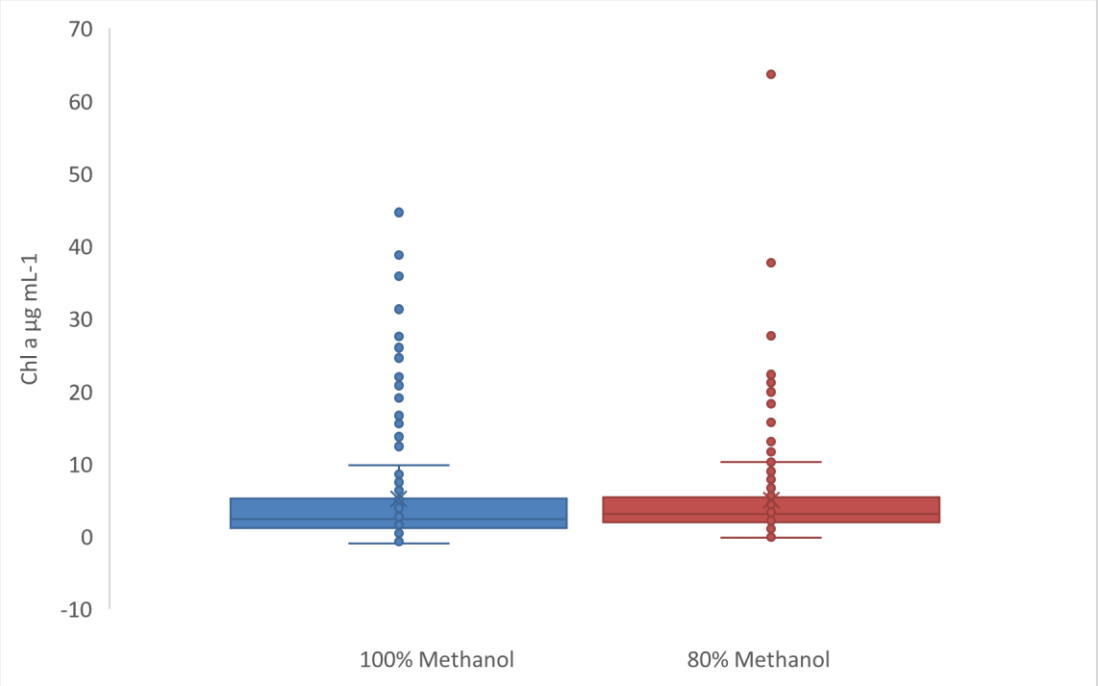


Figure 2.12. Box and whister plot of the comparison of 100% and 80% methanol extractions.

The 80% methanol results were not significantly different from the 100% methanol extraction method using a Mann-Whitney test (*U=49248, p = .174*).

2.5. Toxin qualification and quantification by liquid chromatography coupled to tandem mass spectrometry

2.5.1. The principles and applications of liquid chromatography and mass spectrometry

Liquid chromatography allows the separation of a mixture of chemicals into their constitute compounds (analytes). These analytes are separated by passing a sample through a chromatographic column containing solid silica or polymer based particles coated with an absorbent material (stationary phase). The compounds, solubilised into a liquid sample, are injected into a continuous flow of solvent(s) (mobile phase) which is pumped through the column at high pressure, and enter the column where they partition between the two phases. Analytes are separated by their relative affiliation to both the stationary and mobile phases. This separation tool is termed High Performance Liquid Chromatography (HPLC). The solvent composition of the mobile phase can be varied over time following a sample injection, termed gradient separation, to optimise the separation of analytes present in the original sample solution. Upon eluting from the column, separated compounds, such as microcystins (MCs), are measured using some form of chemical detector, for example a photodiode array detector (PDA) operating in visible or ultra-violet light wavelengths. Compound qualification is determined from the chromatographic retention time, specifically the time for the compound to elute through the column, be detected and qualified.

against external calibration standards containing known concentrations of target analytes. The method is not a ‘turn-key’ ‘push the button’ process, as system pressure, mobile phase solvents and stationary phase chemistries must be researched and developed to the compound(s) under analysis (McMaster 2007). Notwithstanding this HPLC has been a mainstream physiochemical method for the detection of cyanobacterial toxins for over 20 years (Lawton *et al.* 1994), with the versatility to measure microcystins, nodularins (NODs), cylindrospermopsin, anatoxin-*a* and saxitoxins (Metcalf and Codd 2012). The time required to separate compounds by liquid chromatography can be decreased by the application of instrumental systems providing even higher pressures, resulting in the use of rapid ultra-high-performance liquid chromatography (UHPLC) separation techniques.

Compound quantification and qualification can be further refined to a more specific approach, through the coupling of a liquid chromatograph to a mass spectrometer (MS). These detectors enable the determination of the mass-to-charge ratio of ions

formed from analytes, facilitating the measurement of the molecular weight of the target compounds. In triple quadrupole or tandem mass spectrometry (MS/MS) compounds are ionised and separated by mass-to-charge-ratio in the first quadrupole (MS1), targeted compounds are fragmented in a collision cell (MS2) producing daughter ions which are then separated by their mass-to-charge-ratio (MS3). As the ions formed from compounds of interest fragment in predictable ways, the transitions from parent to multiple daughter ions (typically two) provides a good level of specificity to those compounds. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is an extremely powerful tool for the detection and quantitation of variants of microcystin, which range in relative atomic mass from 800 to 1200 g/mol (Metcalf and Codd 2012). Specifically, qualification is achieved through both the comparison of two parent to daughter transitions, termed Selected Reaction Monitoring (SRM) transitions (also known as Multiple Reaction Monitoring; MRM), in samples against those determined in known standard solutions, together with the comparison of chromatographic elution retention times. An analyte peak showing the same two SRM transitions and the same retention time as a peak determined in a reference standard, thereby provides a more specific qualification than any conventional detector such as a PDA. Quantification can again be conducted through the comparison of the most sensitive chromatographic SRM peak areas against those generated in multi-level external calibration standards, with qualification coming from detection of the second SRM peak at the same retention time.

2.5.2. Liquid chromatography and mass spectrometry materials and methods

The method used in this research had the capacity to detect, qualify and quantify twelve variants of microcystin and nodularin (structurally similar to microcystin); specifically MC-LR, Asp^3 MC-LR, Dha^7 MC-LR, MC-RR, MC-LA, MC-LY, MC-LF, MC-LW, MC-YR, MC-WR, MC-HilR and MC-HtyR. All toxin analysis was carried out by ultra-performance liquid chromatography (Acquity standard class UHPLC, Waters, Manchester, UK) coupled to a tandem quadrupole mass spectrometer (Waters, Xevo TQ).

All chemicals were LC-MS reagent grade where possible, sample preparation reagents were HPLC grade. Certified reference materials (CRM) of toxins (MC-RR, MC-LA, MC-LY, MC-LF, MC-LW, MC-YR, MC-WR, MC-HilR, MC-HtyR, MC-LR, Asp^3 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). Calibration standards were prepared in 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 toxin. A seven-level suite of working calibration standards was subsequently prepared through serial dilution of the mixed stock solution using 50% methanol as the solvent, resulting in a calibration range between 0.33 ng/mL to 327 ng/mL. A reference standard of RM-BGA was prepared by extracting 280 mg with 28.0 mL 50% aqueous methanol + 0.1% acetic acid, prior to centrifugation (4,500 rpm; 10 min) and the supernatant collected prior to 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 +60ºC, with samples held in the sample manager at +4ºC. 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 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, a quick rise to 95% B1 and 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 60ºC and followed by a second at 30ºC. New columns were conditioned as per the manufacturer's instructions.

The Waters Xevo TQ tune parameters were as follows: 150ºC source temperature, 600ºC 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 optimized following experiments infusing pure standards into the mass spectrometer in the mobile phase (Table 2.4). Most 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.

Table 2.4. SRM transitions used for MC detection and quantitation

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
Dha^{7} 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

CE = Collision energy

The LC-MS/MS MC and 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. Measurements of microcystins were divided by flow cytometric cell counts to calculate the mass of each per cell in 1 femtograms (fg).

The performance of the MC extraction method was characterised in three variables; time, methanol/H_2O ratio and volume of cells. Cells were harvested from cultures of *Microcystis* PCC 7806 wild type (toxic) at late exponential phase, in commercial BG-11 medium and filtered (Whatman GF/C). Volumes of filtered cells, 1 mL, 100 µL and 1 mL of filtrate were tested, at two-time intervals, 1 and 24 hrs in ratios of 80/20. 70/30 & 60/40 methanol/H_2O v/v. All samples were shaken in a high-speed rotary shaker prior to 0.5 mL aliquots of each filter extract were transferred to 2 ml glass vials with pre-slit screw caps for analysis by UPLC MS/MS.

2.5.3. Liquid chromatography and mass spectrometry results

Chromatographic retention times obtained following the analysis of toxins MC-RR, MC-LA, MC-LY, MC-LF, MC-LW, MC-YR, MC-WR, MC-HilR, MC-HtyR, MC-LR, Asp^3-MC-LR and Nodularin were elucidated from the mixed reference material. Figure 2.12 illustrates the chromatographic peaks measured as shown by the Total Ion Counts (TIC) determined from the sum of both SRM transitions.

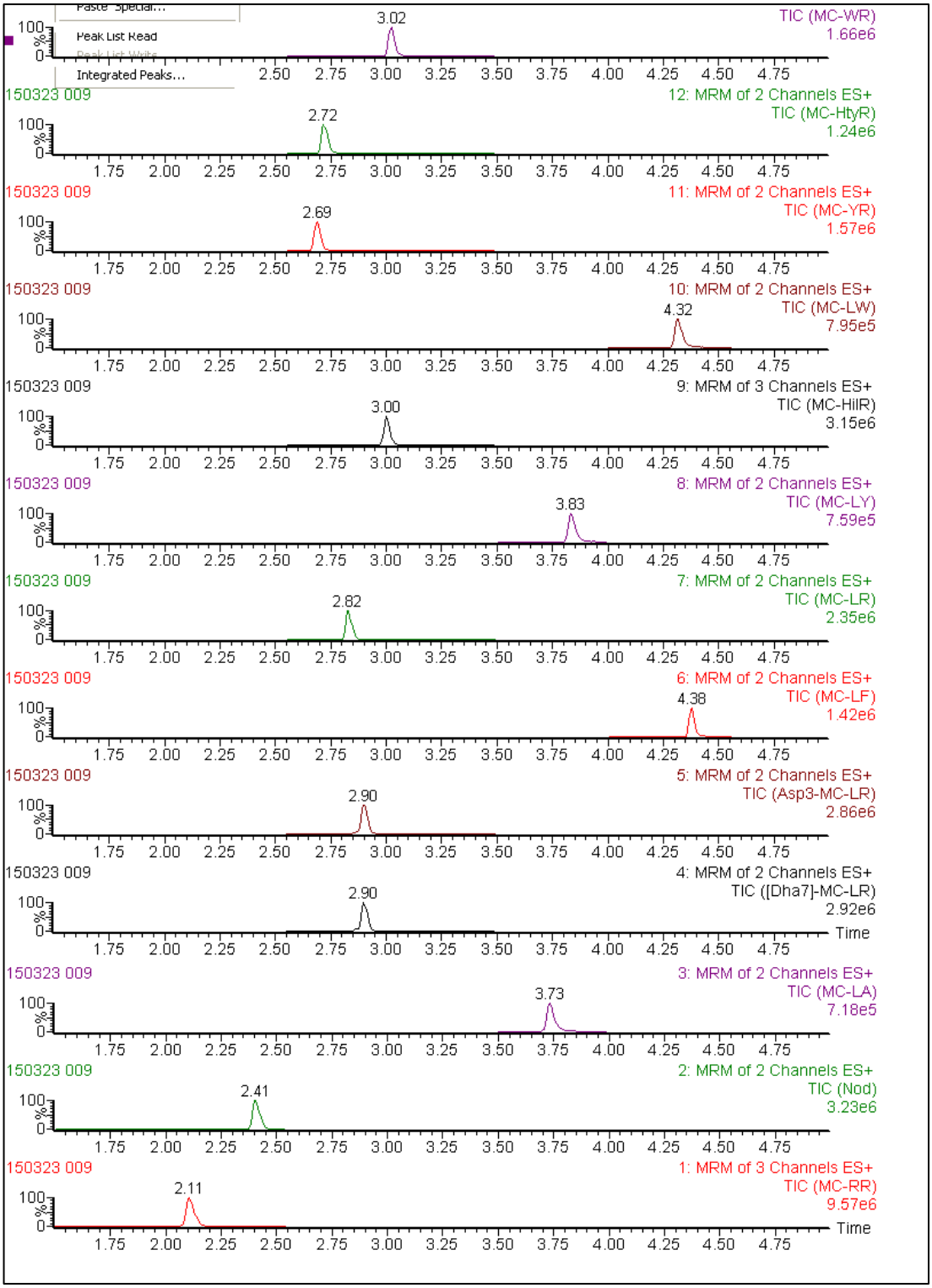


Figure 2.13. TICs from compound specific SRMs for microcystin variants detected in mixed reference material (Toxins MC-RR, MC-LA, MC-LY, MC-LF, MC-LW, MC-YR, MC-WR, MC-HilR, MC-HtyR, MC-LR, Asp^3-MC-LR and Nodularin) showing liquid chromatography retention times.

Variants of microcystin (MC-LR, MC-LR Asp3/Dha7, MC-LA, MC-LY, MC-LF, MC-LW and MC-HilR) were detected in all cyanobacterial strains tested. Higher concentrations of MC-LR and MC-LR Asp^3/Dha^7 were found only in the Microcystis strains PCC7806 wildtype (Figure 2.13.) and CCAP1450/17. In the Anabaena CCAP1446/1A and Oscillatoria CCAP 1459/6 cultures only small amounts of MC-LA. MC-LY, MC-LF and MC-LW were quantified. In the non-toxic Microcystis PCC 7806 mutant, microcystin variants MC-LR, MC-LR Asp3/Dha7, MC-LA, MC-LY, MC-LF and MC-LW were detected with MC-LA, MC-LF, MC-LW in concentrations above 3 µg/L (Table 2.5.).

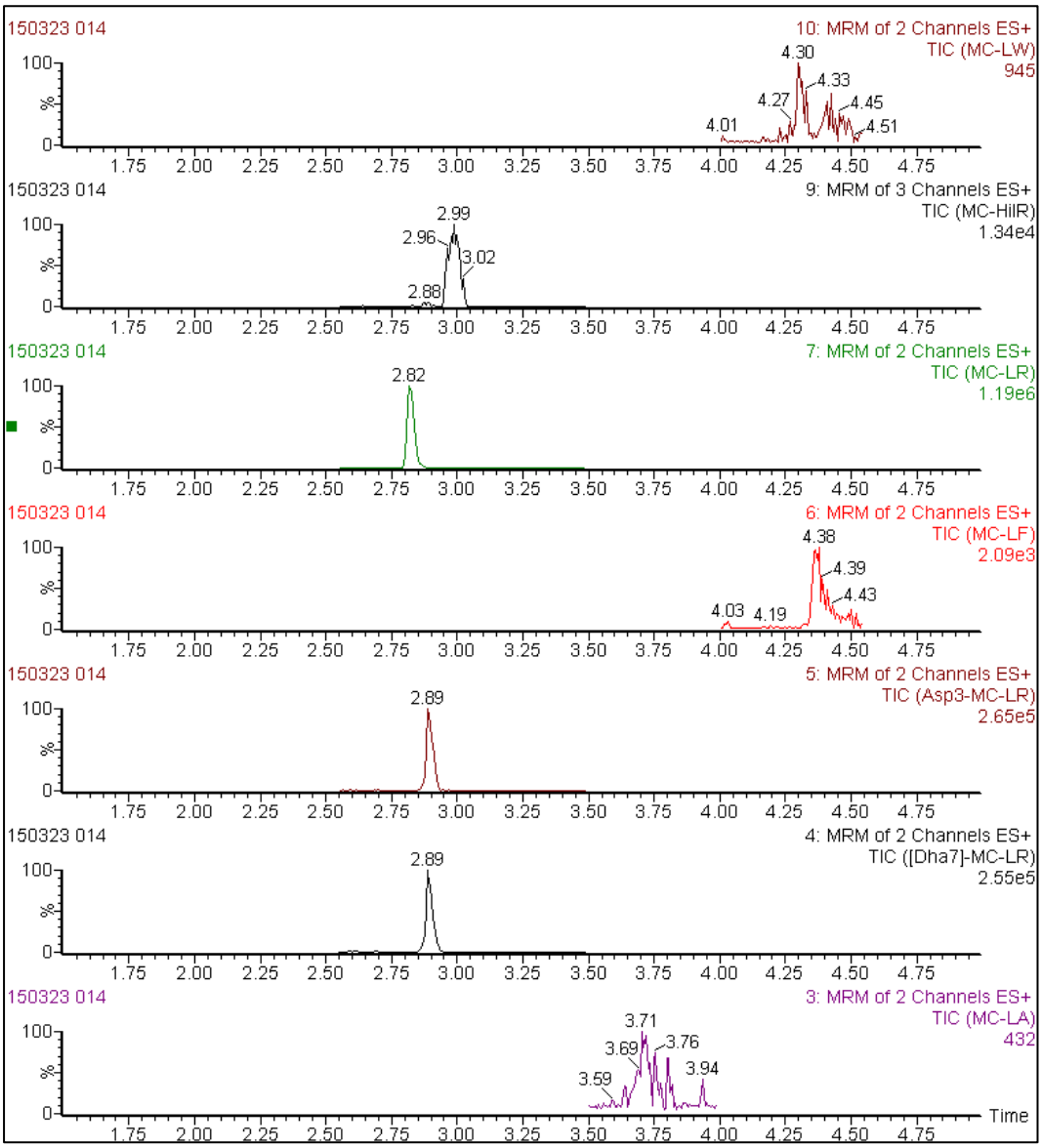


Figure 2.14. TICs of MC variants determined in *Microcystis* PCC7806 wildtype showing chromatographic retention times of MC-LR, Asp^3-MC-LR and Dha^7-MC-LR.

Table 2.5 Concentrations of microcystin variants quantified in 1 ml of medium from each of the five cultures from initial testing (µg/L).

	MC-LR	MC-LA	MC-LY	MC-LF	MC-LW	MC-LR Asp^3/Dha^7	MC-HilR
Microcystis PPC7806 mutant	0.6	3.2	2.6	4.9	3.4	0.4	
Anabaena CCAP 1446/1A		0.6	0.5	1	1.1		
Oscillatoria CCAP 1459/6		0.4	0.2	0.5	0.7		
Microcystis PCC7806 wildtype	141.3	0.2	0.1	0.8	0.6	23.8	2.2
Microcystis CCAP 1450/17 isolation	81			0.3	0.6	13.5	1.2

*shading denotes >3 µg/L.

Results from the microcystin extraction protocol indicated no significant difference in the medipipoupoupoupou0u;oupiuppoipoian MC-LR concentrations extracted at 24hr or 1hr in 60, 70 and 80% methanol, 1 mL & 100 µL volume of cells and 1 mL of filtrate (Figure 2.15.). Independent-samples Mann-Whitney U test U=361, $p>.05$ and n=54.

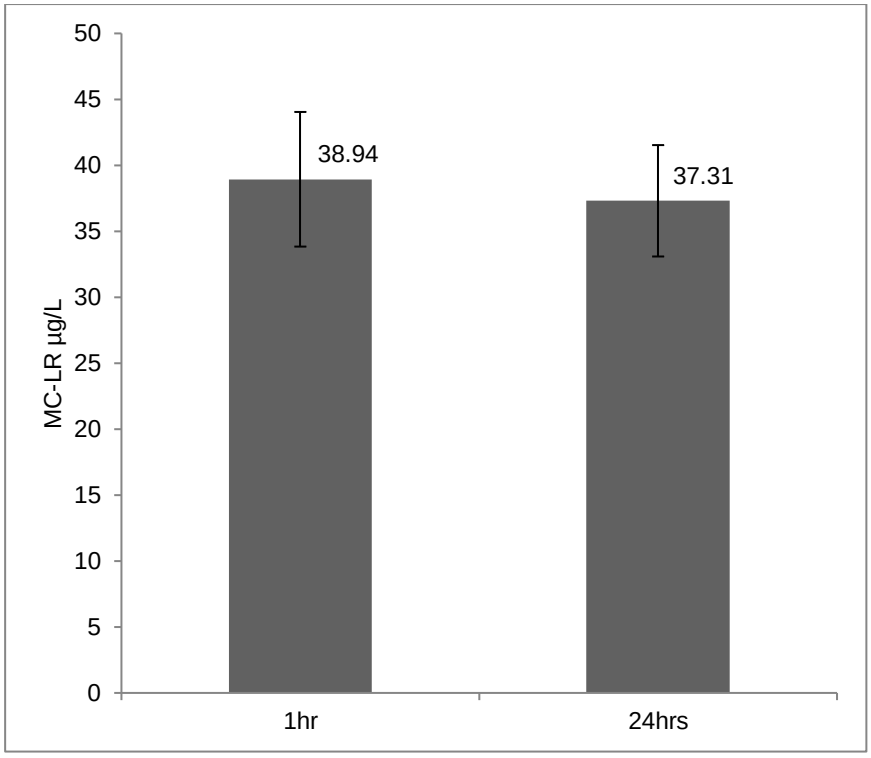


Figure 2.15.. Microcystin-LR extracted from Microcystis PCC 7806 at 1 hour and 24 hours from 60, 70 and 80% methanol, 100 µL & 1 mL volume of cells and 1 mL of filtrate. Mean shown above bars, error bars display standard deviation (n =54).

No difference was identified in MC-LR concentrations extracted from the volume of cells filtered (100 µL vs 1 mL) in 60, 70 and 80% methanol and at 1 hour and 24 hour time intervals (Figure 2.16). Independent samples Mann-Whitney U test *n*=36, U=300 and *p*>.*05*.

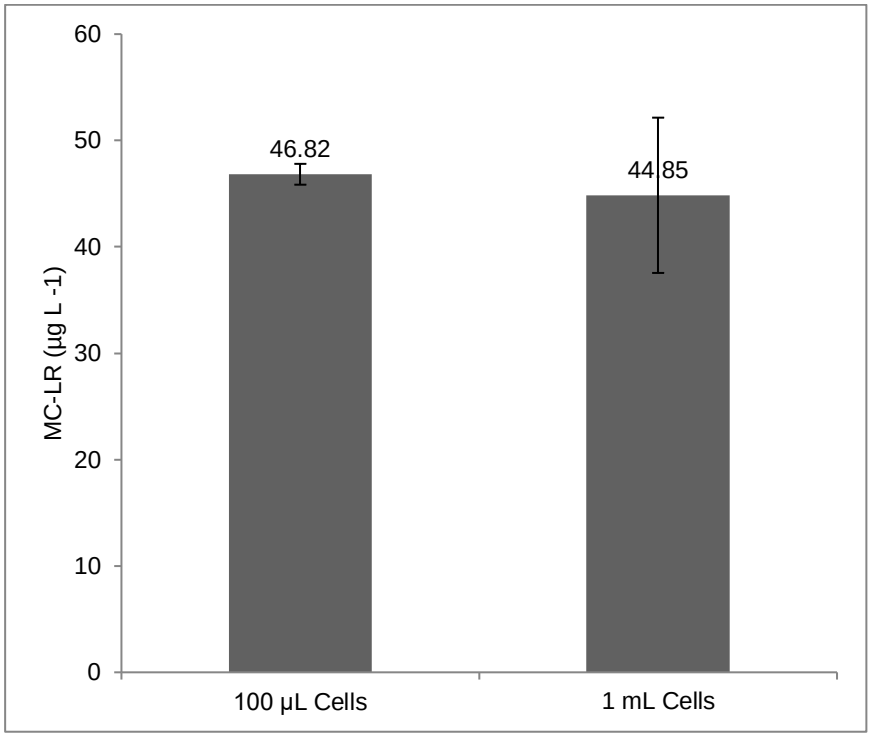


Figure 2.16. Microcystin-LR extracted from *Microcystis* PCC 7806 Microcystin-LR from 100 µL & 1 mL of filtered cells, from 60, 70 and 80% methanol and at 1 hour and 24 hours. Mean shown above bars, error bars display standard deviation (*n* =36).

There was however a statistical difference in the amount of toxin extracted by 80% methanol compared with 70 & 60% methanol, in 1 & 24 hour time intervals in all volumes of cells and filtrate tested (Figure 2.17.), highlighting improved method recovery for the 80% methanol extraction in comparison to other solvent compositions tested. Independent samples Kruskal-Wallis test, n=54, H=8.86, 2 df and p<.05.

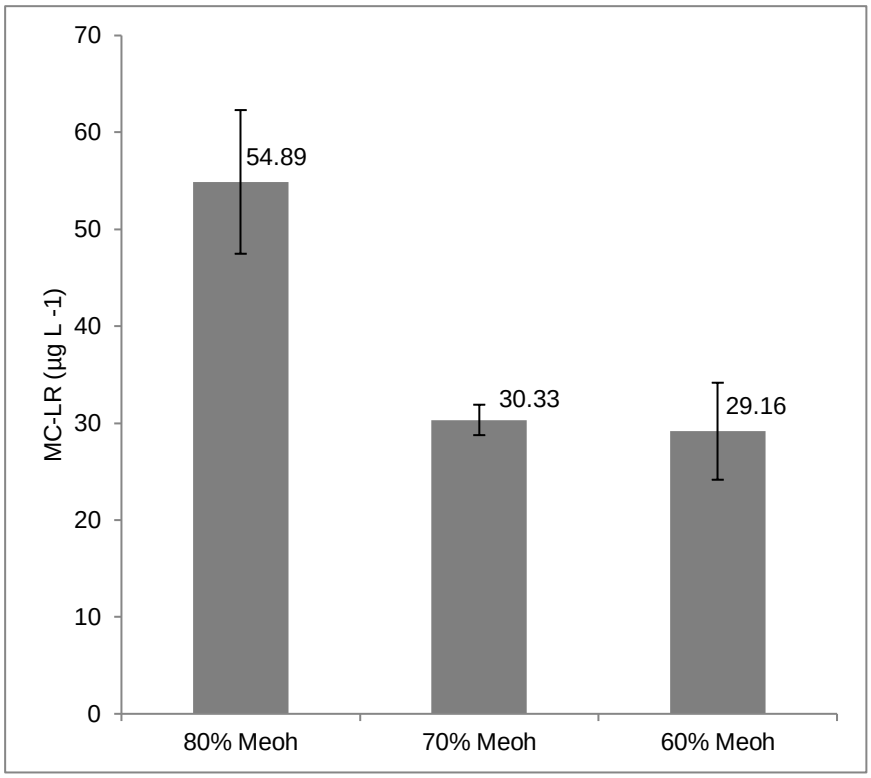


Figure 2.17. Microcystin-LR extracted from *Microcystis* PCC 7806 by 80, 70 & 60% methanol from 100 µL & 1 mL volume of cells and 1 mL of filtrate and at 1 hour and 24 hours. Mean shown above bars, error bars display standard deviation (n =54).

2.6. Discussion

2.6.1. Isolation and purification of Cyanobacteria cells

In this study several techniques were tested, singly and in combination, with the aim of developing a protocol to yield a unialgal culture of *Microcystis*. Investigations into the ecology and physiology of Cyanobacteria is often reliant upon on long-established culture strains which may have been maintained under laboratory conditions for many years. Because of this the biological and ecological representativeness of these laboratory adapted strains has been questioned (Lakeman *et al*., 2009, Alexova *et al*. 2011). The development of simple protocols to isolate fresh cultures from natural populations has been considered beneficial to the study of environmentally representative strains (Hartnell *et al*. 2016). However, isolation of pure cultures from environmental samples is problematic, with frequent co-existence of other cyanobacterial species and contamination of culture media reported as limiting factors (Vaara *et al.* 1979, Castenholz 1988, Rippka 1998); this has led to work on cyanobacteria being restricted to a relatively few representatives (Ferris and Hirsch 1991). In this series of studies, the suitability of 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) were compared to separate cyanobacterial cells from mixed environmental samples.

Mechanical separation by picking and crude selection based upon the well-established methods of Bloch and Blackburn (1996) was unsuccessful. Furthermore, *Microcystis* spp. isolated from Blashford Lakes coexisted with presumptive *Synechococcus* sp. and could not be purified using either mechanical or physiological approaches. Several authors have reported the efficacy of differential effect of resistance or susceptibility to antimicrobials (Ferris and Hirsch 1991, Bolsh and Blackburn 1996). None of the antibiotics and doses applied in this work [chloramphenicol (25 µg), erythromycin (5 µg), fusidic acid (10 µg), oxacillin (5 µg), novobiocin (5 µg), penicillin G (1 unit), streptomycin (10 µg) and tetracycline (25 µg)] enabled successful separation of *Microcystis* from *Synechococcus.* Whilst the literature is variable with respect to the efficacy of antimicrobial mediated purification, in general studies have not reported high levels or consistent success rates using this approach for *Microcystis* sp. (Vaara *et al.* 1979, Bolsh and Blackburn 1996). It is postulated that historic colocation and coexistence of the two strains isolated in this study may partially explain their similar antibiotic susceptibility profiles.

Whitelam and Codd (1983) reported the effective use of density gradients to separate Anabaena spp. from several other microalgal species. Similarly, Bloem and Moed (1985) successfully used Percoll density gradient to separate *Oscillatoria redekei* and *O. agardhii* from diatoms in lake Tjeukemeer, The Netherlands. However, in this study centrifugal separation of the two cyanobacterial using a sucrose gradient was unsuccessful as both cyanobacteria exhibited the same fractionation position in the gradient. Several studies have demonstrated the allelopathic effect of microcystin-LR on aquatic macrophytes (Pflugmacher 2002, Ujvarosi *et al.* 2019, Dong *et al.* 2019). Hu *et al.* (2005) reported toxicological effects of microcystin-RR (MC-RR) on the cyanobacterium *S. elongatus.* Hu and co-workers (2005) reported significant growth inhibition and chlorosis of *S. elongatus at* MC-RR concentrations above 100 μgL^{-1}. Attempts to replicate these effects using microcystin-LR at up to 3x concentrations were also unsuccessful indicating a potential differential effect of microcystin-LR and MC-RR on *Synechococcus,* although this could not be verified in this work. Separation of *Synechococcus* and *Microcystis* was also attempted using an automated cell sorter (BD, FACSAria) resultant cells were discriminated by using a known unialgal Microcystis culture (PCC 7806 wildtype) as a template. Whilst this technique has been reported to achieve excellent discrimination of independent single cell entities in aquatic environments (Yentsch and Pomponi 1986), results in this study were poor and it was apparent that a proportion of the smaller *Synechococcus* cells was remaining attached to the Microcystis mucus sheaths subsequently overgrowing the assumed unialgal cultures (Hartnell *et al.* 2016).

