Bloom formation, species succession, toxin variability and benthic recruitment of cyanobacteria in Lake Rotorua, Kaikoura

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ABSTRACT

Cyanobacteria are an ancient group of photosynthetic prokaryotes that are found in a wide range of habitats including freshwater lakes and rivers. In aquatic ecosystems, under favourable environmental conditions, cyanobacteria can form dense and expansive blooms. The increasing prevalence of cyanobacterial blooms globally has been linked to environmental changes in freshwater ecosystems including anthropogenic eutrophication, catchment modification and climate change. One of the most common genera of bloom-forming cyanobacteria is *Microcystis*. It can produce microcystins, a potent cyanotoxin that poses a risk to human and animal health. It also possesses physiological adaptations that confer a competitive advantage over other phytoplankton and cyanobacteria. One of these is the ability to vegetatively 'overwinter' on sediment surfaces. When conditions are favourable in spring or summer cells are recruited back into the water column and may provide a substantial inoculum for summer blooms.

To better understand the variables that promote *Microcystis aeruginosa* blooms, this study investigated; (i) how environmental variables influence *M. aeruginosa* bloom formation and species succession during a bloom, as well as the seasonal variability of microcystin production, and (ii) how environmental, biotic and ultrastructural changes in the cells influence recruitment of benthic *M. aeruginosa*. The study focused on Lake Rotorua, a small eutrophic lake in Kaikoura, South Island, New Zealand.

To address the first objective, surface water samples were collected weekly or fortnightly from Lake Rotorua between 14 January 2014 and 27 May 2015. Samples were analysed for cyanobacterial cell density and species composition,

total and dissolved nutrients, *mcyE* genotype composition, and microcystin quota. Temperature was measured using loggers at different depths in the water column and weather data were acquired from the Kaikoura weather station (approximately 8 km from the study site). To address the second objective, surface sediment samples were collected from near-edge (containing *Microcystis* and *Aphanizomenon gracile*) and mid-lake (containing predominantly *Microcystis*) sites in Lake Rotorua. A series of laboratory experiments were undertaken which investigated the effect of ammonium (0, 0.1, 0.2, 0.5, 1 and 5 mg N L^{-1}), light intensity (dark, 1.5, 10, 50 and 100 µmol photon m^{-2} s^{-1}) and temperature (4, 13, 16, 19 and 25°C) Changes in cellular structures of *Microcystis* isolated from sediment collected in spring, early and late summer were also analysed using transmission electron microscopy to assess how this influenced recruitment

Cyanobacteria species composition in Lake Rotorua was dominated by *Microcystis aeruginosa*, *Aphanizomenon gracile*, and *Dolichospermum crassum*. Moderate levels of nitrate in the lake appeared to be related to intermittent high-rainfall events during the austral summer of 2014, and may have contributed towards dominance by *A. gracile* and *D. crassum*. While both species are capable of nitrogen-fixation, heterocytes were absent. *M. aeruginosa* blooms subsequently occurred when ammonium concentrations increased, water temperature was high (>27°C), and the total nitrogen: total phosphorus ratio was low. A storm towards the end of the 2014 summer rapidly cooled and mixed the lake, and was followed by bloom collapse. In contrast, an extended drought over summer 2015 resulted in prolonged stratification, increased dissolved reactive phosphorus and very low dissolved inorganic nitrogen concentrations. Cyanobacterial pattern of species succession differed substantially between 2014 and 2015. *Aphanizomenon gracile* and *D. crassum* contained a higher heterocytes frequency compared to 2014 and

M. aeruginosa density remained relatively low. Picocyanobacteria consisting of *Aphanocapsa* sp. were abundant. There was no relationship between toxic genotype abundance and *M. aeruginosa* biovolume or environmental parameters. However, univariate analysis showed that there was a significant positive relationship ($p<0.001$) between microcystin quotas and surface water temperature.

In all benthic recruitment experiments single cells, rather than colonies, accounted for the majority (>55%) of recruited cells and it was speculated this may have been because they had more immediate access to light and nutrients. Linear Mixed Effect Models (LMEMs) analysis showed that *M. aeruginosa* recruitment was significantly lower (ANOVA; $p<0.001$) in near-shore sediment samples, suggesting that *A. gracile* may elicit allopathic effects on *M. aeruginosa.* In mid-lake sediment samples, *M. aeruginosa* recruitment was significantly higher at moderate ammonium concentrations (0.1, 0.2 and 0.5 mg N L^{-1}; ANOVA; $p<0.001$), at two temperatures (16 and 25°C; ANOVA; $p<0.001$) and high light intensities (50 and 100 µmol m^{-2} s^{-1}; ANOVA; $p<0.01$). Under transmission electron microscopy the area of benthic cells occupied by gas vesicles increased significantly (ANOVA; $p<0.001$) for the three samples collected during the study period.

The results highlight the complex successional interplay of cyanobacteria species and their physiological adaptations (e.g., nitrogen fixation, buoyancy regulation). Climate, through its effect on runoff, water temperature and stratification, can lead to successional sequences amongst different cyanobacteria species according to whether the combination of physiological adaptations is advantageous or disadvantageous with environmental change. The benthic recruitment experiments collectively demonstrated that allopathic interactions, ammonium, light and

temperature individually and synergistically regulate gas vesicle synthesis and *M. aeruginosa* recruitment in Lake Rotorua.

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1.1 Introduction

1.1.1 Cyanobacterial blooms

Cyanobacteria, also known as blue-green algae, are photosynthetic prokaryotic organisms found in many aquatic and terrestrial environments (Whitton and Potts, 2012). Cyanobacterial cellular processes primarily rely on water, carbon dioxide, inorganic substances (e.g., nitrogen) and light, as photosynthesis is the principal mode of energy metabolism (Fay, 1983).

In aquatic ecosystems, cyanobacteria occur in many different habitats including in the water column (planktonic), aggregated on the water surface (metaphyton), attached to other algae or macrophytes (epiphyton), or attached to substrates (benthic; Quiblier *et al*., 2013). They can exist as solitary free-living cells or colonial (consisting of few cells to thousands of cells). Most colonies and single cells are microscopic, but their population may be visible when there are large aggregations; as blooms in the water column or mats attached to substrates (Chorus and Mur, 1999). The most common genera of freshwater cyanobacteria genera associated with planktonic bloom formation in the water column are *Microcystis*, *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Planktothrix* and *Nodularia* (Ma *et al*., 2015).

1.1.2 Factors influencing cyanobacteria bloom formation

Cyanobacterial blooms can be harmful to the environment, organisms, and human health. Under favourable conditions, cyanobacteria outcompete other eukaryotic phytoplankton and can form large blooms or scums. The subsequent decay of these blooms can deplete oxygen from the water (Paerl and Otten, 2013). A variety of toxic secondary metabolites may also be produced (e.g., cyanotoxins; Carmichael *et al.*, 2001a; Chorus, 2001). A number of studies have focused on understanding the triggers of bloom formation (Fernández *et al.*, 2015; Paerl and Otten, 2013; Reynolds and Walsby, 1975; Wood *et al.*, 2012; 2011). They have shown that cyanobacterial blooms are driven by a complex interaction of physical, chemical and biological factors, such as temperature, nutrients, stratification and cell physiology (Ahn *et al.*, 2011; Anderson *et al.*, 2002; Reynolds and Walsby, 1975).

Nutrients, such as nitrogen and phosphorus, play an important role on cyanobacteria growth (Ahn *et al.*, 2011), and the increased occurrence of blooms globally has been associated with nutrient over-enrichment, or eutrophication, of inland waters (Anderson *et al.*, 2002). Phosphorus has traditionally been considered the main nutrient limiting primary production and algal biomass accumulation in freshwater ecosystems (Schindler and Fee, 1974; Schindler *et al.*, 2008), especially for nitrogen-fixing cyanobacteria genera (*Dolichospermum* and *Aphanizomenon;* De Nobel *et al.* 1997). However, non-nitrogen-fixing genera (*Microcystis*, *Aphanocapsa*) and nitrogen-fixing blooms are also influenced by nitrogen availability and can proliferate at high N:P ratios (Paerl and Otten, 2013; Paerl, 2014).

Climate change scenarios indicate that lakes and reservoirs will experience increased temperatures, more intense and longer periods of thermal stratification, modified hydrology, and altered nutrient loading (Hamilton *et al*., 2013). These factors are known to influence the incidence of cyanobacterial blooms (Carey *et al*., 2012; Paerl and Huisman, 2009; Paul, 2008; Anderson *et al*., 2002). The increase in temperature increases the vertical thermal stratification of the water column, and also favours the growth of buoyant cyanobacteria in particular (Reynolds and Walsby, 1975).

1.1.3 Cyanobacterial advantages that favour bloom formation

Cyanobacteria have a number of unique characteristics that contribute to their ability to out-compete and dominate planktonic populations, including the ability to grow at higher temperatures than most eukaryotic algae (Briand *et al*., 2004; Paerl *et al*., 1985) and, tolerance to high levels of ultra-violet irradiation (Xu *et al*., 2005; Hu *et al*., 2002), heavy metals (Perales-Vela *et al*., 2006), and low oxygen concentrations (Chorus and Mur, 1999). Some filamentous species produce specialized cells called heterocytes, which fix atmospheric nitrogen. This process can contribute to their success especially when nitrogen supply rates are low (Whitton and Potts, 2000). Under conditions of stress, some species can produce resting spores, called akinetes. These thickened cells provide storage of food (carbohydrates and amino compounds) and after a period of dormancy, followed by appropriate environmental triggers (e.g., a change in temperature), they can germinate into filaments (Baker, 1999). Additionally, some planktonic species contain gas-filled cylindrical structures, gas vesicles, within the cytoplasm that enable them to offset the negative density of other cellular constituents, conferring buoyancy to migrate upwards in the water column (Hayes, 1988). By

being able to regulate buoyancy through a combination of gas vesicles and diurnal changes in photosynthate storage, cyanobacteria may be able to exploit nutrients that are unavailable to other species that have to rely on turbulence to resist sinking (Oliver and Ganf, 2000; Oliver*et al.*, 2012).

1.1.4 Harmful algal blooms

Cyanobacterial blooms are often associated with water quality issues. In addition to causing unpleasant tastes and odours, some species can produce toxic secondary metabolites, commonly referred to as cyanotoxins. These compounds pose a health risk to humans and animals and can have dermatotoxic, hepatotoxic, neurotoxic and/or carcinogenic effects (Carmichael *et al.*, 2001b; Chorus and Mur, 1999). Cyanotoxins vary in chemical structure (Figure 1.1) and can be grouped into cyclic and linear peptides, alkaloids and carbamates (Puddick *et al.*, 2014; Chorus and Mur, 1999). The hepatotoxins include microcystins, nodularins (both are cyclic peptides), and cylindospermopsins (tricyclic alkaloids). Microcystins are also toxic to the central nervous system and reproductive tissues. The neurotoxins include the alkaloid anatoxin-a, the organophosphate anatoxina-a (S), and the saxitoxins, which are alkaloid-carbamates.

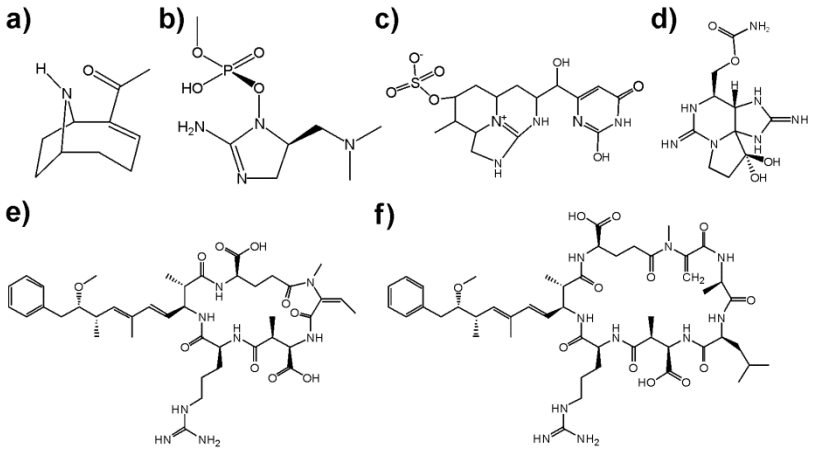

Figure 1.1: Chemical structures of several common freshwater cyanotoxins: (a) anatoxin-a, (b) anatoxin-a(S), (c) cylindrospermopsin, (d) saxitoxin, (e) nodularin-R and (f) microcystin-LR (Wood *et al.* 2015).

Within a species there can be toxic and non-toxic genotypes and these commonly coexist in the environment (Fastner *et al*. 2001; Kurmayer *et al*., 2002; Janse *et al*., 2004). The relative abundance of genotypes can change during a bloom, resulting in marked shifts in cyanotoxin concentrations (Welker *et al*., 2003; Kardinaal and Visser, 2005). The factors driving shifts among toxic and nontoxic strains are not yet well understood (Tonk, 2007).

1.1.5 *Microcystis* blooms

Microcystis is well known as a bloom-forming species of cyanobacteria that occurs globally (Figure 1.2). It is commonly associated with the production of microcystins (Bishop *et al*., 1959; Harke *et al.*, 2016;). In the natural environment, *Microcystis* usually grows in colonies, however, under laboratory conditions it usually grows as single or paired cells (Reynolds *et al.*, 1981). Some studies suggest that the colony formation may be associated with prevention of grazing by

flagellated chrysophytes (Burkert *et al*., 2001), copepods, cladocerans and rotifers (Yang *et al*., 2006; Yang *et al*., 2009) and turbulent mixing may affect the colony size (O'Brien et al., 2004).

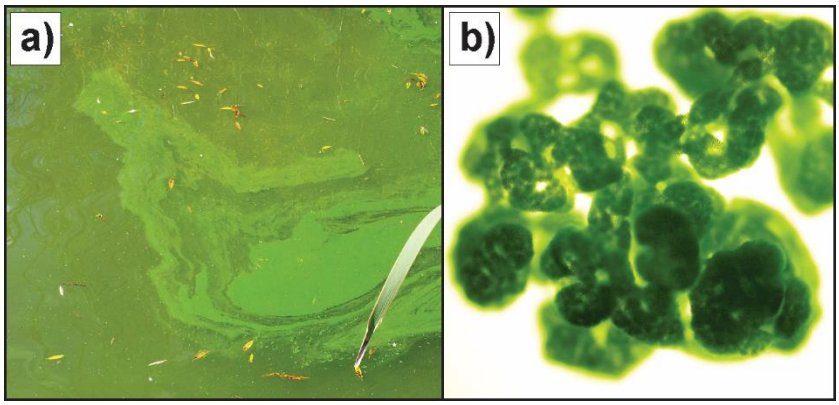

Figure 1.2: a) *Microcystis* scum forming on the surface of a lake and b) *Microcystis* colony at 100× magnification (Photos: J. Puddick (a) and S. Wood (b)).

1.1.6 Life cycle of *Microcystis*

The life cycle of *Microcystis* includes planktonic and benthic stages. During benthic stages *Microcystis* cells accumulate on the sediment where poor light conditions restrict photosynthetic activity (Brunberg and Blomqvist, 2002). This benthic survival of *Microcystis* is commonly referred to as 'overwintering' (Preston *et al*., 1980; Fallon and Brock, 1981). Verspagen *et al*. (2005) investigated seasonal changes in *Microcystis* abundance in the water column and sediment in the Lake Volkerak, Netherlands, and found that recruitment of *Microcystis* cells from the sediments and sedimentation of cells occurred throughout the year, with highest recruitment and sedimentation rates during summer. Their model simulations indicated that the absence of benthic recruitment would reduce the summer bloom biomass by 50%.