In a final attempt to separate the two genera the physiological growth optima were examined (physiological separation). This was driven by the hypothesis, 'could the two cyanobacteria in culture be separated by increasing temperature and decreasing nutrients?' *Microcystis* dominance within a bloom is primarily driven by increased temperature (Rigosi *et al.* 2014), whereas *Synechococcus* dominance is reported to be principally determined by increased nutrients (Agawin *et al.* 2000, Rigossi *et al.* 2014). Increased in temperature and decreased nutrients favoured *Microcystis* proliferation as measured by flow cytometry. Unialgal cultures were obtained and verified by light microscopy by day 15 (Hartnell *et al.* 2016). The *Microcystis* strain isolated was deposited in the Culture Collection of Algae and Protozoa (CCAP, UK) [accession number 1450/17] and was used in subsequent studies throughout this work.

In conclusion, the isolation and purification of cyanobacteria is a time-consuming process, where techniques need to be refined for the target organism. In this work the time invested was rewarded by the establishment of new culture lines for ecological and physiological research into these important organisms. Modification of culture conditions following published eco-physiological niche data was the best method for artificially selecting for the target organism, in this case *Microcystis*, and thereby creating a new culture lineage for study.

2.6.2. Batch culturing of Cyanobacteria

Optimum batch culturing protocols were established for five strains of three cyanobacterial genera. In summary, results demonstrated that BG-11 (Stainer 1971) media, an incubation temperature of 25±1°C and a 12/12 light/dark cycle with an average 35.95 µmol of photons $m^{-2}s^{-1}$ (range 25 – 46 µmol of photons $m^{-2}s^{-1}$) provided the most favourable conditions for all strains. Batch culturing experiments enabled differentiation of 5 phases within the growth of the batch culture, 1. Lag phase, 2. Exponential growth, 3. Decelerating growth, 4. Stationary and 5. Death phase. Growth phases and biomass were determined by light microscopy, flow cytometry and measurement of photosynthetic pigments (chlorophyll-*a*). Whilst all strains grew under the selected conditions, filamentous strains of *Anabaena* and *Oscillatoria* were difficult to enumerate and produced poor levels of repeatability. Variability in growth dynamics of *Anabaena* and *Oscillatoria* grown under laboratory conditions has been reported previously (Collins and Boylen 1982, Robarts and Zohary 1987). As the strains of *Anabaena* and *Oscillatoria* were not toxin producers and not considered important for downstream testing in this work, standardisation of their growth conditions was discontinued.

For PCC 7806 (mutant and wild type) and CCAP 1450/17 *Microcystis* spp. reliable and repeatable results were achieved under laboratory conditions enabling the generation of a standardised growth protocol. Selected conditions were like those reported by other workers for both PCC 7806 and other reference and environmental strains (Alexova *et al.* 2011, Pimental and Giani 2014, You *et al.* 2018). Some authors have reported variations in growth dynamics (and subsequent toxin profiles, protein expression and metabolism) between established reference strains and newly isolated axenic cultures from naturally occurring blooms (Otsuka *et al.* 2001, Alexova *et al.* 2011), with respect to growth (the only parameter determined in this series of studies) this phenomenon was not observed here. BG-11 has been widely

and successfully used for the laboratory batch culture of *Microcystis* spp. and was considered effective here, however its use as a suitable growth medium for all cyanobacterial species, including some species of *Microcystis,* has been questioned (Song *et al.* 1998). *Microcystis* spp. response to high light intensities has been widely studied both under laboratory conditions and *in-situ* (Wiedner *et al.* 2003, Bottcher *et al.* 2001, Hesse and Kohl 2001). In the environment *Microcystis* cells are reported to utilise gas vacuoles to migrate towards the surface followed by a period of rapid growth to generate surface blooms (Reynolds *et al.* 1981). Rapid increases in cell numbers then reduce light availability (and nutrients) (Renaud *et al.* 2011). It is considered likely that this tendency partially explains the observations in these laboratory studies. Likewise, availability of adequate light has been reported extensively to inhibit growth of *Microcystis* spp. (Bottcher *et al.* 2001, Hesse *et al*. 2001, Renaud *et al.* 2011).

In summary, data generated in this series of studies supported the use of an incubation temperature of 25±1°C with 12/12 light/dark cycle at an average of 35.95 µmol of photons $m^{-2}s^{-1}$ (range 25 – 46 µmol of photons $m^{-2}s^{-1}$) for repeatable batch culture of reference strain and environmental isolation of *Microcystis* spp. These standardised conditions were therefore used as the baseline growth parameters for all laboratory-based studies and enabled comparisons between experimental runs in later work.

2.6.3. Estimation of cyanobacterial cell density, size, growth rates and photosynthetic pigment using Flow Cytometry (FCM)

Estimates of cyanobacterial cell density, size, growth rates, phycocyanin and chlorophyll-*a* levels were made using flow cytometry (FCM). In this series of studies laboratory methods used were taken from Chapman (2016) without adaptation. Performance characteristics of the method were verified through application of batch grown *Microcystis* spp., *Anabaena* spp. and *Oscillatoria* spp. at all growth phases and by reference to highly linear calibration curves from mean forward scatter. Application of FCM to *Anabaena* spp. and *Oscillatoria* spp. was more problematic and yielded less repeatable results, due to potentially to their elongated and variable morphology. FCM has been widely used for the estimation of cyanobacteria and is useful for both monitoring and ecological studies (reviewed in Vives-Rego *et al.* 2000 and Poniedzialek *et al.* 2017). It has however been reported previously that filamentous (Poniedzialek *et al.* 2017) and colonial (Cellamore *et al.* 2009) cyanobacteria are difficult to study suing FCM due to their trichome lengths which does not allow individual cells to be distinguished (Poniedzialek *et al.* 2017).

The photopigments phycocyanin and chlorophyll-*a* can be excited in cyanobacteria and detected using FCM (Poniedzialek *et al.* 2017). Phycocyanins are phycobiliproteins found in all cyanobacteria, and some cyptophyta species (Becker *et al.* 2002). In this study FCM detection parameters for phycocyanin and chlorophyll-a following the methods of Chapman (2016) were verified using far-red auto-fluorescence (675nm) gated between 11,000 and 55,000 au, to 500,000 and 950,000 au for phycocyanin for laboratory and environmental samples respectively, and red auto-fluorescence (670nm) gated between 180,000 and 60,000 au, to 2,200,000 and 400,000 au for chlorophyll-*a*. Phycocyanin was used subsequently throughout this work as a proxy for the presence of cyanobacterial species, and together with cell size and yellow auto-fluorescence was used to distinguish *Microcystis*-like cells in environmental samples. This approach has been utilised in several studies estimating cyanobacteria in freshwater bodies (Gregor *et al.* 2006, Ahn *et al.* 2007, Zhang *et al.* 2007) and in studies on cell metabolism (Xiao *et al.* 2011). Gregor *et al.* (2006) used phycocyanin and chlorophyll fluorescence ratios to monitor the occurrence of cyanobacteria in 5 drinking water reservoirs in the Czech Republic. The authors reported successful differentiation between cyanobacteria and eukaryotic algae and suggested that the approach could be used for rapid and semi-quantitative screening in high-throughput water monitoring. Furthermore, utilised phycocyanin

measurements as an alert criterion suggesting that because phycocyanin is a cyanobacteria-specific pigment it was likely to be a better predictor of toxicity.

Additionally, a protocol for determination of chlorophyll-*a* was developed based on spectral absorbance of chlorophyll-*a* (Wellburn 1994) with minor modifications (i.e. using both 80 and 100% methanol). This was applied throughout to assess total chlorophyll-a concentrations in cyanobacteria grown under laboratory conditions and detected in field experiments.

In summary, notwithstanding some difficulties in application of FCM to colonial or filamentous cultures, a standardised protocol to determine cell density and size was produced which was broadly applicable to *Microcystis* sp. and other non-filamentous cyanobacteria (based on size, phycocyanin: chlorophyll-*a* fluorescence ratios). Additionally, a protocol for determination of chlorophyll-*a* was developed based on spectral absorbance of chlorophyll-*a* (Wellburn 1994) with minor modifications (i.e. using both 80 and 100% methanol). These protocols were used throughout all future laboratory experimental and environmental monitoring studies in this thesis.

2.6.4. Liquid chromatography and mass spectrometry

There are a number of methods published in the peer review literature for the determination of cyanotoxins using either HPLC with ultraviolet detection (Lawton *et al*. 1994, Gurbuz *et al.* 2009) and mass spectrometry (MS) (Edwards *et al.* 1993, Dahlmann *et al.* 2003) to detect cyanotoxins from environmental and laboratory samples. More recently ultra-high-performance liquid chromatography (UHPLC), coupled with tandem MS (MS/MS) have been reported to show good performance in a range of matrices (Kalourdis *et al.* 2013, Pekar *et al.* 2016, Turner *et al.* 2018a). Where full performance characteristics are reported, varying degrees of precision have been demonstrated and only a small number of published methods have been subject to validation following international guidelines (Beltran *et al.* 2012, Pekar *et al.* 2016, Turner *et al.* 2018a). Karlsson and co-workers (2005) noted the tendency for performance drift highlighting the needs for rigorous quality control. Because of the range of published approaches to detect cyanotoxins in both environmental and laboratory cell cultures, comparison of datasets generated between independent studies is rarely possible. Therefore, in this series of experiments a single, standardised, fit for purpose approach was developed enabling direct comparisons of toxins produced under laboratory and field samples.

In this study the UHPLC-MS/MS method developed by Turner *et al.* (2018) was optimised for use with environmental and cultured cyanobacteria. The method enabled the quantitation of microcystin analogues: MC-LR, MC-RR, MC-LA, MC-LY, MC-LF, LC-LW, MC-YR, MC-WR, [Asp3] MC-LR, [Dha7] MC-LR, MC-HilR and MC-HtyR in 3 strains of *Microcystis* spp. [*Microcystis* PCC7806 (mutant), *Microcystis* PCC7806 (wild type) and *Microcystis* CCAP 1450/17 (isolation)], *Anabaena* CCAP 1446/1A and *Oscillatoria* CCAP 1459/6. Optimisation studies targeted three extraction variables, time intervals (1 and 24 hr), volume of (cultured) cells (1mL and 100µL) and methanol/H_2O ratios. In brief, results showed no differences in between time or cell volume but did indicate improved toxin recoveries at higher methanol concentrations (80% methanol/20% H_2O). The absence of performance drift between 1 and 24 hr reported here is in contrast with Karlsson *et al.* (2005) who reported substantial signal suppression and enhancement in tissue samples from bivalve shellfish, eider duck livers and rainbow trout. To achieve the required limits of detection for environmental samples, the authors recommended the use of an additional solid phase extraction (SPE) clean up step prior to mass spectrometric analysis. The need for SPE or additional immunoaffinity columns in toxin determination has been reported (Beltran *et al.* 2012). However, it is considered likely that the apparent stability in toxin extraction efficiencies demonstrated in this series of verification studies, was associated with the lack of significant matrix effect when testing water or culture media compared with tissue samples. This has been reported elsewhere (Turner *et al*. 2018a) and thus for downstream analyses no additional sample concentration step was used and direct analysis by LCMS/MS was possible and a 24-hr time interval was used for ease of processing. Starting cell volumes had no impact upon toxin extraction recoveries and therefore in accordance with Turner *et al.* (2018) a standardised volume of 1mL was selected for subsequent sample processing. Several authors have reported differential recoveries dependent upon the methanol/H_2O ratios (Lawton *et al.* 1994, Fastner *et al.* 1998, Turner *et al*. 2018a). Lawton *et al.* (1994) showed good recoveries in samples of cell material with 70% aqueous methanolic extraction whereas, other studies using culture grown *M. aeruginosa* cells suggested optimum solvent ratios of 75% methanol/25% H2O (Fastner *et al.* 1998) and 50% aqueous methanol in samples of raw water (Miles *et al.* 2013). Several alternative solvents have also been tested in cyanotoxin extraction efficiency studies, reviewed in Ame *et al.* (2010). In this study statistically significant ($p<0.05$) differences between 60 and 70% methanol, and 80% were demonstrated for microcystin-LR in type strain *Microcystis* PCC 7806. Extraction efficiency was

significantly improved using 80% methanol in culture grown filtered cells. This finding is in accordance with Turner *et al.* (2018a) where optimum toxin recovery from algal matrix achieved at 80% aqueous methanol. Therefore, for downstream analyses all toxin extractions were carried out using a ratio of 80% methanol/20% H_2O.

In summary, the optimised method showed good performance with respect to recovery, linearity, repeatability and sensitivity for raw water and cell culture matrices for each commercially available microcystin. This method, as applied to bivalve shellfish and water matrices, has subsequently been accredited to ISO/IEC 17025: General requirements for the competence of testing and calibration laboratories (Anon 2015) at the Cefas laboratories by the United Kingdom Accreditation Services (UKAS) [accreditation schedule No. 2293]. It was considered robust and fit for purpose for use in further studies undertaken as part of this thesis.

2.6.5. Conclusions

The work presented here enabled the development of standardised protocols for growth and determination of total cyanobacteria, *Microcystis*-like cells, together with a robust and repeatable method for the quantitation of 12 microcystin analogues. The use of verified, standard protocols were used in all future experimental studies comprising this work and enabled robust and justifiable comparisons to be made both between laboratory generated data and that collected from field experiments.

3. The effects of light, nutrients and temperature on cell growth and toxin content in batch cultures of *Microcystis* strains

3.1. Introduction

3.1.1. General

Microcystis is a genus of unicellular, colonial cyanobacteria comprising several species that under favourable abiotic conditions can form blooms in freshwater systems (Robarts and Zohary 1987, Sejnohova and Marsalek 2013, Briand *et al.* 2012). Some strains of *Microcystis* possess the ability to produce potent toxins known as microcystins (MCs) (Carmichael 1992, Metcalf and Codd 2012). Microcystins are a group of cyclic peptide toxins comprising 5 core and 2 variable amino acids (Metcalf and Codd 2012), to date around 250 microcystin variants have been identified (Spoof and Catherine 2017). The biological role of microcystins is ambiguous (Babica *et al.* 2006), although several authors have postulated that these potent compounds have an anti-predation role conferring competitive advantages (Jang 2007, Ger *et al.* 2016) or, provide a growth advantage by protecting from photo-oxidation in high light conditions (Phelan and Downing 2011, Zilliges *et al.* 2011,) or, induce physiological benefits or allelopathic effects enabling toxin producing strains to exploit scarce resources in their ecological niche (Pearson *et al.* 2004, Holland and Kinnear 2013).

Notwithstanding the uncertain evolutionary biological basis of toxin production, *Microcystis* blooms have been frequently implicated in animal poisonings (Codd *et al.* 2005), tumour promotion (Dittmann and Wiegand 2006) and occasional human fatalities (Kuiper-Goodman *et al.* 1999). As a result, numerous studies have investigated the effects of physical, chemical and biological factors on the toxicity of cells, *in vitro* and *in situ* to understand the triggers for toxin production and modulation. Most studies have focused upon the influence of nitrogen, phosphorous, trace metals, temperature, light and pH, however, few studies have been carried out under controlled conditions using standardised methods (reviewed in Neilan *et al.* 2013). Many such studies have generated conflicting data and therefore both comparisons between studies or extrapolation from the published literature is problematic. Furthermore, many laboratory batch or continuous culture-based approaches have used single laboratory-adapted reference strains which may have lost certain important physiological characteristics and therefore, the environmental representativeness of laboratory adapted strains has been questioned (Lakeman *et*

al. 2009, Alexova *et al.* 2011). The issues of long-term adaption to unrealistic laboratory conditions highlights the importance of including freshly isolated strains in laboratory-based studies.

3.1.2. Effects of abiotic conditions on microcystin production

Factors including light intensity, temperature and nutrient availability have been reported to affect microcystin production in natural environments in two principal ways, firstly by regulating the abundance of microcystin producing strains within a population and secondly by influencing microcystin production by toxin producing strains (Wiedner *et al.* 2003). In laboratory studies using monocultures of cyanobacterial species, microcystin production has been shown to be both positively and negatively affected by photosynthetically active radiation (light) (Sivonen 1990, Utikilen and Gjolme 1992, Wiedner *et al.* 2003) and nitrogen and phosphorous concentrations (Orr and Jones 1998, Song *et al.* 1998, Vezie *et al.* 2002). The impact of temperature on microcystin production *in vitro* and *in situ* has been widely studied (Davis *et al.* 2009, Dziallis and Grossart 2011, Bui *et al.* 2018). However, overall there is little agreement between study results, with contradictory data generated suggesting that increased temperatures may either increase (Wood *et al.* 2017, Celeste *et al.* 2017), decrease (Bui *et al.* 2018) or have variable impact (Song *et al.* 1989, Wiedner *et al.* 2003) upon toxin content. These conflicting data may be attributable to several factors, including the use of differing methodologies for determination of toxin contents including mouse bioassay, enzyme-linked-immunosorbent assay (ELISA), HPLC etc. All of which variously suffer from a lack of standardised study design and the use of a wide range of cyanobacterial species obtained from reference collections and diverse geographic and temporal isolations. In addition, the reported variability within microcystin biosynthesis pathways presents issues for study comparability (Kaebernick *et al.* 2002).

3.1.3. Relationship between abiotic parameters, growth phase and microcystin production

Despite the challenges of comparing diverse studies and methodologies, several authors have attempted to use both batch and continuous culture systems to explain and model the relationships between microcystin production, culture methods and the physiological conditions of cells (Orr and Jones 1998, Long *et al.* 2001, Wiedner *et al.* 2003, Puddick *et al.* 2015, Celeste 2017). The role of batch culture growth phase in toxin production has also been examined to understand how changes in growth and cell division rates (factors impacted by abiotic parameters) influence microcystin production rates and cellular content (Orr and Jones 1998, Long *et al.* 2001). Several authors have reported that microcystin production is restricted to the phase of growth when cell concentration is increasing (Orr and Jones 1998, Long *et al.* 2001). These findings have helped to inform theories and models on the function of microcystins and to predict toxin concentrations under various abiotic conditions (Celeste 2017). In a comprehensive series of studies Long and co-workers (1998, 2001) used a generalised linear model to predict cellular microcystin content from estimated *Microcystis aeruginosa* growth rates. This model has been reported to explain the conflicting results observed from many batch culture investigations (Neilan *et al.* 2013) and was therefore further examined in this study.

3.1.4. Aims of the study

The aim of this study was to further understand the role of temperature, nutrient conditions and light intensity on the production of the toxic cyclic peptide (microcystin) in two strains of *Microcystis* sp.

Firstly, a series of controlled, replicated batch culture experiments were used to examine the impact of light intensity, nutrient limitation and temperature on microcystin production and growth in two strains of *Microcystis* sp. in batch cultures. Cell densities and size were measured using a harmonised flow cytometry (FCM) method, a rapid automated method of counting and discriminating between single cells in a population. Microcystin determination was undertaken using an ISO 17025 accredited Ultra-High-Pressure Liquid Chromatography (UHPLC) coupled to Tandem Mass Spectrometry (MS/MS) for the discrimination of 12 microcystin variants (Turner *et al.* 2018a). Both methods improved standardisation of approach and enabled robust comparisons between experimental runs.

Secondly, the assumption that a laboratory-adapted reference strain would exhibit different behaviours to a more recently isolated strain was tested, using a type strain of *Microcystis aeruginosa* (PCC 7806 toxic wildtype) and a presumptive *Microcystis* sp. (CCAP 1450/17) recently isolated from a lake in the South of England.

Finally, the dynamics of total microcystin production and cellular content with growth rates, and to an extent cell size was explored to hypothesize the potential role of microcystin in cellular physiology.

3.2. Materials and methods

Two strains of *Microcystis* spp. were used to investigate the effects of light, nutrients and temperature on the growth and toxin content in controlled laboratory batch cultures. Selected strains were the established reference culture PCC 7806 toxic wildtype and a recently deposited strain, CCAP 1450/17 (Hartnell *et al*. 2016). Culture conditions were as Section 2.3.1, with the addition of two incubator temperatures of 20±1°C and 30±1°C (in addition to standard temperature of 25±1°C). Using flow cytometry (FCM) cell count estimations (Section 2.4.2.) all batch cultures were inoculated at a cell density of ±2,500,000 per mL. Growth characteristics and toxin profiles and levels were measured at 3 and 4 day intervals over the first 21 days whilst the cultures were in lag and exponential growth phases. Microcystin toxins were qualified and quantified by ultra-high-pressure liquid chromatography (UHPLC) coupled to tandem mass spectrometry (MS/MS) methodology is detailed in section 2.5.2.

Standard BG-11 media with variable nitrogen (N) and phosphorous (P) content were made up from stock solutions (Table 2.3.) (Stainier 1971). The ratio of N and P was modified in two ways;

1. Sodium nitrate was substituted for sodium bicarbonate to produce a phosphorous rich media (N- media) as some nitrogen was provided by the ammonium ferric citrate green.
2. The quantity of di-potassium phosphate was reduced by 25% to produce a nitrogen rich media (P- media). Compound quantities, elemental molar concentrations and N:P ratios are presented in Table 3.1.

Table 3.1. Modification of BG-11 from stock solutions to produce nitrogen and phosphorous rich media.

Media		Compound (g/L)			Molarity (mMol)		N:P ratio	
		$NaNO_3$	K_2HPO_4	$C_6H_9FeNO_7$	N	P	N	P
Standard (Control)	BG-11	15	4	0.6	0.24755	0.01422	17.40	1
Nitrogen- (N- media)	BG-11	0	4	0.6	0.00032	0.01422	1	44.33
Phosphorus- (P- media)	BG-11	15	1	0.6	0.24751	0.00355	69.60	1

Data and statistical analysis were undertaken using MS Excel and IBM SPSS 23.

3.3. Results

The effect of three light intensities (high, M=117, medium, M=36 and low, M=15 µmol photons m^{-2} s^{-1}) and five treatments, BG-11 at 25°C (Control), P- media, N- media, 20 & 30°C, on the growth and toxin production from batch cultures of *Microcystis* strains PCC 7806 and CCAP 1450/17.

3.1.1. Cell growth and size

Cell density

Highest cell density (maximum in any experimental procedure) of 32,903,481 was recorded from strain CCAP 1450/17 at high light intensity in P- media at 25°C (P- media) and the highest mean of 15,390,689 cells mL^{-1} was recorded in the same treatment. The lowest mean cell density of 4,756,887 cells mL^{-1} from CCAP 1450/17 was recorded at low light in BG-11 media at 25°C (Figure 3.1.A.).

For strain PCC 7806 a maximum of 30,699,926 cells mL^{-1} was recorded at high light in BG-11 media at 25°C (Control) and the highest mean cell density of 14,127,126 was recorded at medium light in P- media at 25°C. The lowest mean cell density of 4,683,652 cells mL^{-1} was recorded at high light in BG-11 at 20°C (Figure 3.3.A.).

Statistical differences between groups were observed in PCC 7846 and CCAP 1450/17 at high and low light intensity when tested by ANOVA to the $p<0.05$ level. A Tukey HSD *post hoc* test revealed cell densities from PCC 7806 at high light in BG-11 media at 20°C to be statistically different from control and 30°C, at low light intensity only the control was statistically different to P- media (Figure 3.1.A). Mean cell densities from CCAP 1450/17 at high light P- media significantly from 20°C treatments and at low light P- media treatment significantly differed from all other treatments (Figure 3.3.A.).

Growth rates

In strain CCAP 1450/17 the highest growth rate 0.402 $(k)d^{-1}$ and lowest growth rate, -0.330 $(k)d^{-1}$ were recorded at high light in BG-11 at P- media and 30°C respectively. In PCC 7806 the highest of 0.355 $(k)d^{-1}$ at high light in BG-11 at 30°C, but the lowest -0.165 $(k)d^{-1}$ was from high light in P- media (Figure 3.1.B and 3.3.B.).

When tested by ANOVA to the $p<0.05$ level, no statistical differences were observed between treatments in any light intensity or *Microcystis* strain.

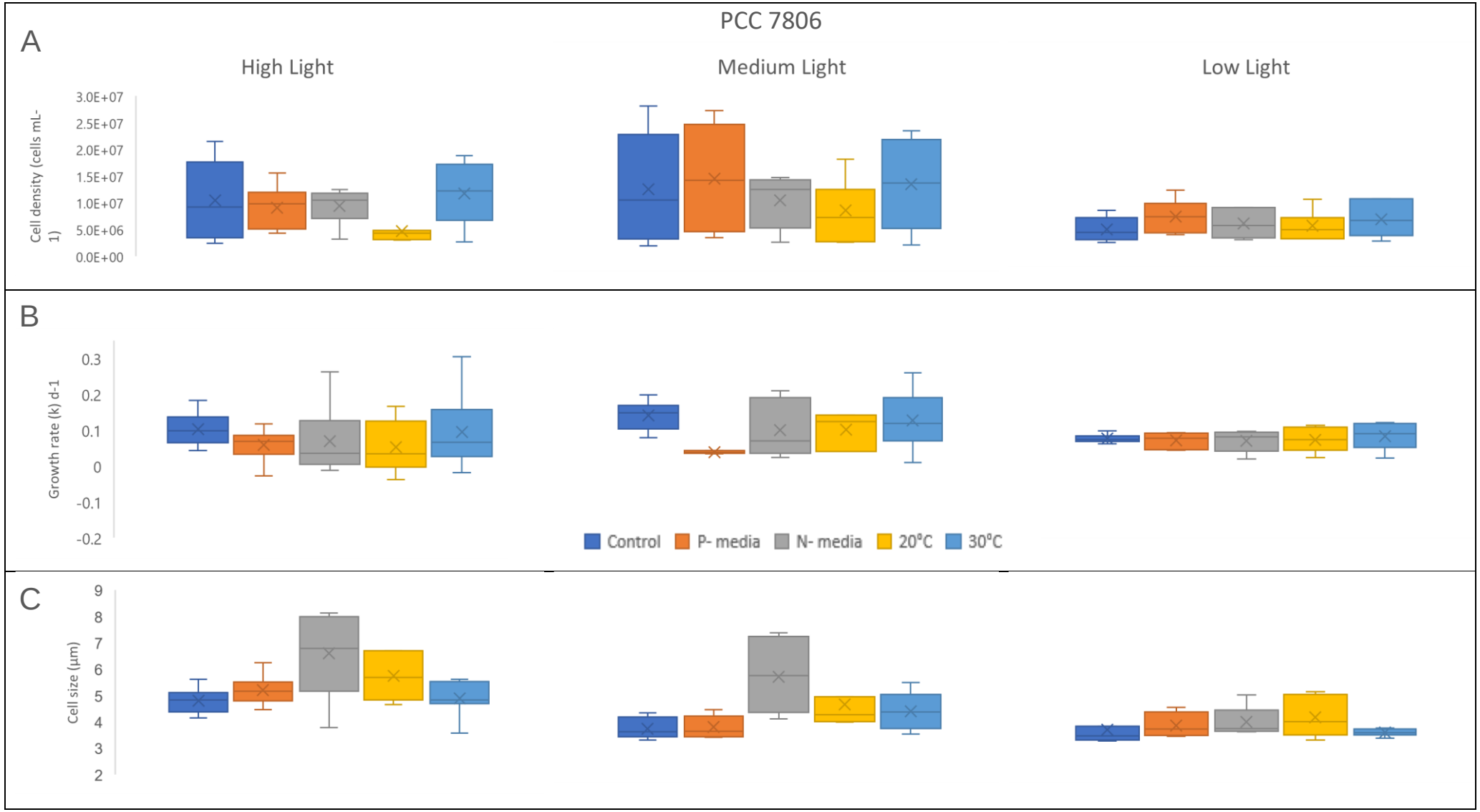


Figure 3.1. Box and whisker plots to show the effects of three light intensities (high=117, medium=36 and low=15 µmol of photons m^{-2} s^{-1}) on *Microcystis* strain PCC 7806 from five treatments, Control (BG-11 media at 25±1°C), P- media (Phosphorus minus BG-11 media at 25±1°C), N- media (Nitrogen minus BG-11 at 25±1°C) 20°C (BG-11 media at 20±1°C) and 30°C (BG-11 media at 30±1°C). Results from measurements of A Cell densities (cells mL^{-1}) n = 105 B Growth rates ((k)d^{-1}) n = 90 and C Cell size (µm) n = 105. Boxes upper and lower quartiles, median is marked by a horizontal line inside the box. Whiskersextend to the highest and lowest observations.

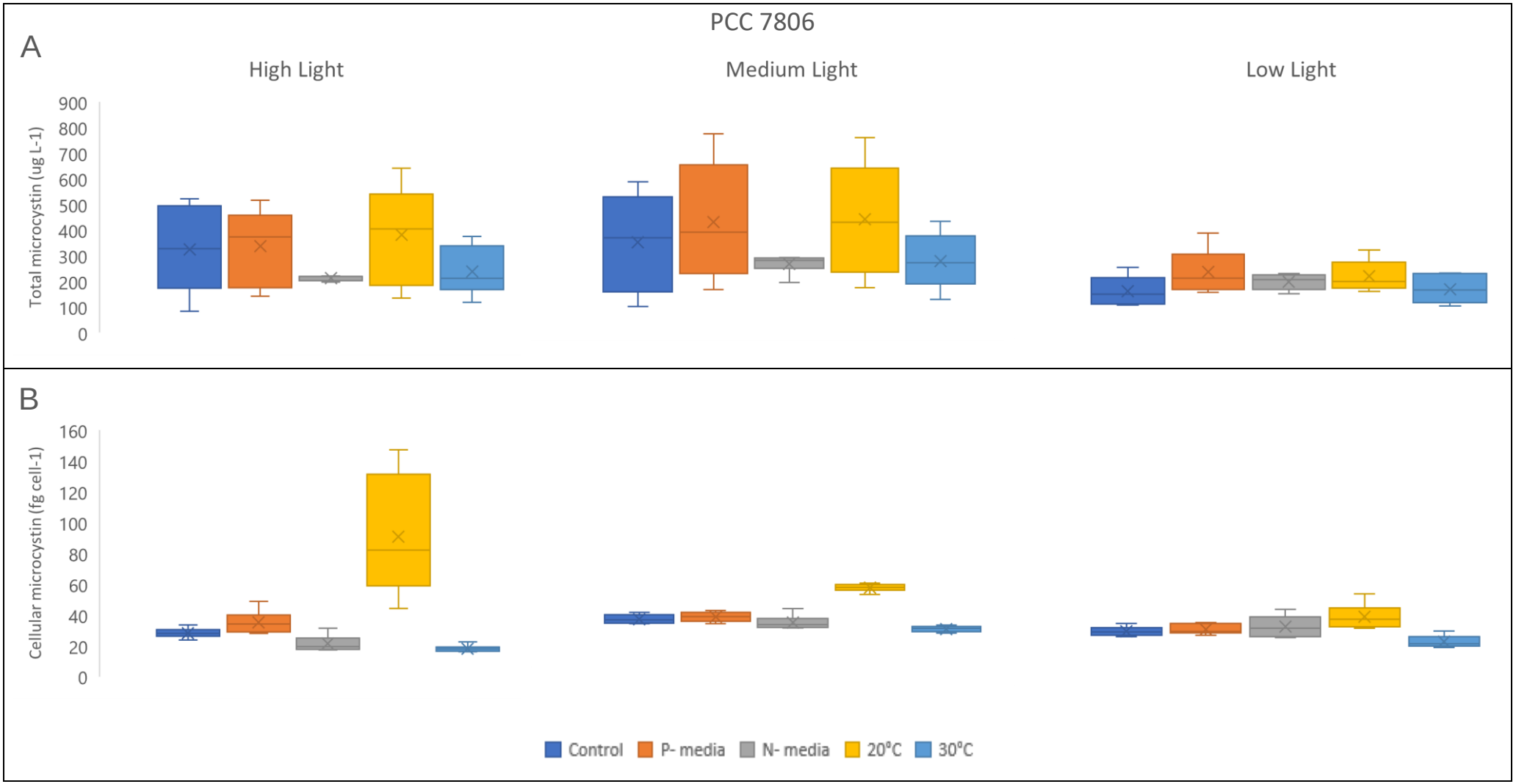


Figure 3.2. Box and whisker plots to show the effects of three light intensities (high=117, medium=36 and low=15 µmol of photons m^{-2} s^{-1}) on *Microcystis* strain PCC 7806 from five treatments, Control (BG-11 media at 25±1°C), P- media (Phosphorus minus BG-11 media at 25±1°C), N- media (Nitrogen minus BG-11 at 25±1°C) 20°C (BG-11 media at 20±1°C) and 30°C (BG-11 media at 30±1°C). Results from measurements of A Total microcystin concentrations (µg mL^{-1}) n = 264 B Cellular microcystin content (fg $cell^{-1}$) n = 89. Boxes upper and lower quartiles, median is marked by a horizontal line inside the box. Whiskersextend to the highest and lowest observations.