The variables that trigger *Microcystis* recruitment from the sediment to the water column are still unclear, however, many studies suggest that environmental variables are important. Yamamoto (2009) investigated the effects of environmental factors on *Microcystis* recruitment in a shallow pond. In a laboratory experiment, the sediment was incubated with 15 mL of distilled water under a temperature gradient ranging from 5°C to 25°C, with a light supply of 50 µmol photon m^{-2} s^{-1} on a 12 h : 12 h light-dark cycle. Benthic recruitment was enhanced at and above 15°C and cell buoyancy likely played a role in recruitment. Other studies have suggested that temperature is important in the recovery of *Microcystis* colony buoyancy (Tsujimura *et al*., 2000; Brunberg and Blomqvist, 2003; Verspagen *et al*., 2005). Tsujimura *et al*. (2000) considered that the buoyancy of most of the *Microcystis* in natural sediments from Lake Biwa, Japan, changed at the beginning of summer when the water temperature exceeded 20°C. Ståhl-Delbanco *et al*. (2003), in a lake study, investigated how the recruitment of *Microcystis* from the sediments responded to different levels of nitrogen and phosphorus, and variations in grazing. They suggested that the high nutrient concentrations (up to 3,000 µg L^{-1} of dissolved inorganic nitrogen) in combination with low N:P ratios favour *Microcystis* recruitment.

Another environmental variable that is believed to trigger benthic recruitment is light. Reynolds *et al*. (1981), under laboratory conditions, showed that when colonies suspended from the benthos were exposed over 17 days to continuous light, at intensities of 7.6 or 22.7 µmol m^{-2} s^{-1}, and controlled temperature (20 ±1°C) the colonies approximately doubled in concentration and also increased considerably in mean diameter. Colonies kept in the dark did not change in concentration or size. Conversely, Brunberg and Blomqvist (2002), studying the

survival of benthic *Microcystis* in the laboratory under light/dark conditions, reported that light exposure (30 µmol m^{-2} s^{-1}) led to supersaturated oxygen conditions and a very poor survival of *Microcystis* whereas under dark conditions and low concentrations of oxygen, there was much greater survival. Head *et al*. (1999) investigated the response of benthic recruitment to environmental conditions in a temperate mesotrophic lake and did not find any significant differences in the numbers of cyanobacteria undergoing vertical movements along a depth transect which encompassed a range of temperature, oxygen, and irradiance levels, suggesting that the variations in environmental conditions with depth in the lake were not important.

Verspagen *et al*. (2004) suggest that benthic *Microcystis* recruitment may be facilitated by a passive process resulting from resuspension of the cells/colonies from the sediment as a result of wind-induced mixing or bioturbation. Ståhl-Delbanco and Hansson (2002) observed in a long-term laboratory experiment that high activity of the isopod *Asellus* sp. increased the benthic recruitment through resuspension of *Microcystis* spp. and *Anabaena* spp.

Current literature suggests that there is no single over-arching environmental factor triggering the recruitment of *Microcystis*. However, temperature, light, nutrients, resuspension, and bioturbation may collectively impact on *Microcystis* recruitment based on results from field studies and simulation experiments (Tan *et al*., 2008).

1.1.7 Bloom succession

Cyanobacteria blooms are often characterized by seasonal succession of dominant species. Many factors can influence the spatial and temporal variability of cyanobacteria populations, including temperature (including climate change),

grazing pressure, nutrients, light intensity, stratification, and competition amongst cyanobacteria species and with other eukaryotic algae. *Microcystis* bloom dynamics are usually characterised by succession patterns associated with competition with other cyanobacteria species, such as *Aphanizomenon* (Miller *et al*., 2013) and *Dolichospermum* (Ma *et al.*, 2015).

Recent studies in shallow Lake Dianchi in China have shown that temperature and nitrogen availability are the main factors influencing *Aphanizomenon* and *Microcystis* succession (Wu *et al.*, 2016). The spring-summer succession patterns of *Aph. flos-aquae* and *Microcystis* from 2009 to 2012 were suggested to result from changes in nitrogen concentration and temperature, however, physiological plasticity of the organisms, together with climate change, may have also had some effect. In another study also looking at the succession of cyanobacterial species, Fernández *et al.* (2015) found that the variables that related most closely to bloom formation were total phosphorus and climatic variables (e.g., temperature and solar irradiation). They observed that phosphorus was a limiting factor for *Dolichospermum* (basionym *Anabaena*) *circinalis* development but never limited *Microcystis* growth. While temperature and solar radiation impacted the growth of both species, solar radiation had the greatest impact once the decrease in light promoted bloom collapse. In general, cyanobacteria succession remains understudied, as much of the focus has been on monospecific blooms (Wu *et al*. 2015; Fernández*et al*., 2015). Additionally, there is limited knowledge on the drivers of genotype succession (Miller *et al*., 2013).

1.1.8 Microcystins

Of the known cyanotoxins, the hepatotoxic microcystins are the most commonly detected and/or targeted worldwide. Microcystins are synthesised by many strains

of planktonic, benthic and terrestrial cyanobacteria genera (e.g., *Microcystis*, *Dolichospermum*, *Nodularia*, *Oscillatoria*, *Nostoc*, *Cylindrospermopsis*, *Planktothrix*, *Oscillatoria*, *Radiocystis*, *Arthrospira*, *Nostoc*, *Hapalosiphon*, *Fischerella*, *Phormidium* and *Plectonema* (Chorus and Mur, 1999; Fiore *et al.*, 2009; Mohamed *et al.*, 2006; Sivonen and Jones, 1999). *Microcystis* is the most common microcystin producing and bloom forming genus.

Microcystins are cyclic heptapeptides (Figure 1.3) containing both D- and L-amino acids plus *N*-methyldehydroalanine and a unique ß-amino acid side-group, 3-amino-9-methoxy-2-6, 8-trymethyl-10-phenyldeca-4, 6-dienoic acid (Adda). Microcystin isoforms differ primarily at the two L-amino acids, and due to the presence or absence of the methyl groups on D-erythro-ß-methylaspartic acid (D-MeAsp) and/or *N*-methyldehydroalanine (Mdha). However, substitutions of all moieties within microcystin have been reported (Pearson *et al.*, 2010). To date, at least 130 different microcystin congeners have been characterized (Puddick, 2013). Varying levels of toxicity have been reported for each microcystin congeners. For example, the LD_{50} of the most common congener, microcystin-LR, is 50 µg per kilogram of body weight in mice (Krishnamurthy *et al*., 1986), while the rarer microcystin-RR requires a significantly higher dose of 600 µg per kilogram of body weight in mice to produce the same lethal effect (Watanabe *et al*., 1988).

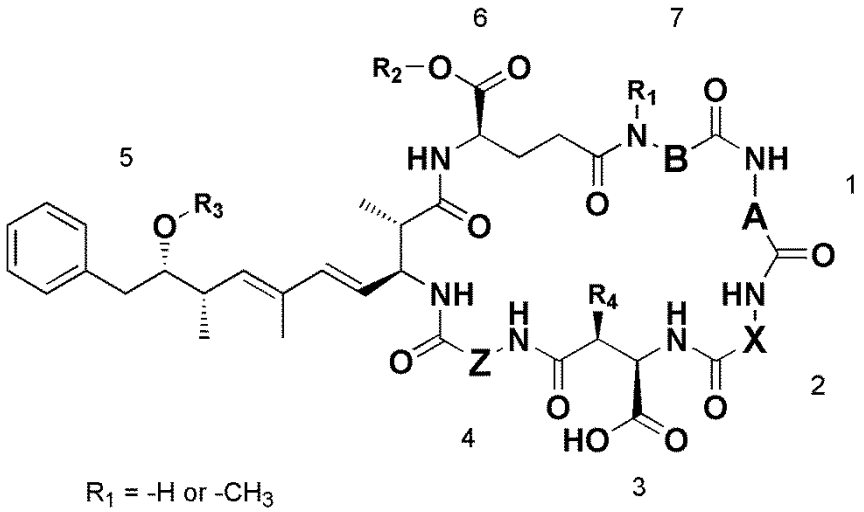

Figure 1.3: General chemical structure of microcystin (Puddick, 2013).

Microcystins inhibit eukaryotic protein phosphatases type 1 and 2A, which results in a shift towards higher phosphorylation of target proteins, such as tumor suppressor proteins. This binding is inhibitory, highly specific and irreversible, resulting in liver disease as well as nephro- and neuro-toxicity (Feurstein *et al.*, 2009). In liver cells, intermediate filaments of the cytoskeleton are hyperphosphorylated, leading to cellular disruption (Carmichael, 1992; Chorus and Mur, 1999). Microcystins are synthesized non-ribosomally by the thiotemplate function of a large multifunctional enzyme complex containing both non-ribosomal peptide enzymes (NRPS) and polyketide synthase (PKS) domains. The *mcy* gene cluster encodes for these biosynthetic enzymes (Pearson *et al.*, 2010). In *Microcystis*, the *mcy* gene cluster (Figure 1.4) spans 55 kb and comprises 10 genes arranged in two divergently transcribed operons, *mcyA-C* and *mcyD-J* (Pearson *et al.*, 2010; Tillett *et al.*, 2000).

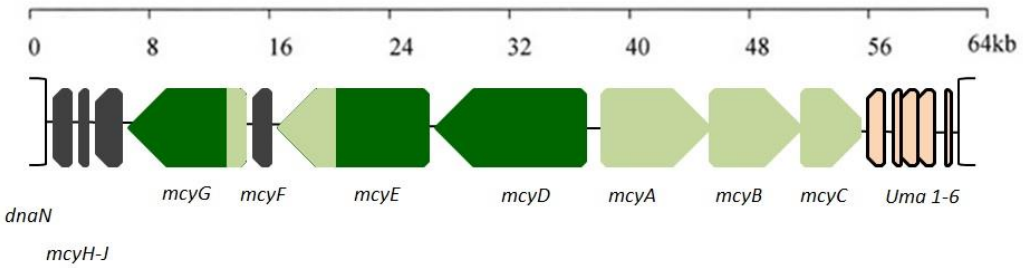

Figure 1.4: Gene cluster (mcy) responsible for microcystins synthesis (modified from Tillet *et al*., 2000).

Numerous cases of hepatotoxic intoxication involving animals have been reported, with *Microcystis* and microcystins, respectively, the most common genera and cyanotoxins involved (Chorus and Mur, 1999; Codd *et al.*, 2005b). The most dramatic case involving microcystins was the death of over 65 hospital patients in 1996 due to contamination of water used in a hemodialysis clinic in Brazil (Carmichael *et al.*, 2001b).

In New Zealand, microcystins have been identified in over 60 water bodies and *Microcystis* is the most common genus responsible for microcystin production (Hamill, 2001; Wood *et al.*, 2006). In a survey of cyanotoxins in New Zealand between 2001 and 2004, Wood *et al*. (2006) found microcystins in 75 samples from 27 lakes, and among other genera *Microcystis* was associated with over 80% of the microcystin production. Ingestion of microcystins from *Microcystis* blooms has caused multiple cattle deaths in New Zealand (Wood pers. comm.).

1.1.9 Regulation and ecological functions of microcystins

Despite many decades of research, the ecological function of microcystins is still unclear. Some of the more commonly proposed hypotheses include; involvement in photosynthesis, an intra-cellular function or signalling functions in a quorum sensing-like manner, protection against grazing pressure, and iron scavenging

(Kaebernick and Neilan, 2001; Schatz *et al*., 2007; Wood *et al*., 2011, Gan *et al*., 2011; Neilan *et al*., 2013; Yang *et al*., 2013). Current literature on each of these is reviewed below.

Photosynthesis: Kaebernick *et al*. (2000) observed changes in *mcy* operon transcription (non-quantitatively) with varying light intensity, and proposed the microcystin synthetase gene cluster is regulated by light quality, either directly or via another regulatory factor, and that transcription requires different thresholds of light intensity for initiation and up-regulation. Jahnichen *et al*. (2007) studied the impact of inorganic carbon availability on microcystin production and suggested that microcystins may be involved in enhancing efficiency of the adaptation of the photosynthetic apparatus to fluctuating inorganic carbon conditions. Neilan *et al*. (2013) also proposed a link between toxin production and photosynthesis, suggesting that the regulation of toxin-producing genes and toxin production changes with light appears to be universal among cyanobacteria. Young *et al*. (2008) reported that microcystins were specifically localized in the nucleoplasmic region of the cells and were also associated with major inclusion bodies, with a preferential association with the thylakoids, region where photosynthesis take place, suggesting a role for microcystin in photosynthesis and/or photoregulatory processes.

Potential role as a signalling molecule and relationship with cell density: Dittmann *et al*. (2001) identified a microcystin related protein (*mrpA*) that shares similarities with proteins thought to play a role in modulation and that are upregulated via a quorum sensing mechanism in *Rhizobium*. This protein was only present in a wild-type culture and not the inactivated mutant. Kehr *et al*. (2006) provided further evidence for this process by demonstrating interactions

between microcystin and a lectin (MVN) isolated from *Microcystis.* MVN is believed to be involved in the aggregation of single *Microcystis* cells into colonies. The identification of an 84 bp region of homology between the *mcyA/D* promoter and the upstream region of the MVN lectin protein, indicates that microcystins may be accepted as a signalling compound after which the expression of both MVN and its binding partners are up-regulated. Schatz *et al*. (2007) showed that the release of microcystin from lysed cells into the extracellular environment induced a significant up-regulation of *mcyB* and resulted in an accrual of microcystins in remaining *Microcystis* cells.

Wood *et al.* (2011) studied microcystin production in Lake Kaikoura, South Island, New Zealand. During a period of increased microcystin synthesis *Microcystis* cell density increased ca.30 fold. Wood *et al.* (2012) then used mesocosm experiments in which cell density was manipulated. They reported that after a certain cell density threshold there was a substantial increase in intracellular microcystin production (ca. 20-fold), over a period of 240 min. They suggested that the *Microcystis* cell abundance and/or mutually correlated environmental parameters (e.g., pH, DO) were responsible for the enhanced microcystin synthesis. Pereira and Giani (2014) investigated whether cell density influenced the production of bioactive compounds produced by four cyanobacterial strains under laboratory conditions. The strains produced 17 different peptides and 14 were identified, including microcystins, aeruginosins, cyanopeptolins and microviridins. The results suggested that cell density had a significant effect on peptide production as cellular quotas of the compounds were greater in the higher-density treatments. The authors also suggested that the results may indicate a role of a quorum sensing function for microcystins.

An intra-cellular function for microcystins: Zilliges *et al*. (2011) recently presented data to support an intracellular protein-modulation function for microcystins. They observed that responses were most pronounced in *Microcystis* sp. during acclimation to high light and oxidative stress conditions.

Colony formation: Gan *et al*. (2012) found that high concentrations of microcystins in the environment significantly enhanced *Microcystis* spp. colony size, and that the degradation of the extra-cellular microcystins in the environment dramatically decreased colony size. They also observed that microcystin induced the production of extra-cellular polysaccharides, which contributed to cell colony formation in the culture medium and up-regulated genes related to its synthesis, however, microcystin did not influence *Microcystis* growth rate.

Nitrogen: Orr and Jones (1998) studied the relationship between microcystin production and cell division rates in nitrogen-limited *Microcystis aeruginosa* cultures and observed that microcystin production decreased at an identical rate to that of the reduction in cell division when the culture became nitrogen limited, suggesting that microcystin production is controlled by environmental effects related to the rate of cell division. Recently, Puddick (2013) investigated the effect of nitrogen concentration on microcystin congeners and reported a correlation of changes of microcystin congener abundance with nitrogen supply, showing that a decrease in the abundance of the arginine-containing microcystin congeners coincided with a decrease of nitrate concentration. Further evidences to support this hypothesis comes from Beversdorf *et al*. (2015) who demonstrated that nitrogen speciation and inorganic carbon availability might be important drivers of *Microcystis* population dynamics and that an imbalance in cellular carbon: nitrogen ratios may trigger microcystin production. Also, recent studies have

begun to interrogate the regulatory regions of the *mcy* operon, which contains known transcriptional start sites (Kaebernick *et al*., 2002), including a binding site for the global nitrogen regulator, *NtcA* (Ginn *et al*., 2010). Ginn *et al*. (2010) reported transcriptional activation of *mcy*B and *ntcA* by *M. aeruginosa* under N stressed conditions, and Sevilla *et al*. (2010) showed that an excess of nitrogen, in the form of nitrate, increased growth but did not influence *mcy* transcription or microcystin production.