Cell size

The largest mean cell size (8.29 µm) was measured in CCAP 1450/17 at high light in N- media at 25°C, the smallest mean (3.08 µm) was measured at low light in BG-11 media at 30°C (Figure 3.3.C.). Strain PCC 7806 produced similar results with the largest mean cell size (8.18 µm) measured at high light in N- media at 25°C, the smallest mean (3.08 µm) was measured at low light in BG-11 media at 25°C (Control) (Figure 3.3.C.).

Statistical differences in cell size were observed between treatments in both strains and all light intensities when tested by ANOVA to the *p<0.05* level. Tukey HSD *post hoc* tests revealed that in PCC 7806 in high light N- media significantly different to the control, P- media and 30°C, in medium light N- media significantly different to all other treatments and 20°C was additionally different to control and P- media. At low light N- media was different to control & 30°C. (Figure 3.1.C.). In CCAP 1450/17 at high and medium light N- media was significantly different to all other treatments and 20°C was different to 30°C, in low light N- media was significantly different to control, P- media and 30°C (Figure 3.3.C.).

3.1.2. Toxin profile and levels

Total microcystin concentrations

The highest levels of total microcystins, comprising MC-LR + dm-MC-LR were extracted from strain CCAP 1450/17 (total = 1021.92 & mean = 495.98 µg mL^{-1}) at medium light intensity at the 20°C treatment (Figure 3.2.A.). A maximum was recorded in PCC 7806 also with the variants MC-LR + dm-MC-LR (total = 881.84 & mean = 406.62 µg mL^{-1}) at medium light intensity in the P- media (Figure 3.4.A.).

Statistical differences in total microcystin concentrations were observed between treatments in both strains and all light intensities when tested by ANOVA to the *p<0.05* level. Tukey HSD *post hoc* tests revealed that in PCC 7806 in high light N- media significantly different from all treatments except 30°C which was statistically different from 20°C. At medium light intensities 20°C was different from all but P- media which significantly different to N- media treatment. At low light P- media, N- media & 30°C were significantly different to the control and 30°C was different to N- & P- media. (Figure 3.2.A.). In CCAP 1450/17 at high light N- media was significantly different to all other treatments, except 30°C which was different to P- media and 20°C. At medium light intensity 20°C was significantly different to all but P- media, which was different to the other treatments. In low light N- media and 20°C were significantly different to all treatments (Figure 3.4.A.).

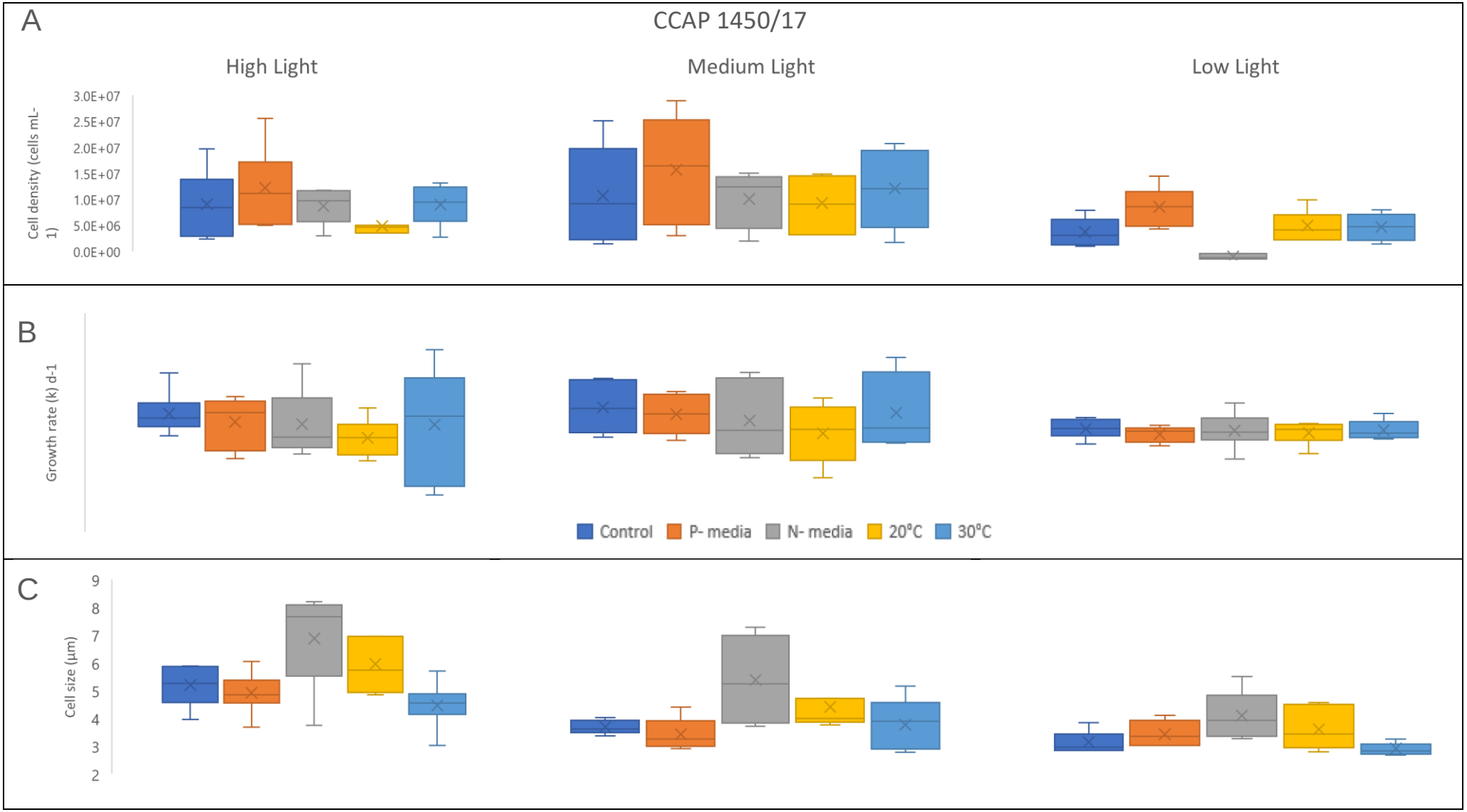


Figure 3.3. Box and whisker plots to show the effects of three light intensities (high=117, medium=36 and low=15 µmol of photons m-2 s-1) on Microcystis strain CCAP 1450/17 from five treatments, Control (BG-11 media at 25±1°C), P- media (Phosphorus minus BG-11 media at 25±1°C), N- media (Nitrogen minus BG-11 at 25±1°C) 20°C (BG-11 media at 20±1°C) and 30°C (BG-11 media at 30±1°C). Results from measurements of A Cell densities (cells mL-1) B Growth rates ((k)d-1) and C Cell size (µm). Boxes upper and lower quartiles, median is marked by a horizontal line inside the box. Whiskersextend to the highest and lowest observations.

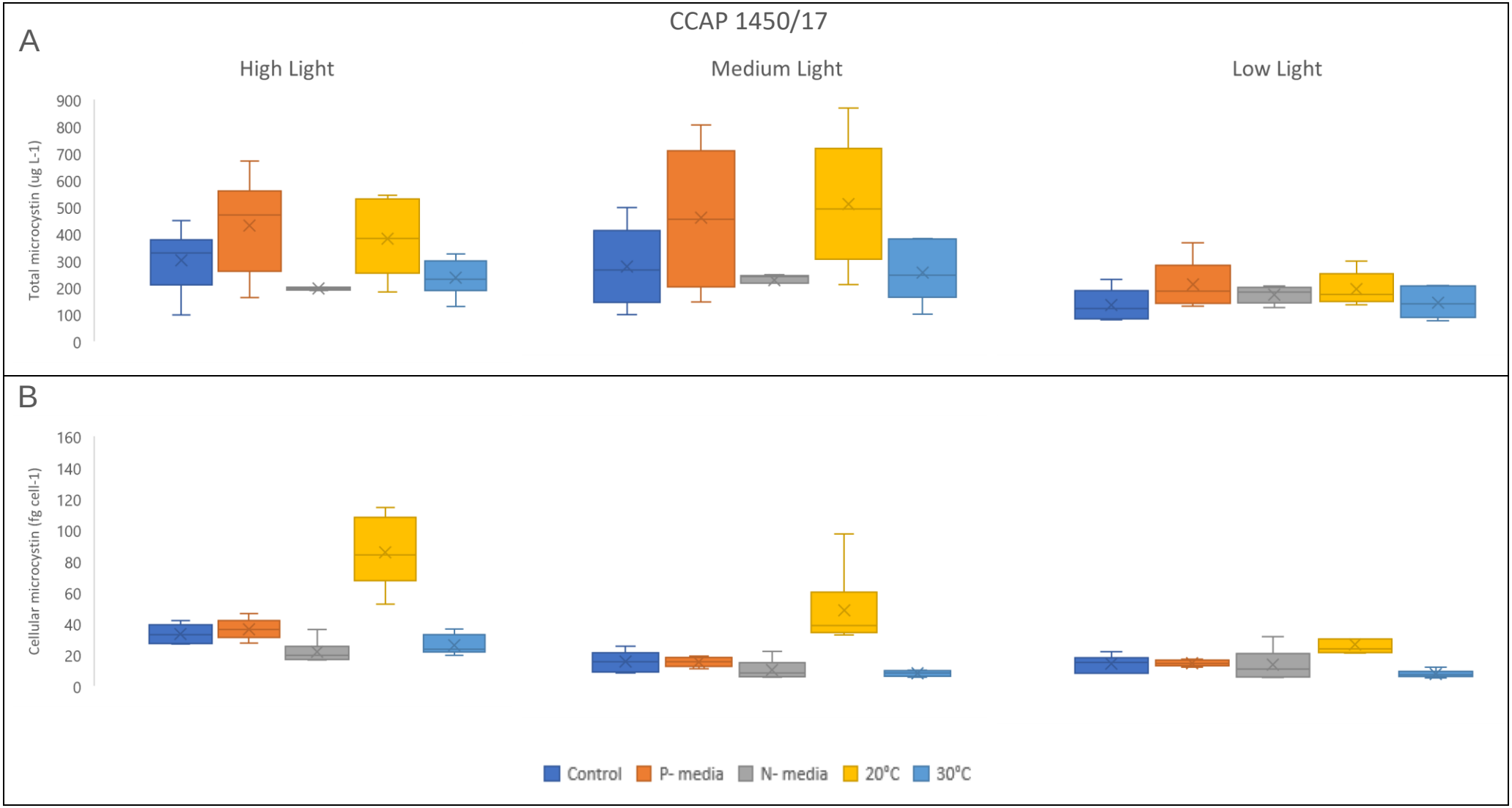


Figure 3.4. Box and whisker plots to show the effects of three light intensities (high=117, medium=36 and low=15 µmol of photons m^{-2} s^{-1}) on *Microcystis* strain CCAP 1450/17 from five treatments, Control (BG-11 media at 25±1°C), P- media (Phosphorus minus BG-11 media at 25±1°C), N- media (Nitrogen minus BG-11 at 25±1°C) 20°C (BG-11 media at 20±1°C) and 30°C (BG-11 media at 30±1°C). Results from measurements of A Total microcystin concentrations (µg mL^{-1}) B Cellular microcystin content (fg $cell^{-1}$) n = 89. Boxes upper and lower quartiles, median is marked by a horizontal line inside the box. Whiskersextend to the highest and lowest observations.

Cellular microcystin content

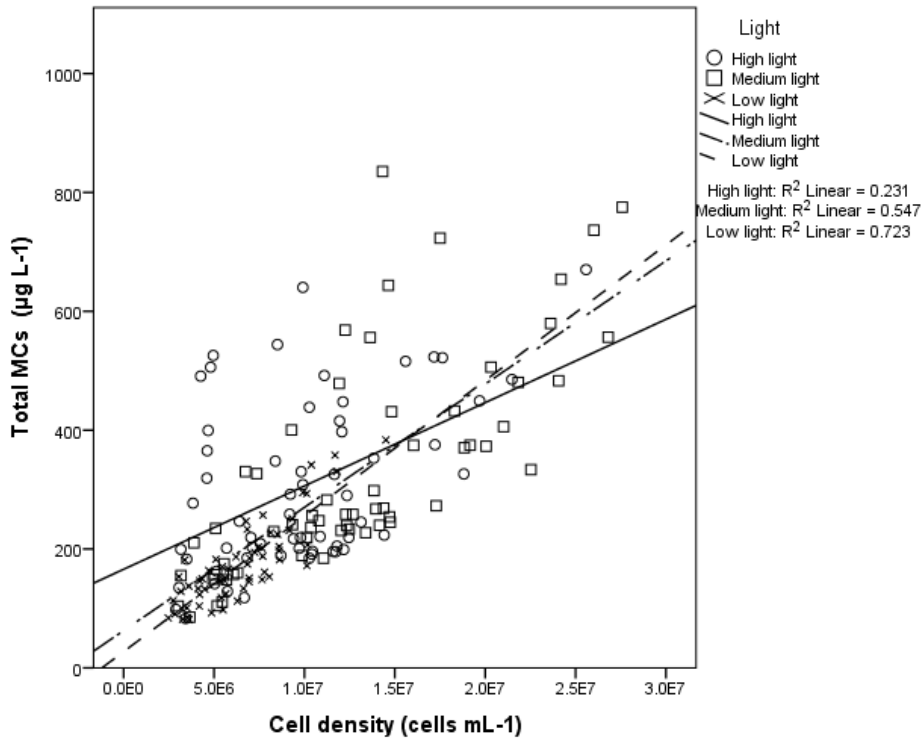
Highest cellular microcystin content was recorded in strain PCC 7806 of 225.54 fg $cell^{-1}$ at high light in the 20°C treatment (Figure 3.2.B.) and in strain CCAP 1450/17 of 210.34 fg $cell^{-1}$ was recorded at medium light in the 20°C treatment (Figure 3.4.B.).

Statistical analysis of mean cellular microcystin content by ANOVA to the $p<0.05$ level revealed differences between groups in both strains at all three light intensities. Tukey HSD *post hoc* tests revealed that 20°C was statistically different from all other treatments in both strains at high and medium light intensities. The treatment 20°C was different from all treatments in CCAP 1450/17 at low light but not in PCC 7806, where it was not different from the control (Figure 3.2.B. and 3.4.B.).

3.1.3. Relationship between cell density and toxin production

Light intensity

Figure 3.5. describes the regression relationship between cell density and, total and cellular microcystin concentration (strain separation did not impact the regression – data not shown). Total microcystins were positively correlated with an increase in cell density at all light levels ($R^{2\ low} = 0.603$, $R^{2\ med} = 0.509$, $R^{2\ high} = 0.203$), although the relationship was less linear at high light levels. Regression analysis of cellular microcystin and cell density showed slight negative correlation with light levels most evident at high light intensities ($R^{2\ low} = 0.143$, $R^{2\ med} = 0.112$, $R^{2\ high} = 0.239$).



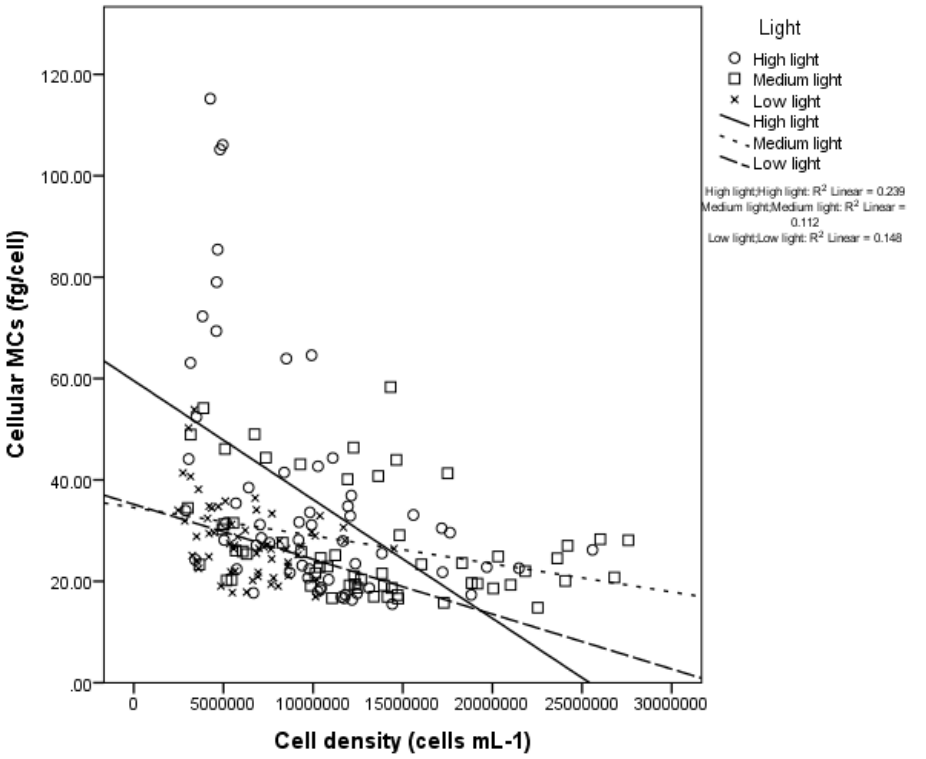


Figure 3.5. Regression relationship between cell density and, total and cellular microcystin concentration.

Temperature

Figure 3.6 shows the regression relationship between cell density and, total and cellular microcystin concentration (data for individual strains not shown). Total microcystins were closely, positively correlated with an increase in cell density at all temperatures ($R^{2\ 20°C}$= 0.614, $R^{2\ 25°C}$ = 0.833, $R^{2\ 30°C}$ = 0.865). Regression analysis of cellular microcystin and cell density at all temperatures showed slight negative correlation ($R^{2\ 20°C}$= 0.084, $R^{2\ 25°C}$ = 0.096, $R^{2\ 30°C}$ = 0.170).

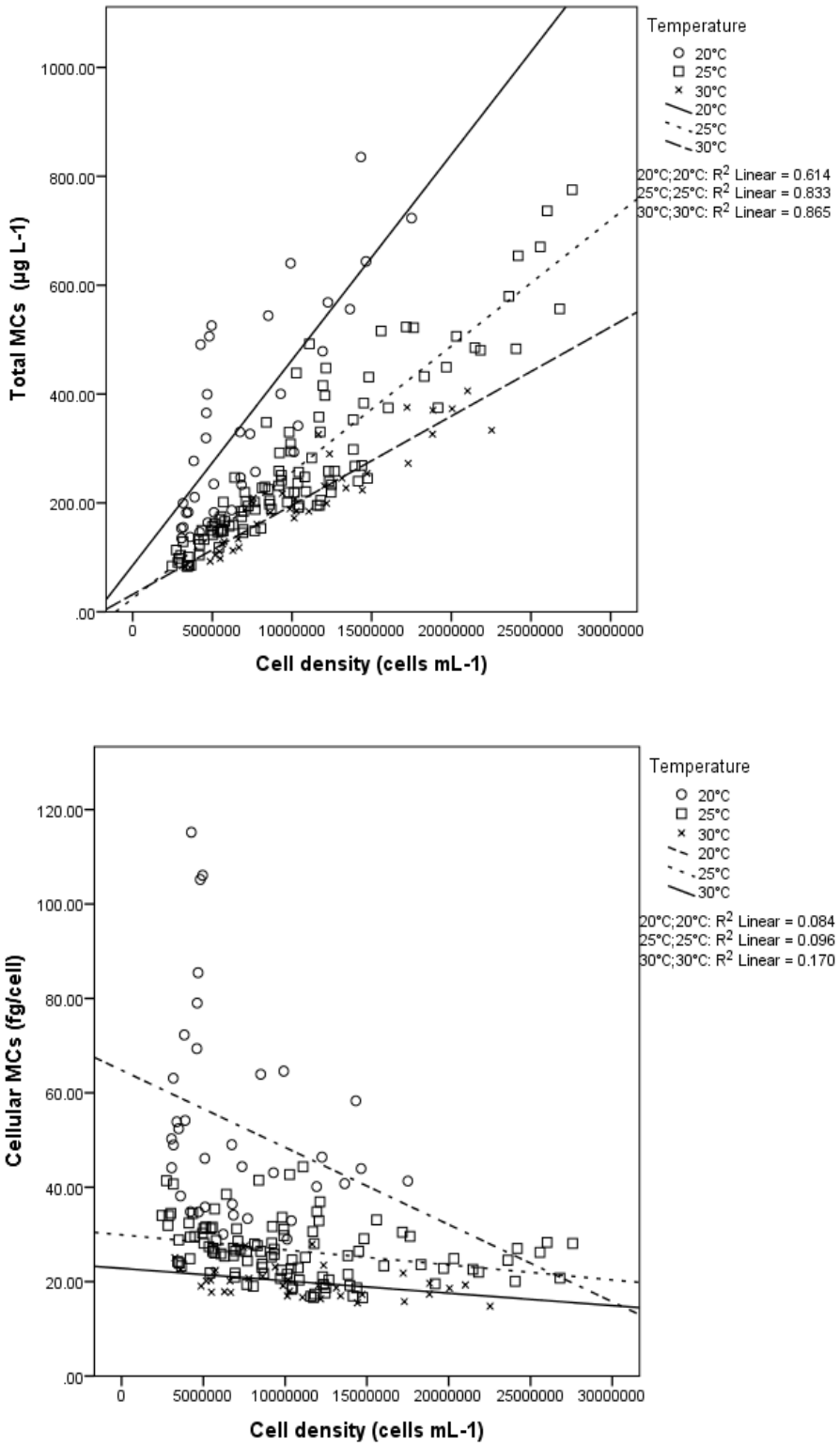


Figure 3.6. Regression relationship between cell density and, total and cellular microcystin concentration.

Nutrients

Figure 3.7. shows the regression relationship between cell density and, total and cellular microcystin concentration (data for individual strains not shown). Total microcystins were generally closely, positively correlated with an increase in cell density under all nutrient conditions, with stronger linearity in both nutrient modified medium ($R^{2\ BG11}$= 0.384, $R^{2\ N\text{-}lim}$= 0.752, $R^{2\ P\text{-}lim}$ = 0.896). Regression analysis of cellular microcystin against cell density at all temperatures showed negative correlation ($R^{2\ BG11}$=0.128, $R^{2\ N\text{-}lim}$= 0.755, $R^{2\ P\text{-}lim}$ = 0.030), a strong negative association between cellular microcystin production and cells grown under nitrogen negative medium.

To examine this phenomenon, further pairwise regression analysis revealed that under high light conditions both strains responded significantly differently in phosphate and nitrogen modified medium with respect to total microcystin concentrations relative to low and medium light levels (p<0.000) (effect was more pronounced in PCC 7806 than CCAP 1450/17) (Figure 3.13.) (total microcystin $R^{2\ PCC\ 7806\ BG11[high]}$ = 0.139, $R^{2\ PCC\ 7806\text{- }N\text{- media [high]}}$ = 0.044, $R^{2\ PCC\ 7806\ P\text{- media [high]}}$ = 0.905; $R^{2\ CCAP\ 1450/17\ BG11[high]}$ =0.087, $R^{2\ CCAP\ 1450/17\ N\text{- media [high]}}$ =0.124, $R^{2\ CCAP\ 1450/17\ P\text{- media [high]}}$ =0.846). The cellular microcystin response was significantly different between nutrient conditions and light levels (p<0.000), with a strong negative correlation observed under nitrogen negative conditions ($R^{2\ BG11[high]}$ = 0.319, $R^{2\ N\text{- media [high]}}$ = 0.903, $R^{2\ P\text{-media [high]}}$ = 0.023; $R^{2\ BG11[med]}$ =0.166, $R^{2\ N\text{- media [med]}}$ =0.968, $R^{2\ P\text{- media [med]}}$ =0.003, $R^{2\ P\text{-media [low]}}$ = 0.136, $R^{2\ BG11[low]}$ =0.835, $R^{2\ N\text{- media [low]}}$ =0.019) at all light levels (Figure 3.8.), no differences were observed between strains.

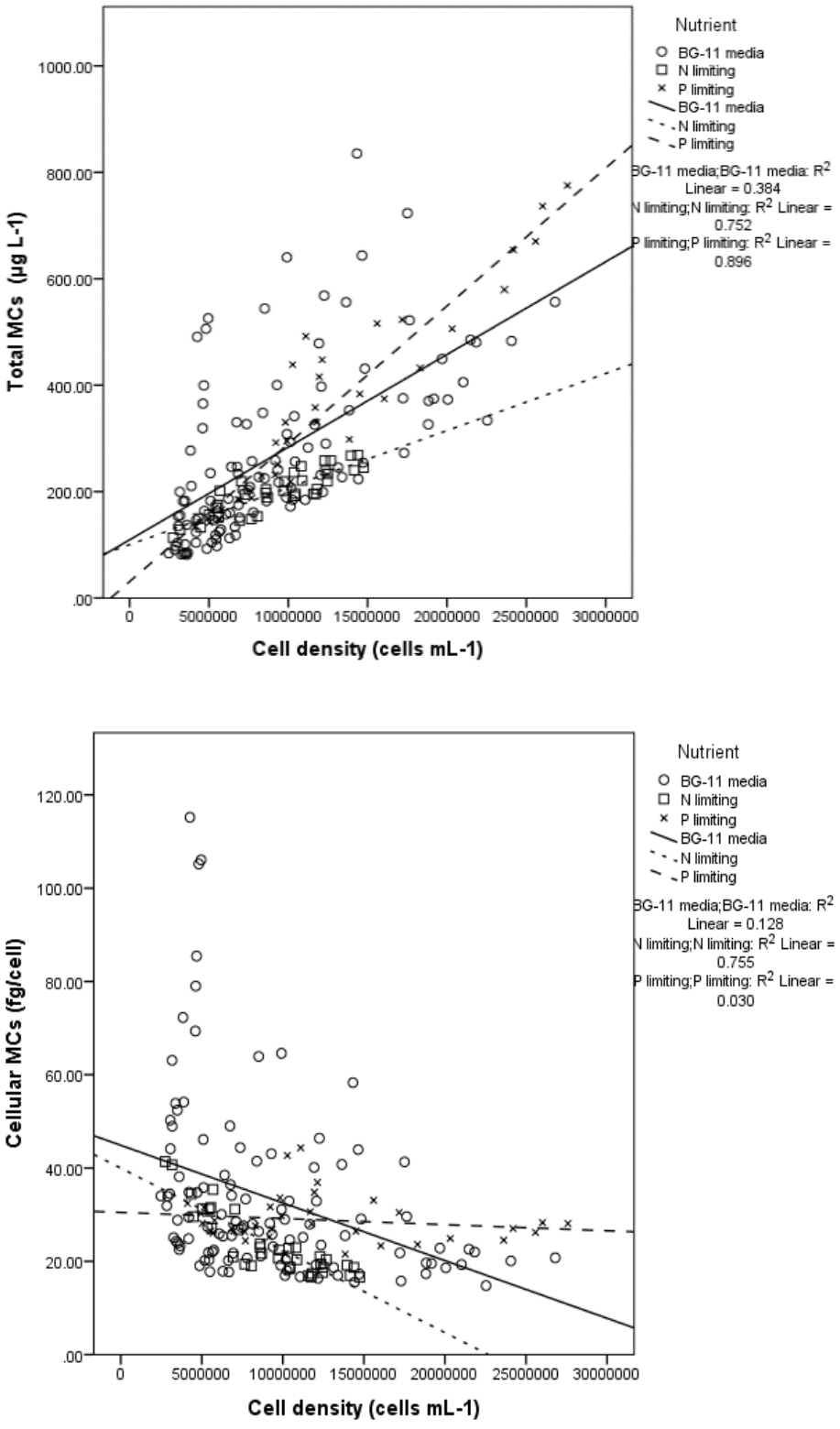


Figure 3.7. Regression relationship between cell density and, total and cellular microcystin concentration under different nutrient conditions.