Protection against grazing: Several studies have suggested that microcystins may prevent grazing. For example, DeMott *et al*. (1991) demonstrated that the microcystins produced by *Microcystis* inhibit the feeding rate of the crustacean zooplankton *Daphnia pulicaria*, suggesting that they evolved as a chemical defence against grazers. Jang *et al*. (2003, 2004, 2008) showed that the production of microcystin by *Microcystis* increased in response to direct cues from exposure to micro-crustaceans and phytoplanktivorous fish or indirect chemical cues from feeding. However, since the *mcyE* gene cluster appears to have been present in ancestral cyanobacteria species that existed prior to the mesoproterozoic period (Rantala *et al*., 2004; Kurmayer and Christiansen, 2009), it seems unlikely that this is the main role of microcystins.

Iron scavenging compounds: Multiple studies have suggested a role of microcystins as an iron-scavenging molecule. Utkilen and Gjolme (1995) and Lyck *et al*. (1996) showed that toxic *Microcystis* strains possess more efficient Fe uptake systems compared with non-toxic cyanobacteria strains. Utkilen and Gjolme (1995) demonstrated that microcystin is an intracellular chelator which inactivates free cellular Fe^{2+} and that microcystin is produced by an enzyme (synthetase) whose activity is controlled by the amount of free Fe^{2+} present.

Sevilla *et al*. (2008) used RT-QPCR to explore the effect of iron on *mcyD* expression and for the first time established a correlation between increased transcription of a *mcy* gene and microcystins synthesis. They observed that in iron-depleted cells the *mcyD* gene was upregulated approximately 2.5-fold (compared to iron-replete cells and only during the late exponential phase).

1.2 Study objectives

1.2.1 General objective

The overarching objective of this study was to elucidate how environmental variables influence cyanobacteria species succession and specifically *M. aeruginosa* bloom formation in a small, eutrophic lake, including quantifying the seasonal variability of microcystin production and benthic recruitment of *M. aeruginosa*.

1.2.2 Specific questions

Chapter 2 addressed three main questions:

(i) How are physical and chemical processes within a shallow eutrophic lake affected by contrasting temperature and precipitation regimes over consecutive summers?

(ii) Do physical and chemical differences affect cyanobacterial biomass, composition and succession, and which parameter/s are the key drivers of any observed changes?

(iii) How does the relative abundance of toxic/non-toxic *M. aeruginosa* genotypes and toxin quota vary between the two summers and what were the possible drivers of these differences?

Chapter 3 addressed three questions related to benthic recruitment of *M. aeruginosa*:

(i) How does benthic recruitment vary between near-shore and mid-lake sites?

(ii) Do variations in ammonium concentration, light or temperature trigger recruitment of benthic *M. aeruginosa*?

(iii) Do ultrastructural changes occur within *M. aeruginosa* cells during the recruitment period?

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Chapter 2

Contrasting cyanobacterial communities and microcystin concentrations from extreme weather events: insights into potential effects of climate change

2.1 Introduction

The occurrence of cyanobacterial blooms appears to be increasing in lakes across the globe (Garcia-Pichel *et al.*, 2003; Harke *et al.*, in press; Paerl and Otten, 2013). Cyanobacterial blooms can have negative environmental impacts including reductions in water clarity and dissolved oxygen, which may suppress macrophyte growth and affect invertebrates and fish (Paerl, 2014; Smith, 2003). Many bloom-forming species of cyanobacteria also produce natural toxins (cyanotoxins) which pose chronic and acute health risks to humans and animals (Chorus, 2001; Codd *et al.*, 2005). Blooms and their associated toxins also have economic and social consequences such as loss of tourism opportunities and increased costs associated with upgrades to water treatment plants (Hamilton *et al.*, 2014; Kouzminov *et al.*, 2007; Steffensen, 2008).

Increases in anthropogenic eutrophication have historically been considered to be the main drivers of cyanobacterial proliferations, with the relative importance of both nitrogen (N) and phosphorus (P) inputs debated for many decades (e.g. (Ahn *et al.*, 2011; Conley *et al.*, 2009; Lewis and Wurtsbaugh, 2008; Schindler *et al.*, 2008; Smith, 1983). In recent years the impacts of global climate change on the

distribution and intensity of cyanobacterial blooms has attracted considerable scientific and media attention (Brookes and Carey, 2011; Carey *et al.*, 2012; Kosten *et al.*, 2012; Paerl and Huisman, 2008; Paerl and Huisman, 2009; Paerl and Paul, 2012). Most climate change models predict that aquatic systems will experience increases in temperature, thermal stratification and water column stability, all factors associated with promoting cyanobacterial blooms (Jöhnk *et al.*, 2008, Kosten *et al.*, 2012, Trolle *et al.*, 2011, Wagner and Adrian, 2009). Less attention has been given to how other variables linked to climate change, such as variations in precipitation, extended droughts or increased carbon dioxide levels, might affect cyanobacterial biomass (O'Neil *et al.*, 2012, Reichwaldt and Ghadouani, 2012). For example, prolonged and intense precipitation events can mobilize nutrients and sediments on land and increase nutrient enrichment of receiving waterbodies (Paerl *et al.*, 2006).

Understanding the effects of climate change on cyanobacterial blooms is complicated by successional patterns in cyanobacterial communities. For example, Fernández *et al.* (2015) tracked the succession of *Microcystis* spp. and *Dolichospermum* (basionym *Anabaena*) *circinalis* during a bloom, and identified phosphorus, temperature and solar radiation as key factors in regulating shifts in their relative composition. Despite this and other examples (Cai and Kong, 2013; Ni *et al.*, 2012; Rajaniemi-Wacklin *et al.*, 2008), most research to date has focused on monospecific blooms and cyanobacterial succession remains understudied and poorly understood (Fernández *et al.*, 2015). A further layer of complexity is that among cyanobacterial species known to produce cyanotoxins, both toxic and non-toxic genotypes can exist. Blooms are usually comprised of both and these can currently only be distinguished in the natural environment via molecular techniques (Kurmayer and Kutzenberger, 2003). Factors that promote

one genotype to dominate over another are also largely unknown (Rinta-Kanto *et al.*, 2009; Van De Waal *et al.*, 2011) and predicting how these might shift with climate change is extremely challenging (Wood *et al.*, 2015). Furthermore, the amount of toxin produced by toxic strains is variable (Vezie et al., 1998), and factors such as temperature (Dziallas and Grossart, 2011; Kleinteich *et al.*, 2012) and nutrients (Harke and Gobler, 2013; Horst *et al.*, 2014) may be at least be partly involved in regulating production and therefore also influenced by climate change. Assessing within genotype variability was beyond the scope of this study but could be explored using high-throughput sequencing of variable genes, or via isolation and characterisation of strains from samples.

To date, most research investigating how variables linked with climate change might affect cyanobacterial biomass, species succession, genotype abundance and toxin production has been based on models, or culture-based studies and there is uncertainty around the applicability of these data to real-world situations (Wood *et al.*, 2015). Natural population surveys undertaken over consecutive years with contrasting and extreme climatic conditions may provide valuable insights to address this uncertainty. Lake Rotorua (South Island, New Zealand; Fig. 1) is a shallow eutrophic lake which experiences annual cyanobacterial blooms between spring and autumn (Wood *et al.*, 2012a; 2011). During the summer of 2014, the North Canterbury region of New Zealand (where Lake Rotorua is located) experienced a series of rainfall events including intense precipitation from tropical cyclones Lusi (March 2014) and Ita (April 2014) as they transitioned into sub-tropical weather systems over New Zealand. In contrast, this region experienced a severe drought in the summer of 2015, with the second lowest total annual rainfall measured since records began 75 years before present. These contrasting weather patterns provided a unique opportunity to explore how differences in climate

affect physical and chemical parameters in a shallow eutrophic lake, and the subsequent influence on cyanobacterial biomass, community composition and toxin concentrations.

The aim of this study was to use a dataset collected at weekly or fortnightly frequency from Lake Rotorua between January 2014 to May 2015 to address the following three questions: (i) How are physical and chemical conditions within a shallow eutrophic lake affected by contrasting temperature and precipitation regimes over consecutive summers? (ii) Do physical and chemical changes affect cyanobacterial biomass, composition and succession, and which parameter(s) are the key drivers of any observed changes? We focused specifically on *Microcystis*, as this is the only known toxin-producing cyanobacteria genus in the lake (Wood *et al.*, 2012a; 2011). Our final question was: (iii) Did the relative abundance of toxic and non-toxic *M. aeruginosa* genotypes, and toxin quota vary between the two summers and what were the possible drivers of these differences?

2.2 Material and Methods

2.2.1 Study site and sample collection

Lake Rotorua (South Island, New Zealand 42°24′05S, 173°34′57E) is a small (0.55 km^2), shallow (max. depth 3 m), eutrophic lake in the northeast of the South Island of New Zealand (Flint, 1975). Its catchment comprises mixed land use of low-intensity grazing, regenerating native scrub and bushland, and exotic weeds and trees. Inflow is largely from surface run-off during rainfall events. During high rainfall there is discharge from a small outflow at the southern end of the lake (Fig. 2.1).

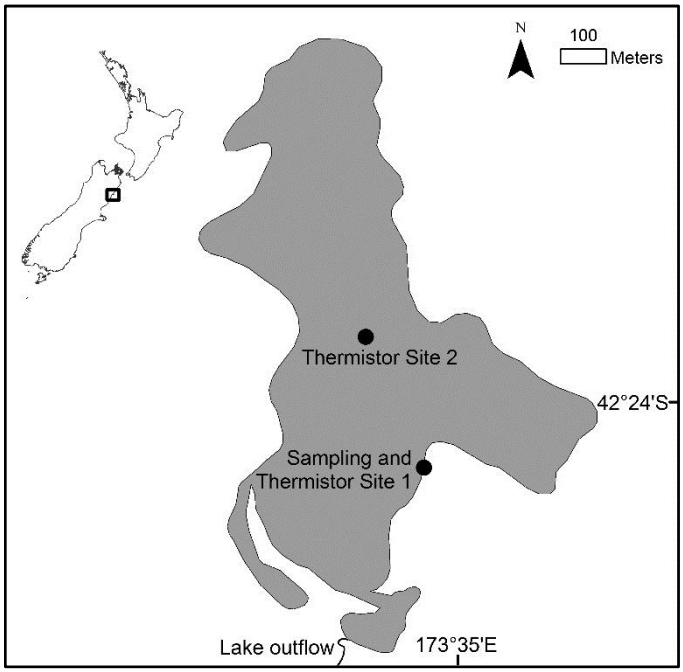

Figure 2.1: Map of Lake Rotorua (South Island, New Zealand) showing the sampling site and thermistor chain sites 1 (January to April 2014) and 2 (May 2014 to May 2015). Inset: map of New Zealand showing the location of the lake.

Temperature was measured at 5 or 15 min intervals using temperature loggers (HOBO®, Onset USA). Initially this was undertaken from the end of a floating pontoon (January to April 2014; Fig. 2.1) with loggers at the lake surface (0 m), and 0.3, 0.45, 0.9 and 1.5 m below the water surface. Thereafter the chain was positioned at a central point in the lake (Fig. 2.1) with loggers at the lake surface, and 0.8, 1.0, 1.4, 1.7, 2.0 and 2.6 m below the surface. To enable the data loggers to be maintained at a constant distance from the surface regardless of fluctuations in lake level, the thermistor chain was attached to a buoy floating on the surface of the lake. Daily mean rainfall data at the Kaikoura weather station (situated approximately 8 km from the study site) were obtained from the Cliflow database of the National Institute of Water and Atmospheric Research, New Zealand. The topography between the lake and the weather station is variable and coastal winds at the location of the meteorological station may be variously attenuated and altered by this topographic effect.

Surface water samples (400 mL) were collected weekly or fortnightly from the end of a floating pontoon of 15 m length on the eastern side of the lake (Fig. 2.1) between January 2014 and May 2015. Samples were stored chilled and in the dark, and were received at the laboratory within 24 h. Samples were subsequently well mixed and six sub-samples were taken: (1) 45 mL of pre-filtered lake water (20-µm netting) was syringe-filtered (Whatman GF/C) and the filtrate was frozen (-20 °C) for dissolved nutrients analysis; (2) 45 mL of lake water was frozen (-20 °C) for total nutrients analysis; (3) 15 mL of lake water was preserved using Lugol's Iodine for microscopic analysis; (4) 15-60 mL was filtered on to Whatman GF/C filter which were stored frozen (-20 °C) for DNA extraction; (5) 5 mL of pre-filtered lake water (20-µm net filter) was syringe-filtered (Whatman GF/C) and the filtrate was frozen (-20 °C) for extracellular microcystin analysis; (6) 5 mL of lake water was frozen (-20 °C) for total microcystin analysis.

2.2.2 Laboratory analysis of weekly/fortnightly samples

Total and filtered nutrients were analyzed on a Lachat QuickChem® Flow Injection Analyser (FIA+ 8000 Series, Zellweger Analytics, Inc.) using the methods provided in Apha (2005). The nutrient species were analyzed as ammonium (NH_4-N), nitrite (NO_2-N), nitrate (NO_3-N), total nitrogen (TN), dissolved reactive phosphorus (DRP) and total phosphorus (TP). The limits of detection were 0.002 mg L^{-1} for NH_4-N, 0.001 mg L^{-1} NO_2-N, and NO_3-N, and 0.004 mg L^{-1} for TN, DRP, and TP.

Cyanobacterial identification and enumeration was undertaken using an inverted microscope (IX70, Olympus). Samples were mechanically ground (Tissue Grinder, Wheaton, USA) for ca. 30 s to break up *M. aeruginosa* colonies and filamentous species, to assist in accurate enumeration. Subsamples (0.5-1 mL)

were settled in Utermöhl chambers (Utermöhl, 1958), and cyanobacteria cells and heterocytes were enumerated by scanning 1 to 2 transects at 400-600× magnification. Species identification was undertaken with reference to Baker *et al.* (2002), McGregor and Fabbro (2001), Wood *et al.* (2005) and Komárek and Anagnostidis (1999). Biovolumes were determined for each species by using median cell dimensions (n=50) measured at 1000× magnification under oil immersion (Olympus BX51) and volumetric equations of geometric shapes closest to each cell shape (Sun and Liu, 2003).

Microcystis sp. culture CAWBGX (RotoD) isolated from Lake Rotorua (Rogers, 2014), was grown in a glass flask (500 mL) in MLA medium (Bolch and Blackburn, 1996a) under a light regime of 90 µmol s^{-1} m^{-2} with a 12 h:12 h light/dark cycle, at a temperature of 18°C ± 1°C. When the culture was in the exponential growth phase, a subsample (60 mL) was filtered (Whatman GF/C) and the filter stored frozen (-20 °C) for DNA extraction and used to prepare a standard curve for the quantitative polymerase chain reaction (QPCR) assay used to assess toxic genotype concentrations. A second subsample (10 mL) was preserved using Lugol's Iodine and the cell concentration was determined as described above.