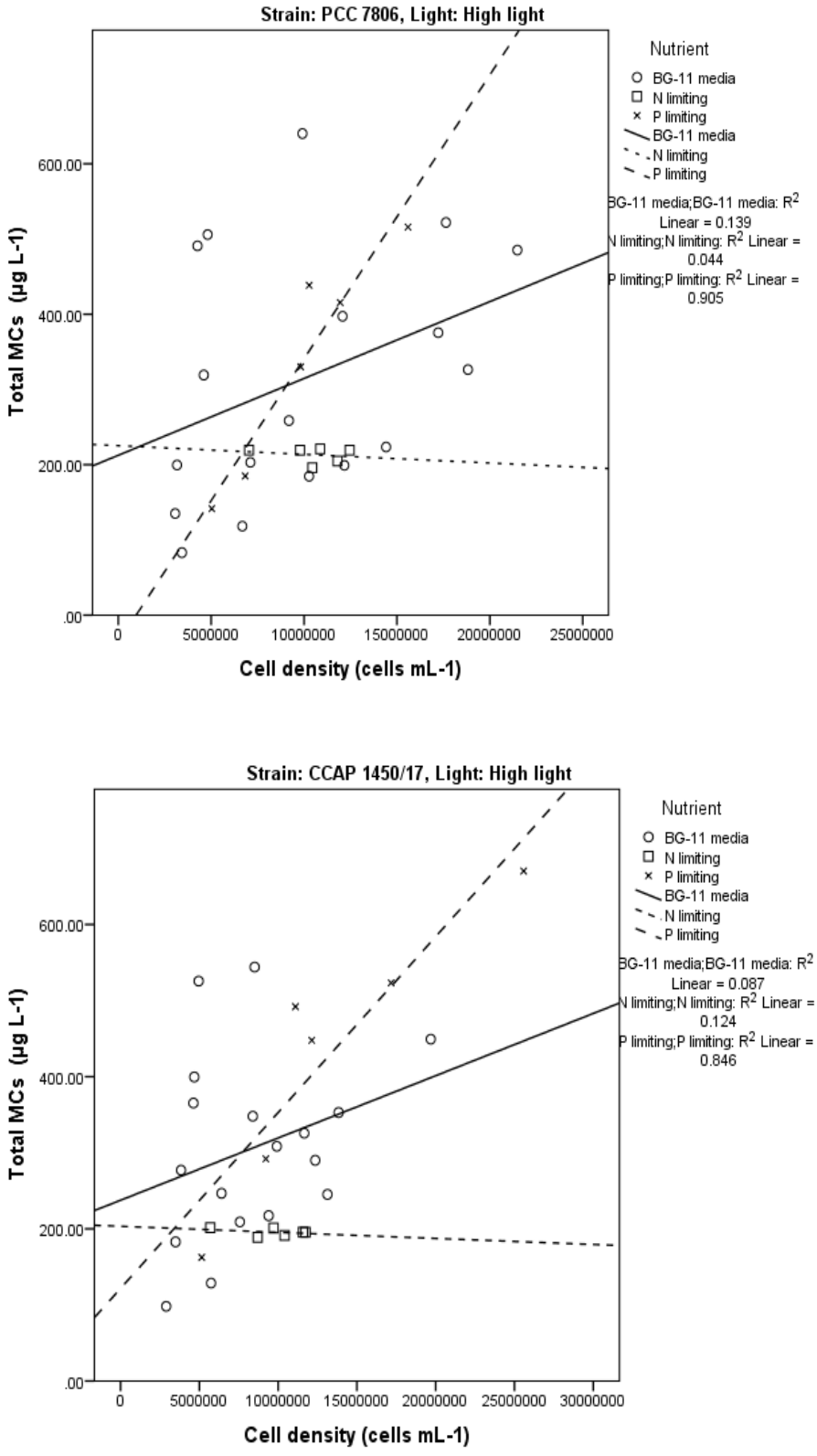


Figure 3.8. Relationship between total microcystin and cell density at high light levels for strains PCC 7806 and CCAP/17 grown under different nutrient conditions.

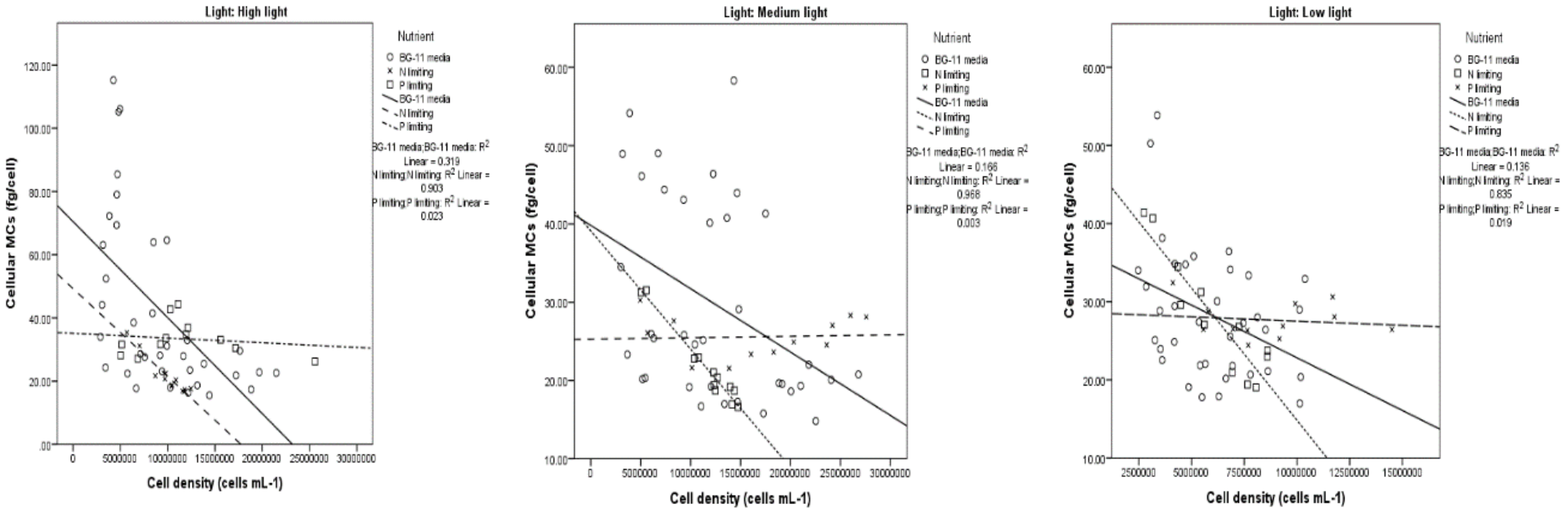


Figure 3.9. Relationship between cellular microcystin production and cell density at high(M=117 µmol photons m^{-2} s^{-1}), medium(M=36 µmol photons m^{-2} s^{-1}) and low(M=15 µmol photons m^{-2} s^{-1}) light intensities for cells grown under different nutrient conditions.

3.1.4. Difference between Microcystis strains within experimental treatments

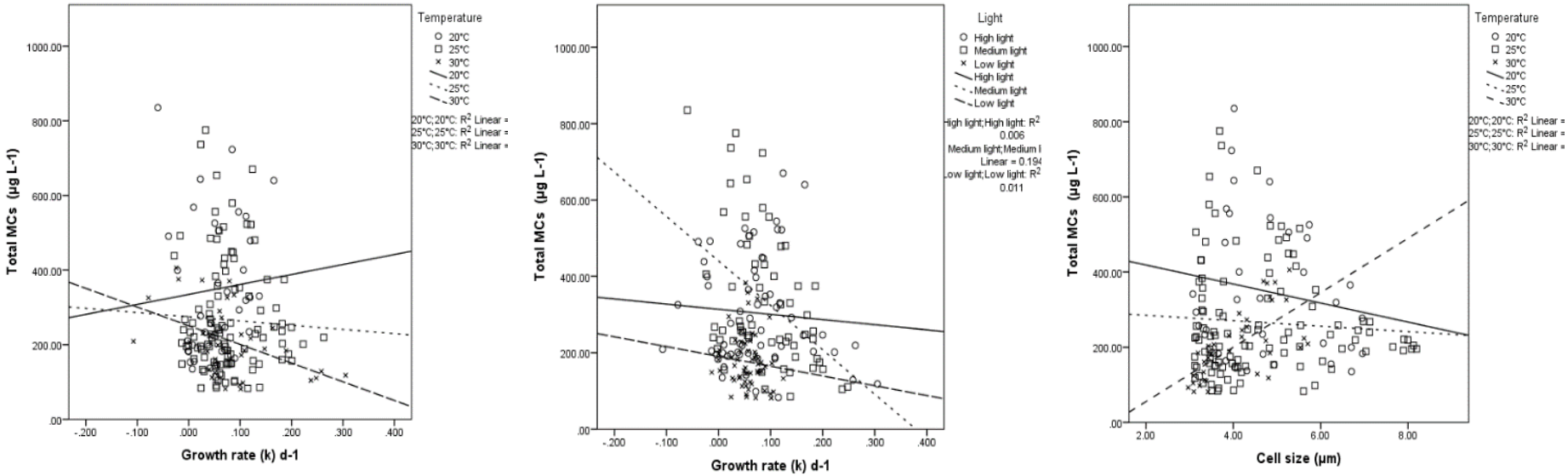
Difference between strains PCC 7806 and CCAP 1450/17 with all experimental treatments of light (high, medium & low), Nutrients (BG-11, P-media & N- media) and temperature (20, 25 and 30°C) were tested using Student *t*-tests. Few significant differences between strains were indicated however cellular microcystin content at 30°C were significantly different ($p < 0.05$) and highly significantly different for total microcystin concentrations in P- and N- media ($p > 0.001$), verifying the response described in section 3.3.3.2.

3.1.5. Cellular growth rates and size

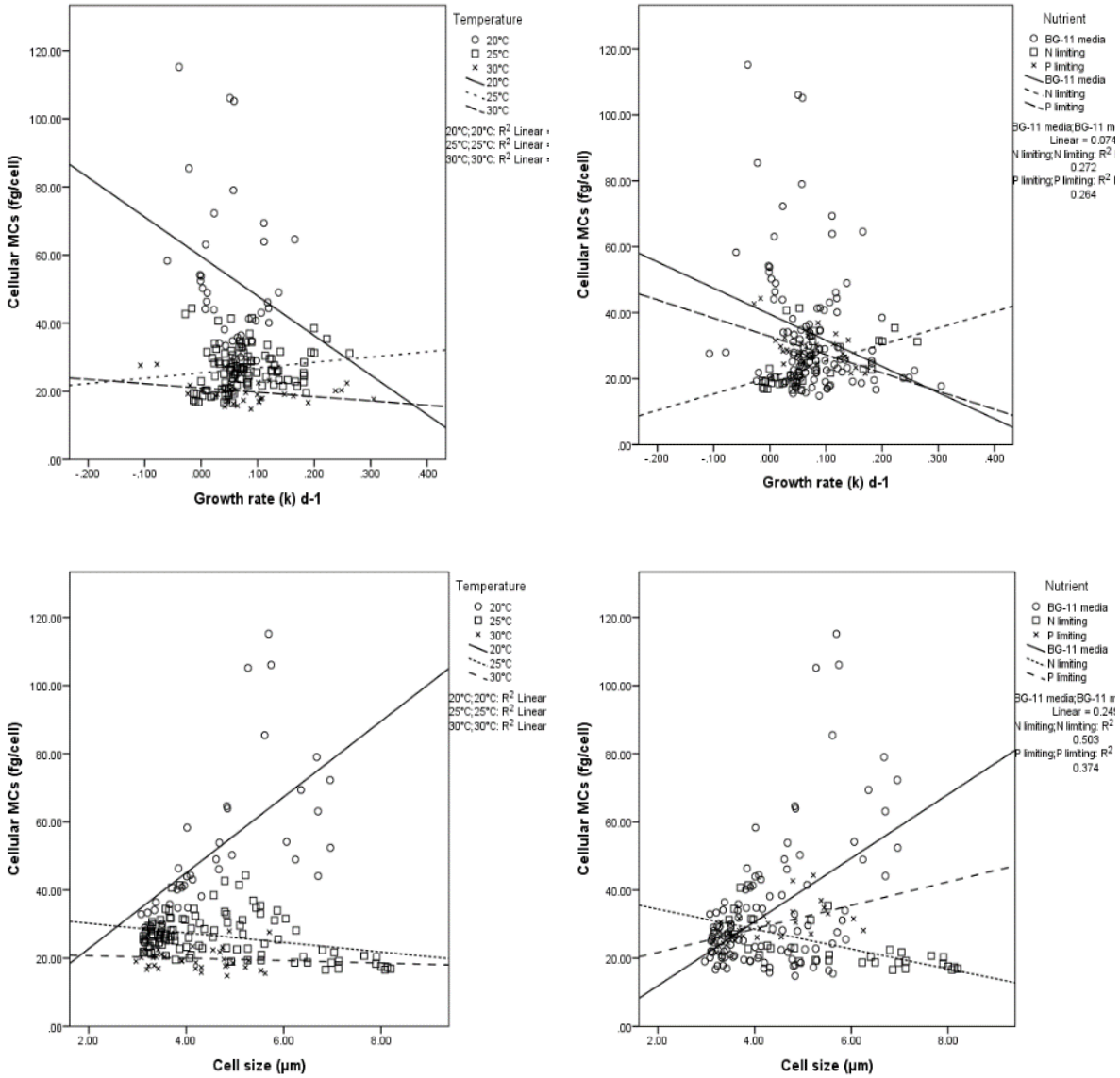
Relationship between growth rates, cell size and toxin production

A univariate general linear model was applied to the data to test the null hypothesis that there were no differences between growth rates or cell sizes of each strain and either total microcystin or cellular microcystin levels under the various abiotic test conditions. No differences between the two strains were identified (all $p > 0.1$). Applying the model to cells grown under different temperature, light or nutrient regimes indicated that light and temperature had a significant impact on total microcystin production and growth rates, but not under different nutrient conditions ($p<0.01$). Significant associations were also indicated between cell size, total microcystins and temperature ($p<0.01$). Cellular microcystin levels were highly significantly different with growth rates and cell size under varying nutrient and temperature regimes ($p<0.001$). Where the model indicated significant differences in effects between, relationships were plotted (Figure 3.10 & 3.11.). Regression plots enabled visualisation of relationships and lines of best fit. For growth rates and total microcystin production differences in the relationships between the responses to light and temperature were confirmed with a slight negative correlation between total microcystin production and growth rates under high light intensities ($R^{2\,low} = 0.006$, $R^{2\,med} = 0.002$, $R^{2\,high} = 0.225$), and minor responses to light observed ($R^{2\,low} = 0.011$, $R^{2\,med} = 0.194$, $R^{2\,high} = 0.006$). Cell size revealed more notable effects with smaller cells tending to be associated with higher total microcystin concentrations, except for those cultures grown at higher temperatures where a moderate positive relationship between increased cell size and total toxin concentration was observed ($R^{2\,30°C} = 0.426$). For cellular microcystin content minor differences in responses to culture

conditions were also observed. At higher temperatures (30°C), cellular content tended to decrease as growth rates increased ($R^{2\ 30°C}$= 0.109), this phenomenon was not observed at 20 and 25°C. Cellular microcystin content under nitrogen and phosphate negative conditions produced contrasting results with a slight positive correlation between cellular toxin content and growth rates under nitrogen negative conditions and a negative relationship observed for cells grown under phosphate limitation relative to BG-11 controls ($R^{2\ BG11}$=0.074, $R^{2\ N\text{-}lim}$= 0.272, $R^{2\ P\text{-}lim}$ = 0.264). The strongest relationship were observed for cellular content and cell size at both temperature ($R^{2\ 20°C}$= 0.379, $R^{2\ 25°C}$ = 0.106, $R^{2\ 30°C}$ = 0.008) and medium($R^{2\ BG11}$=0.249, $R^{2\ N\text{-}lim}$= 0.503, $R^{2\ P\text{-}lim}$ = 0.374). A trend for increased microcystin cellular content with increased cell size at 20°C but not at 25 or 30°C was indicated. Whereas under nitrogen limitation conditions smaller cells were associated with decreased cellular content relative to either phosphate limitation conditions or controls



Figures 3.10. Regression variable plots for experimental data where general linear model indicated significance of effect between variables for total microcystin production.



Figures 3.11. Regression variable plots for experimental data where general linear model indicated significance of effect between variables for cellular microcystin content.

3.4. Discussion

In this study the singular and synergistic effects of light intensity, temperature, and nutrient conditions were measured for two strains of *Microcystis* spp. on the levels of total microcystin produced in batch cultures and on the microcystin content per cell. The aims of the study were to understand the role of light intensity, temperature and nutrient conditions on the production of the toxic cyclic peptide (microcystin) in two toxin producing strains of *Microcystis*, a laboratory reference strain (PCC 7806) and one recently isolated from a freshwater lake in the south of England (CCAP 1450/17) grown in batch culture systems. Cell growth and size were determined by flow cytometry and the microcystin concentrations and cellular content were quantified by a robust, repeatable and reproducible IEC/ISO 17025 accredited protocol (Turner *et al.* 2018a), which enabled high confidence in the comparability of data between experimental runs. The results of this study confirmed complex interactions between abiotic parameters and were suggestive of additive effects with respect to total and cellular toxin concentrations. Relatively little difference was observed between the two strains, suggesting that although data generated through study of established culture collection isolates have frequently been questioned due to the presumption that laboratory adapted strains exhibit atypical behaviours (Lakeman *et al.* 2009), particularly *M. aeruginosa* PCC 7806 (Alexova *et al.* 2011), in this study PCC 7806 was a valid study organism.

Broadly, microcystin production, but not microcystin cellular content, and cell density were closely, positively correlated, with temperature, light and nutrient constituents all effecting cell density maxima. Total toxin concentrations were consistently highest at lower temperatures (20˚C) at medium (36 µmol of photons m^{-2} s^{-1}) and high (117 µmol of photons m^{-2} s^{-1}) light intensities. Conversely, at low temperatures and medium light intensity, cellular toxin contents were lower suggesting density dependent effects with increases in cell numbers resulting in net decrease in individual cellular toxin production. Responses to nutrient limitation conditions were interesting and complex. Overall, total toxin levels were lowest under nitrogen negative media conditions suggesting that nitrogen is an essential component of toxin production. An inverse linear relationship between growth rates (and to a lesser extent a negative correlation with cell size) with respect to cellular microcystin content influenced both by temperature and nutrients, potentially as a result of metabolic processes or stress induced by rapid cell division was observed. The potential for microcystins as induced compounds, modulating cyanobacterial stress responses or acting as quorum sensing chemicals has not been widely studied in cyanobacteria and suggests an important avenue for future study.

3.2.1. Effects of Light intensity

The results clearly show an effect of light intensity on cell density, total microcystin concentrations and cellular microcystin content in both laboratory reference strain, *M. aeruginosa* (PCC 7806) and an environmental isolate of presumptive *M. aeruginosa* (CCAP 1450/17). Several trends were identified, firstly, that for both strains total microcystin levels were highest at 36 µmol of photons m^{-2} s^{-1} (medium), with high light intensity (117 µmol of photons m^{-2} s^{-1}) apparently resulting in a decline in microcystin concentrations. Secondly, cellular microcystin content, again for both strains, were highest at 20˚C, under medium and high light intensities. Thirdly, that under low irradiance (15 µmol of photons m^{-2} s^{-1}), apart from nitrogen negative media, both total microcystin concentrations and cellular content were reduced. Finally, growth rates and cell densities were highest under medium light levels 36 µmol photons m^{-2} s^{-1} potential indicative of closer to optimum conditions for *Microcystis* physiology, but growth rates were not predicative of microcystin levels.

The relationships between light intensity and microcystin concentrations has been studied in both laboratory batch or continuous cultures or in the field (Watanabe and Oishi 1985, Hesse *et al.* 2001, Wiedner *et al*. 2003, Xie *et al.* 2016) and has generated a wide range of often conflicting responses. It has been suggested that microcystins may play a role in light adaptation processes (Hesse *et al.* 2001, Renaud *et al.* 2011) and several authors have shown that microcystin production is inversely correlated with disadvantageous culture conditions, postulating that the high metabolic costs associated with toxin production are casual in reduced toxin production (Tilman 1977, Sivonen 1990, Sedmak and Kosi 1998, Babica *et al*. 2006). Xie *et al.* (2016) proposed that there were two possible impact pathways with respect to light intensities; the authors suggested that in mixed cultures, low light conditions limited microcystin production by deregulating toxin production, or by reducing the numbers or growth rates of toxigenic strains, particularly in the presence of high nitrates.

Conversely, Renaud *et al.* (2011) showed that in laboratory grown mixed cultures under conditions of low light intensity (20 µmol of photons m^{-2} s^{-1}), toxigenic strains became dominant suggesting that microcystin production was not a disadvantage in competition for limited resources. The authors went on to demonstrate that number of copies of the gene responsible for microcystin production, *mcyD*, (Kaebernick *et al.* 2000), also increased under low light conditions however the authors reported variable results from other strains revealing a complex picture and concluded that production of microcystins *per se* could not explain the dominance of strains under conditions of low light intensity. In a series of monoculture experiments examining the impact of various light intensities regimes, Kaebernick *et al.* (2000) used RNase protection assays to measure *mcyB* and *mcyD* transcription high (68 µmol of

photons m^{-2} s^{-1}), medium (31 µmol of photons m^{-2} s^{-1}) and low light (16 µmol of photons m^{-2} s^{-1}) and red and blue light at 23˚C. The authors showed that high light intensities and red light triggered gene upregulation at specific threshold intensities, with low light and blue light reducing transcription but corresponding differences in cellular microcystin content were not observed using a PP2A assay based on the inhibition of protein phosphatase activity; although it was noted that the assay used did not show a high level of repeatability which may have accounted for this finding. In the current study low (15 µmol of photons m^{-2} s^{-1}) and medium (36 µmol of photons m^{-2} s^{-1}) light intensities were comparable to those employed by Kaebernick *et al.* (2000) and there was some indication of similar effects of light intensity.

In this study cellular microcystin content for PCC 7806 at 15 µmol of photons m^{-2} s^{-1} (low) was reduced relative to controls whereas significant increased total and cellular microcystins were demonstrated at 36 µmol of photons m^{-2} s^{-1} (medium) at 20˚C. Furthermore, this effect was significantly more pronounced at high light intensities (117 µmol of photons m^{-2} s^{-1}) these findings are broadly in accordance with Kaebernick *et al.* (2000) in that cellular microcystin content (total microcystins) were greatest under medium and high light, and lowest at low light conditions. Similar observations were made for the environmental strain (CCAP 1450/17), with higher (but not significant) cellular microcystin content demonstrated at 20˚C under medium light conditions. The finding that elevated microcystin levels and corresponding cellular content were observed at light intensities of greater than 35 but less than 75 µmol of photons m^{-2} s^{-2} are in accordance with several other workers who have reported microcystin maxima production with this range (Utkilen and Gjolme 1995, Wiedner *et al.* 2003). Furthermore, whilst the data did not reveal saturation irradiances, higher light intensities showed some suppressive effect on microcystin cellular content and to an extent total microcystins, under all conditions except for cultures grown at 20˚C, this latter finding in accordance with previous studies carried out on *M. aeruginosa* (van der Westhuizen and Elof 1985, Deblois and Juneau 2010). Taken together this study shows a clear relationship with total and cellular toxin content and irradiances which was broadly independent of growth rates. Maxima mean toxin concentrations were demonstrated at medium irradiances, with lowest levels at low light levels these data in accordance with several studies in the published literature. However, the contradictory findings reported by several authors, reveal a complex picture. It is however difficult to compare data generated from multiple studies due to large differences in experimental design, culture methods, cyanobacteria strains and the performance characteristics of methods to determine total and cellular microcystin contents.

3.2.2. Effects of Temperature

The results demonstrated a clear increase in cell density and growth rates as temperatures increased from 20 ˚C to 30˚C for both laboratory reference strain, *Microcystis aeruginosa* (PCC 7806) and environmental isolate of presumptive *M. aeruginosa* (CCAP 1450/17) which was independent of irradiance. Conversely, mean cell size was higher at 20˚C than 30 ˚C. At all light intensities (low, medium and high), both total microcystin production and cellular microcystin content were higher, often significantly so at 20˚C compared to 30˚C. These findings are in accordance with van der Westhuizen and Elof (1985) in studies of *M. aeruginosa* (UV-006). The authors demonstrated that cells were most toxic when grown at 20˚C, and revealed marked reduction in toxicity (as measured by mouse bioassay) in cells grown at temperatures above 28˚C. Similarly, to the results presented here, van der Westhuizen and Elof (1985) further reported that optimal conditions for growth (as determined by cell density) did not correlate with toxin production. Likewise, Deblois and Juneau (2010) showed an inverse correlation between microcystin levels and cell specific division rate. The authors further demonstrated that the electron transport rate (ETR), used as a measure of photosynthetic light reactions, and correlating with cell division rates, modulated microcystin production at a cellular level. Comparable data have recently been published by Walls *et al.* (2018) from a series of laboratory studies on *Planktothrix agardhii*, demonstrating that total microcystin contents were highest at between 20 and 25°C but declined rapidly at 30°C. The authors reported that a temperature threshold could be used to forecast bloom severity and toxin release in eutrophic lakes.

Davis *et al*. (2009) further demonstrated that increased experimental temperatures significantly increased growth rates of toxin producing strains but did not produce the same effect on non-toxic strains. The authors postulated that elevated temperatures (within an environmentally relevant range) yielded more toxic *Microcystis* cells (and *mcyD* copies per cell) relative to non-toxic strains. Davis *et al.* suggested that either scenario introduced the potential for yielding more toxic blooms, however cell toxin contents were not reported and therefore it was not possible to assess fully in the context of cell density, growth rates and microcystin levels. Walls *et al.* (2018), further explored the hypothesis that temperature modulated microcystin production and release from cyanobacterial cells. The authors concluded that maxima toxin levels were released into the water column at temperatures between 20 and 25˚C (several degrees higher than the selected strain optima) and were inversely proportional to algal biomass and cell density. These data support the hypothesis that there may be potential temperature triggers, where toxic cyanobacteria release a greater proportion of toxins into the water column which, whilst not corresponding to conditions that promote the highest growth rates, may have a suppressive effect on other non-toxic stains within a population thus

providing a physiological advantage to toxic strains. It has previously been postulated these temperature mediated triggers may induce positive feedback on the frequency and severity of harmful blooms over non-toxic strains in a population (Briand *et al.* 2008, Kleinteich *et al.* 2012, Walls *et al.* 2018), promoting intracellular toxin synthesis (Davis *et al*. 2009) and subsequent toxin release (Walls *et al.* 2018). The data generated in this series of laboratory batch culture experiments, partially support this hypothesis, demonstrating that toxins are preferentially produced from cells at lower temperatures (within the optima range for the species) and are inversely related to growth rates.

One increasing observation from this study was that mean cell size, as determined by flow cytometry, was greater in cells grown at 20˚C than at 30˚C and thus corresponded to higher toxin quotient. This observation supports early work of Orr and Jones (1998) who reported a direct linear correlation between cell biovolume, cellular division and microcystin production. Furthermore, Kumayer *et al.* (2003) reported that larger size classes of Microcystis colonies (>100 µm) showed the highest proportion of microcystin-producing genotypes, and the highest microcystin cell content. In more recent studies, Islam and Beardall (2017) showed that cultures of toxic strains of *M. aeruginosa* exhibited consistently higher biovolumes relative to non-toxic strains. This finding, which is consistent with larger cell sizes, has been further explored in other toxic and non-toxic strains of cyanobacteria. Zarontenella *et al.* (2018) demonstrated that induced outer membrane vesicles (OMV) and extracellular vesicles (EVs) were generated as a response to early stress conditions in toxin producing *Cylindrospermopsis raciborskii* but not in non-toxic strains. The authors suggested that EVs permitted translation of cell to cell signalling compounds in some cyanobacteria, in a similar way to mechanisms observed in some aquatic bacteria, functioning as adaptive/protective responses to rapid changes in environmental conditions (Gamalier *et al.* 2016). Further examination of this response is required in a range of cyanobacteria species to explain this phenomenon.

3.2.3. Effect of Nutrients

The data generated in this study revealed several trends with respect to nutrient ratios (at 25˚C). The responses of both PCC 7806 (reference strain) and CCAP 1450/17 (environmental isolate) to nitrogen and phosphorous negative media, relative to control, treatments were generally similar in scale, apart from CCAP 1450/17 cell density under low light (15 µmol of photons m^{-2} s^{-1}). Total microcystins were significantly depressed in N- media compared to cells grown in P- medium and to cells grown in the control medium (BG-11). The cellular microcystin content were elevated in cells grown under P- media, with a corresponding negative correlation

between cellular toxin content and nitrogen limitation; both were impacted by irradiance. Cell size, growth rates and cell densities were all affected by nutrient availability but with no discernible pattern, except for a notable effect of increased cell size under N- media at high and medium light intensities, and corresponding depression of cellular microcystin content. Cell densities were elevated under P- media relative to control groups at all irradiances, an effect that was particularly apparent for the environmentally adapted strain (CCAP 1450/17), with parallel elevation of total (but not cellular) microcystins presumably due to the dilution effect of increased cell numbers.

Numerous studies have noted the requirement of nutrients (N and P) for the growth of cyanobacteria (Trimbee and Prepas 1987, Watson *et al.* 1997, Orr and Jones 1998, Vézie *et al.* 2002, Davis *et al.* 2009, Briand *et al.* 2012), many suggesting that high levels of nitrogen and phosphorus may favour toxic strain proliferation over non-toxic ones (Vezie *et al.* 2002, Flores and Herrero 2005) thus selecting for toxic bloom formation in water bodies subject to periodic nutrient enrichment and eutrophication (Paerl and Otten 2013). This study demonstrates a close, positive relationship between cell density and total microcystin concentrations in strains grown under nitrogen replete conditions (e.g. control and P- media), this effect was not observed in cells starved of nitrogen. Conversely, phosphorus limitation did not appear to impair microcystin production, in fact under all irradiance regimes total microcystins were slightly elevated relative to control groups in P deprived batch cultures. These observations, together with the significant reduction in total, and to a lesser extent cellular, microcystin in nitrogen deprived cultures supports the hypothesis that nitrogen rather than phosphorus is a significant modulator of microcystin production (Downing *et al.* 2005, Monchamp *et al.* 2014, Pimentel and Giani 2014). At the transcriptome level, it has been reported that approximately one third of the genome is differentially expressed under N-conditions, with significant decrease in transcription of microcystin synthetase gene, genes related to photosynthesis and genes related to nitrogen assimilation. This downregulation is concurrent with a decrease in microcystin concentrations (Harke and Gobler 2013, Harke and Gobler 2015). Furthermore, under low phosphorous conditions, little or no effect on transcription of microcystin synthetase gene but substantial induction of the Pho regulon (responsible for the suite of genes responsible for peptide tailoring and transport and harvesting of organic phosphorus) was noted (Harke and Gobler 2013).

In a further study to examine daily dynamic transcriptome changes in toxic *M. aeruginosa*, Harke and Gobler (2015) showed that downregulation of the microcystin synthetase gene under nitrogen depletion was restored following nitrogen restoration consequent with increased cellular microcystin content. These findings offer a partial explanation for the data generated in this series of studies, and it is plausible that under nitrogen depleted conditions

Microcystis cells divert metabolic resources to capturing nitrogen rather than synthesizing nitrogen rich metabolites, such as microcystins. This observation supports earlier work of Orr and Jones (1998) who suggested that microcystins were not secondary metabolites but that they performed a functional role as intracellular nitrogenous compounds in toxigenic strains. It is worthy of note however, that whilst responsivity of the microcystin synthetase gene cassette to nitrogen has been reported by previously (Ginn *et al.* 2010, Pimentel and Giani 2013) confounding data have been generated to date highlighting the potential limitations from drawing conclusions based on single (or low numbers) of gene function in intricate biological systems. It is likely that substantial additional work will be required across a range of disciplines including transcriptomics, proteomics, genomics, biochemistry and modelling to more fully elucidate the complexity of *Microcystis* physiology.