DNA was extracted from GF/C filters using a Power Soil DNA Isolation Kit (MO BIO, USA) according to the protocol supplied by the manufacturer. All DNA samples from the Lake Rotorua samples were screened in duplicate for inhibition using an internal control assay. In this assay, salmon sperm DNA is spiked into the samples at a known concertation and differences in the cycle threshold between a control and samples are assessed. Each 12.5 µL reaction contained 6.25 µL KAPA Probe Fast QPCR Kit Master Mix (2×), 1 µL of primers targeting

the internal transcribed spacer region 2 of the rRNA gene operon of *Oncorhynchus keta* salmon sperm (0.4 µM, Sketa F2 and Sketa R3, IDT, USA; Haugland *et al.*, 2005), 1 µL TaqMan probe synthesised with a FAM reporter dye at the 5´-end and a Black Hole Quencher 2 at the 3´-end (0.2 µM; Sketa P2, IDT, United States (Haugland *et al.*, 2005)), 1 µL extracted salmon sperm DNA (15 ng; Sigma, United States) and 1 µL of template DNA. The cycling profile was 95°C for 3 min, followed by 50 cycles at 95°C for 3 s and 58°C for 10 s. When inhibition (defined as a shift in cycle threshold) was observed samples were diluted (10-fold) and re-analysed.

Quantitative PCR, used to enumerate the copy numbers of *mcyE* genes, was undertaken in triplicate for each sample in a 12.5 µL of reaction mix containing 6.25 µL KAPA Probe Fast qPCR Kit Master Mix (2×), 1 µL of primers targeting a region within the *mcyE* open reading frame of the microcystin synthase gene (0.4 µM, mcyE-F2 and MicmcyE-R8; (Vaitomaa *et al.*, 2003), 0.2 µL of McyE probe (Rueckert and Cary, 2009) and 1 µL of template DNA per sample. DNA from CAWBGX(rotoD) was used to generate five-point standard curves ranging from 10.8×10^{6} to 10.8×10^{2} cells mL^{-1}. Each point of the standard curve was analysed in triplicate for each QPCR run conducted. The standard curve generated was linear (R^2>0.99) and PCR efficiency was >0.8.

Subsamples collected for extracellular microcystin were centrifuged (10,000 × *g*, 5 min) and the supernatant was placed in a liquid chromatography (LC) vial. Subsamples for total microcystin analysis were freeze-thawed and sonicated (30 min, 60 kHz) four times after adding formic acid (final concentration 0.1% v/v). The extract was clarified by centrifugation (10,000 × *g*, 5 min) and the supernatant was placed in a LC vial. Previous study has shown that this extraction

procedure has high efficiency (Rogers *et al.*, 2015) Microcystin samples were analysed directly or diluted (1/10 to 1/100 with 50% methanol containing 0.1% formic acid) by LC-tandem mass spectrometry (LC-MS/MS) as described in Puddick *et al.* (2016). Compounds were separated on an Acquity I-Class ultra-performance liquid chromatography system (Waters Co.) using a C_{18} column (Waters Acquity BEH-C_{18}, 1.7-µm, 50×2.1 mm) maintained at 40°C in a column oven. The microcystin/nodularin congeners assessed were nodularin-R, MC-RR, didesmethyl MC-RR, desmethyl MC-RR, MC-YR, MC-LR, didesmethyl MC-LR, desmethyl MC-LR, MC-AR, MC-FR, MC-WR, MC-RA, MC-RAba, MC-LA, MC-FA, MC-WA, MC-LAba, MC-FAba, MC-WAba, MC-LY, MC-LW and MC-LF. Multiple reaction monitoring channels assessing the *m/z* 135 fragment ion from the protonated molecular cations were used to quantify each toxin ($[M+2H]^{2+}$ for MC-RR and variants; $[M+H]^{+}$ for the others).

Primary standards of the microcystin congeners -RR, -YR, -LR were purchased from DHI Lab Products (Denmark) and a quality control sample containing a broad range of microcystin congeners (Puddick *et al*., 2014) was analysed during each sample run to confirm the retention time and signal response of each microcystin being assessed. The amount of microcystin per toxic cell (microcystin quota) was calculated by summing the concentration of all congeners in the samples, subtracting the extracellular microcystin values from the total microcystin values and dividing this value by the *mcy*E copies mL^{-1} (as determined using QPCR).

2.2.3 Data and statistical analysis

Thermocline depth (z_t) was quantified as:

$$z_t = \max\left(\frac{\Delta T}{\Delta z}\right) \quad (1)$$

(Hoare and Spigel, 1987), where *T* is the temperature of water and *z* is water depth. An a priori cut-off of ≥ 5°C m^{-1} was used to delineate the presence of a thermocline. Profiles for temperature and thermocline depth were plotted using the software R (R Development Core Team, 2014, Zuur *et al.*, 2013).

Differences in environmental variables between the summers of 2014 and 2105 were evaluated for the period of January to May in each year. These were tested using a one-way ANOVA analyses with year as a fixed factor. Response variables were log transformed if necessary, to fulfil the assumptions of normality and heterogeneity of variance. This was tested by inspecting the q-q plots of the residuals of the ANOVA and using a Shapiro-Wilk test.

The relationship between cyanobacterial community composition (measured as biovolumes) and environmental variables was analysed using multivariate multiple regression (Mcardle and Anderson, 2001), using the DistLM routine with PRIMER 6 and PERMANOVA (Anderson *et al.*, 2008). A marginal test was used where individual variables were fitted separately to test their relationship with the cyanobacterial data (ignoring other variables), followed by a stepwise selection procedure, conditional on variables already included in the model and using the Akaike Information Criterion (AIC) with a correction for finite sample sizes (AICc) selection criteria. The conditional test identifies the subset of variables that best predicts the observed pattern in cyanobacterial community composition. Both the conditional and marginal tests were undertaken with 4,999 permutations using Bray-Curtis similarities of the log+1 transformed cyanobacterial biovolume data. Draftsman plots were used to check multi-skewness among environmental variables and all were log+1 transformed prior to the analysis and included: NO_2-

N, NO_3-N, NH_4-N, DRP, DIN:DRP, TN, TP, TN:TP, average weekly surface water temperature and total weekly rain.

The relationship between *M. aeruginosa* biovolume, percentage of toxic *M. aeruginosa* cells, microcystin quota and selected environmental variables was modelled using generalized linear models within the software R (R Development Core Team, 2014, Zuur *et al.*, 2013). *Microcystis aeruginosa* biovolume and microcystin quotas were modelled using gamma errors and a log link, whereas the percentage of toxic *M. aeruginosa* cells was modelled using beta error and a logit link (Pinheiro and Bates, 2000, Zuur *et al.*, 2013).

Initial data exploration was conducted to identify outliers, homogeneity of variances, normality, excess of zeros, collinearity and the relationship between predictors and response variables following the protocol described in Zuur *et al.* (2010). Covariates were transformed if necessary to avoid right-skewness and achieve unimodal distributions. Collinearity among predictor variables was initially checked by calculating the variance inflation factor (VIF) for each covariate and sequentially dropping the covariate with the highest VIF until all were <5 (Zuur *et al.*, 2010). To avoid over-parametrization of the models only TN:TP, log(DIN:DRP) and surface water temperature were included. Additionally for the analysis of the percentage of toxic *M. aeruginosa* cells and microcystin quota, *M. aeruginosa* biovolume was included as a covariate in the initial models. Models were selected using a stepwise process based on generalised AIC and validated by inspecting the normalised quantile residuals.

2.3 Results

Surface water temperature in Lake Rotorua ranged from 5.7°C (24 July 2014) to 33°C (4 February 2014), and 5.7°C (24 July 2014) to 25.9°C (22 February 2014)

at 2.6 m depth (Fig. 2.2). When the thermistor chain was positioned close to the shore (January to April 2014) diurnal stratification was apparent with the presence of a shallow surface thermocline (0.1-0.5 m; Fig. 2.2). From May 2014 to June 2015, when the thermistor chain was positioned mid-lake, a relatively persistent thermocline at was present at ca. 1-1.2 m from October to December 2014, and April to May 2015 (Fig. 2.2), and at ca. 1.8-2 m in summer (January to May).

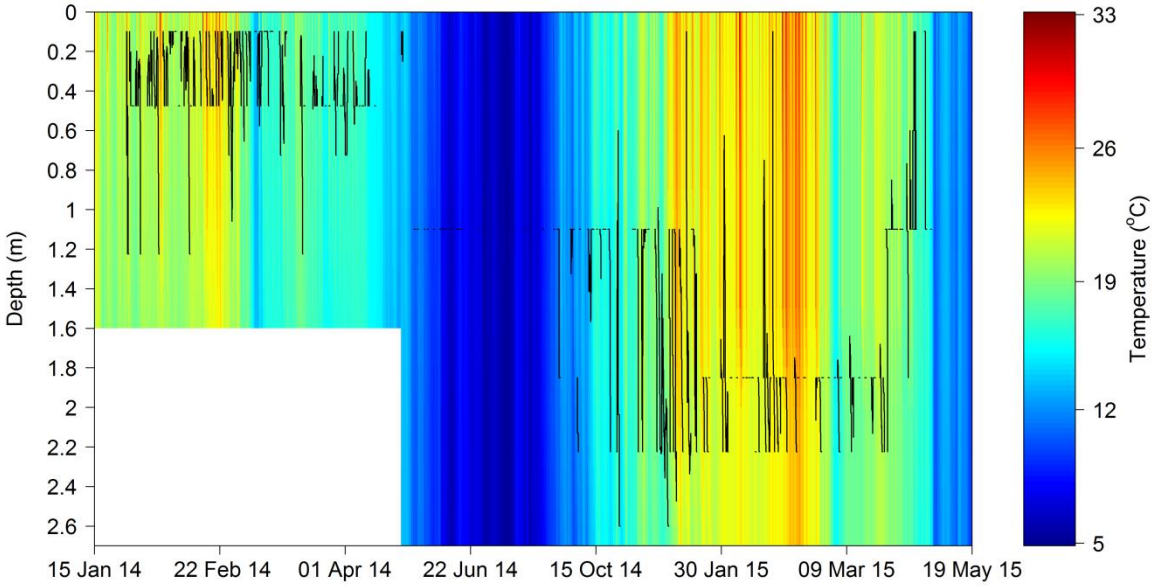

Figure 2.2: Temperature contour plot from Lake Rotorua (Kaikoura, New Zealand) over the 17-month study period (January 2014 to May 2015). From January to April 2014 the thermistor chain was positioned off the end of the floating pontoon, thereafter it was positioned at a central point in the lake (See Fig. 2.1 for locations). The white section on the plot is due to the shallower depth of the initial deployment at the pontoon. The black line shows the calculated position of the thermocline.

Between January and May 2014 total rainfall was 540 mm compared to 82 mm during the same period in 2015. During this period in 2014 there were five large rainfall events (>20 mm per day).The two largest were associated with tropical cyclones Lusi (15 March, 79 mm) and Ita (17 April, 138 mm; Fig. 2.3).

From January 2014 to May 2015, NH_4-N concentrations in the surface water were mostly low (<0.09 mg L^{-1}; Fig. 2.4A). Several exceptions occurred during the warmer water periods on 26 February 2014 (0.23 mg L^{-1}), 30 April 2014 (0.11 mg L^{-1}), 6 November 2014 (0.4 mg L^{-1}), 3 March 2015 (0.2 mg L^{-1}) and 1 May 2015 (0.2 mg L^{-1}). Also, during the winter period of July to August 2014, NH_4-N concentrations were generally above 0.2 mg L^{-1} (Fig. 2.4A). Nitrite and nitrate concentrations were low (<0.05 mg L^{-1}) during both summer periods but increased markedly from March to September 2014 (0.1 to 0.7 mg L^{-1}). Surface water DRP concentrations were low (average 0.005 mg L^{-1}) from January 2014 to November 2014, with the highest concentrations (0.02 mg L^{-1}) on 11 March 2014 and 6 November 2014 (Fig. 2.4B). Concentrations of DRP were higher from December 2014 to May 2015 (average 0.02 mg L^{-1}), with three notable peaks on 29 December 2014 (0.05 mg L^{-1}), 20 January 2014 (0.04 mg L^{-1}) and 26 March 2015 (0.05 mg L^{-1}).

Total phosphorus and TN were higher during the summer months (January to May) of both years (Fig. 2.4A and 2.4B). Over the period from January to May 2014, the DIN:DRP ratio was highly variable (2.05 to 108, average of 33) and in 2015 it was consistently low (1.3 to 24, average of 6) (Fig. 2.4C). The TN:TP ratio was <5 from January 2014 to April 2015, and increased to >15 from May to July 2014. From November 2014 to the end of the study, TN:TP was relatively constant ca. 10-15 (Fig. 2.4C).

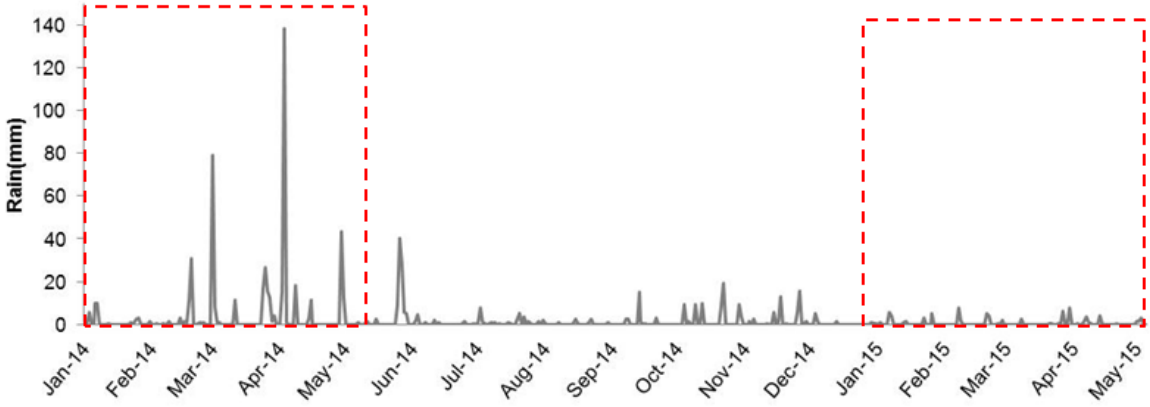

Figure 2.3: Total daily rainfall at the Kaikoura weather station between January 2014 and May 2015. The red boxes show the two time periods used for comparative purposes in this study.