3.5. Conclusions

The data generated in this series of studies revealed something of the complex and dynamic role played by microcystins in *Microcystis* spp. under laboratory conditions. Whilst it is unwise to extrapolate from laboratory to field situation the results observed in this study had considerable resonance with those generated in section 4 (field observations) with respect to impact of exogenous physical and chemical factors. The advantages of using standardised flow cytometry and an IEC/ISO accredited methodology with well-defined performance characteristics (Section 2), and a high degree of repeatability and reproducibility were clear, enabling robust comparisons between experimental runs and good confidence in the quality of test results. Many of the observations here, particularly with respect to cell density dependency of microcystin concentrations, obligate requirements of nitrogen for toxin production, and increased toxin synthetase at lower growth temperatures are in accordance with previous studies. Indications of the uncoupling of growth rates and microcystin concentrations, observed here, have reported previously (Celeste 2017) but are a relatively unusual finding within the published literature and worthy of further study. The apparent inverse relationship between cell size, and nitrogen availability and toxin production was a novel observation and is worthy of further study with respect to predictions of toxic events.

In summary, the data show that microcystins are not actively synthesized under conditions of nitrogen depletion (N- media) and whilst levels correlate well with cell density (observed at growth optima and sub-optima temperatures), production is not intrinsically linked to growth rates. It is considered likely that microcystins perform a functional role under nitrogen replete conditions, potentially conferring competitive advantages over non-toxic strains, which may be metabolically expensive and is substantially reduced under nitrogen deprivation and/or when cells are rapidly dividing. Substantial further work would be required to further test this hypothesis, but it is likely that advances in transcriptomics will be useful in unravelling the functional properties of microcystins, and revealing their relationships with abiotic parameters, which in turn will assist in predictions and mitigation of harmful blooms.

4. Long-term monitoring of cyanobacteria and cyanotoxins in two lowland freshwater reservoirs in southern Britain

4.1. Introduction

4.1.1. General

Cyanobacteria blooms are a global problem in freshwater ecosystems (Codd 2000, Paerl *et al.* 2001, Paerl and Paul 2012). A range of factors have been reported to influence the abundance and likelihood of bloom formation in closed aquatic systems, notably increased temperature and nutrient enrichment (Robarts and Zohary 1987, Paerl and Huisman 2008, O'Neil *et al.* 2012, Rigosi *et al.* 2014). A proportion, estimated as between 40-70%, of cyanobacteria blooms are reported to occur concomitantly with elevated levels of cyanobacterial toxins (microcystins) (Lawton and Codd 1991, Chorus 2001, Turner *et al*. 2018b). Microcystins can have detrimental impacts on public and animal health; and require management strategies to mitigate risks. Understanding the frequency, distribution and drivers of microcystin occurrence could assist in the implementation of targeted and cost-effective solutions to maintain the safety of drinking water and the recreational use of freshwater environments.

4.1.2. Environmental assessment and monitoring for cyanobacteria

Cyanobacterial toxins are known to be responsible for intoxication in humans and animals globally. Most frequently reported are wild animal, livestock and pet deaths with numerous accounts in the literature, from both more and less economically developed nations (Lawton and Codd 1991, Kuiper-Goodman *et al.*1999*,* Van Ginkel 2001, Alonso-Andicoberry *et al.* 2002, Qin *et al.* 2010). In most countries, including England, Wales and Scotland, control plans for public health risks associated with exposure to cyanobacterial toxins are based on assessments of cyanobacterial cell presence and number in the event of bloom formation. In the UK assessments and management recommendations are made by national agencies (the Environment Agency (EA) in England and the Scottish Environmental Protection Agency (SEPA) in Scotland). In both devolved administrations samples are collected reactively from the water column in response to visual bloom occurrence and cyanobacterial species are counted microscopically to determine the number of cells per millilitre of water. Samples containing > 20,000 cells mL^{-1} trigger actions such as preventative closures or restrictions on usage enforced by the owner of the water body. The presence of scums automatically indicates

action, as scum formation is known to increase the likelihood of adverse health effects by factors of up to 1,000 (WHO 2017) and would typically, in the UK, result in measures to prevent exposure of humans and animals (Water UK 2006). Systematic, or risk-based routine monitoring of water bodies for cyanobacteria is not undertaken in the UK, consequently the incidence, intensity and seasonality of cyanobacterial blooms is not well known (Krokowski *et al.* 2012, Turner *et al.* 2018b). Furthermore, whilst the presence of elevated cyanobacterial cells enables identification of potential risks, toxin production during blooms formation is not certain (Lawton and Codd 1991, WHO 2017). In a recent study on the levels of microcystins in freshwater bodies in England, Turner *et al.* (2018b) found that only 18% of samples containing cyanobacterial cells exceeding action state thresholds contained microcystins above the WHO medium health criterion of 20 µg L^{-1}. Therefore, management actions driven by elevated cyanobacterial cell counts may be unnecessary when blooms are formed from non-toxic species and may have unnecessary detrimental economic impacts.

4.1.3. Determination of microcystin profiles in freshwater systems

To date very few studies have been undertaken to examine the prevalence and levels of microcystin toxins and variants globally, thus relatively little is known about the distribution of microcystin profiles in freshwater systems. In one notable exception, a recent study the European Multi-Lake Survey analysed toxin profile data from 26 European countries from lakes with a history of eutrophication, together with many environmental parameters. The authors reported substantial direct and indirect effect of temperature on toxin concentrations and toxin quota, concluding that whilst few geographical patterns could be discerned, increasing lake temperatures could drive changes in the distribution of cyanobacterial toxins, possibly selecting for a few toxic species (Mantzouki *et al.* 2018). In a study on the array of microcystins determined during cyanobacterial blooms in Lake Victoria, Tanzania, East Africa, Miles *et al.* (2013) reported a distinctive, complex toxin profile signature during bloom events which has also been confirmed in Ugandan and Kenyan regions of Lake Victoria (Okello *et al.* 2010, Miles *et al.* 2012). In a systematic study to assess microcystins in freshwater lakes in England Turner *et al.* (2018b) revealed complex toxin profiles with occurrence of toxin clusters unrelated to cyanobacterial species and no correlation with environmental parameters. These data are suggestive of complex ecosystems, with levels and signatures of microcystin and variants potentially influenced by geographical range but with impact of environmental factors unclear.

4.1.4. Predicting toxic cyanobacteria in aquatic environments

It is widely reported that factors such as light intensity, temperature and nutrient content (phosphate and nitrate) availability as well as hydrodynamics may influence the occurrence and density of cyanobacterial blooms in closed water bodies (Lawton and Codd 1991, Oliver *et al.* 2012). A number of studies have attempted to model both cyanobacterial concentrations using meteorological, hydrological and environmental parameters (Downing *et al.* 2001, Howard *et al.* 2002, Carvalho *et al.* 2011, Rigosi *et al.* 2015). In most studies the predictive ability of models with respect to risk management has been limited not least because the relationship between the presence and increase in cyanobacterial cells is not ubiquitously or directly correlated with an increase in the occurrence of toxins (Sivonen and Jones 1999, Mekebri *et al.* 2009, Turner *et al.* 2018b). Notwithstanding this, Carvalho *et al.* (2011), demonstrated that statistical models applied to phytoplankton data from 134 lakes in the UK could be used to describe lakes that may be susceptible to cyanobacterial blooms events. It is evident that understanding the key environmental drivers that favour cyanobacterial abundance and potentially toxic events would facilitate proactive rather than reactive monitoring and management strategies to reduce the public and animal health risks.

4.1.5. Aims of the study

In this study a range of biological and chemical factors were examined together with direct measurements of cyanobacteria cell concentrations, phycocyanin levels, *Microcystis* cell levels, and total microcystins and microcystin variants in two freshwater reservoirs in the South of England over a twelve-month period. The overarching aims of the study were threefold, to;

- Assess the frequency and concentrations of cyanobacteria, *Microcystis* spp. and the presence of toxins in two freshwater lakes in the South of England,
- To examine the levels and toxin profiles of microcystins in the two lakes, and to discern any relationships between the abundance of cyanobacteria and *Microcystis* spp. and the potential toxicity,
- To use these data collected over a twelve-month monitoring period combined with simple statistical modelling to predict the likelihood of toxicity in the two lakes based upon biological and chemical measurements.

4.2. Study site and methods

4.2.1. Longham Lakes, Bournemouth, Dorset

Longham Lakes, consists of two freshwater reservoirs, used as a nature reserve and recreational fishery within the borough boundaries of Bournemouth (Figure 4.1.). The two lakes located at national grid reference SZ 06237 98079 are man-made, fed by the River Avon and provide an auxiliary water supply to the Bournemouth-Poole conurbation. The northern lake was completed in 2003, has a perimeter of 1,400m and an area of 97,000 m^2. The southern lake is connected to the northern lake, it was completed in 2010, has a perimeter of 2,050m and an area of 250,000 m^2. The maximum depth for both lakes is approximately 14m and they both have an average depth of 2.9m. Longham Lakes is managed by Bournemouth Water which is part of South West Water, lake water chemistry and phytoplankton are constantly monitored and weekly water samples are taken.

4.2.2. Chemical and biological measurements

Frequent water samples and measurements were taken at the northern lake (Lake 1) and southern lake (Lake 2) at the points marked in Figure 4.2., from the surface and at 1m intervals until the bottom, subsurface samples collected using a 2.2 L horizontal sampler. Samples and measurements were taken weekly in the spring, summer, autumn and dropped down to once every three weeks intervals in the winter. Chemical and biological parameters were measured by a multiparameter probe (6600 V2, YSI) measuring:

- Dissolved oxygen (mg L^{-1}),
- turbidity (NTU),
- Phycocyanin fluorescence (a proxy for cyanobacteria abundance) (RFU or cells mL^{-1}),
- temperature (^oC),
- pH,
- salinity (ppt),
- depth (m).

The measurement of the photosynthetic pigment phycocyanin is used as a proxy for cyanobacteria as the pigment is exclusive to the phyla. Most multi-parameter probes are equipped with chlorophyll sensors, unfortunately, the chlorophyll sensor was unserviceable during most of the period of this study.

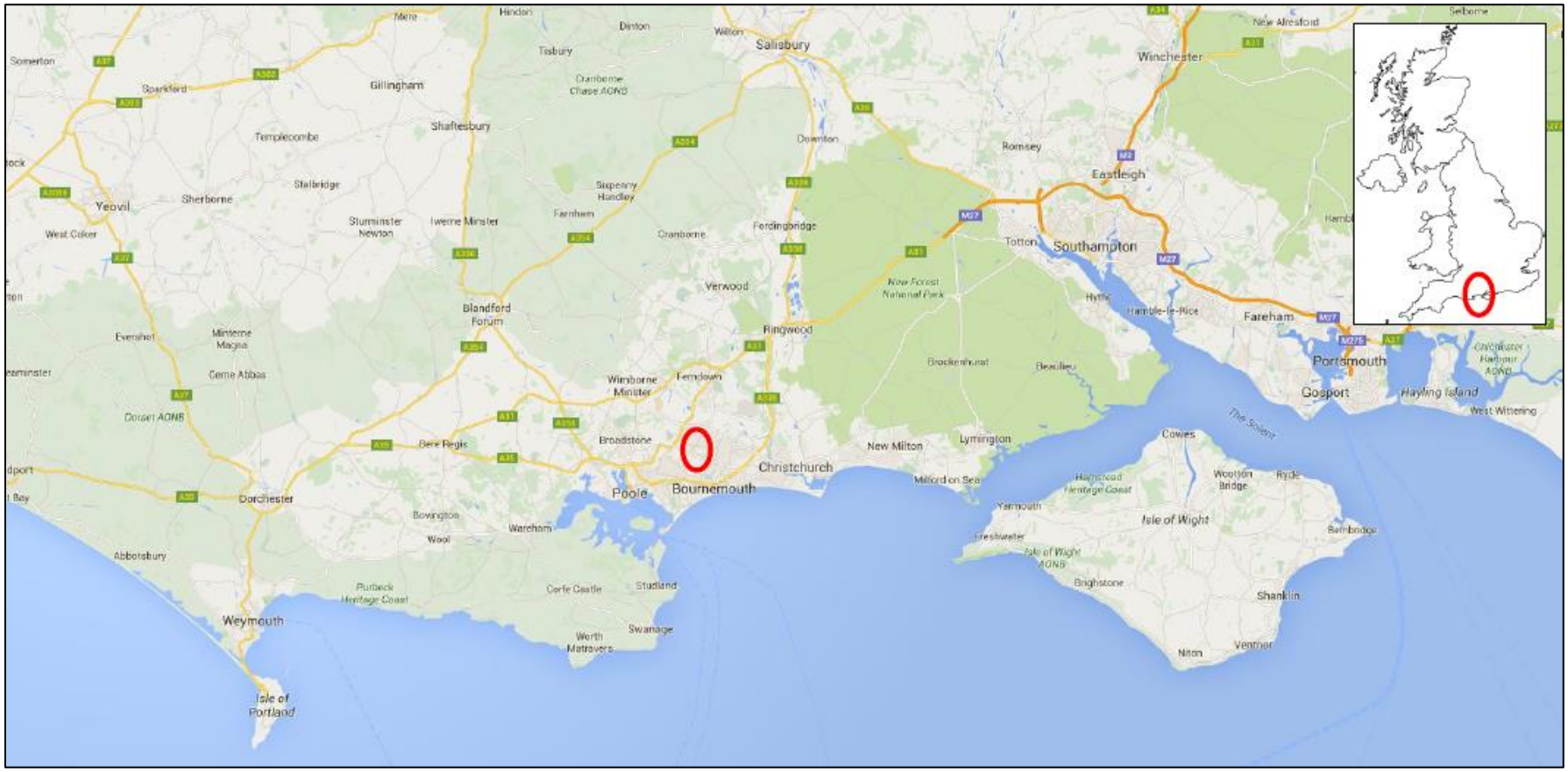


Figure 4.1. Location of the environmental monitoring site, Longham reservoirs, Dorset, UK.

Figure 4.2. Aerial view of Longham Lakes with sampling point marked A. Lake 1 (northern) and B. Lake 2 (southern).

4.2.3. Identification and enumeration of phytoplankton

In the laboratory, water samples from each depth were homogenised and ~5 mL was aliquoted into a 10mL plastic test tube. Cyanobacteria cells in water samples counted by flow cytometry (Accuri C6, BD) threshold 80,000 au FSC-H, flow rate 100 µl/min, core size 25 µm for 5 minutes. The protocol has been used previously by BU to identify and enumerate *Microcystis* spp. from environmental samples (Chapman 2016, Section 2.).

For identification and enumeration of phytoplankton by light microscopy, 15mL of homogenised top sample was aliquoted into a 15mL centrifuge tube. The tube was sealed then inverted and the lid struck on the bench several times to burst any gas flotation vesicles with in cells. Centrifuge tubes were then placed upright and stored at 4-6ºC in the dark for two days for the phytoplankton to settle out. The top 14mL was carefully removed by pipette to not disturb the sedimented phytoplankton, the remaining 1mL was vortexed and transferred to a Sedgwick-rafter counting chamber. The phytoplankton in a minimum of 10 of the 1000 grid squares were identified and enumerated by light microscope (Olympus, BX51) at 40x and 100x magnification, cell densities per mL were calculated by the equation:

$$C_D = \frac{G_C}{G_N} \, x \, \frac{S_R}{L_S}$$

Where, C_D equals cell densities (mL^{-1}), G_c equals the number of cells counted in each Sedgwick-rafter grid, G_N equals the number of grids counted, S_R equals the total number of grids in a Sedgwick-rafter counting chamber and L_S equals the volume of lake water sampled.

4.2.4. Cyanobacterial toxin analysis

Cyanotoxins were analysed by filtering 200 mL of water from each depth in triplicate (Whatman, CFC), filter papers were wrapped individually in aluminium foil and preserved at -80ºC. On analysis, filter papers were subjected to three cycles of freeze-thawing before submersion in 10mL of 80% aqueous methanol. Samples were left in the dark at 4-6ºC for 24 hours, before ~0.5mL was aliquoted into a LCMS certified autosampler vial. Cyanotoxins were qualified and quantified by ultra-high-pressure liquid chromatography coupled to tandem mass spectrometry, as per the protocol in chapter 2. Lake cyanotoxin (Microcystins) concentrations in micro-gram per litre calculated using the equation:

$$L_C = \frac{S_C}{(L_S/S_V)}\ x\ F$$

Where, *Lc* equals Lake microcystin concentration (ng L^{-1}), S_C equals LCMS sample concentration (ng mL^{-1}), L_S equals the volume of lake water sampled, S_V equals solvent volume and *F* is the factor required convert L_S to one litre.

4.2.5. Photosynthetic pigment extraction.

Concurrently with toxin analysis, 3mL of solvent resulting from the extraction of the filter paper was aliquoted into a cuvette to measure pigment absorption in a spectrophotometer (Varian, Cary 50) at 470, 652.4 and 665.2 nm for each triplicate. Chlorophyll *a* (C_a) and *b* (C_b) concentrations were calculated according to the following equation (Wellburn 1994):

$$C_a = 16.72A_{665.1} - 9.16A_{652.4}$$

$$C_b = 34.09A_{652.4} - 15.28A_{665.2}$$

Total carotenoids (C_{x+c}) were calculated using the equation (Wellburn 1994):

$$C_{x+c} = \frac{1000A_{470}\ -\ 1.63C_a\ -\ 104.96C_b}{221}$$

The photosynthetic pigment concentrations from 80% aqueous methanol were compensated from the 100% method of Wellburn (1994) as per the method described in chapter 2.

4.3. Results

Flow cytometry and Sonde probe measurements were taken from the 16th of May 2016 until the 31st of May 2017. Toxin and photosynthetic pigment samples were collected and analysed between the 16th May 2016 and the 28th November 2016. In both instances data from each depth were combined to produce an average for each measurement at the time of sampling, except for turbidity (NTU), where the lake bottom measurement was disregarded due to sediment disturbance from the horizontal sampler. For assessment of seasonality data were separated into two groups, spring/summer comprising measurements taken in March to August and autumn/winter in September to February.

4.3.1. Chemical and biological water conditions at northern and southern lakes

Tables 4.1 and 4.2 show collated data collected over the 12-month study period. Mean, medium, maxima and minima for temperature, turbidity, dissolved oxygen, pH, total microcystins, *microcystis*-like cells, phycocyanin fluorescence, chlorophyll *a* and *b* and total carotenoids are given for the two lakes. Water temperatures at lake 1 ranged between 5.57ºC in January and 21.64ºC in August, a temperature range of 16.07ºC, with a mean water temperature of 14.96ºC. In lake 2, water temperatures ranged between 5.81ºC in January and 21.51ºC in August, a temperature range of 15.7ºC, with a mean water temperature of 15.13ºC. Over the measurement period pH averaged 8.4 in lake 1 (range 7.9 to 9.0) and 8.5 (range 8.1 to 9.0) in lake 2. In lake 1 the pH maxima and minima were recorded on the same sampling day (30/08/2016) from surface and bottom measurements. Maximum and minimum dissolved oxygen levels were 24.16 mg L^{-1} and 6.14 mg L^{-1} in lake 1, and 18.98 mg L^{-1} and 9.44 mg L^{-1} in lake 2. Substantially larger reductions in dissolved oxygen were observed in lake 1 (18.02 mg L^{-1}) compared to lake 2 (9.54 mg L^{-1}), lowest levels were measured during September, highest in April and March at Lake 1 and 2 respectively. The average turbidity of lake 1 was 2.25 NTU (range 8.90 to -0.40 NTU) and of lake 2 1.51 NTU (range 8.2 to -1.6 NTU). Median and mean chlorophyll *a* and *b* levels were higher at lake 1 then lake 2 (median = 2.398 mg mL^{-1}, mean = 3.821 mg mL^{-1} in lake 1 and median = 0.969 mg mL^{-1}, mean = 1.315 mg mL^{-1} lake 2). Maximum chlorophyll *a* levels were observed in lake 1 during September (15.373 mg mL^{-1}), highest recorded levels at lake 2 were 4.056 mg mL^{-1}. Chlorophyll *b* measurements were also consistently higher at lake 1 than lake 2 with maximum at both sites recorded in July and August at lakes 1 and 2 respectively. Maximum and minimum chlorophyll *b* measurements were observed between a 10-day period in July, with a 4.33 mg mL^{-1} change observed.

Table 4.1. Biological and chemical measurements in Longham Lake1 (Northern), sampled between 16/05/2016 and 31/05/2017.

	Mean	Median	Maximum	Date	Minimum	Date	Notes
Total microcystins (µg L^{-1})	0.497	0.00	1.922	14/07/2016	0.00	10 Sampling days	
Microcystis-like cells (cells mL^{-1})	6874	2826	51384	14/07/2016	251	23/05/2016	
Phycocyanin (Cyanobacteria cells mL^{-1})	1425	836	7649	30/08/2016	109	13/06/2016	
Temperature (ºC)	14.96	16.51	21.64	15/08/2016	5.57	09/01/2017	
Turbidity (NTU)	2.25	1.50	8.90	05/09/2016	-0.40	31/05/2017	
Dissolved Oxygen (mg L^{-1})	12.19	12.07	24.16	03/04/2017	6.14	12/09/2016	
pH	8.44	8.47	9.29	30/08/2016	7.52	30/08/2016	Maximum from surface, minimum from bottom measurements
Chlorophyll a (mg mL^{-1})	3.821	2.398	15.373	20/09/2016	0.44	14/11/2016	
Chlorophyll b (mg mL^{-1})	2.296	2.206	6.752	30/08/2016	0.41	14/11/2016	
Total Carotenoids (mg mL^{-1})	1.200	0.676	6.295	13/10/2016	-0.18	03/11/2016	

Table 4.2. Biological and chemical measurements in Longham Lake 2 (Southern), sampled between 16/05/2016 and 31/05/2017.

	Mean	Median	Maximum	Date	Minimum	Date	Notes
Total microcystins (µg L-1)	1.524	0.000	7.089	28/09/2016	0.000	9 Sampling days	
Microcystis-like cells (cells mL-1)	1403	1012	12204	07/03/2017	258	19/12/2016	
Phycocyanin (Cyanobacteria cells mL-1)	1924	705	10290	30/08/2016	20	03/08/2016	
Temperature (˚C)	15.13	15.88	21.51	30/08/2016	5.81	09/01/2017	
Turbidity (NTU)	1.51	0.70	8.20	21/03/2017	-1.60	31/05/2017	
Dissolved Oxygen (mg L-1)	12.74	12.99	18.98	07/03/2017	9.44	20/09/2016	
pH	8.52	8.54	8.97	07/03/2017	8.06	09/01/2017	
Chlorophyll a (mg mL-1)	1.315	0.969	4.056	14/07/2016	0.042	14/11/2016	
Chlorophyll b (mg mL-1)	1.294	1.143	4.367	14/07/2016	-0.038	04/07/2016	
Total Carotenoids (mg mL-1)	0.260	0.135	1.467	23/05/2016	-0.251	20/09/2016	

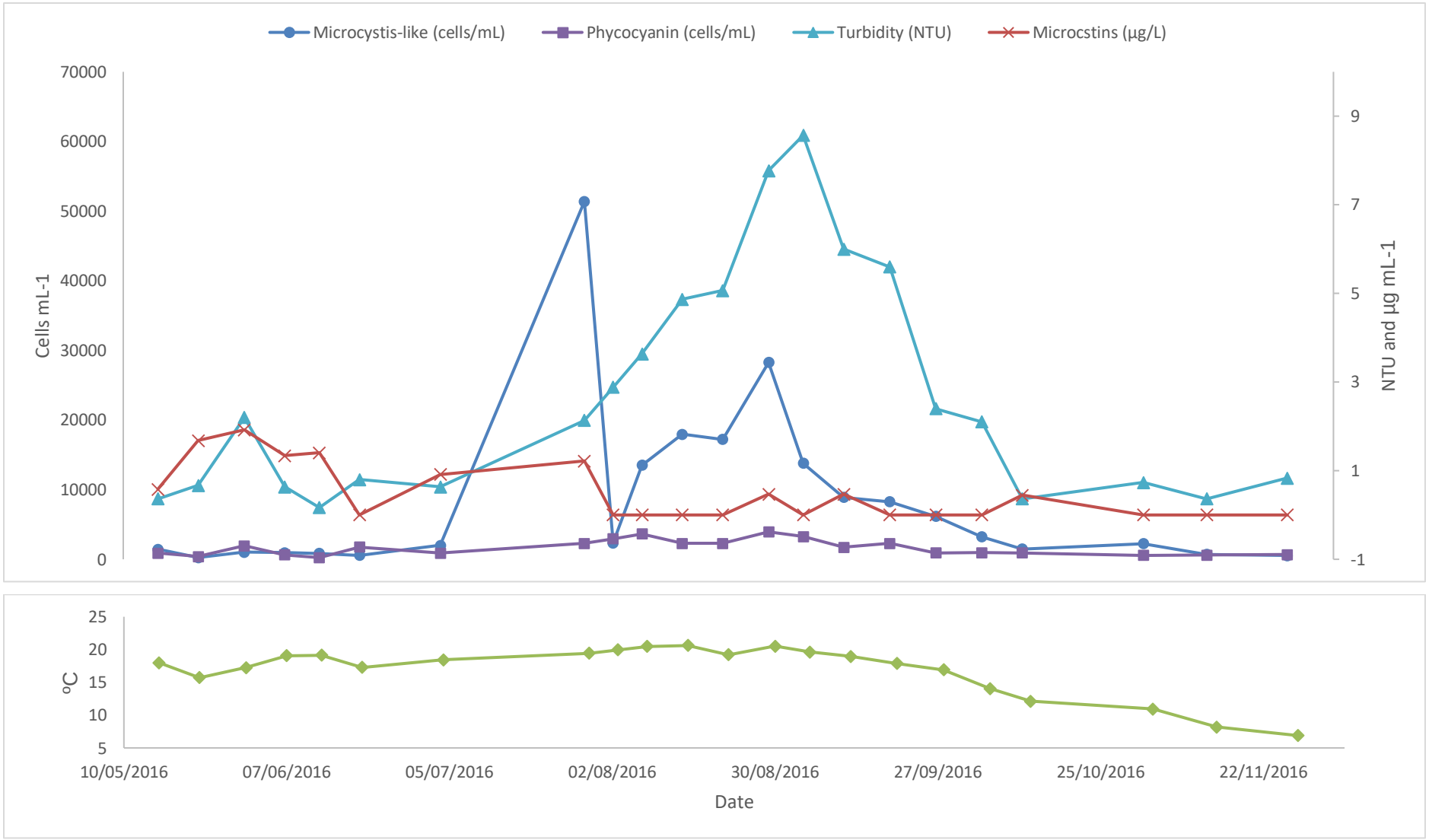


Figure 4.3. Seasonal variation recorded in Longham lake 1 (Northern) of *Microcystis*-like cells mL^{-1} by FCM, Sonde probe measurements of phycocyanin (a proxy for cyanobacterial cells) (left-hand axis), temperature (lower graph) and turbidity and total microcystins quantified by LCMS (right-hand axis).

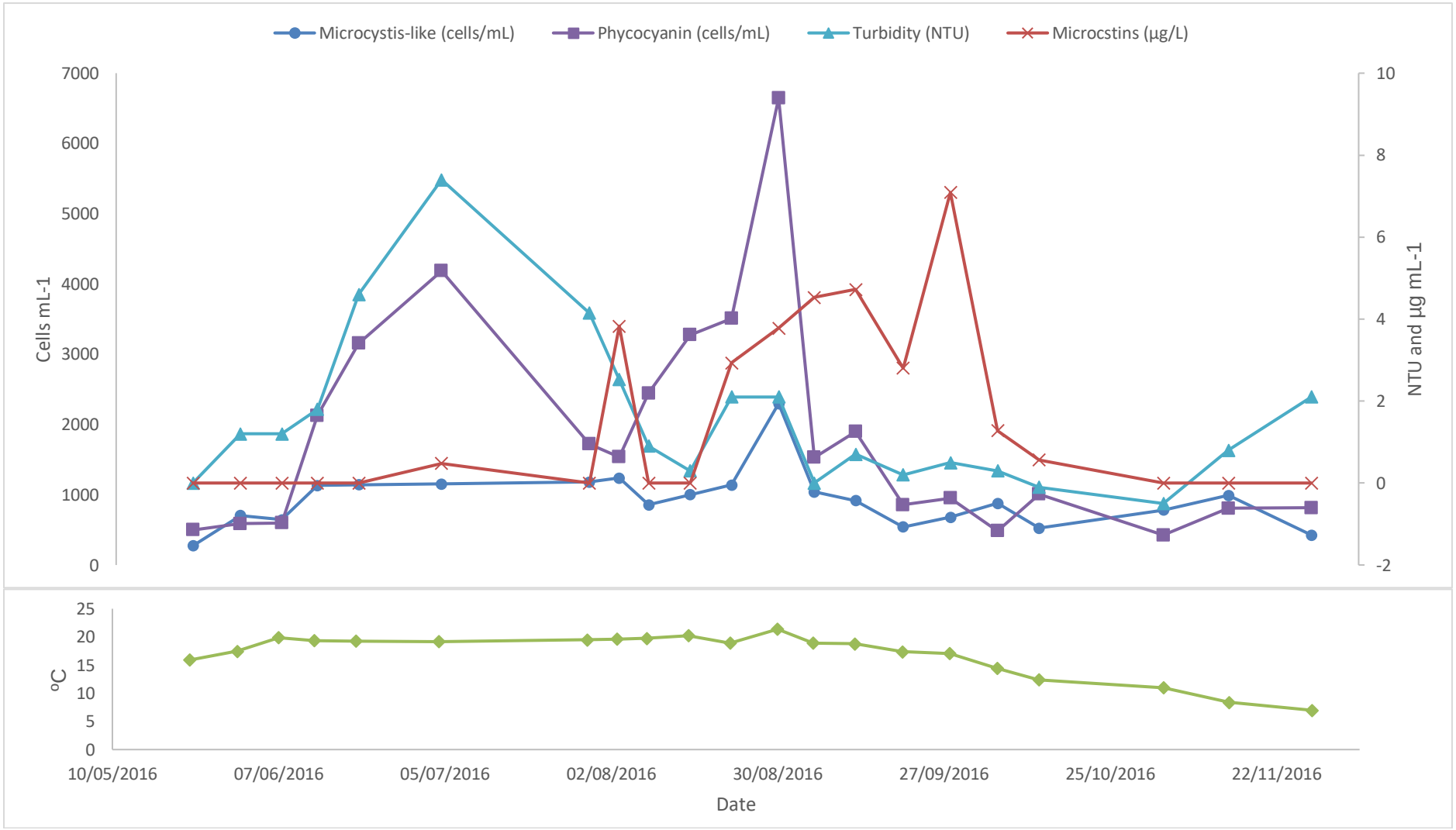


Figure 4.4. Seasonal variation recorded in Longham lake 2 (Southern) of *Microcystis*-like cells mL^{-1} by FCM, Sonde probe measurements of phycocyanin (a proxy for cyanobacterial cells) (left-hand axis), temperature (lower graph) and turbidity and total microcystins quantified by LCMS (right-hand axis).

A null hypothesis that no differences between biological and chemical measurements were observable between the two lakes was tested at the P=0.05 significance level using a series of Student's t-tests. No significant differences between temperature, pH or turbidity were observed between the two lakes over the study period (P>0.05), however significant and highly significant differences between the two lakes across the sampling period were observed for dissolved oxygen (P< 0.001), chlorophyll *a* and *b* levels (P<0.01, P<0.001) and carotenoids (P< 0.001) with dissolved oxygen demonstrating the most significantly different parameter.

4.3.2. Identification, distribution and enumeration of phytoplankton at northern and southern lakes by microscopy

A wide range of phytoplankton species were identified at both lakes between August 2016 and May 2017 by light microscopic examination. Whilst there was correspondence between the species identified in both sampling locations, species assemblages were consistently different between months and between lakes. In lake 1, thirteen species or species groups were identified, whereas in lake 2 twelve species or species groups were present. Only one species, *Coelophaerium* spp. was found in just one lake (lake 1). The pie charts (Figure 4.5) illustrate the species diversity and relative abundance of identification throughout the nineteen sampling occasions at lakes 1 and 2. Figure 4.4 is a collection of micrographs showing the major species identifications at 10x magnification.

Lake 1 **Lake 2**

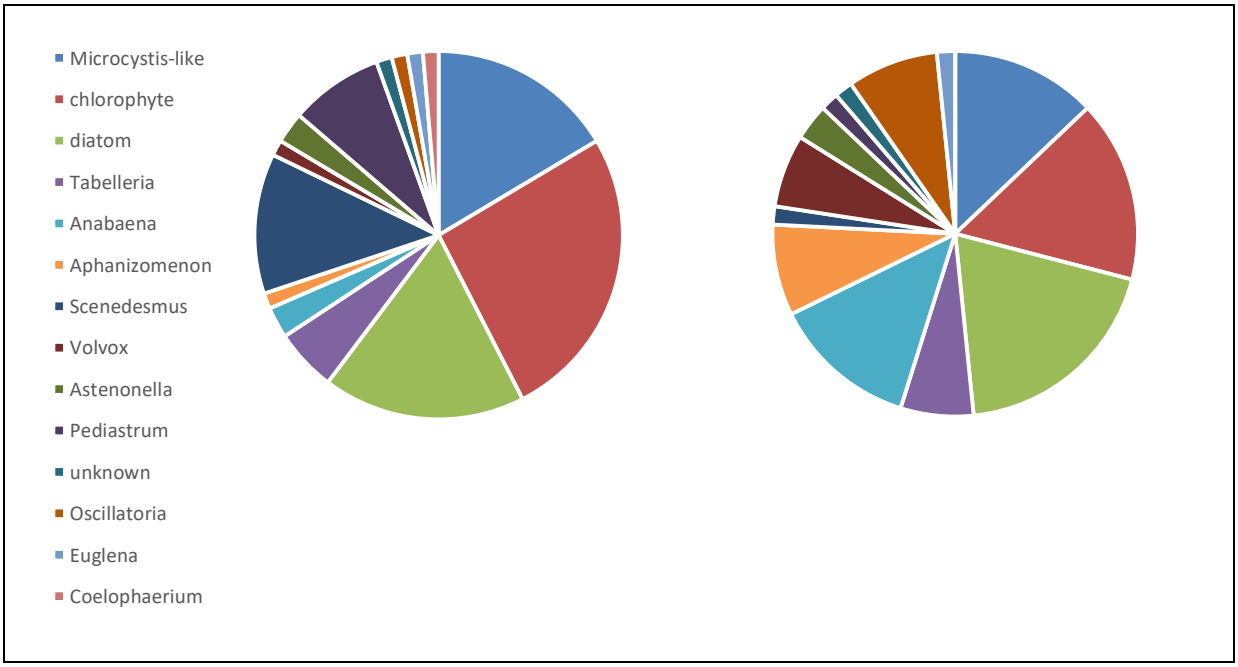


Figure 4.5. Distribution of Microcystis-like cells, Cyanobacteria and Phytoplankton in Lakes 1 and 2.

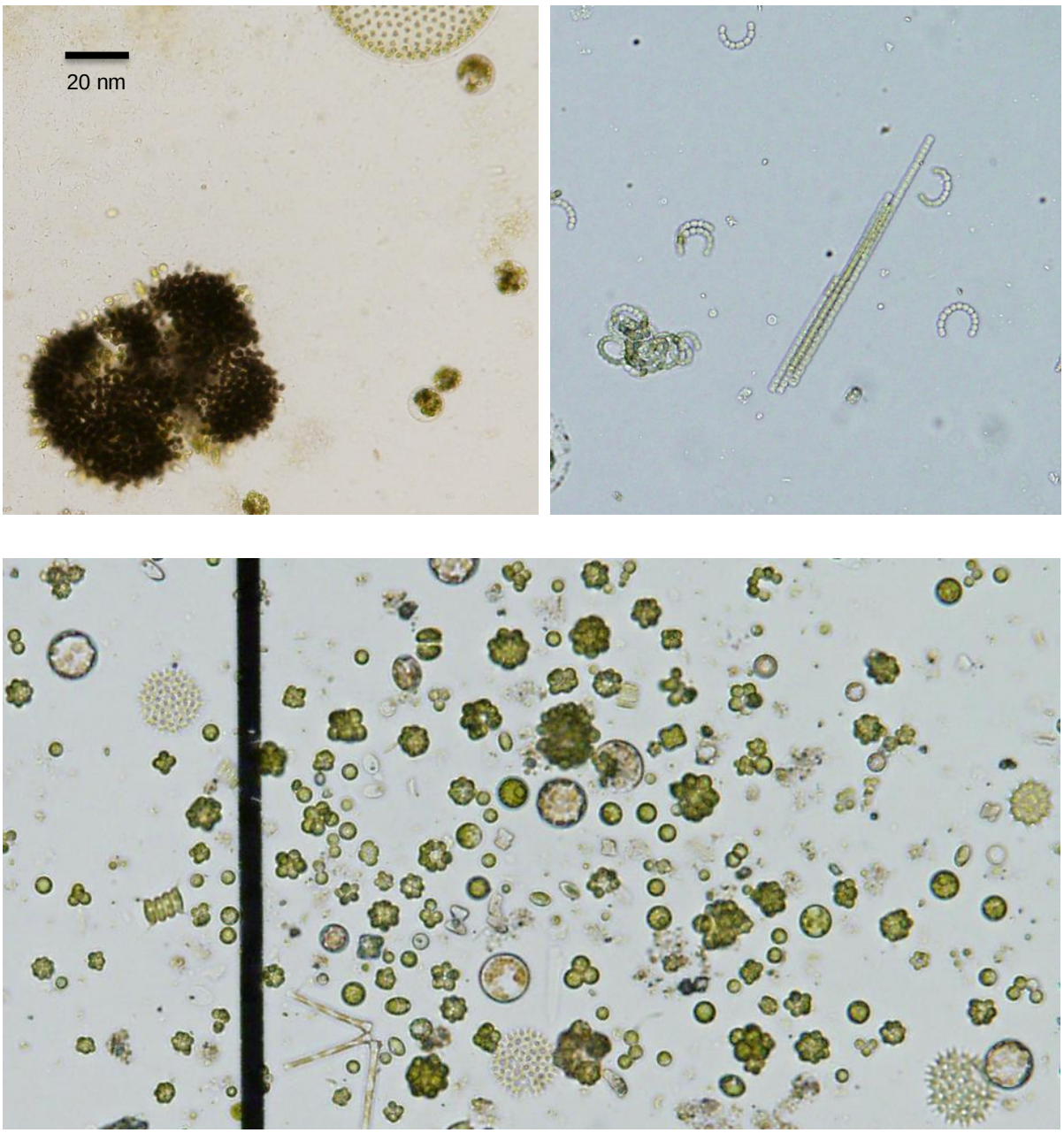


Figure 4.6. Micrographs from Longham Lakes, Dorset. A. Colony of *Microcystis* sp. and *Volvox* sp. (top right) from Lake 1 (18/08/16). B. *Anabaena* sp. (spiral filaments) and *Aphanizomenon* sp. (straight filaments) from Lake 2 (12/09/16). C. Image of phytoplankton community from Lake 1 (22/08/16), including; *Scenedesmus* sp., *Volvox* sp., *Asterionella* sp. and *Pediastrum* sp.

The species identified microscopically at both lakes were classified into functional groups according to the following groupings:

- Phytoplankton - chlorophytes, diatoms, *Tabellaria* spp., *Scenedesmus* spp., *Volvox* spp., *Asterionella* spp., *Euglena* spp., *Pediastrum* spp. unidentifiable species and all cyanobacterial cells,
- Cyanobacteria – *Anabaena* spp., *Aphanizomenon* spp., *Coelosphaerium* spp. and *Oscillatoria* spp., including *Microcystis*-like cells
- *Microcystis*-like cells - *Microcystis*-like cells only.

Figure 4.7 describes the relative frequency of occurrence assessed by light microscopy of the cyanobacterial species, *Microcystis*-like cells and non-toxin producing phytoplankton in lakes 1 and 2. Species/species type profiles at the two lakes showed considerable differences with proportionally greater frequency of identifications of cyanobacteria at lake 2 (82%) compared to lake 1 (18%). Identification of *Microcystis*-like cells was 60% at lake 1 and 40% at lake 2, and for phytoplankton species 61% at lake 1 and 39% at lake 2.

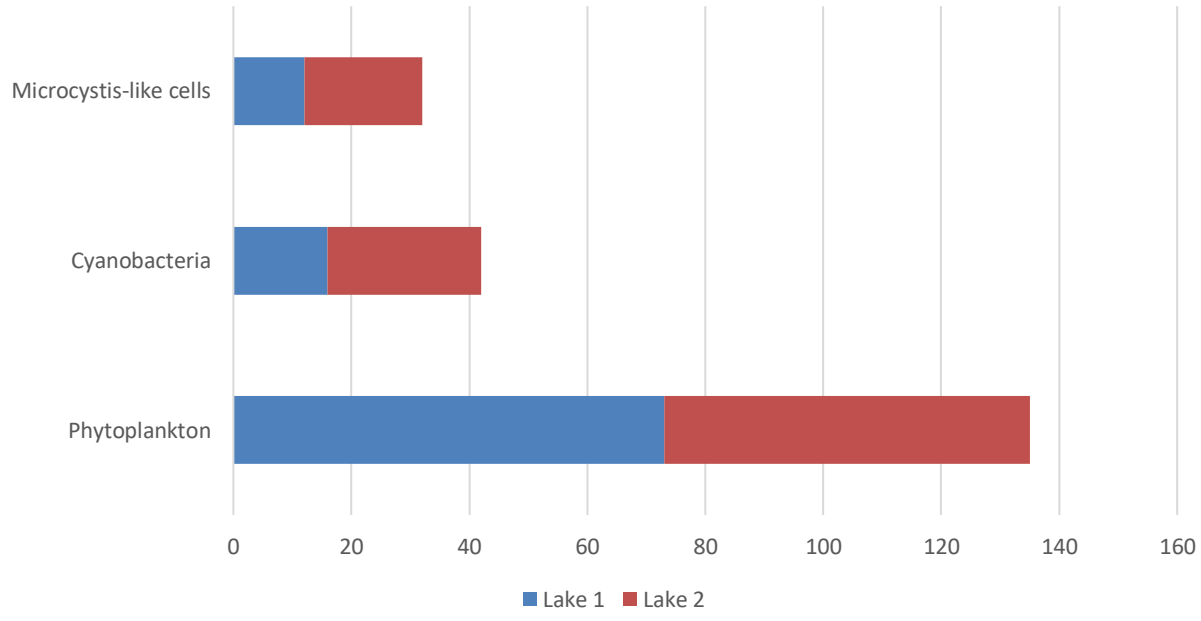


Figure 4.7. Relative frequency of occurrence of cyanobacterial species, *Microcystis*-like cells and phytoplankton at the two lakes.

To determine phytoplankton community structure over the study period counts of phytoplankton, cyanobacteria and *Microcystis*-like cells were logarithmically transformed (Figures 4.8. and 4.9.). Generally, the counts for lake 2 showed more variability and were higher for all determinants than in lake 1. Students *t*-tests applied to counts for total phytoplankton, cyanobacteria and *Microcystis*-like cells ($P<0.05$) revealed significant differences in estimated cell counts for total phytoplankton between the lakes but no other significant differences were demonstrated between counts for cyanobacteria and *Microcystis*-like cells and (or the sum of *Microcystis*-like cells and cyanobacteria – total cyanobacteria) between the lakes. On four occasions however, the total cyanobacterial counts exceeded the UK 20,000 cells mL^{-1} trigger actions states. More than 20,000 cells mL^{-1} were estimated on three occasions between the 15th and 30th August 2016 and in May 2017 (31/05/17). Toxin profiles for these sampling occasion are discussed in section 4.3.3.

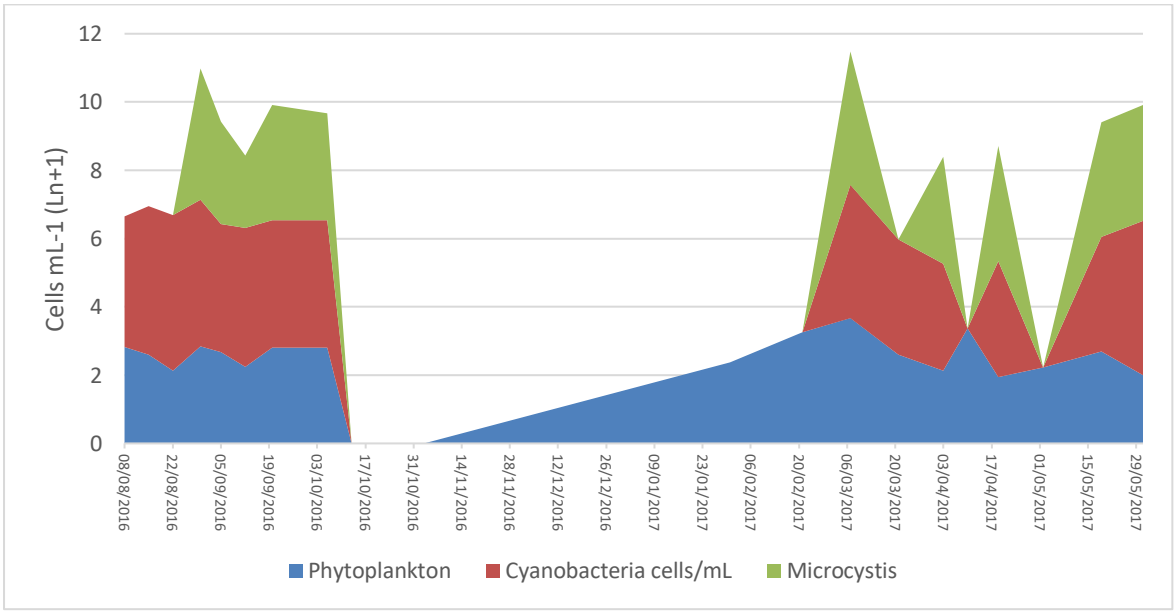


Figure 4.8. Logarithmically (Ln+1) transformed cell counts for cyanobacteria, *Microcystis*-like cells and phytoplankton at Lake 1.

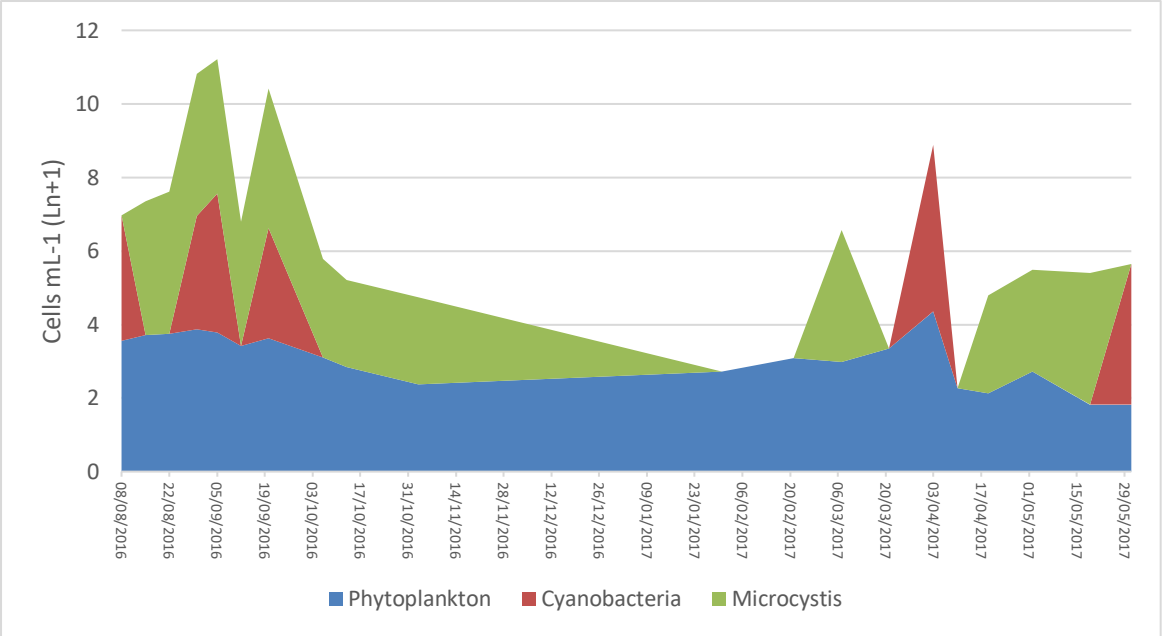


Figure 4.9. Logarithmically (Ln+1) transformed cell counts for cyanobacteria, *Microcystis*-like cells and phytoplankton at Lake 2.

1.1.1 Comparison of counts of Microcystis*-like cells by flow cytometry and microscopic methods*

In both lakes counts of *Microcystis*-like cells by flow cytometry were consistently higher and no zero counts were registered as compared to counts by light microscope (Figure 4.8). A strong correlation between the two methods was observed in lake 1 when tested with a Pearson product moment correlation (PC=0.763, P=0.001). But, correlation was not observed between the two methods in lake 2 (PC=-0.048, P=0.864).

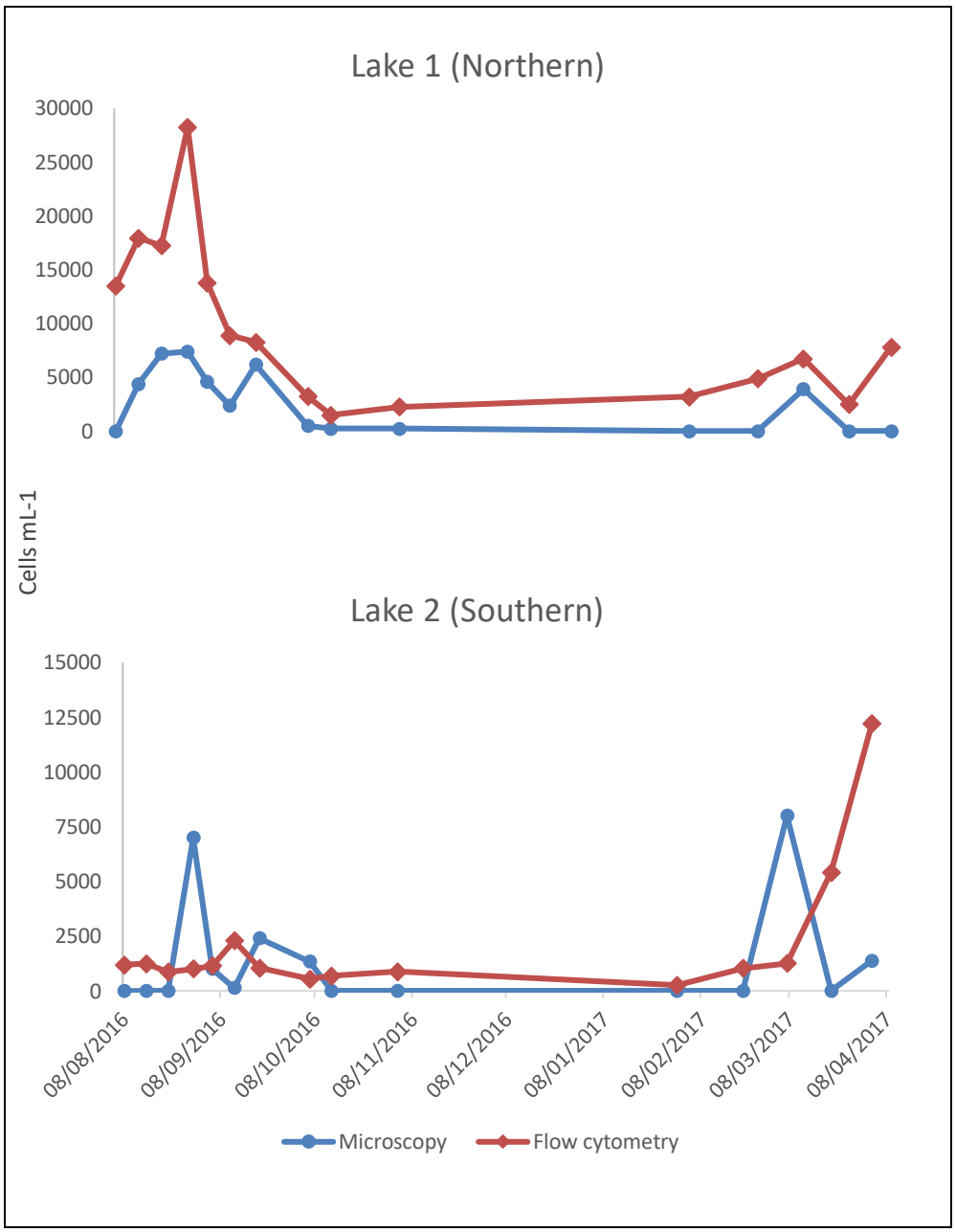


Figure 4.10. Comparison of counts of *Microcystis*-like cells in both lakes at Longham as counted by flow cytometry and microscope methods over the study period.

4.3.3. Determination of Microcystis-like cells and phycocyanin (Cyanobacteria spp.), and levels of microcystins

Figures 4.1 and 4.2 show the *Microcystis*-like cells and total microcystins with phycocyanin (cyanobacteria cells), turbidity and temperature measured over the study period at both lakes. *Microcystis*-like cells were detected in both lakes by FCM throughout the sampling period, increasing in July/August in lake 1 and in August in lake 2. An order of magnitude more *Microcystis*-like cells were detected in Lake 1 than lake 2. Mean *Microcystis*-like cells in lake 1 were 6,874 mL^{-1} with a median of 2,826 and range of 251 (23/05/2016) to 51,384 mL^{-1} (14/07/2016). In Lake 2 mean *Microcystis*-like cells were 1,403 mL^{-1} with a median of 1,012 and range of 258 (19/12/2016) to 12,204 mL^{-1} (07/03/2017).

Microcystin variants were detected in both lakes but were consistently lower in lake 1 than lake 2. Total microcystin variants and quantities detected are shown in Figures 4.11. & 4.12. and Tables 4.1 & 4.2. In total 7 microcystin variants were detected in Lake 1 these comprised MC-LR, MC-LA, MC-LY, MC-LF, MC-LW, MC-YR and Asp^3 MC-LR / [Dha^7] MC-LR. The microcystin variant detected at the highest concentrations at lake 1 was MC-LF in June and July. Six microcystin variants were detected in Lake 2 (MC-LR, MC-RR, MC-LA, MC-LY, MC-YR and Asp^3 MC-LR / [Dha^7] MC-LR). In Lake 2 MC-YR was detected at highest concentrations during August and September, similar but slightly lower levels of variant MC-LR was detectable between August and October. Mean total microcystins were 0.497µg L^{-1} (0.000 to 1.922µg L^{-1}) in lake 1 and 1.524µg L^{-1} (0.000 to 7.089 µg L^{-1}) in lake 2. Maximum levels in Lake 1 were detected between 23/05/2016 and 14/07/2016, microcystins were rarely detected between 03/08/2016 and 28/11/2016. Maximum levels in Lake 2 were detected between 03/08/2016 and 28/09/2016, microcystins were rarely detected between 23/05/2016 and 17/07/2016. No microcystins were detected in both lakes between 03/11/2016 and 28/11/2016.

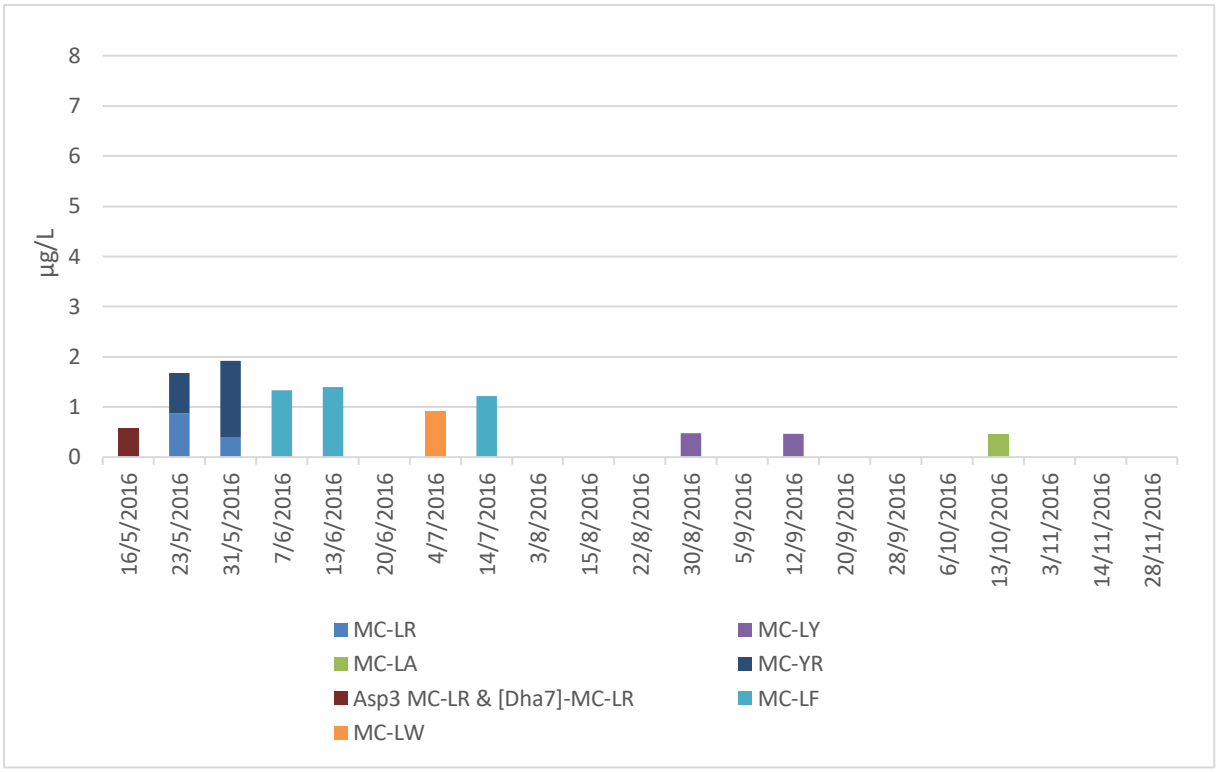


Figure 4.11. Microcystin variants qualified and quantified from water samples collected at Longham lake 1 (Northern) by LC-MS.

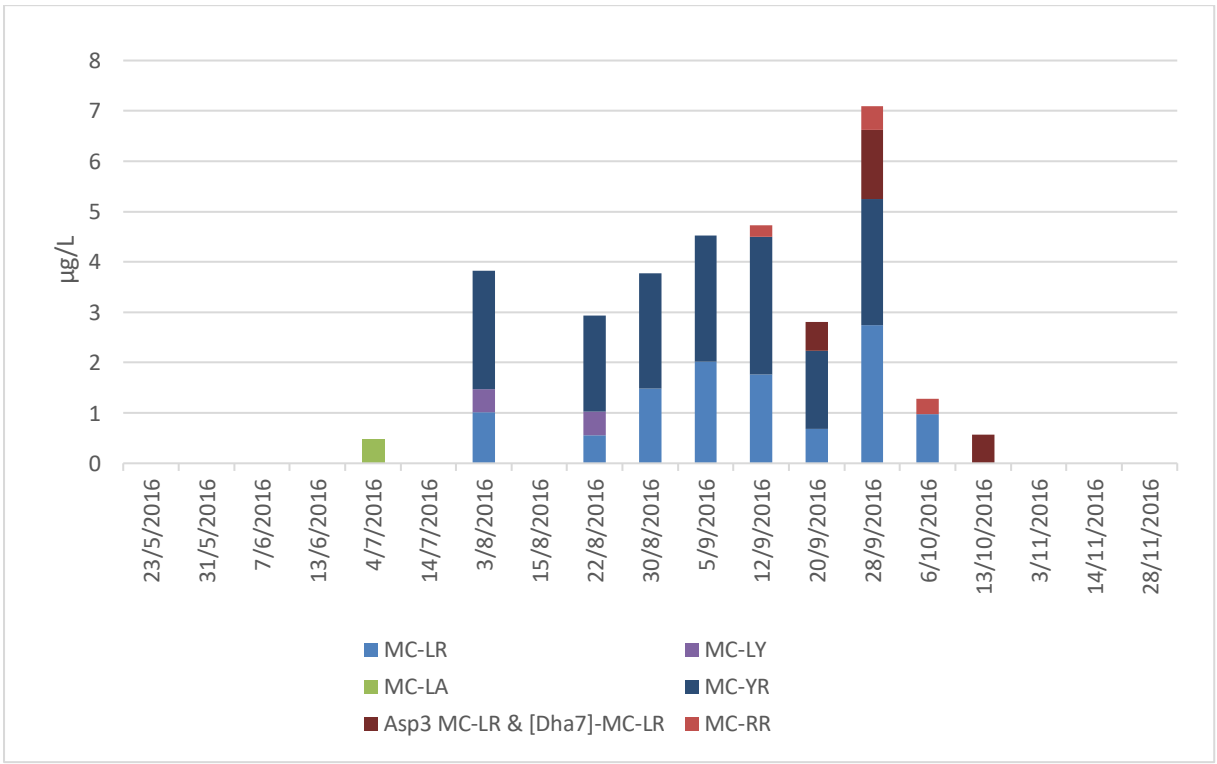


Figure 4.12. Microcystin variants qualified and quantified from water samples collected at Longham lake 2 (Southern) by LCMS.

A null hypothesis that no differences between *Microcystis*-like cells, total microcystins and phycocyanin (cyanobacteria cells) measurements were observable between the two lakes was tested using a series of Student's t-tests P=0.05. No significant differences between phycocyanin measurements were observed between the two lakes over the study period (P>0.05), however, total microcystins and *Microcystis*-like cells were significantly and highly significantly different between the two lakes respectively (P<0.01, P<0.001).