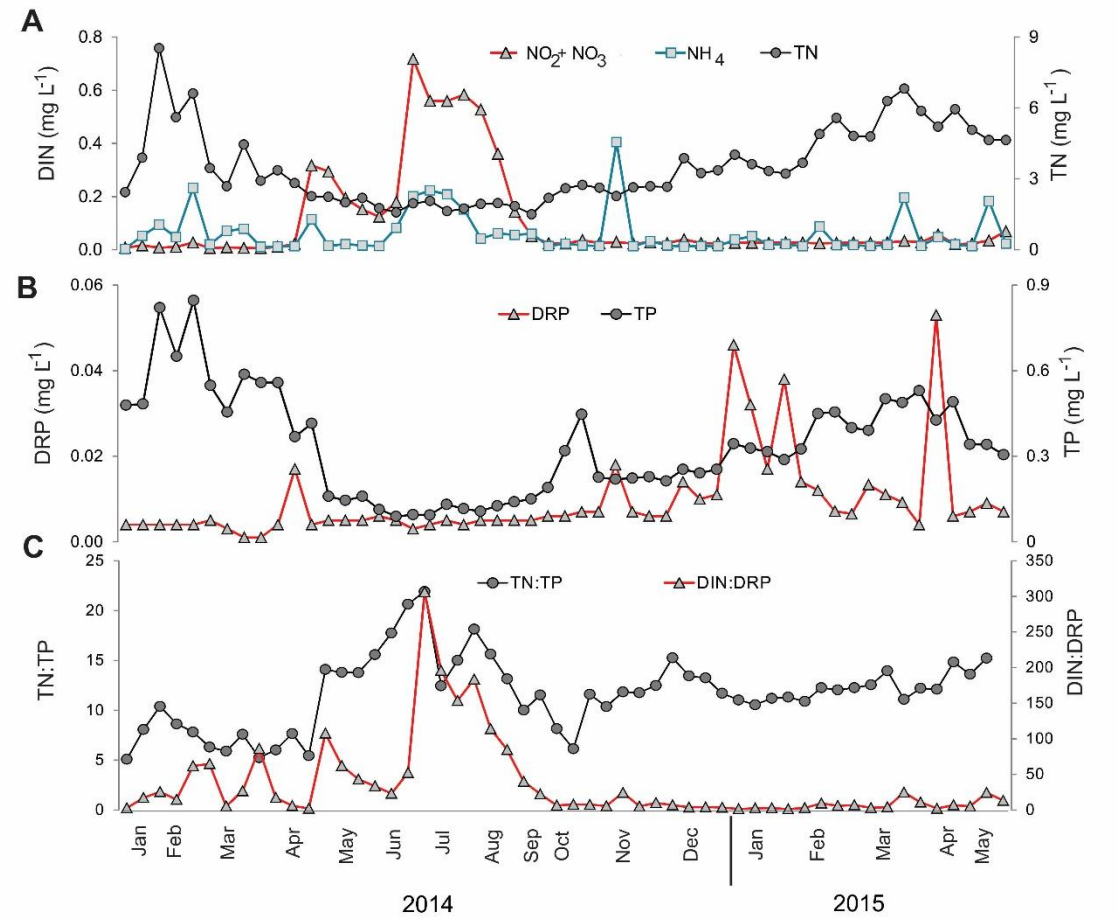

Figure 2.4: Nutrient concentrations for Lake Rotorua (Kaikoura, New Zealand) over the 17-month study period of January 2014 to May 2015; A) ammonium (NH_4-N), nitrate+nitrite (NO_2+NO_3-N) and total nitrogen (TN) concentrations, B) dissolved reactive phosphorus (DRP) and total phosphorus (TP) concentrations, and C) TN:TP and dissolved inorganic nitrogen (DIN):DRP ratios.

The ANOVA analysis identified significant differences in summer periods of 2014 and 2015 (assessing only the months of January to May for each year) for a number of physical and chemical parameters (Fig. 2.5). Higher surface water temperatures ($p<0.01$); and lower rainfall ($p < 0.05$) were observed in 2015 compared to 2014 (Fig. 2.5). Comparing these two periods, there was an increase in the mean TN and DRP concentrations ($p < 0.01$) and TN:TP ratio ($p < 0.001$), and a significant decrease in the DIN:DRP ratio ($p < 0.01$).

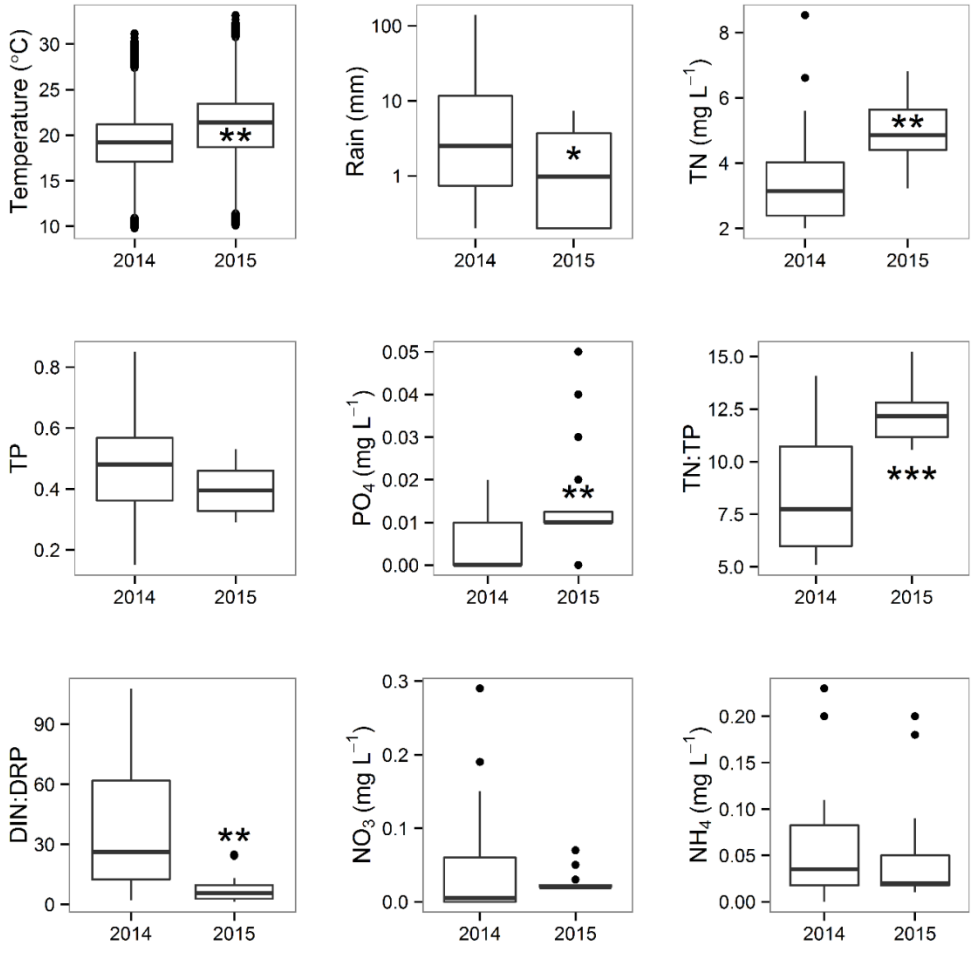

Figure 2.5: Boxplot of environmental variables between January and May for 2014 and 2015. Solid black line shows median, box shows 1st and 3rd quartiles, whiskers extend to the last data point within 1.5 times the inter-quartile range where those data exist. Black dots are outliers beyond this range. Note the log scale for rainfall. Significant difference between years is indicated: *p < 0.05, **p < 0.01 and ***p < 0.001. TN = total nitrogen, TP = total phosphorus, DRP = dissolved reactive phosphorus, DIN = dissolved inorganic nitrogen, NO_3= nitrate-N and NH_4 = ammonium-N.

During the 17-month study, four cyanobacterial species were detected in Lake Rotorua; *Aphanocapsa* sp., *Aphanizomenon gracile, Dolichospermum crassum* and *Microcystis aeruginosa*. Between 29 January and 22 February 2014, *Dolichospermum crassum* dominated the waterbody, reaching a maximum biovolume of 87 mm^3 L^{-1} on 5 February 2014 (Fig. 2.6A). *Aphanizomenon gracile* was present at moderate levels (<8 mm^3 L^{-1}) and *Aphanocapsa* sp. at low levels (<0.1 mm^3 L^{-1}) during this period (Fig. 2.6A). *Microcystis aeruginosa* was observed in samples from 5 February 2014 and became dominant on 5 March 2014 (Fig. 2.6A).

Over the winter to early spring period of June to October 2014, total cyanobacterial biovolumes were <1 mm^3 L^{-1} (Fig. 2.6A). From 15 October 2014 to 9 February 2015 the majority of the total cyanobacterial biovolume was *Aphanizomenon gracile* (Fig. 2.6A). From 25 February 2015, *D. crassum* accounted for the greatest biovolume of the four species for the remainder of the study period, reaching a maximum of 23 mm^3 L^{-1} on 22 April 2015. This was in contrast to the same period of the previous summer when *D. crassum* was only present at low levels (<1 mm^3 L^{-1}) (Fig. 2.6A). *Microcystis aeruginosa* was present in low concentrations (<0.8 mm^3 L^{-1}) from 23 October to 26 November 2014. It was not present again until 29 December 2014 and was then observed in all samples until the end of the study, but *M. aeruginosa* biovolumes never exceeded 3 mm^3 L^{-1} (Fig. 2.6A). Between 9 February and 26 March 2015 *Aphanocapsa* was present in high concentrations, with biovolumes of ca. 1 mm^3 L^{-1} and a maximum of 3.5 mm^3 L^{-1} (Fig. 2.6 A).

Between January and May 2014 heterocytes occurred in low abundance in *D. crassum* filaments and none was observed in *A. gracile* filaments (Fig.2.6B). In

contrast, heterocytes were common in *D. crassum* and *A. gracile* filaments between October 2014 and May 2015 (Fig. 2.6B). Interestingly, heterocyte frequency in *Dolichospermum crassum* was highest prior to the peak biomass of this species, whereas the highest heterocyte frequency in *A. gracile* occurred after its peak biomass (Fig. 2.6B).

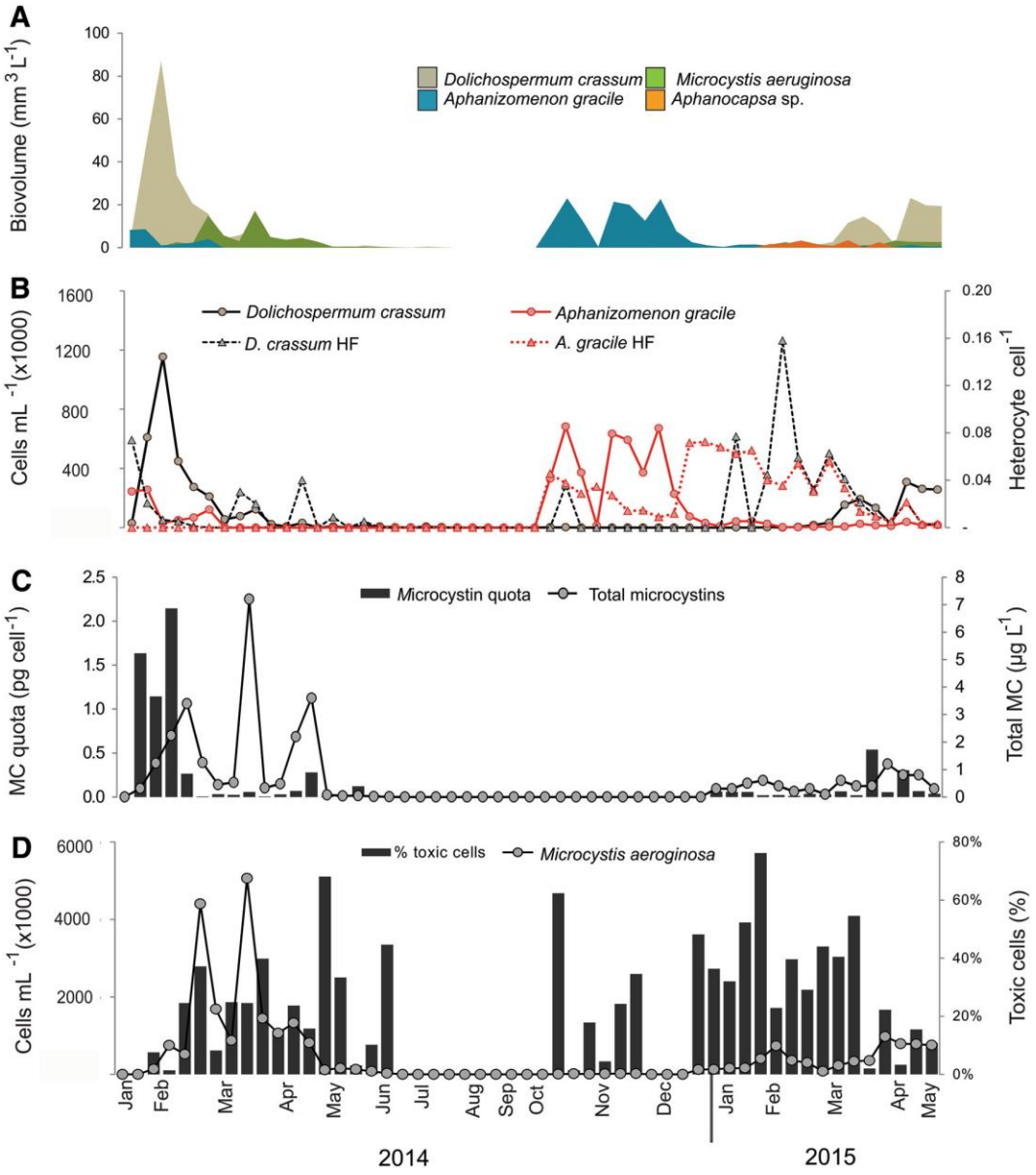

Figure 2.6: (A) Cyanobacterial biovolumes, (B) heterocyte frequency (HF), (C) microcystin (MC) quotas of toxic genotypes and total microcystin concentrations, and (D) *Microcystis aeruginosa* concentrations and percentage toxic genotypes in water samples from Lake Rotorua (Kaikoura, New Zealand) over the 17-month study period (January 2014 to May 2015).

Multivariate regression analysis identified six parameters that individually had a significant relationship with the cyanobacterial community composition; NO_3-N, TN, TP, TN:TP, DIN:DRP and surface water temperature (Table 2.1A). The sequential model showed that the four variables together explained 53.6% of the total variation of cyanobacterial community composition (Table 2.1). The univariate regression analyses indicated a significant ($p<0.001$) negative relationship between *M. aeruginosa* biovolume and TN:TP (Fig. 2.7A).

Table 2.1: Results of non-parametric multiple regression of cyanobacterial community composition on individual environmental variables for: A) each variable taken individually (ignoring other variables) and, B) step-wise selection of variables, where variation explained by each variable is added to the model conditional on variables already in the model. %Var: percentage of variance in species data explained by that variable; Cum. (%): cumulative percentage of variance explained. P values in bold are considered significant (< 0.05).

Variable	F	P	%Var	% Cum.
A)Marginal test				
Nitrite	1.3	0.28	2.4	
Nitrate	10.9	**<0.001**	17.3	
Dissolved reactive phosphorus (DRP)	1.7	0.16	3.3	
Ammonium	0.9	0.43	1.7	
Total Nitrogen (TN)	21.6	**<0.001**	29.3	
Total Phosphorus (TP)	23.2	**<0.001**	30.8	
TN:TP	5.0	**<0.01**	8.7	
Dissolved inorganic nitrogen:DRP	4.9	**<0.01**	8.6	
Surface water temperature	10.9	**<0.001**	17.4	
Rain	0.6	0.63	1.1	
B) Sequential test				
TP	23.2	**<0.001**	30.8	30.8
Temperature	13.6	**<0.001**	14.5	45.4
TN	5.0	**<0.01**	5.0	50.4
DRP	3.4	**<0.05**	3.3	53.6
Nitrite	2.3	0.08	2.1	55.8

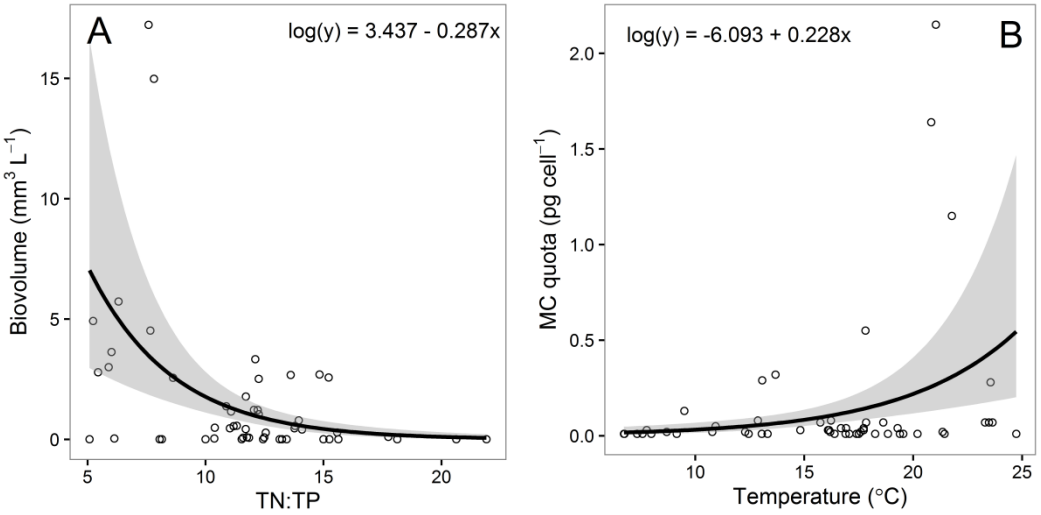

Figure 2.7: Relationship between: (A) *Microcystis aeruginosa* biovolume and ratio of total nitrogen to total phosphorus (TN:TP), and (B) microcystin (MC) quota and surface water temperature.

Microcystins were detected in 43% of the samples (Fig. 2.6C). Six microcystin variants were detected during the course of the study; -RR, desmethyl-LR, didesmethyl-LR, -FR, -WR, and -LA, although -FR, -WR and -LA were only detected on one occasion (26 April 2014; data not shown). Total microcystin concentrations were generally higher in 2014 than 2015, with the maximum concentration of 7.2 µg L^{-1} measured in the sample from 19 March 2014 (Fig 2.6C). The highest microcystin quotas were measured between 29 January 2014 and 12 February 2014 (1.14 to 2.14 pg $cell^{-1}$; Fig. 2.6C) with all other quotas <0.54 pg $cell^{-1}$. Marked temporal variability in microcystin quotas was observed, for example, there was a 280-fold decrease within a two-week period (2.14 pg $cell^{-1}$ on 12 February 2014 to 0.01 pg $cell^{-1}$ 26 February 2014; Fig. 2.6C).

Microcystis aeruginosa genotype composition was highly variable in 2014 (between 1 and 68% were toxic; Fig. 2.6D). Although no microcystins were detected, toxic genotypes were measured using QPCR between 29 October and 26

November 2015 (1 to 62%; Fig. 2.6D). During the summer of 2015, the abundance of toxic genotypes was more stable (32-76%) declining in relative abundance from the end of March 2015 to the conclusion of the study (Fig. 2.6D).

Univariate regression analysis identified a significant positive relationship ($p<0.001$) between surface water temperature and microcystin quota and indicated a significant ($P<0.001$) negative relationship between *M. aeruginosa* biovolume and TN:TP (Fig. 2.7B). There were no significant relationships between the environmental variables explored and the percentage of toxic *M. aeruginosa* cells.

2.4 Discussion

(i) Variability in physical and chemical conditions between summers

An increase in the frequency and intensity of rainfall events is one of the predicted outcomes of climate change for some regions of the globe (IPCC, 2007). However, few studies have investigated the direct effect of rainfall on cyanobacterial biomass and composition (reviewed in Reichwaldt and Ghadouani, 2012). High intensity rainfall events have been shown to cause either a decrease in biomass, or a complete collapse of the bloom due to de-stratification, increased turbidity, dilution and flushing (Jacobsen and Simonsen, 1993; Jones and Poplawski, 1998). These studies concur with the observations in Lake Rotorua, where the most extreme rainfall event (17 April 2014) led to cooling and complete mixing of the water column (Fig. 2.2) and coincided with a rapid decrease in cyanobacterial biomass (<3 mm^3 L^{-1}, which was low on subsequent sampling occasions for around six months, until spring; Fig. 2.6A). Although discharge in the lake outflow was not directly measured, the authors were at the lake on 18 April 2014 and observed an increase in lake level by >1 m from observations on the previous day and substantial discharge in the previously dry outflow. Both the

lake water outflow had become highly turbid. In contrast, over the same period in 2015 cyanobacterial biovolume remained high (> 22 mm^3 L^{-1}) until sampling ceased in June (Fig. 2.6A).

Both prolonged rainfall and intense rainfall events may promote cyanobacterial blooms through increased particulate and soluble nutrient loads entering lakes (Bormans *et al.*, 2005; Tóith and Padisák, 1986). In Lake Rotorua there was a marked spike in NO_2-N+NO_3-N concentrations in the first sampling (30 April 2014) which was conducted after the 17 April 2014 rainfall event (Figs 2.3 and 2.4A). Changes in TP were not observed, suggesting that most of the particulate material flushed into the system had settled within this two-week period. Interestingly, possible effects of run-off from less intense rainfall events earlier in the summer were not apparent in the lake nutrient analysis. For example, there was no increase in NO_2-N+NO_3-N concentrations following a significant (79 mm) rainfall event on 15 March 2014 (Figs. 2.3 and 2.4A). I speculate that the high cyanobacterial biomass in the lake at this time would have rapidly taken up incoming soluble nutrients (Takamura *et al.*, 1987), resulting in no detectable change in NO_2-N +NO_3-N in samples from 19 March 2014 (Fig. 2.6A). This rapid uptake of DIN by cyanobacteria is the most likely explanation for the lack of significant difference in DIN between the summers of 2014 and 2015 as the sampling was not frequent enough to capture these shifts. Analysis of the data from winter provides further evidence to support this. In winter when cyanobacterial biomass was low, rainfall events (e.g., 9 June 2014) usually resulted in a marked increase in water column NO_2-N+NO_3-N concentrations (Fig. 2.4A).

One of the most notable differences between the summer periods of 2014 and 2015 was the almost complete absence of heterocytes in *A. gracile* and *D. crassum* filaments in 2014, whereas the heterocyte frequency (i.e., relative to vegetative cells) was relatively high in these species throughout 2015 bloom (Fig. 2.6B). The development of heterocytes in response to low concentrations of DIN has been observed in the field (Laamanen and Kuosa, 2005; Walve and Larsson, 2007; Wood *et al.*, 2010b) and in laboratory cultures (Zapomělová *et al.*, 2008). The absence of substantial rainfall events in summer 2014 likely reduced the delivery of DIN from catchment sources and created strong DIN limitation on biomass, reinforcing dominance of N-fixing cyanobacteria in the early stages of the bloom.

Predicted increases in dry periods may promote the proliferation of many cyanobacterial species through increased water temperature and greater water column stability (Jöhnk *et al.*, 2008; Kosten *et al.*, 2012; Wagner and Adrian, 2009). As predicted, the dry summer of 2015 resulted in significantly warmer surface water temperatures (average 1.75°C greater than 2014 in the period of January to May; Fig. 2.5). The two different positions of the thermistor chain in Lake Rotorua in 2014 and 2015 means a direct comparison of water column temperatures were not possible. There was a prolonged and relatively stable period of stratification (ca. 1.9 m depth) from mid-January to mid-March in 2015 (Fig. 2.2). Whilst we did not measure dissolved oxygen, we suggest that this stable stratification would have allowed development of an anoxic hypolimnia promoting the release of nutrients (e.g., DRP and NH_4-N) from the bottom sediment (Trolle *et al.*, 2010). Surface water concentrations of DRP were significantly higher in 2015, with the spikes in surface concentrations largely

corresponding to periodic de-stratification events (Fig. 2.2), likely caused by wind-driven mixing and convective cooling during the progression of fronts. Similar large spikes were evident for NH_4-N, possibly due to rapid N uptake.

(ii) Cyanobacterial biomass, composition and succession

In contrast to predictions from climate models and previous culture- or field-based studies, the warmer water temperatures, prolonged stratification and higher concentrations of DRP in 2015 did not result in an increase in cyanobacterial biomass in Lake Rotorua. The average cyanobacterial biomass between January and May 2015 (10.7 mm^3 L^{-1}) was almost one-half of that in the same period in 2014 (20 mm^3 L^{-1}). Closer examination of the cyanobacterial data provides several possible explanations to this conundrum. Firstly, an average cyanobacterial biovolume of over 10 mm^3 L^{-1} is still extremely high and there was a persistent dense cyanobacterial bloom during most of the summer in 2015. Secondly, the 2014 data is skewed by one extremely high biovolume value (87 mm^3 L^{-1} or 1.2 million cell mL^{-1}; 2 February 2014). Removal of this data point reduced the average biovolume across the January to May period to 15 mm^3 L^{-1}, however, as noted below this event appears to have had a profound influence on cyanobacterial succession in 2014.

The multiple regression sequential model identified temperature, TP and TN and as the three most important variables related to cyanobacteria composition over the study period. The temperature at which maximum replication rate occurs varies among cyanobacterial species (Reynolds, 1989; Reynolds, 2006). Additionally, changes in temperature also have indirect effects, or are a response to other events such as stratification, or cooling due to large rainfall events. There is little doubt that temperature plays a direct and indirect role in structuring the

cyanobacterial community in Lake Rotorua and this is discussed in further detail below for specific species. The importance of N versus P in explaining total cyanobacterial biovolume and that of specific cyanobacterial taxa has been demonstrated and discussed for many decades (e.g., Dolman *et al.*, 2012; Smith, 1983; Vrede *et al.*, 2009; Watson *et al.*, 1997). Although the multiple regression identified N and P as important, careful consideration of temporal variability, internal and external fluxes and uptake rates are needed to understand their role in structuring the succession of cyanobacterial communities.

Within the two summers there was a distinct succession in the cyanobacterial species. In 2014, *D. crassum*, and to a lesser extent *A. gracile*, initially dominated the biomass (Fig 2.6A). Although both species are known nitrogen-fixers, the near complete absence of heterocytes in 2014 indicated that no active fixation was occurring and that there was sufficient DIN to support growth. In the sampling immediately after the extremely high biomass event on 2 February 2014 (which was predominantly *D. crassum*), there was a marked increase in NH_4-N, which may have resulted from the lysis of at least some of this biomass (as previously noted by Wood *et al.* (2010b). *Microcystis* is extremely adept at utilising NH_4-N (Blomqvist *et al.*, 1994; Dai *et al.*, 2012), and this in concert with other promoters of *M. aeruginosa* dominance such as high water temperature (Chu *et al.*, 2007a, Davis *et al.*, 2009), was likely to have initiated the bloom of this species. A further possibility could be that *M. aeruginosa* has the ability to store excess phosphorus intracellularly as polyphosphate granules, that it enable its persistence in conditions that do not allow for the accumulation of significant biomass (Saxton *et al.*, 2012). However, the present study has no data to support this theory. After a peak in biomass in February, *M. aeruginosa* declined as DIN decreased. The rainfall event of 15 March 2014 appeared to provide new soluble

nutrients, promoting *M. aeruginosa* growth, until cyclone Lusi (17 April 2014) curtailed the bloom, most likely through washout of the biomass. *Microcystis aeruginosa* biovolume was markedly lower in 2015, but peaks also coincided with increased concentrations of NH_4-N in the water. However, in these instances we suggest that the increased concentrations were likely the result of NH_4-N release from the sediment, as they coincided with short-term mixing events following a period of sustained stratification. The likely causes for the lower *M. aeruginosa* biovolume observed in 2015, compared to 2014, are that in 2015 the NH_4-N pulses were of lower magnitude and did not occur during a period of elevated water temperature, and the TN:TP ratio was consistently high (see discussion below).

The univariate analysis indicated an inverse relationship between *M. aeruginosa* biomass and a TN:TP ratio (Fig. 2.7A). Although early research suggested that this non-N fixing species would be favoured by higher TN:TP ratios, there is now increasing evidence that corroborates my findings of high biomass associated with low TN:TP (e.g., Dolman *et al.*, 2012; Lehman *et al.*, 2008; Marinho and De Oliveira and Azevedo, 2007). For example, Takamura *et al.* (1992) investigated a *Microcystis* bloom in Lake Kasumigaura, and showed that TN:TP ratios were mostly <10 during the bloom, but exceeded 20 after the disappearance of the bloom, a pattern also observed in Lake Rotorua. Miller *et al*. (2013) also observed a similar pattern of species succession in Lake Mendota, where the succession started with *A. gracile*, followed by *D. crassum* and then *M. aeruginosa*, what could be explained by temperature, since *Aphanizomenon* and *Doclichospermum* have a lower optimal growth temperature then *Microcysits*. Beversdorf *et al.* (2013) noted a similar pattern to that observed in Lake Rotorua during their

analysis of a 15-year dataset from the North Temperate Lakes Long Term Ecological Research Program. They found increased *M. aeruginosa* biomass associated with low N:P ratios, while *Aphanizomenon*, which potentially fixes N, was more likely to occur with higher N:P ratios. Marinho and De Oliveira E Azevedo (2007) provide evidence to show that low N:P ratios can be the consequence of high-uptake capacity and greater relative uptake rates of N than P by *Microcystis*, causing a decrease in the ratio. A similar result was also shown by Xie *et al.* (2003) although in their experimental setup both N and P were supplied in high concentrations.

One of the most notable features of the cyanobacterial community in spring 2014 (October to December) was the near monospecific bloom of *A. gracile*. I did not sample over the same period in 2013, preventing an inter-annual comparison. During this period in 2014, the DIN:DRP ratio was extremely low, which may favour N-fixing species, and this may be one of the reasons for the dominance of *A. gracile* during this period (e.g., Vrede *et al.*, 2009). Among N-fixing species, their capacity to fix N varies. For example, Dolman *et al.* (2012) noted differences in the N-fixation rate per heterocyst for a variety of nostocalean species. Higher rates of N-fixation in concert with preference for cooler temperatures (Reynolds, 1989; Zhou *et al.*, 2015) could explain the dominance of *A. gracile* during this period. Allelopathy may be a further possible reason for its dominance as it was observed in Chapter 3 benthic *M. aeruginosa* recruitment in Lake Rotorua was significantly inhibited when *A. gracile* was present in the sediment. Extracts from other *Aphanizomenon* species are known to inhibit cellular processes in other algae (e.g., Suikkanen *et al.*, 2004).

Aphanocapsa sp. was present only in very low numbers in 2014, yet was abundant between January and April 2015. *Aphanocapsa* sp. is the smallest (diameter 0.9 μm) of the cyanobacteria species that have been observed in Lake Rotorua. The large surface area to volume ratio of picocyanobacteria has been proposed as a reason for their dominance when nutrient concentrations are relatively low (Legendre and Rassoulzadegan, 1995; Raven, 1998). Thus picocyanobacteria dominance in Lake Rotorua is consistent with low DIN concentrations between January and April 2015, but not with the elevated DRP concentrations. In the Baltic Sea, small colony-forming Chroococcales co-occur with N-fixing Nostocales (Andersson *et al.*, 2015; Hajdu *et al.*, 2007). These researchers suggested that the Chroococcales utilized nitrogen released from the N-fixing species, which may explain the high abundance of species with N-fixing capacity even though the DIN availability was low.

In summary, the data clearly show that different cyanobacterial species have diverse responses to varying temperature and stratification, as well as different forms, concentrations and ratios of nitrogen and phosphorus. My results point to the need to differentiate cyanobacterial biomass into species that better reflect their varied physiological attributes, as well to better resolve fluxes of nutrients that affect the competitive attributes of these species.

(iii) Variability and drivers of toxic and non-toxic Microcystis genotypes and microcystin quotas

Previous field-based studies have reported positive correlations between toxic *Microcystis* genotype abundance and TP (Rinta-Kanto *et al.*, 2009, Yu *et al.*, 2014), NO_3-N (Yoshida *et al.*, 2007) and water temperature (Conradie and Barnard, 2012). In the present study no relationships were identified between the

abundance of toxic genotypes and *M. aeruginosa* biomass or the environmental variables included in our model. In both summers studied there was peak number of toxic genotypes (Fig. 2.6D). Although the statistical analysis undertaken in the present study did not identify any significant relationships this increase might be related to other variables that couldn't be measured in this study. A longer-term study is recommended. To avoid over-parameterising the model only a limited set of variables were incorporated, including those previously identified as important (or integrators of them such as TN:TP and DIN:DRP). Based on the data in the present study and the contrasting conclusions from previous studies we suggest that predicting temporal shifts in toxic and non-toxic genotype abundances in cyanobacterial blooms is extremely challenging and it is likely that variables that drive these shifts vary temporally and spatially within and between lakes. Another challenge is that within toxic and non-toxic genotypes there is genetic variability, and different strains can produce different amounts of toxins. Analysis of this was beyond the scope of the present study. Future studies could investigate this using PC-IGS and/or 16S-23S ITS analysis and this would avoid missing important variability that occurs by lumping species with strain-level diversity together (Miller *et al*., 2013).