Levels of microcystins in lake 2 increased following a peak in phycocyanin in August and a sharp elevation in *Microcystis*-like cells preceded a microcystin peak in September. Despite higher *Microcystis*-like cells recorded in lake 1, no correlation was observed between *Microcystis*-like cells and microcystins. Considerable differences between the two lakes with respect to the presence and distribution of microcystin variants and levels detected was observed. In both lakes a moderate relationship between phycocyanin, *Microcystis*-like cells and temperature was demonstrated with elevated levels of both proportional to increased temperature (Figure 4.13.). Regression analysis to test the relationship between temperature and *Microcystis*-like cells/ phycocyanin showed R^2 values ranging between 0.08 for temperature and Microcystis-like cells across both lakes and seasons to 0.83 for *Microcystis*-like cells in lake 1 during the winter/autumn period. Phycocyanin (Cyanobacterial cells) in Lake 2 were considerably higher than lake 1, during June and July phycocyanin increased alongside turbidity. There was a tendency for turbidity and phycocyanin to increase together in June/July in both lakes. Lake 1 phycocyanin measurements were lower than lake 2 throughout the sampling period. In both lake systems *Microcystis*-like cells increased together with turbidity.

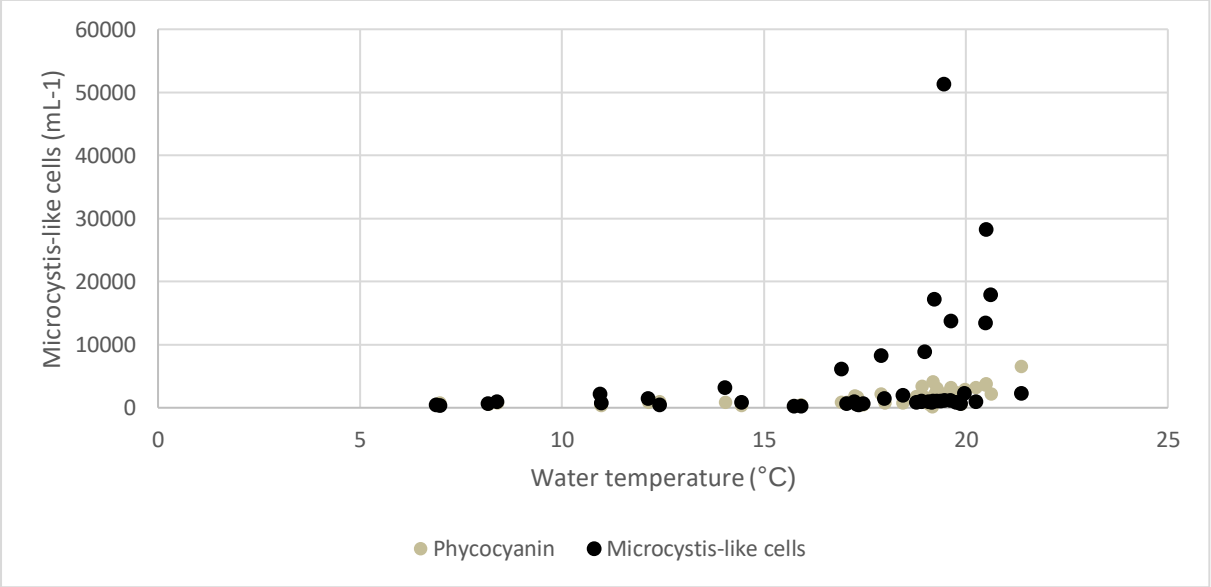


Figure 4.13. Relationship between water temperature, phycocyanin and Microcystis-like cells.

4.3.4. Seasonality

Figures 4.1 and 4.2 show the levels of *Microcystis*-like cells, microcystins and phycocyanin with temperature and turbidity at lakes 1 and 2 across the entire study period. The potential relationships between seasonality (March to August - spring/summer; September to February - autumn/winter) and data for all measured parameter were tested using Student t-tests. At lake 1 no significant differences between dissolved oxygen, turbidity, chlorophyll a and b, total carotenoids, phycocyanin or *Microcystis*-like cells could be demonstrated between arbitrary seasons, however differences between pH, microcystins ($p<0.05$) and temperature ($P<0.01$) were observed. At lake 2, turbidity, dissolved oxygen chlorophyll b, *Microcystis*-like cells or microcystins were not significantly different between seasons, however pH, chlorophyll a, phycocyanin ($p<0.05$) and temperature and carotenoids ($p<0.01$) were significantly different.

4.3.5. The ability of chemical and biological parameters to predict presence of microcystins, Microcystis-like cells and phycocyanin.

Tables 4.3 and 4.4 show the Pearson product moment correlation of environmental and biological measurements from lakes 1 and 2. No correlation could be determined between total microcystins and any other measured parameters at lake 1. Highly to moderate significant correlations were however observed for *Microcystis*-like cells and phycocyanin and temperature, turbidity, pH, chlorophyll a and b and total carotenoids. The strongest predictor of *Microcystis*-like cells was total carotenoids whereas for phycocyanin chlorophyll *b* provided the best correlation. In lake 2, moderate correlation between microcystins and dissolved oxygen was observed ($P<0.05$), with a decrease in dissolved oxygen predictive of detection of microcystins, but no other parameter provided predictive ability. For *Microcystis*-like cells and phycocyanin at lake 2, high to moderate correlation coefficients between temperature and pH were observed.

Table 4.3 Pearson correlation coefficients of environmental and biological measurements from Longham lake 1 (Northern) (n=22).

	Total Microcystins (ng mL^{-1})	*Microcystis*-like cells (cells mL^{-1})	Phycocyanin (Cyanobacteria (cells mL^{-1})	Temperature (^oC)	Turbidity (NTU)	Dissolved Oxygen (mg L^{-1})	pH	Chlorophyll *a*	Chlorophyll *b*
Microcystis-like cells (cells mL^{-1})	.009 .970								
Phycocyanin (Cyanobacteria (cells mL^{-1})	-.296 .181	.574** .005							
Temperature (^oC)	.205 .360	.428* .047	.594** .004						
Turbidity (NTU)	-.332 .131	.484* .022	.800** .000	.510** .015					
Dissolved Oxygen (mg L^{-1})	-.006 .977	.122 .588	.238 .286	.269 .226	-.122 .588				
pH	.007 .976	.438* .041	.751** .000	.624** .002	.509* .016	.681** .000			
Chlorophyll *a*	-.180 .422	.646** .001	.773** .000	.487* .022	.890** .000	-.108 .632	.547** .008		
Chlorophyll *b*	-.227 .309	.610** .003	.858** .000	.638** .001	.918** .000	-.080 .722	.570** .006	.920** .000	
Total Carotenoids	-.105 .643	.661** .001	.610** .003	.301 .174	.675** .001	-.049 .828	.458* .032	.914** .000	.726** .000

** Correlation is significant at the 0.01 level (2-tailed)

* Correlation is significant at the 0.05 level (2-tailed)

Table 4.4 Pearson correlation coefficients of environmental and biological measurements from Longham lake 2 (Southern) (n=21).

	Total Microcystins (ng mL^{-1})	*Microcystis*-like cells (cells mL^{-1})	Phycocyanin (Cyanobacteria (cells mL^{-1})	Temperature (^oC)	Turbidity (NTU)	Dissolved Oxygen (mg L^{-1})	pH	Chlorophyll *a*	Chlorophyll *b*
Microcystis-like cells (cells mL^{-1})	.243 .289								
Phycocyanin (Cyanobacteria (cells mL^{-1})	.149 .521	.835** .000							
Temperature (^oC)	.276 .226	.494* .023	.552** .009						
Turbidity (NTU)	-.143 .537	-.141 .542	-.016 .947	-.196 .394					
Dissolved Oxygen (mg L^{-1})	-.466* .033	.225 .327	.197 .391	-.177 .443	.230 .317				
pH	-.153 .508	.530* .013	.560** .008	.350 .120	.083 .719	.743** .000			
Chlorophyll *a*	.170 .460	.348 .122	.396 .075	.631** .002	-.074 .751	-.239 .297	.304 .180		
Chlorophyll *b*	.255 .265	.382 .088	.331 .142	.493* .023	-.041 .860	-.227 .322	.169 .464	.820** .000	
Total Carotenoids	-.120 .605	.079 .735	.209 .362	.472* .031	-.068 .770	-.229 .319	.179 .436	.776** .000	.319 .158

** Correlation is significant at the 0.01 level (2-tailed)

* Correlation is significant at the 0.05 level (2-tailed)

Table 4.5. Linear regression of all chemical and biological variables to predict the occurrence of Total Microcystins in Longham Lake 1 (Northern). Greatest effect on correlation in highlighted cell.

	R squared value	Standard error of the Estimate
All variables	0.607	0.546
Without *Microcystis*-like cells	0.584	0.539
Without Phycocyanin (Cyanobacteria cells)	0.560	0.554
Without Temperature	0.530	0.572
Without Turbidity	0.599	0.528
Without Dissolved Oxygen	0.436	0.627
Without pH	0.462	0.612
Without Chlorophyll a	0.600	0.528
Without Chlorophyll b	0.601	0.527
Without Total Carotenoids	0.597	0.530

Table 4.5. Linear regression of all chemical and biological variables to predict the occurrence of Total Microcystins in Longham Lake 2 (Southern). Greatest effect on correlation in highlighted cell.

	R squared value	Standard error of the Estimate
All variables	0.655	1.697
Without *Microcystis*-like cells	0.625	1.695
Without Phycocyanin (Cyanobacteria cells)	0.643	1.653
Without Temperature	0.653	1.629
Without Turbidity	0.649	1.638
Without Dissolved Oxygen	0.587	1.777
Without pH	0.654	1.628
Without Chlorophyll a	0.548	1.860
Without Chlorophyll b	0.546	1.863
Without Total Carotenoids	0.469	2.015

To further explore the predictability of multiple chemical and biological parameters on the presence and concentration of total microcystins multiple linear regression analyses were conducted (Tables 4.5 & 4.6). The strength of the relationship between the dependent variable (microcystin levels) was tested against all independent variables (biological and chemical parameters) together and using a stepwise approach. The best fit was obtained using all independent variables, explaining between 60% (lake 1) and 65% (lake 2) of the variability. This regression model further indicated that dissolved oxygen was the best predictor in lake 1 of microcystin presence in lake 1 and total carotenoids most closely associated in lake 2. However, in both lakes the difference in effect on the fitted regression was marginal, and combined measurement data better explained the microcystin levels than any single biological or chemical factor.

4.4. Discussion

In this study relationships between biological and chemical parameters, phytoplankton, cyanobacterial taxa with specific reference to *Microcystis* spp., using microscopy and flow cytometry were examined in two low land lakes in southern England. The total microcystins, microcystin variants and toxin profiles were also determined using ultra-high-pressure liquid chromatography coupled to mass spectrometry, over an approximately 12-month period. The aim of this work was to identify any associations between the abundance of cyanobacteria and *Microcystis* spp. and toxicity, and to use environmental data to attempt to predict the likelihood of toxic events. The results of this study revealed a complex microbial community and suggested the importance of both bottom-up and top-down control in regulating these systems.

The phytoplankton assemblages found at both lakes were similar with respect to species identified but significant differences were found both in frequency of identifications by light microscopy, and cell counts (by microscopy, flow cytometry and multi-parameter probe). Cyanobacteria and *Microcystis* spp. were present in both lakes throughout the study period, moderate to good correspondence between microscopic and flow cytometric counting methods for *Microcystis* spp. was observed. On several occasions, between April and August, cyanobacterial cell counts exceeded the UK threshold levels of >20,000 cells mL^{-1} (once in lake 1 and 3 times in lake 2). The cyanobacterial population structure differed significantly between the lakes with *Microcystis* spp. more abundant in lake 1 and the dominance of other cyanobacterial species in lake 2.

Microcystins were detected and quantified in both lakes during the study period. Levels of microcystin variants were significantly higher in lake 2 than lake 1, suggesting that cyanobacterial species other than *Microcystis* were responsible for toxin production. Microcystin levels did not exceed the WHO medium health threshold of 20 µg L^{-1} during the study period. In 2017 WHO published a chronic health threshold level of 1 µg L^{-1} MC-LR for lifelong drinking water consumption, 16% of samples from lake 2 exceeded the MC-LR chronic threshold value. The results of this study revealed the complexity, intricacy and fragility of freshwater phytoplanktonic community structures which have identified several areas for further study.

4.4.1 Phytoplanktonic community structure, distribution and prevalence of cyanobacteria and Microcystis *spp.*

In recent years many studies have examined the phytoplankton community composition and diversity in lakes around the world to provide information on population ecology, linkages between community structure and environmental variables, potential toxicity and potential applications of aquatic microorganisms (Lindstrom 2001; Kim *et al.* 2006; Acinas *et al.* 2008; Marshall 2013, Rosen and Short 2013, Singh *et al.* 2014). These studies, and others, have provided substantial insight into the phytoplanktonic and phytobacterial components in a variety of aquatic systems but are all characterized by the complexity of community structures and indicate often cryptic interactions between and within microbial taxa, the function of which are relatively unknown.

In this study microscopic analysis of water samples revealed at least thirteen taxa (and some unidentifiable species) in the two lakes during the study period. The most prevalent species identified at both lakes throughout the sampling period were *Microcystis*–like cells, chlorophytes, diatoms, *Tabellaria* spp. and *Anabaena* spp. together accounting for over half of the taxa recorded. Only one species, presumptive *Coelosphaerium* spp. appeared only in lake 1. The phytoplankton taxa and frequency of detection of cyanobacteria and *Microcystis*–like cells by light microscopic examination is in accordance with number of studies on phytoplankton taxa studies in natural lakes, reservoirs and rivers in the United States (Marshall 2013), Sweden (Lindstrom 2001) and The Netherlands (Johnk *et al.* 2008). Likewise, the distribution of taxa identified in the two lakes were very similar to that observed in a contemporary study of >100 samples from 70 natural lakes and reservoirs in England targeted as part of the Environment Agencies cyanobacteria response programme (Turner *et al.* 2018b). Whereas Turner *et al.* (2018b) focused on water samples exceeding the UK threshold action level (20,000 cells ml^{-1}) (72% of samples tested) this study applied a routine monitoring approach for the key bloom forming genera of cyanobacteria (*Microcystis* spp., *Anabaena* spp., *Oscillatoria* spp. and *Aphanizomenon* spp. (O'Neil *et al.* 2012, Oliver *et al.* 2012).

Total cyanobacterial levels exceeding the UK threshold were observed on four occasions during the sampling period (5%). Three exceedances occurred in the Southern lake (lake 2), the taxonomic identification of samples containing higher levels of cyanobacteria (>20,000 cells ml^{-1}) in lake 2 revealed that *Microcystis* spp. were low or absent. Similarly, in lake 1, whilst *Microcystis* spp. were detected more frequently and in relatively higher numbers, in the single sample exceeding the UK

threshold levels, *Microcystis* spp. counts were very low. In lake 1 levels of *Coelosphaerium* sp. were estimated to peak at 34,000 cells ml^{-1}.

The presence of a *Coelosphaerium* sp. bloom is interesting as although not thought to be toxic (no concurrent toxicity was found in this study) certain species have been frequently associated with the periodic production of musty or earthy odours and tainted shellfish flesh in Japan (Godo *et al*. 2017) and aquaculture systems (Schrader *et al.* 2011). Geosmin, a secondary metabolite resulting from the cyclisation of farnesyl diphosphate, is found variously in several cyanobacterial species (Giglio *et al.* 2008) however no reports of odour during sampling occasions, from either the lake bailiff or from recreational users of the water course during the period were noted (Ian Hayward, South West Water, pers. comm.) although it is postulated that the *Coelophaerium* sp. may be associated with ichthyo-eutrophication (as discussed below).The relatively high levels of cyanobacteria in lake 2 and the significant differences between the lakes with respect to *Microcystis*-like cells in in lake 1 during the summer and early autumn was intriguing as the two lakes were very similar with respect to most environmental parameters and were contiguous (see section 4.2.1.).

The major identifiable difference between the two water bodies was the presence of a managed fishery at lake 1 stocked with omnivorous fishes; Common carp (*Cyprinus carpio*), bream (*Abramis brama*), tench (*Tinca tinca*) and pike (*Esox lucius*), and with substantial natural populations of roach (*Rutilus rutilus*), and rudd (*Scardinius erythrophthalmus*). Larval, fry and fingerlings of stocked species favour zooplankton, with certain high nutrient species (rotifers etc) making up a substantial component of adult fishes' food supply. Larval stages and young age classes of *R. rutilus* and *S. erythrophthalmus,* which would have been numerous during summer months, are almost exclusively zooplanktoniverous with a feeding preference for rotifers (Zapletal *et al.* 2013). Rotifers and other small zooplankton such as cyclopoid copepods and cladocerans, are selective grazers that can coexist with bloom forming cyanobacteria and are reported to periodically exhibit top-down control (Van Wichelen *et al.* 2016). Interestingly, several authors have suggested that *Microcystis* spp. represent a less attractive foodstuff for zooplankton due, *inter alia* to colony formation (Rohrlack *et al.* 2001, Lotocka 2001, Van Wichelen *et al.* 2016) which together with a reduction in grazing pressure on other cyanobacterial taxa may have created a competitive advantage for the *Microcystis*-like cell populations in lake 1. Roach abundance has previously been implicated in ichthyo-eutrophication of reservoirs in The Czech Republic (Zapletal *et al.* 2013), this has been attributed to a range of factors but may also include reduction in grazing rates by zooplankton which in turn create

advantageous conditions for blooms (Zurawell *et al.* 2005). Furthermore, eutrophication can disrupt microbial loops and therefore may inhibit antagonistic microorganisms (viruses, bacteria, microalgae, microfungal and amoeboid taxa) present within *Microcystis* colonies in the operation of bottom-up controls (Van Wichelen *et al.* 2016).

4.4.1. Relationship between Microcystis spp., cyanobacteria and toxin production

Quantifiable levels of toxins were detected in 48% of samples analysed, identical frequencies of toxin detection being observed but with total toxin levels and toxin profiles significantly different between the 2 lakes. Taken across the two study sites, toxin concentrations ranged from 0 to 7.1 µg L^{-1} and were usually but not exclusively detected in samples where microscopic and/or instrumental (optical phycocyanin measurements or flow cytometry) analyses had demonstrated the presence of potentially toxin producing cyanobacteria. In terms of toxin concentrations these data are like those reported by several authors for natural lakes and reservoirs in the absence of scums in the Lower Great Lakes (Dyble *et al.* 2008), England and Wales (Turner *et al.* 2018b) and selected European water bodies (Mantzuoki *et al.* 2018).

In lake 1 total microcystins were consistently approximately an order of magnitude lower than in lake 2, despite proportionally higher isolation frequencies and levels of *Microcystis*-like cells. *Microcystis*-like cells, which as measured by microscopic methods, did not exceed 8,000 cells mL^{-1} during the study and constituted a relatively minor fraction of the total estimates of cyanobacteria. It is therefore possible that *Microcystis*-like cells at either lake were not present in high enough levels to enable quantitation of toxins in samples. Alternately the apparent lack of toxicity of *Microcystis* spp. in this study could be indicative of the presence of non-toxin producing strains of *Microcystis* spp. in lake 1 (and probably lake 2, where Microcystis-like cells were broadly similar in magnitude) and suggest that toxins detected in this study were produced by species other than *Microcystis.* It is well documented that the *Microcystis* blooms vary in their toxin profiles (Sivonen 2009, WHO 2017, Turner *et al.* 2018b). The ability for microcystin production in *Microcystis* spp. (and by analogy other cyanobacterial species) is genetically determined (Janse *et al.* 2004, Meyer *et al.* 2017) and differentiation between toxin producing and non-toxin producing genotypes can be determined by the presence of mobile genes (Meyer *et al.* 2017).

Several studies have reported that strains isolated from geographically and temporally distinct *Microcystis* spp. populations are clonal and are therefore likely to be either microcystin producers or non-producers (Tillett *et al.* 2001, Paerl and Huisman 2008). Furthermore, some authors have reported considerable variations between both the amounts and toxic variants produced by individual cells (Meyer *et al.* 2017). It is considered possible therefore that *Microcystis*-like cells detected in this study were non-microcystin producing genotypes. However, it is also important to consider the limitations of the testing methods which also may introduce uncertainty in data interpretation.

Flow cytometric methods used here enabled the precise and accurate quantitation of monocultures of *Microcystis aeruginosa in vitro* (Section 3.), however these methods remain un-validated in 'real world' samples and thus species other than *Microcystis* (*Microcystis*-like spp.) such as species of picoplankton may interfere with quantification. Likewise, microscopic identification and cell counting is subjective and has been reported to introduce substantial variability and uncertainty (Mantzuoki *et al.* 2018) even when measurements and interpretations are made by well trained, supervised taxonomists (Pawlik-Skowronska *et al.* 2013).

The hypothesis that species other than *Microcystis* spp. were responsible for toxin production in the study lakes is supported by the presence of other well characterised cyanobacteria specifically, *Anabaena* spp., *Aphanizomenon* spp. and *Oscillatoria* spp at elevated numbers and in lake 2. Whilst the levels of these genera were not quantified, at lake 2 on the 3 occasions where the UK threshold action limits were exceeded, less than 8.5% of the estimated cyanobacterial populations comprised *Microcystis*-like cells. In a review of cyanobacterial bloom components in the UK, Howard *et al.* (1996) recorded that the dominant species in addition to *Microcystis* spp., were *Oscillatoria, Plantothrix, Anabaena, Pseudoanabaena, Snowella* and *Gomphospaeria.* The dominance of these species amongst phytoplankton communities in samples from natural lakes and reservoirs has been subsequently confirmed in England and Wales (Krowkoski and Jamieson 2002, Turner *et al.* 2018b) and Scotland (Krowkoski *et al.* 2012). *Anabaena* spp., *Aphanizomenon* spp. and *Oscillatoria* spp., are well known as toxin producing species and common members of phytoplanktonic communities (reviewed in Sivonen 2009).

In this study the maximum total microcystin levels recorded were 7.1 µg L^{-1} mean levels in lake 1 were 0.497 µg L^{-1} and 1.524 µg L^{-1} in lake 2, Student t tests indicated that total microcystins and *Microcystis*-like cells were significantly and highly significantly different between the two lakes respectively (total microcystins higher in

lake 2; *Microcystis*-like cells higher in lake 1). Of the multiple microcystin variants described, MC-LR has been most extensively studied and is reported to be between 3 and 10 times more toxic than other microcystin congeners (Dyble *et al.* 2008). Analysis of the microcystin toxin profiles between the two study lakes indicated differences in variants both in terms of variants, proportions and levels. In lake 1, where relatively low levels of microcystins were determined MC-LR was detected at low levels (<1 µg L^{-1}) and the dominant variant was the more hydrophobic MC-LF. This finding is in accordance with Turner *et al.* (2018b) who reported MC-LF as the highest mean proportion of profiles from *Aphanizomenon* and *Oscillatoria* spp. in analyses of freshwater bodies in England and Wales. This adds some support to the premise that *Microcystis*-like cells present in lake 1 were non-toxin producers and is worthy of further study. In lake 2, MC-YR was the dominant congener closely followed by MC-LR, with MC-RR and Asp^3 appearing towards the end of the period of toxin prevalence. Microcystin profiles determined in samples taken from weekly monitoring during August and September were similar in relative proportions indicating potentially that clonal or semi-clonal populations were responsible for toxin production. Similar data have been generated from studies in Greece (Zervou *et al.* 2017), throughout Europe via the European Multi Lake Survey (Mantzouki *et al.* 2018) and Finland (Halinen *et al.* 2007), the latter associated with toxins produced by species of *Anabaena.*

In summary whilst data on the relative proportions of microcystin variants in measured by LC-MS/MS are relatively sparse, levels and variants of microcystins presented here are consistent with the recorded literature and can help to unravel the structure and function of cyanobacterial populations.

4.4.2. Predicting the presence of microcystins using chemical and biological parameters

In recent years many authors have attempted to understand drivers for elevated cyanotoxin levels and/or occurrence of cyanobacterial blooms in natural lakes, reservoirs (Mantzouki *et al.* 2018, Mowe *et al.* 2015, Ferguson *et al.* 2008, Elliot 2012) and aquaculture systems (Sinden and Sinang 2016). In a large-scale study assessing the continental scale distribution of cyanotoxins across Europe Mantzouki *et al.* (2018) demonstrated that temperature (rather than classical nutrient availability or euphotic depth) was generally responsible for distributional characteristics of

cyanotoxins. Likewise, concordant observations with respect to temperature were recorded by Elliot (2012) in this review article assessing the potential impacts of climate change on pelagic freshwater cyanobacteria. The author demonstrated increased relative cyanobacteria abundance concurrently with increased water temperature, together with decreased flushing rates and increased nutrient loading. Similarly, Sinden and Sinang (2016) identified temperature, in combination with elevated water pH as key environmental factors influencing proliferation of cyanobacteria and toxicity in Malaysian aquaculture ponds. In the present study the presence of correlations between a range of biological and chemical parameters were tested against presence of microcystins. Perhaps not surprisingly given the low levels of microcystins detected in lake 1, no correlations were observed. For lake 2 where moderate levels of microcystins were present during the summer and autumn, water temperatures did not correlate, however a decrease in dissolved oxygen was closely associated with presence and levels of microcystins. A decrease in dissolved oxygen concurrent with decomposition of cyanobacterial blooms has been reported previously (O'Neil *et al.* 2012, Paerl and Otten 2013) and thus has the potential to indicate onset of toxicity derived from lysed cells within a rapidly blooming population. Perturbations in dissolved oxygen are frequently used as an indicator of water quality and eutrophication.

Conversely, with respect to the correlations of microcystin production, in a comprehensive review of biological and chemical factors Dai *et al.* (2016) postulated that light intensity and temperature were the more important physical factors with nitrogen and phosphorus as the critical chemical drivers of harmful algal blooms and microcystins. The authors also noted the complex interactions with biotic factors, suggesting that predator-prey relationships in phytoplanktonic communities may promote microcystin production and release (Dai *et al.* 2016). To explain the strength of the association between the measured parameters and their potential future ability to inform predictive models for microcystin events stepwise multiple linear regression analysis was applied to total microcystins and multiple chemical and biological parameters. Combined independent variables (i.e. chemical and biological parameters) explained approximately 60-65% of the variability at lakes 1 and 2 respectively, stepwise analysis further suggested the importance of dissolved oxygen in microcystin predictions. Using a similar statistical approach as a precursor to the development of empirical predictive models for cyanobacterial Beaulieu *et al.* (2013) showed that total nitrogen and water temperature provided the best model and explained 25% of cyanobacterial biomass. Using these explanatory variables, the

authors developed competing path models which showed that both nitrogen and temperature were indirectly (and directly) linked to cyanobacteria by interactions with total algal biomass. Model outputs predicted an average doubling of cyanobacterial biomass with a 3.3°C rise in water temperature. In contrast Carvalho *et al.* (2011) showed that significant explanatory variables were dissolved organic carbon and pH, and that furthermore nutrient concentrations were not a primary explanatory variable. Due to time constraints it was not possible to extend the statistical analysis to develop predictive models in this study however the relatively high levels of variability explained by the biological and chemical parameters via regression analysis indicated promise and areas for future investigation.

In summary, there is little agreement within the published literature with respect to unequivocal environmental drivers for toxin production from cyanobacterial taxa, the complexity of phytoplankton communities, cryptic ecological interactions together with the presence of non-toxin producing cyanobacterial strains and bottom-up/top-down controls throughout the trophic levels make this a remarkably rich and productive area for future study. The development of predictive models remains challenging, the multifaceted trophic relationships that influence cyanobacterial population dynamics and toxin production coupled with a high level of variability within cyanobacterial populations present major obstacles to making predictions at an environmentally relevant scale (Oliver *et al*. 2012). However, improved predictive ability and modelling will provide more efficient, proactive management of water bodies impacted by toxic cyanobacteria and in turn have positive public and animal health benefits.

5. Synthesis, evaluation and conclusions

Increases of blooms of toxic cyanobacteria are a threat to freshwater resources globally, with factors causing these reported to include anthropogenic climate change and eutrophication. Cyanobacteria have a long evolutionary history, significantly contributing to global primary production and yet relatively little is understood about their eco-physiology. As a phylum cyanobacteria demonstrate complex life histories and physiological traits potentially allowing them to out-compete eukaryotic algae at increased water temperatures and elevated nutrient levels. They produce a diverse array of compounds, some of which, are potent toxins, these cyanotoxins are produced by many species and strains, but the drivers of toxin production remain unknown. Where risk management has been undertaken to safeguard human, animal and environmental health, risk mitigation strategies have been frequently based on cyanobacteria cell counts, on the assumption that all blooms have the potential to be toxic. Whilst this approach is broadly protective, in certain instances it can incur unnecessary economic and social costs. Furthermore, it assumes on the presence of a linear relationship between increased cell numbers and toxicity which may not always be the case. Increased understanding of the complex interactions between abiotic and biotic factors, which mediate toxin production would improve the management of future cyanobacterial blooms. Thus, the main aim of the research project was to investigate the effects of biotic and abiotic factors on the growth and toxicity of cyanobacterial cells. This was accomplished by conducting laboratory-based experiments with *Microcystis* spp. and through monitoring cyanobacteria, toxins and a range of environmental parameters in two lowland freshwater reservoirs.

5.1. Methodological development: cell isolation, assessment of cell growth and toxin content

Many cyanobacterial reference strains have been held in laboratory collections for extended periods and as such have been serially sub-cultured in artificial media which may have altered their physiological, metabolic and genomic characteristics. Essentially, this can create atypical ‘type’ strains, and many workers have questioned the ecological relevance of using such isolates. Therefore, an early aim of this work was to isolate *Microcystis* sp. from the environment to provide fresh isolates for subsequent experiments. Several established isolation techniques were attempted. In brief, cyanobacterial cells were easily isolated from freshwater systems and grown successfully in the laboratory. However, the purification of monocultures was problematic and *Microcystis* cultures were consistently found to be contaminated with

another cyanobacterium, specifically *Synechococcus*. The solution was the development of a novel, yet simple procedure which exploited the eco-physiological differences between the two cyanobacteria (Section 2.2., Appendix 7.1.). The resultant presumptive *Microcystis aeruginosa* isolate (CCAP1450/17) was deposited and used in subsequent laboratory comparative experiments alongside the well characterised *Microcystis aeruginosa* reference strain (PCC7806). Interestingly although some differences the responses to abiotic factors were apparent between CCAP1450/17 and PCC7806 (Section 3.3.4.) both isolates responded similarly suggesting that PCC7806 remains a suitable model organism for study.

Batch culturing of uni-cellular *Microcystis* was successful, the edition of a three-light level incubator using LED's allowing the precise investigation on the effects of light and triplicate batch cultures delivered reproducible results. Supplementary work to produce and manipulate batch cultures of *Anabaena* CCAP1446/1A and *Oscillatoria* CCAP 1459/6 were unsuccessful due primarily to difficulties in homogenising the cultures for chlorophyll-a extraction and, lack of adequate repeatability and reproducibility data from flow cytometry and light microscopy making monitoring cell growth and size impracticable. Additionally, as neither strain was a substantial producer of nodularin or the microcystins (Section 2.5.3) work on these strains was discontinued.