Marked variability (>200-fold) in microcystin quota was observed over a relatively short (ca. 2 week) time frame in this study. Some of this variation may be attributed to a community shift within the microcystin-producing strains as variability can occur amongst toxic genotypes, with each producing different microcystin quotas and variants under the same conditions (Krüger *et al.*, 2010; Saker *et al.*, 2005). As the QPCR assay used in this study only discriminates between toxic and non-toxic genotypes, no information on strain-level diversity

was collected during the study. Wood *et al.* (2016) observed high variability in microcystin quotas over short distances (ca. 1 m) in Lake Rotorua samples collected within several minutes of each other and demonstrated this was not due to shifts in toxin-producing strains, but demonstrated a correlation with *Microcystis* cell density. In the present study no relationship was observed between microcystin-quota and *M. aeruginosa* biovolumes. However, the sampling techniques varied from surface water grab samples in the present study (i.e. bulk water samples of volume 400 mL) to highly discrete, low-volume surface water collections used by Wood *et al.* (2016). Meissner et al. (2013) recently demonstrated that microcystin and nodularin synthesised in response to high light stress bind rapidly and covalently to proteins, thereby making them undetectable using standard methanol extraction techniques. This could have influenced the results of the present study but all samples were extracted in the same manner, therefore relative differences in microcystin quotas among time points should be comparable. Although in my study I didn't investigate microcystin production by *Dolichospermum*, previous study in Lake Rotorua showed that there is no *mcy*E gene present nor microcystin production associated with *D. crassum* (Wood et al., 2011), and when *D. crassum* was present in high abundance on this study, there was no (or very low) microcystin detectable in the lake samples.

The univariate modelling used in the present study identified a significant positive relationship between microcystin quotas and surface water temperature. This corroborates with earlier studies which have shown that microcystin concentrations are maximal around 20 to 25 °C (Amé and Wunderlin, 2005; Van Der Westhuizen and Eloff, 1985, Van Der Westhuizen *et al.*, 1986, Watanabe and

Oishi, 1985) and highlights the possibility that predicted global increases in surface water temperature may lead to higher microcystin quotas. It is also possible that the higher surface water temperatures selected for toxin-producing strains which have higher microcystin quotas, but as noted above I was not able to ascertain this using the QPCR assay.

The discrepancy between times of maximal *M. aeruginosa* biovolumes, toxic genotype abundance, and microcystin quotas highlights the challenges for water managers trying to estimate periods of highest risk when reliant solely on the cell concentration (or biovolume) data as recommended by numerous guidelines (e.g., NHMRC, 2008; WHO, 2003; Wood *et al.*, 2009). For example, the second highest total microcystin concentrations detected in a water sample (3.6 µg L^{-1}, 30 April 2014) occurred when *M. aeruginosa* biovolumes were moderate (2.8 mm^3 L^{-1}), toxic genotype composition was low (16%), but the microcystin quota was at intermediate levels (0.2 pg $cell^{-1}$). To provide greater assurance in estimating risk from cyanotoxin exposure, measurements of known toxins and/or bioassays that assess potential toxicity, and cyanobacterial identification and enumeration are recommended. In order to improve predictive models we need to know what factors drive: A) transcription of the microcystin operon, B) succession of toxin versus non- toxic genotypes, and C) growth of different cyanobacterial species. These data are essential to enable a progression from current models of *Microcystis* biomass or specific physiological attributes of cyanobacteria (see Oliver et al., 2012), to genotype composition and microcystin production, including how these variables might respond under predicted climatic change scenarios. Moreover, I suggest that while generalised prediction on how *Microcystis* biomass might alter under predicted climatic change scenarios are

feasible, understanding how genotype composition and microcystin production will vary is not currently possible.

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Chapter 3

Intracellular, environmental and biotic interactions trigger recruitment of benthic *Microcystis* (Cyanophyceae) in a shallow eutrophic lake

3.1 Introduction

Microcystis is a cosmopolitan cyanobacterial genus that forms surface blooms in eutrophic waterbodies (Harke *et al.*, 2016). It has gained notoriety because of its ability to produce cyclic heptapeptides known as microcystins. Microcystins can be hepato-, nephro- and neuro-toxic (Feurstein *et al.*, 2010; Mackintosh *et al.*, 1990; Yu, 1995) and ingestion of contaminated water has caused human and animal fatalities (Carmichael *et al.*, 2001a; Codd *et al.*, 2005a; Šejnohová and Maršálek, 2012).

In the environment *Microcystis* cells are usually organized in colonies that can consist of many thousands of cells aggregated together with a mucus secretion. One of the reasons for the global success of *Microcystis* is that cells within these colonies contain gas vesicles which enable them to regulate their buoyancy and gain access to vertically separated resources such as light and nutrients (Walsby, 1994). This provides them a competitive advantage over other phytoplankton (Ibelings *et al.*, 1991). Buoyancy is primarily regulated through photosynthesis, where excess energy is stored as a carbohydrate ballast and the colonies sink, until

respiration sufficiently uses up these stores and cells become buoyant again (Visser *et al.*, 1995).

Another related feature of *Microcystis* that may account for its global dominance is its ability that vegetative *Microcystis* cells have to overwinter in the bottom sediment (Fallon and Brock, 1981; Preston *et al.*, 1980), differently from other species such as *Aphanezomenon* and *Dolichospermum* which produce specialised cells (akinetes) in order to overwinter. In temperate environments summer *Microcystis* blooms dissipate in autumn as temperature decreases and colonies sink to the bottom sediments due to an increase in cellular carbohydrate content (Visser *et al.*, 1995). Colonies overwinter on the sediment surface until spring or summer when they are recruited back into the water column which can provide a substantial inoculum for summer blooms (Preston *et al.*, 1980; Torres and Adámek, 2013; Verspagen *et al.*, 2005). However, there is uncertainty as to whether recruitment is an active process triggered by intracellular changes in buoyancy, or a passive process induced by resuspension, or a combination of both mechanisms.

Amongst those advocating for intracellular changes, two scenarios are postulated; gas vesicle synthesis and a reduction in carbohydrate ballast. Gas vesicle synthesis is supported by the work of Šmarda and Maršálek (2008) and Šmarda (2009) who demonstrated that overwintering *Microcystis* cells contain no gas vesicles. They documented an increase in their abundance from spring to mid-summer when recruitment occurs. Whilst there is limited knowledge on the variables which stimulate gas vesicle synthesis, temperature, light and nutrient have been postulated as triggers (Caceres and Reynolds, 1984; Ståhl-Delbanco *et al.*, 2003; Tan *et al.*, 2008; Tsujimura *et al.*, 2000; Walsby, 1994). Conversely, other

researchers have shown that benthic *Microcystis* cells always contain gas vesicles (Reynolds *et al.* 1981) and that cells remain photochemically active on the sediment surface whilst overwintering (Verspagen *et al.*, 2004). Under this scenario, rather than gas vesicle synthesis, cells become positively buoyant in spring or summer as environmental conditions such as temperature or light increase and enhance activity levels in combination with depletion of carbohydrate stores.

Others have suggested that benthic recruitment is passively induced by physical mixing by processes such as wind resuspension or bioturbation (Misson and Latour, 2012; Ståhl-Delbanco *et al.*, 2003; Torres and Adámek, 2013; Verspagen *et al.*, 2004; Verspagen *et al.*, 2005). However, unless cells are physiologically active and already contain gas vesicles it seems unlikely that these physical processes alone would explain observed levels of recruitment and allow cells to remain positively buoyant.

Variability in recruitment rates from different habitats within lakes has been documented (Hansson *et al.*, 1994; Livingstone and Reynolds, 1981; Reynolds *et al.*, 1981; Tsujimura *et al.*, 2000). Traditionally, deep-water environments have been thought to be the most important, as these sites receive the largest proportion of settling cells (Hansson *et al.*, 1994; Tsujimura *et al.*, 2000). Verspargen *et al*. (2005) observed that *Microcystis* benthic population was almost one order of magnitude smaller in the shallow parts than in the deep parts of the lake. The results indicated that after autumnal sedimentation, benthic *Microcystis* are gradually transported from shallow to deep sediments, a process also known as sediment focussing.

Although deep sediments seem to have the highest densities of benthic cells, Brunberg and Blomqvist (2003) showed much higher recruitment rates from shallow bays and suggested that this phenomenon could be due to early access to increased light in these habitats. Few studies have investigated whether there are variable recruitment rates amongst different habitats in shallow lakes. Whilst shallow lakes may vary little in depth, in those where cyanobacteria are dominant, scums are more likely to accumulate along shorelines or in bays, potentially providing a substantial inoculum.

To date research on benthic recruitment has focused mostly on Northern Hemisphere lakes where *Microcystis* persists throughout the summer. Lake Rotorua (South Island, New Zealand) is a shallow eutrophic lake which experiences summer blooms that are initially dominated by nitrogen fixers (*Dolichospermum* and *Aphanizomenon*) before a succession to *Microcystis* blooms later in summer ((Wood *et al.*, 2012a, 2010a). Water temperature in Lake Rotorua is generally warmer (e.g., minimum surface temperature in 2014 was 5.8°C) than the lakes where benthic recruitment studies have previously been undertaken. Because of the observed cyanobacterial succession patterns and less extreme climatic conditions we hypothesised that triggers of benthic recruitment in Lake Rotorua may differ from those previously identified. We investigated three key questions: (i) Does recruitment vary between near-shore and mid-lake sites in this shallow lake? (ii) Do variations in ammonium concentration, light or temperature trigger benthic recruitment of *Microcystis*? (iii) Do ultrastructural changes occur within *Microcystis* cells during the recruitment period? To answer these questions we undertook a series of laboratory experiments using sediment collected from near-shore (ca. 0.5 m depth) and mid-lake (ca. 2.6 m depth) sites.

Microcystis recruitment was recorded under different ammonium concentrations, light intensities and temperatures. Transmission electron microscopy (TEM) was used to assess the cellular structures of *Microcystis* cells isolated from sediment samples collected from spring to late summer.

3.2 Material and Methods

3.2.1 Sampling site and sample collection

Lake Rotorua (42°24′05S, 173°34′57E) is a small (0.55 km), shallow (max. depth 3 m), eutrophic lake in the northeast of the South Island of New Zealand (Flint 1975). It is surrounded by farmland, regenerating scrub and native bush, and has one outflow at its southern end (Fig. 3.1).

Sediment samples were collected for the laboratory experiments with a Ponar grab sampler (0.0225 m^2) from six sites on 10 October 2014; three near-shore sites (ca. 0.5 m depth; sites 1-3; Fig.3.1) and three mid-lake sites (ca. 2.6 m depth; sites 4-6; Fig. 3.1). Samples (ca. 40 mL) from the top layer (ca. 1 cm) of the sediment were transferred to sterile tubes (50 mL) and stored in the dark at 4 °C and transported to the laboratory within 24 h.

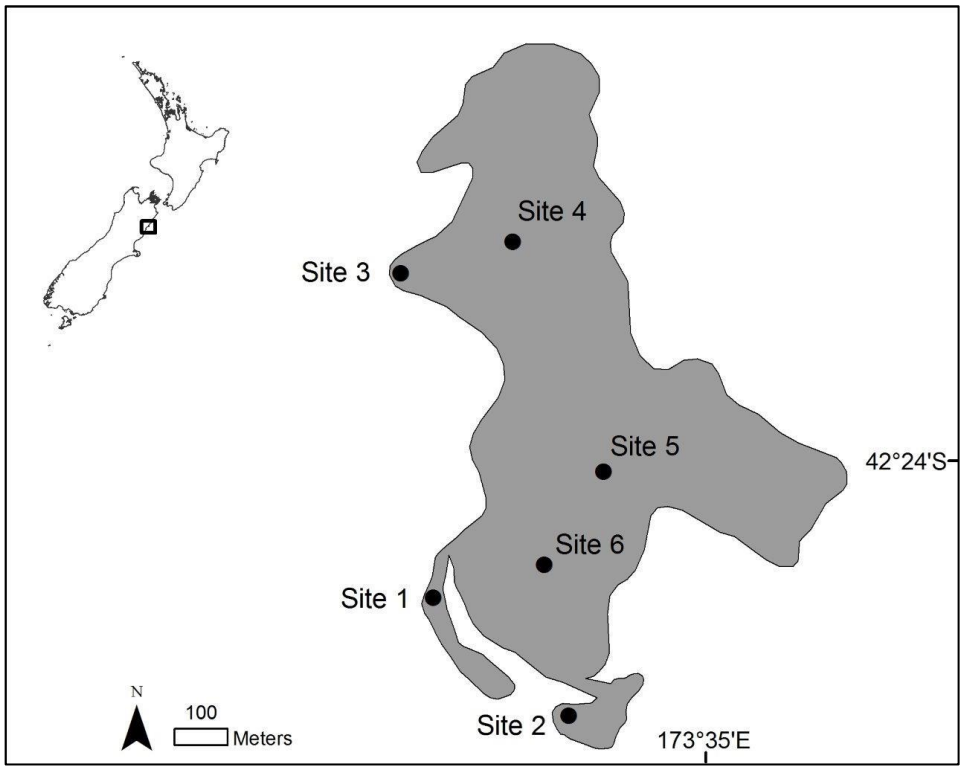

Figure 3.1: Map of Lake Rotorua (Kaikoura, New Zealand) and the six sediment sampling sites. Inset: map of New Zealand showing location of the lake.

3.2.2 Enumeration of cyanobacteria in sediment

Subsamples (35 mL) were pooled for the three near-shore sites and the three mid-lake sites. An aliquot (2 mL) from each pooled sediment sample was added to Percoll® solution (5 mL; Sigma-Aldrich, USA) and nitrogen-free MLA medium (5 mL; (Bolch and Blackburn, 1996b) in 15 mL tubes. The tubes were centrifuged (600 × *g*, 15 min) to separate cyanobacterial cells from the sediment. The upper layer (ca. 2 mL) was removed and immediately preserved with Lugol's iodine. Preserved samples were pipetted into 12-well plates (COSTAR, USA) and allowed to settle (ca. 3 h). *Microcystis aeruginosa* single cells and *Aphanizomenon gracile* cells were enumerated from 1-2 transects, at 400× or 600× magnification using an inverted microscope (IX70, Olympus). *M. aeruginosa* colonies were assessed by scanning the entire well at 200× magnification. The colonies were categorised according to their sizes (CS1 = 2-10, CS2 = 11-50, CS3 = 51-100, CS4 = 101-300 and CS5 ≥ 300 cells). The

approximate number of cells in each *M. aeruginosa* size class was calculated by multiplying the number of colonies in each size class by the mid-point of each bin, e.g., for CS1 a mid-point of 6 cells was used.

3.2.3 Experimental setup

Ammonium experiment

Subsamples (2 mL) from the pooled near-shore and mid-lake sediment samples were pipetted separately into 60 mL gamma-sterilised polystyrene culturing containers (ThermoFisher Scientific, New Zealand) containing aliquots (40 mL) of Milli-Q water (18.2 MΩ cm) or Milli-Q supplemented with ammonium chloride at concentrations of 0.1, 0.2, 0.5, 1 and 5 mg N L^{-1}. Controls and treatments were tested in triplicate. The culture containers were incubated at 19°C (± 1°C) at an irradiance of 5 µmol photons m^{-2} s^{-1} on a 12 h:12 h light/dark cycle.