Flow cytometry (Accuri, c6) provided a relatively low cost and straightforward technique for measuring cell density and cell size. This technique had the advantage that data could be re-examined for multiple parameters after collection and similar techniques were suitable for use in the laboratory and field. In the field, combining chlorophyll *a, b* and total carotenoids extraction and toxin extraction reduced the workload whilst providing triplicate data for photosynthetic pigment extraction. The liquid chromatography coupled to tandem mass spectrometry method developed, facilitated the separation and detection of many cyanotoxins in a total run time of 5.5 min (Section 2.5.). As such, the method provided a rapid assessment of important toxin variants, facilitating the ability to analyse hundreds of samples in a relatively short space of time. The limits of detection of the method also compared well with those generated by other cyanotoxin detection methods, enabling the quantitation of low concentrations of microcystins which would not have been detected using other methods (Section 4.3.4.). Without this rapid and sensitive methodology, the data generated in this study would have been less useful.

5.2. The effects of light, nutrients and temperature on cell growth and toxin content in batch cultures of *Microcystis* strains

Microcystis is an important bloom forming cyanobacterium and the organism first associated with the toxin microcystin. The ecological role of the microcystins remain elusive, although many researchers have proposed plausible yet conflicting theories. However, within blooms there will be toxic and non-toxic strains and light, nutrients and temperature will affect the proportion of toxic strains and the amount of toxins produced by those toxic strains. In this series of studies both stains tested (PCC 7806 & CCAP 1750/17), produced the highest cell densities and growth rates at medium light intensity (36 µmol of photons m^{-2} s^{-1}) indicating optimum irradiance (Section 3.4.1.). Relatively little difference was observed between the two strains, suggesting that in this study PCC 7806 was a valid study organism or that CCAP 1450/17 had acclimatised to laboratory conditions. A positive linear relationship between microcystin concentrations and cell densities was consistently observed. Nitrogen availability was an obligate requirement for microcystin production, but phosphorous replete conditions were not needed (Section 3.4.2.). Toxin production was negatively correlated with growth rates and cell size under nitrogen replete conditions. Cellular microcystin content was significantly higher at 20°C compared to 25 & 30°C. (Section 3.4.2.). These data confirmed that nitrogen, but not phosphorus is required for microcystin production and although toxin concentration was positively correlated with cell density, production is independent of growth rates. Furthermore, smaller cells appeared to show higher toxin contents, which is a novel observation. Toxin production was significantly higher at lower temperatures (20°C) questioning the widely accepted paradigm that increased water temperatures will favour toxic bloom formation.

The results confirmed complex interactions between light, nutrients and temperature, but were suggestive of additive effects with respect to microcystin production. Although the functional role of microcystins is far from clear, from these results it is postulated that under nitrogen replete conditions, microcystins perform an eco-physiological function, perhaps conferring an advantage over non-toxic strains, which may be metabolically costly and reduced under nitrogen deprivation and/or when cells are rapidly dividing. Further work focusing on mixed batch culture experiments would be required to test this hypothesis and the use of transcriptomics would assist in better explaining the functional role of microcystins.

5.3. Long-term monitoring of cyanobacteria and cyanotoxins in two lowland freshwater reservoirs in southern Britain

The results of monitoring the physically connected Longham Lakes, Bournemouth, UK over a twelve-month period indicated complex bottom-up and top-down control in controlling cyanobacterial taxa abundance and structure, the latter potentially mediated by the presence of omnivorous fishes. The cyanobacterial population structures differed significantly between the lakes, with *Microcystis*-like cells more abundant in lake 1 and the dominance of other cyanobacterial species in lake 2. Cyanobacterial cell counts exceeding the UK threshold levels of >20,000 cells mL^{-1} were recorded on four occasions. Toxins were detected in both lakes during the study period but did not correspond with highest levels of *Microcystis*-like cells Microcystin levels did not exceed the WHO medium health threshold of 20 µg L^{-1} during the study period although low threshold values were reached in 16% of samples. Levels of total microcystins were significantly higher in lake 2 than lake 1 (Section 4.4.1) and community structure and abundance data strongly suggested that cyanobacterial species other than *Microcystis* were responsible for toxin production. (Section 4.4.2.).

Examination of the environmental data collected alongside biological sampling showed that approximately 60-65% of the variability could be explained by combined independent variables (i.e. chemical and biological parameters). Time constraints and data gaps (particularly with respect to nitrogen availability (Section 3) prevented development of predictive models in this study however the relatively high levels of explained variability indicated promise and areas for future investigation.

The results of this study generated data broadly consistent with published studies from Northern European water bodies. A lack of consensus with respect to drivers for toxin production from cyanobacterial taxa exists, but here it was shown that multiple diverse species of cyanobacterial are implicated in toxin production, and that furthermore *Microcystis* cell numbers alone are not predictive of toxin concentrations. Trophic relationships that influence cyanobacterial population dynamics and toxin production require further study to generate data to inform predictive models, which will ultimately provide water quality managers with tools to enable efficient, active management of water bodies.

5.4. Overall conclusion

The aim of this work was to investigate the role and interactions of biotic and abiotic factors on cyanobacteria levels and toxin concentrations under laboratory and field conditions. In order to achieve this, a suite of techniques was applied and, in some cases, developed, these included batch culturing, flow cytometry and liquid chromatography coupled to tandem mass spectrometry. Each approach yielded different but complimentary information on aspects of cyanobacterial biology, physiology and toxicology. Notably the application of standardised, well controlled and, in the case of toxin quantification, ISO 17025 accredited methods, for flow cytometry and mass spectrometry to both laboratory and field sampling assisted in results interpretation and comparability between studies.

Extrapolation between laboratory studies and field conditions was challenging but some consistency between observations were apparent particularly with respect to toxin production, water temperatures and total cyanobacterial (but not *Microcystis*) cell densities. Laboratory studies revealed complex abiotic and biotic interactions between toxin production and cell physiology and, were suggestive of a potential functional physiological role for microcystins under nitrogen replete conditions at optimum irradiance and lower temperatures. Interestingly, temperatures above 20° did not result in elevated toxin production, whereas growth rates and cell density maxima were observed at higher temperatures. These observations suggest that increased bloom frequency mediated by higher water temperatures (consequent to climate change) may not automatically be associated with increased toxicity. Field studies were more difficult to interpret but indicated the presence of substantial populations of non-toxic *Microcystis* spp. and substantial toxin producing non-*Microcystis* taxa. Some indications that omnivorous fish may provide important top down controls were also noted. Although, simple modelling techniques explained almost two thirds of the variability observed in the environmental monitoring studies, the absence of some important data from field studies, notably nutrient availability, potentially limited the attempts to more fully explain toxin concentration and should be included in future investigations.

In conclusion, this series of studies has enhanced the understanding of cyanobacteria and toxin production under laboratory and field conditions, it has in part explained the biotic and abiotic factors that influence cyanotoxin production and thus has, in this respect, successfully achieved the aim. Relationships between temperature, light and nutrients, and cyanobacterial physiology were further revealed, but for the strains studied, the assumption that increased water temperatures will inevitably increase the frequency of toxic blooms was not confirmed. Several areas for further investigation have been suggested throughout this series of studies which will further clarify the role of microcystin in cyanobacterial biology and the relationships between toxin production and environmental parameters, including those as a result of climate change and eutrophication. This will assist in predictions and mitigation of harmful blooms, providing more efficient and proactive management of water bodies impacted by toxic cyanobacteria and have positive public and animal health benefits.

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7. Appendices

7.1. Publication: Exploiting eco-physiological niche to facilitate the separation of the freshwater cyanobacteria *Microcystis* sp. and *Synechococcus* sp.

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Note

Exploiting eco-physiological niche to facilitate the separation of the freshwater cyanobacteria *Microcystis* sp. and *Synechococcus* sp.

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ABSTRACT

In a novel approach to separate the co-occurring freshwater cyanobacteria *Microcystis* and *Synechoccous*, 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*.

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

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of *Microcystis* were contaminated with smaller *Bacillus* cells (Fig. 1, A.). These were found to be photosynthetic by epifluorescence microscopy (Fig. 1, B.) and the red auto-fluorescence signal from flow cytometry (Fig. 2, C.). The non-target cyanobacterial cells were identified as *Synechococcus* and tentatively assigned to *Synechococcus elongatus* (John Day, Culture Collection of Algae and Protozoa, pers. comm.). Therefore further techniques were required to separate the two cyanobacteria.

2. Other separation techniques

The contaminated cultures of *Microcystis* were subjected to additional techniques to attempt separation from the *Synechococcus*. 1) A 2 mL subsample of culture was homogenised and serially diluted to −3 log, the final dilution was spread on 10 solid agar petri dishes at 1 or 5% and 10 solid agarose petri dishes at 1 or 5%. All agar and agarose plates were prepared using 100% BG-11 adapting the work of Shirai et al. (1989). 2) Ten additional agarose petri dishes were prepared with the addition of antibiotic discs (Mastring-S, M13) to test for difference in resistance or susceptibility between *Microcystis* and *Synechococcus*. Antibiotics and dose applied; chloramphenicol (25 μg), erythromycin (5 μg), fusidic acid (10 μg), oxacillin (5 μg), novobiocin (5 μg), penicillin G (1 unit), streptomycin (10 μg) and tetracycline (25 μg). 3) A 5 mL subsample was centrifugally separated in a sucrose gradient using 5, 10, 20, 30 and 40% in 1 mL volumes. Each sucrose concentration was placed in a 50 mL conical flask with 20 mL 100% BG-11. The aim was to separate the cyanobacteria by using differences in buoyancy through an increasingly viscose media. 4) The next approach was to place 5 mL in a 50 mL conical flask with 20 ml of 100% BG-11 and the addition of 7 μg microcystin-LR (Sigma-Aldrich, 33893) to test susceptibility of *Synechococcus* to the *Microcystis* toxin. 5) The cyanobacteria were separated using an automated cell sorter (BD, FACSAria) into densities of 100, 1000, 10,000 and 100,000 cells per mL, respectively and placed into 20 mL of 100% BG-11 in 50 mL conical flasks. Cells were discriminated by using a known unialgal *Microcystis* culture (PCC 7806 wild type) as a template. In all the techniques attempted culture flasks and plates were incubated as per the parameters described in step one. The relative cell growth of the cyanobacteria was monitored by light microscopy and flow cytometry for up to three weeks.

None of the techniques attempted were successful in separating the *Microcystis* sp. cells from the *Synechococcus* sp. cells. The solid plating technique there was no observed difference in the resistance or susceptibility between the two cyanobacteria to all antibiotic types and doses. The sucrose gradient centrifuging also failed due to both cyanobacteria having the same fractionation position in the gradient. Not one of the cell densities obtained from automated cell sorting showed any growth when incubated, the cyanobacteria could have been adversely affected by fluid acceleration, electrical shock or photo-bleaching (light shock). In techniques where cyanobacterial growth was observed, it was apparent that a proportion of the smaller *Synechococcus* cells was remaining attached to the *Microcystis* mucus sheath and subsequently overgrowing the assumed unialgal cultures.

The main drivers of cyanobacterial blooms are increasing water temperature and increased nutrient input (Paerl et al., 2001, Schindler et al., 2008). Rigosi et al. (2014) conducted an analysis of data collected from over 1000 lakes in the United States and reported evidence of the growth of cyanobacteria genera responding differently to temperature and nutrients. For example, *Microcystis* dominance within a bloom is primarily driven by increased temperature, whereas *Synechococcus* dominance is primarily driven by increased nutrients. This posed the hypothesis, could the two cyanobacteria in culture be separated by increasing temperature and decreasing nutrients?

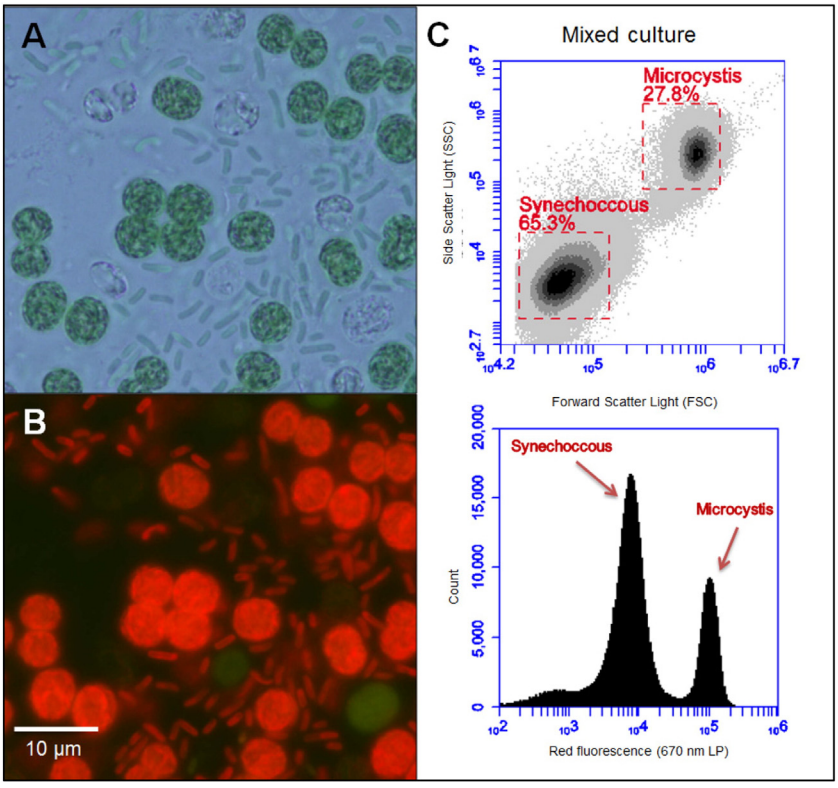

Fig. 1. (A) True colour micrograph of isolated *Microcystis* sp., spherical cells with darker green pigment, and *Synechococcus* sp., smaller *Bacillus*-shaped cells with lighter green pigment. (B) The same image through epifluorescence microscopy, red fluorescence indicates chlorophyll content in both cell types and green fluorescence indicates chlorotic (non-photosynthesising) cells. (C) Flow cytometry cytogram showing *Microcystis* cells and *Synechococcus* cells and red auto-fluorescence histogram, smaller peak *Microcystis* larger peak *Synechococcus*.

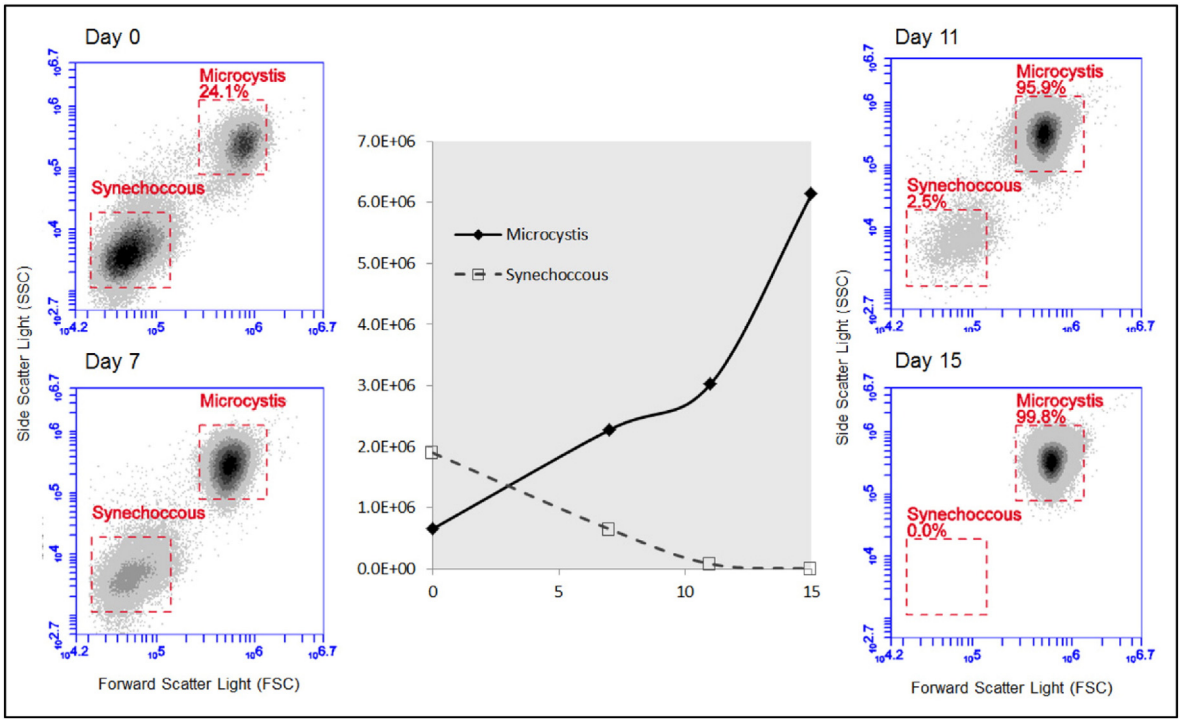

Fig. 2. The cell densities of *Microcystis* and *Synechococcus* in 20% BG-11 medium at 32 °C monitored over a 15 day period by flow cytometry, density plot cytograms of cell size forward scatter light (FSC) and cell granularity, side scatter light (SSC). FSC signal threshold 20,000 arbitrary units (au), with gates on red fluorescence (670 nm LP) between 2000 and 300,000 au and far-red auto-fluorescence (675 nm ± 12 nm) between 1000 and 800,000 au to remove noise from the light signal.

3. Step two (physiological separation)

Five decreased concentrations of BG-11 at 40, 30, 20, 10 and 0% were put into 5 test tubes in 5 mL volumes and 0.5 mL of mixed culture was transferred into each. The test tubes were placed in an incubator (AlgaeTron, AG230) at 32 ± 1 °C, on a 12 h light/dark sine wave regime provided by white and infra-red LED's at 20 μmol quanta m^{-2} s^{-1}. The relative cell growth of both cyanobacteria was monitored by flow cytometry over 15 days.

The increase in temperature and decrease in nutrients were conditions that favoured the target cyanobacteria. Flow cytometry data showed an increase of *Microcystis* cells and a reciprocal decrease in *Synechococcus* cells over 15 days (Fig. 2.).

The effect was seen in all concentrations of BG-11, with the greatest *Microcystis* biomass in 20 and 30%. Inspection by microscopy and return to 100% BG-11 and 25 °C culture conditions confirmed a unialgal culture had been isolated. The *Microcystis* strain isolated from 20% BG-11 is now deposited in the Culture Collection of Algae and Protozoa (CCAP, UK) accession number 1450/17.

The isolation and purification of cyanobacteria is a time consuming process, where techniques need to be refined for the target organism. Time invested is, however, rewarded by the establishment of new culture lines for ecological and physiological research into these important organisms. We conclude that modifying culture conditions in accordance with published eco-physiological niche requirements was the best method for artificially selecting for the target organism, in this case *Microcystis*, and thereby creating a new culture lineage.

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7.2. A novel bioassay to test the toxicity of microcystins on the greater wax moth larva, *Galleria mellonella,* using toxic and non-toxic *Microcystis* strains

The toxicity of microcystins (MC), in particular MC-LR to mammals has been traditionally investigated using *in vivo* mammalian toxicity assays, usually mice (Watanabe and Oishi 1985, Zurawell *et al.* 2005). The use of live vertebrates for toxicity testing is being reduced by the Organisation for Economic Co-operation and Development's (OECD) 3R's (Replacement, Reduction and Refinement) policy of which the UK is a signatory. Live vertebrate eco-toxicological testing is replaced with the use of phytoplankton and invertebrates and reduced and refined with *in vitro* assays. However, the relative toxicity of other MC variants, which may increase in response to eutrophication and climate change, is an area where research is required (Faassen and Lurling 2013). The larva of the greater wax moth, *Galleria mellonella,* is a model organism used in the toxicological testing of pathogenic bacteria (Desbois and Coote 2012), due to similarities in insects' immune system to those of mammals (Champion *et al.* 2009).

The aim of this experiment was to assess the potential use (proof of concept) of the *G. mellonella* model as an additional non-vertebrate ecotoxicology test for microcystins and to test MC extraction methods for future eco-toxicological studies.

Materials and methods

Microcystin extraction

Cells harvested from batch cultures of *Microcystis* PCC 7806, toxic wild type and non-toxic mutant at late stationary phase when MC's per cell are at their highest. For each 15 mL of culture centrifuged at 2400 rpm for 15 minutes, bottom 2 mL of concentrated cells removed and counted by FCM. Cells lysed by sonification in 3 x 10 second bursts (SoniPrep 150, MSE) then centrifuged at 4000 rpm for 30 minutes at 15°C, supernatant removed and centrifuging repeated. Supernatant aliquots of 550 µL preserved at -18°C for toxicology bioassay and toxin analyse by LCMS. For toxicity testing aliquots were defrosted and freeze dried (Thermo Scientific, SPD 121P) for 90 minutes. The residue dissolved in phosphate buffered saline (PBS) to a concentration of 10 mg per litre.

Range finding and test concentrations/doses.

The average MC-LR content per cell over the growth cycle of wild type PCC 7806 is 10.04 fg, with a standard deviation of 4.95 fg, therefore, the MC-LR content of each fulcrum tube should be between 5.09 and 15.99 µg. Taking the median value of 10.04 µg each tube will contain

enough MC-LR to be lethal to 10 *G. mellonella* larvae with an average weight of 200 to 300 mg.

There were 5 concentrations/doses, with the highest concentration predicted to result in 100% fatality and the lowest have no lethal effect (Table 1.). Five larvae were injected for each concentration, with 5 injected with PBS (injection control) and 5 not injected (incubation control). Dilution series repeated with non-toxic PCC 7806 mutant strain.

Table 1. Test concentrations, dilution ratios and volumes

	Conc 1	Conc 2	Conc 3	Conc 4	Conc 5
Concentration	10 mg/L	3.2 mg/L	1 mg/L	0.32 mg/L	0.1 mg/L
Dilution	1:1	1:3.125	1:10	1:31.25	1:100
Volume (taken from previous concentration)	100 µL	32 + 68 µL	10 + 90 µL	32 + 68 µL	10 + 90 µL

G. mellonella injection

Larvae were stored at 15°C and each concentration and control set of 5 larvae weighed. Each larva was held over a pipette tip (1 or 5 mL depending on ease) and 10 µL of concentration injected by Hamilton syringe into the right foremost leg. The syringe was flushed with 70% ethanol between injections. Each set of larvae was placed in a filter paper lined petri dish and incubated at 37°C. Injected larvae and controls were monitored for survival at 1, 6, 24 and 48 hrs.

Statistical analysis

The lethal concentration 50 (LC_{50}), where the probability of survival is 50%, was calculated by Probit regression analysis of mortality percentage using SPSS 21 statistical software (IBM). The lethal dose 50 (LD_{50}) was calculated using the known dose and average weight of the larvae to calculate the mass of toxin for 1 kg.

Results

In the toxic microcystin concentration series there was 100% mortality in the highest and second highest concentrations, no mortality was observed in the other concentrations. Probit

regression analysis predations were obtained (Figure 1.) and LC_{50} and LD_{50} values calculated for 24 & 48 hrs (Table 2.).

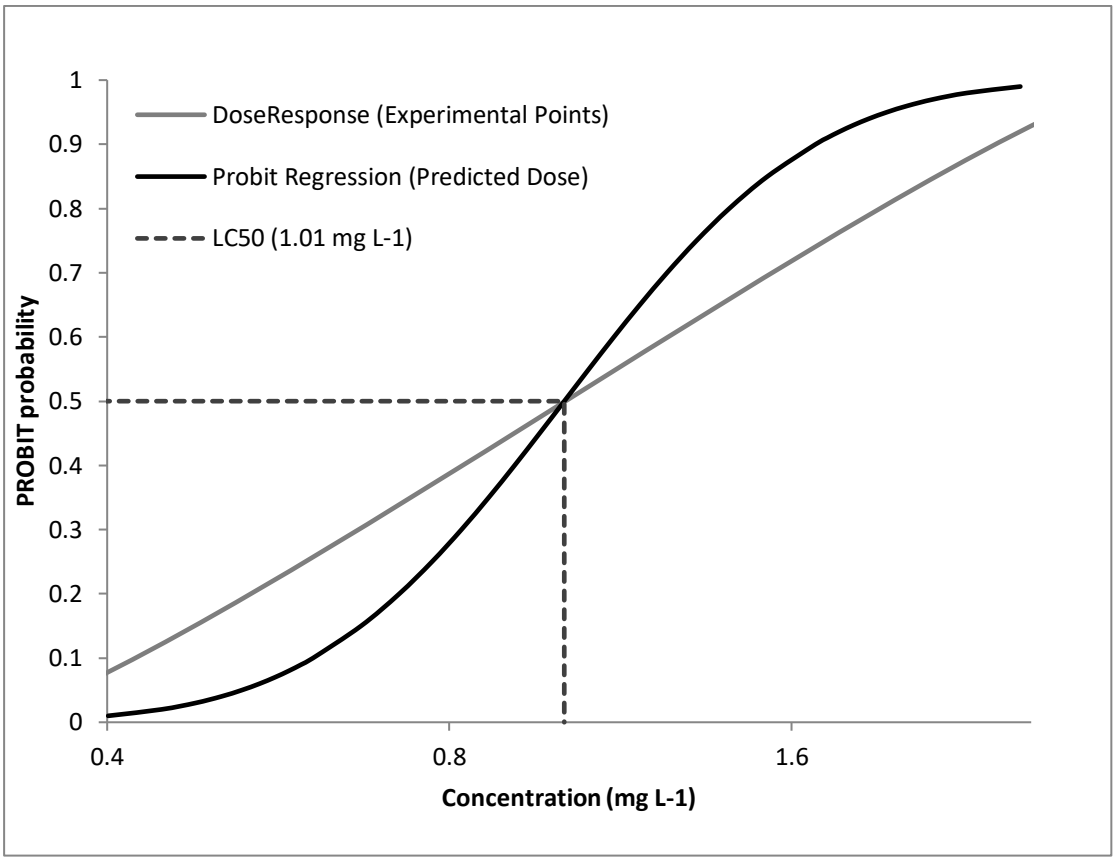

Figure 1. Probit survival analysis for toxic wild type Microcystis culture PCC 7806

For the non-toxic control, however, there was 80% mortality in the highest concentration and 20% in the second. Probit regression analysis yielded higher relative concentration values for LC_{50} and LD_{50} (Figure 2, Table 2.), indicating that total microcystins are toxic to *G. mellonella* larva.

Table 2. Lethal concentration 50 for toxic wild type and non-toxic mutant cultures

Culture	Time	LC50 mg/L	LD mg/kg
Toxic *Microcystis* PCC 7806 (Total MC's)	24 hrs	2.31	0.95
	48 hrs	1.01	0.42
Non-Toxic *Microcystis* PCC 7806 (Relative concentrations)	24 hrs	5.56	2.29
	48 hrs	5.25	2.16

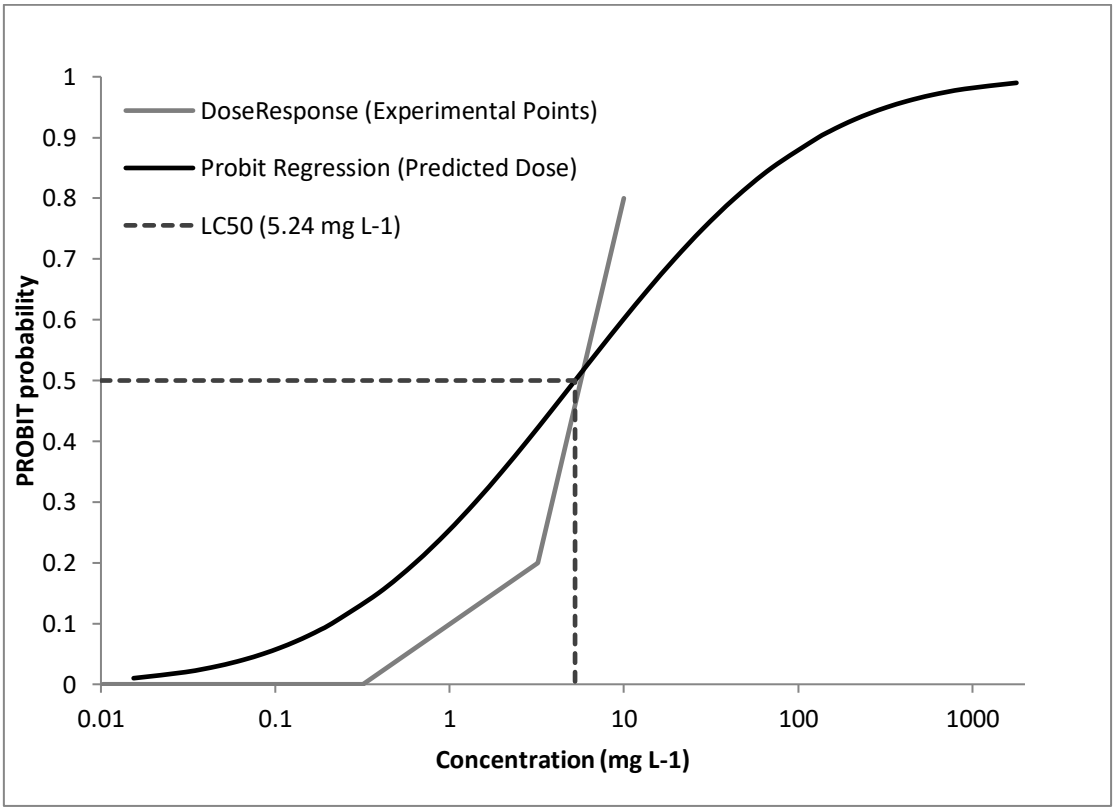

Figure 2. Probit survival analysis for non-toxic mutant Microcystis culture PCC 7806

Discussion and conclusions

The larvae of the wax moth, *Galleria mellonella,* were not complex of difficult toxicological test organisms to work with. Injection was straightforward and in the initial injection controls there was less than 2% mortality, also mortality was extremely low whilst the larvae were stored for over 4 weeks.

The values obtained for LD_{50} 24 and 48hrs of 0.95 and 0.42 mg/kg for total microcystin differ from reported MC-LR of 3.9 and 1.9 mg/kg for 24 and 48hrs in Lepidoptera larvae (Denley and Wilkins 1995). The difference may be attributed to the method of MC detection, tandem mass spectrometry was used in this study and or the method of MC extraction. The method used here was crude, with many other compounds, notable photosynthetic compounds (chlorophylls, phycocyanin etc.) not removed in the extraction process. These compounds would also account for the mortality recorded in the non-toxic mutant cultures. Additional problems from the extractions was the small amount of total MC's extracted, this was why only 5 larvae were used at each concentration than the usual 10 and additional larvae would make statistical analysis more robust.

Overall, this study has highlighted the suitability of *G. mellonella* larvae as eco-toxicological test organisms, for biotoxins and manmade compounds. The extraction process can be improved to yield higher quality MC's in greater quantities, also reference materials are available which should be suitable.

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