To determine whether dissolved nutrients from the sediments influenced the concentrations in the water, an additional three culture containers were filled with Milli-Q water (40 mL) and set-up as described above. After 48h, water samples (30mL) were collected, filtered (Whatman GF/C), and stored at -20°C. These were analysed with a Lachat QuickChem® flow injection analyser (FIA+ 8000 Series, Zellweger Analytics, Inc.) using APHA (2012) 4500 methods for ammoniacal nitrogen (NH_4-N), nitrate (NO_3-N), nitrite (NO_2-N) and dissolved reactive phosphorus (DRP).

Temperature and light intensity experiments

Subsamples (2 mL) of the pooled near-shore and mid-lake sediment samples were pipetted into 40 mL aliquots of Milli-Q water in culture containers (two sets of 30). For the temperature experiments, the culture containers were incubated in

triplicate at low irradiance (5 µmol m^{-2} s^{-1}) and at five different temperatures; 4, 13, 16, 19 and 25 °C, using temperature controlled cabinets (SKOPE, 1000K). For the light experiments, the triplicate culture containers were incubated at 19 °C in the dark or at four different light intensities; 1.5, 10, 50 and 100 µmol m^{-2} s^{-1} (12 h:12 h light/dark cycle). The different light intensities were achieved by varying the numbers of layers of high density polyethylene cloth covering the culture containers. Light intensities were measured using a photometer (LI-COR, 250A). The mesh structure reduces the light penetration but doesn't change wavelength of the light reaching the cultures.

Sampling of benthic recruitment experiment

For all experiments, subsamples (10 mL) were collected from the surface of each culture container after 1, 3, 6, 9 and 12 days, and were immediately preserved with Lugol's iodine. Each container was carefully supplemented with Milli-Q water (10 mL) or the appropriate ammonium chloride solution (ammonium experiment only) to replace the sampled volume, taking care to avoid mixing of sediment. The addition of chloride at these low levels would be highly unlikely to have any influence on recruitment. Aliquots of the preserved samples (5 mL) were pipetted into 12-well plates (COSTAR, USA), allowed to settle (ca. 3 h) and enumerated as described above.

Statistical Analysis

Linear Mixed Effect Models (LMEMs) were used to test for interactions between habitat (near-shore and mid-lake) and the different environmental variables (ammonium, temperature and light intensity). The three environmental variables and habitat were tested as fixed effects; time was the continuous covariate and sample was a randomised effect to account for repeated measures. The LMEM

analysis was undertaken separately on samples from the near-shore and mid-lake habitats using the total *M. aeruginosa* concentration data. The p-values were obtained using ANOVA and adjusted post-hoc pair-wise tests (Chi-squared test) were undertaken.

3.2.4 Assessment of the ultrastructure of benthic *M. aeruginosa*

To identify changes in the ultrastructure of benthic *M. aeruginosa* cells (gas vesicles, polyphosphate bodies and structured granules), sediment samples were collected from site 1 (Fig. 3.1) on 10 October 2014, 26 January 2015 and 29 March 2015 as described above. Sediments were sub-sampled (10 mL) and preserved with 2% glutaraldehyde in 0.1M sodium cacodylate (40 mL) for later TEM imaging. Samples were stored in the dark at 4 °C and processed within 48 h. Surface water samples (250 mL) were collected on the same days, preserved with Lugol's (which allows cells to settle to the bottom of counting chambers enabling enumeration with an inverted microscope), and stored in the dark at 4 °C. Cyanobacteria in these samples were identified and enumerated as described above.

In the laboratory, sediment samples were homogenised by gently inverting 10 times. To separate *M. aeruginosa* cells from the sediment, subsamples (ca. 6 mL) were suspended in Percoll solution (1:1 Percoll/MLA medium, adapted from Van Liere and Mur (1978)) and centrifuged (600 ×*g*, 15 min). The supernatant containing the cells (ca. 0.5 mL) was transferred to 1.5 mL tubes and centrifuged (600 ×*g*, 10 min) with 2% glutaraldehyde in 0.1M sodium cacodylate (1.5 mL) to remove residue sediment. The supernatant was transferred to a new 1.5 mL tube, centrifuged (1,000×*g*, 5 min), the supernatant discarded and fresh 2% glutaraldehyde in 0.1 M sodium cacodylate (1.5 mL) was added. The samples

were gently mixed on a rotating wheel (1 rpm, 1 h, 20 °C), centrifuged (1,000×*g*, 5 min) and the supernatant replaced with 0.1 M sodium cacodylate (1.5 mL). This process was repeated twice to give a total of three washes.

The supernatant was removed and samples were fixed with 1% osmium tetroxide in 0.1M sodium cacodylate (1 h, gentle mixing, 20 °C). Samples were centrifuged (1,000×*g*, 5 min) and the supernatant replaced with 0.1M sodium cacodylate (1.5 mL). The process was repeated twice more to give a total of three washes. The samples were then dehydrated through a series of ethanol concentrations (50%, 70%, 90%, 2 × 100%) and infiltrated with Spurrs resin, initially diluted 1:1 with 100% ethanol. Infiltration involved three changes of resin over two days. To assist in concentrating the cells in the resin, the samples were centrifuged (1,500×*g*, 15 min). For the embedding process in the final resin, the resulting pellet was carefully resuspended in a small volume of the remaining resin supernatant and resin with cells was transferred by pipette to a polyethylene embedding capsule (BEEM capsule). This technique has been used previously (Verspagen *et al*. 2005; Mission and Latour, 2012) to separate benthic cells from the sediment. To ensure minimum loss of specimen during the transfer, the processing vial (a 1.5 mL tube) was 'rinsed' repeatedly with small volumes of fresh resin that was then used to top up the BEEM capsule. The resin was cured at 60 °C for 48 h.

The cured block was sectioned using a Leica UC6 Ultramicrotome. Sections (0.5 µm-thick) were initially screened for cells under a light microscope (CX31, Olympus). Sections showing cells were recut (80nm-thick) and placed onto Formvar-covered slot grids. The sections were contrasted with uranyl acetate and lead citrate using an LKB 2168 Ultrostainer. The sections were viewed on a

Philips CM100 TEM and images captured using an Olympus/SIS MegaView 3 camera.

Using Standard CellSens (Version 1.0, Olympus), 30 cells from each of the three samples were assessed to measure the biovolume and the percentage of cell area occupied by gas vesicles, polyphosphate bodies, and structured granules. The cell structures were visually identified based on previous descriptions (Reynolds, 1981; Šejnohová and Maršálek, 2012). Differences in the mean biovolume and the cellular components among collection dates were assessed using one way ANOVA and Tukey's honestly significant difference (HSD) post hoc tests in R Studio for Windows (Version 0.98.501, Intel Corp).

3.3 Results

3.3.1 Recruitment experiment

Total *M. aeruginosa* cell densities in the pooled near-shore and mid-lake sediment samples were 3,750 and 3,600 cells mL^{-1}, respectively. Fifty-seven percent of cells in the near-shore sample were single cells, compared to 18% for the mid-lake sample. The remainder of the cells were colonies (CS1-CS4 size classes; Appendix Table 3.1). *Aphanizomenon gracile* filaments (24,800 cells mL^{-1}) were present in the near-shore samples, but none were observed in mid-lake samples.

Average nutrient concentrations from the three sediment samples with Milli-Q, which were used to assess the effect of nutrient inputs from the sediment, were all relatively low: NH_4-N = 0.05 mg L^{-1}, NO_3-N = 0.2 mg L^{-1}, NO_2-N = 0.02 mg L^{-1} and DRP = 0.01 mg L^{-1} and minimally influenced experimental nutrient concentrations.

In all experiments and all treatments *M. aeruginosa* recruitment was observed on day 1 (Fig.3.2). *M. aeruginosa* densities were always lower (ca. 2-5 fold) on day 1 using the near-shore sediment compared to the mid-lake sediment (Fig. 3.2). At the conclusion of the experiment *M. aeruginosa* cell densities were similar in the near-shore samples among experiments (max. day 12: ca. 2,000 cells mL^{-1}; Fig. 3.2). In contrast, total *M. aeruginosa* cell density in the mid-lake samples varied, for example, were highest in the 100 µmol m^{-2} s^{-1} light treatment (5,380 cells mL^{-1}; 72% of cells in original sediment sample), followed by the ammonium treatment at 0.1 mg N L^{-1} (4,660 cells mL^{-1}; 65%), and lowest in the treatment at 13°C (1,570 cells mL^{-1}; 21%; Fig. 3.2; Appendix Table 3.2).

Single cells of *M. aeruginosa* were dominant in the near-shore and mid-lake samples in all experiments at each time point (Fig. 3.3a-f; Appendix Figs. 3.1-3). Single cells represented >80% of total *M. aeruginosa* density for the ammonium and light intensity experiments, and >55% for the temperature experiment in the near-shore samples at day 12 (Fig. 3.3a-f). In all mid-lake samples single cells comprised over 90% of the total *M. aeruginosa* density at day 12 (Fig. 3.3). The range of colony sizes was greatest in the temperature experiment, e.g., after 12 days in the 25 °C treatment all six colony size categories were present (Appendix Fig. 3.2).

Cumulative *M. aeruginosa* recruitment increased significantly with time across all treatments in the ammonium, temperature and light experiments (time: $p<0.001$, Fig. 3.2). The effect of treatment significantly interacted with habitat type (ammonium, temperature or light × habitat: $p<0.001$, Fig. 3.2).

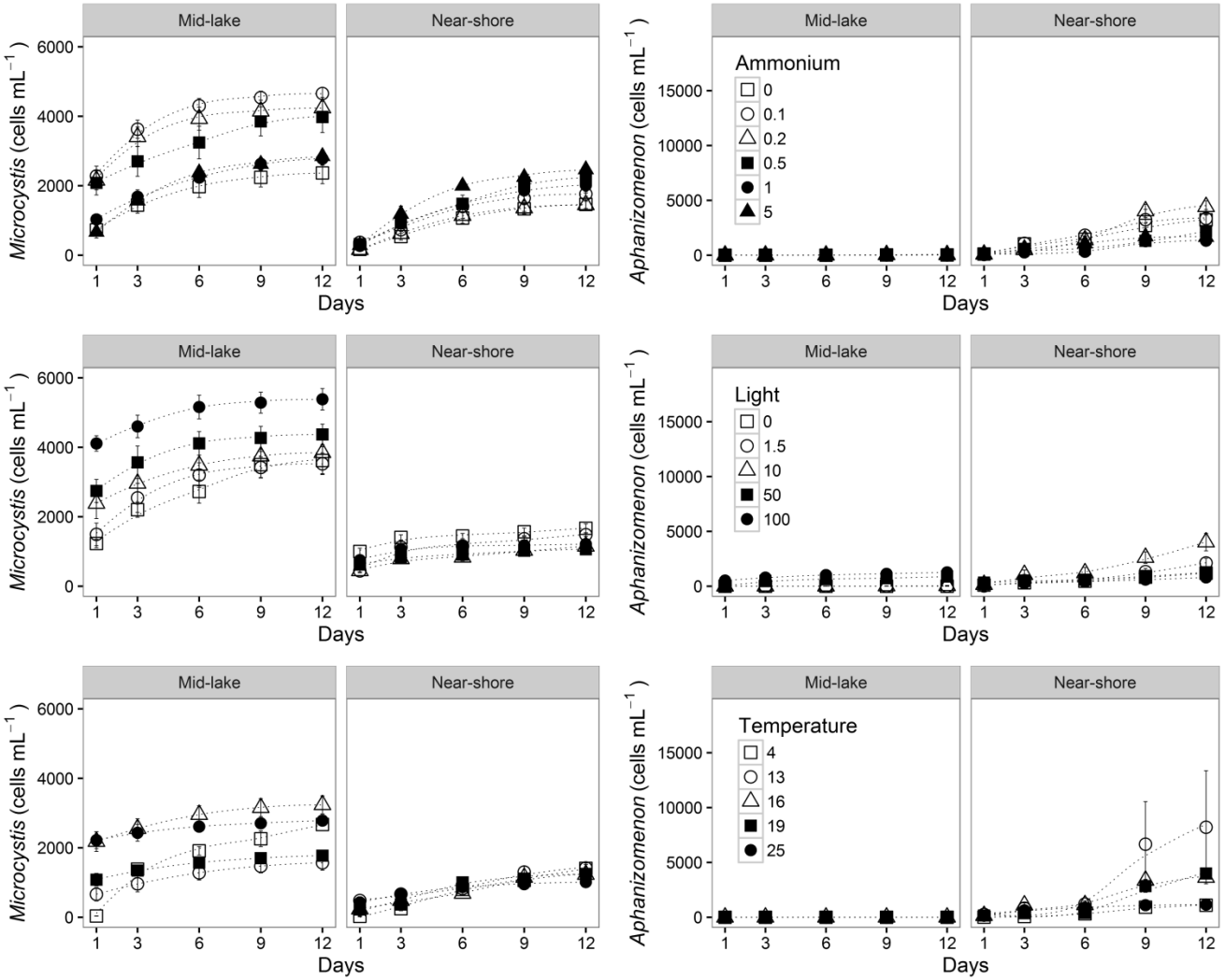

Figure 3.2: Total cumulative cell density of *M. aeruginosa* (left) and *Aphanizomenon gracile* (right) in the (top panel) for ammonium (mg N L-1), (mid panel) light intensity (µmol m^{-2} s^{-1}), and (bottom panel) temperature (°C) experiments over the 12-day sampling period for the mid-lake and near-shore sediment samples. Note: Y-axis scales differ between the left and right panels.

Adjusted post-hoc pair-wise tests showed there was no significant difference within treatments in near-shore samples. However, in the mid-lake samples *M. aeruginosa* recruitment was significantly higher in samples: (1) exposed to ammonium concentrations of 0.1, 0.2 and 0.5 mg L^{-1}compared to the 0 mg L^{-1}, 1 and 5 mg L^{-1} ($p<0.01$), (2) kept at 16 and 25°C compared to 4, 13 and 19 °C ($p<0.001$), and (3) exposed to light intensities of 50 and 100 µmol m^{-2} s^{-1} compared to 0, 1.5 and 10 µmol m^{-2} s^{-1} ($p<0.001$), whilst recruitment in the 100 µmol m^{-2} s^{-1} was also higher than at 50 µmol m^{-2} s^{-1} ($p<0.01$; Supplementary Information Table 3.3).

Aphanizomenon gracile (>800 cells mL^{-1} on day 12) recruitment was observed in all experiments and treatments using near-shore sediment samples, while there was very little recruitment from mid-lake samples (<1300 cells mL^{-1} on day 12; Fig. 3.2). After 12 days the highest recruitment was observed in near-shore treatments at 13 °C (8,210 cells mL^{-1}), followed by ammonium at 0.2 mg N L^{-1} (4,420 cells mL^{-1}), and light at 10 µmol m^{-2} s^{-1} (4,020 cells mL^{-1}).

In general, the *M. aeruginosa* recruitment rate for all experiments was highest on day 1 (Fig. 3.4). In contrast, the recruitment rate of *A. gracile* in near-shore samples was highest on day 9 in the ammonium and temperature experiments for all treatments, except for at 25 °C which had the highest recruitment rate on day 3. The highest *A. gracile* recruitment rate was recorded on day 1 for light treatments of 50 and 100 µmol m^{-2} s^{-1}, and day 12 for 0, 1.5, and 10 µmol m^{-2} s^{-1} (Fig. 3.4). Recruitment rates of *A. gracile* in the mid-lake samples were negligible.

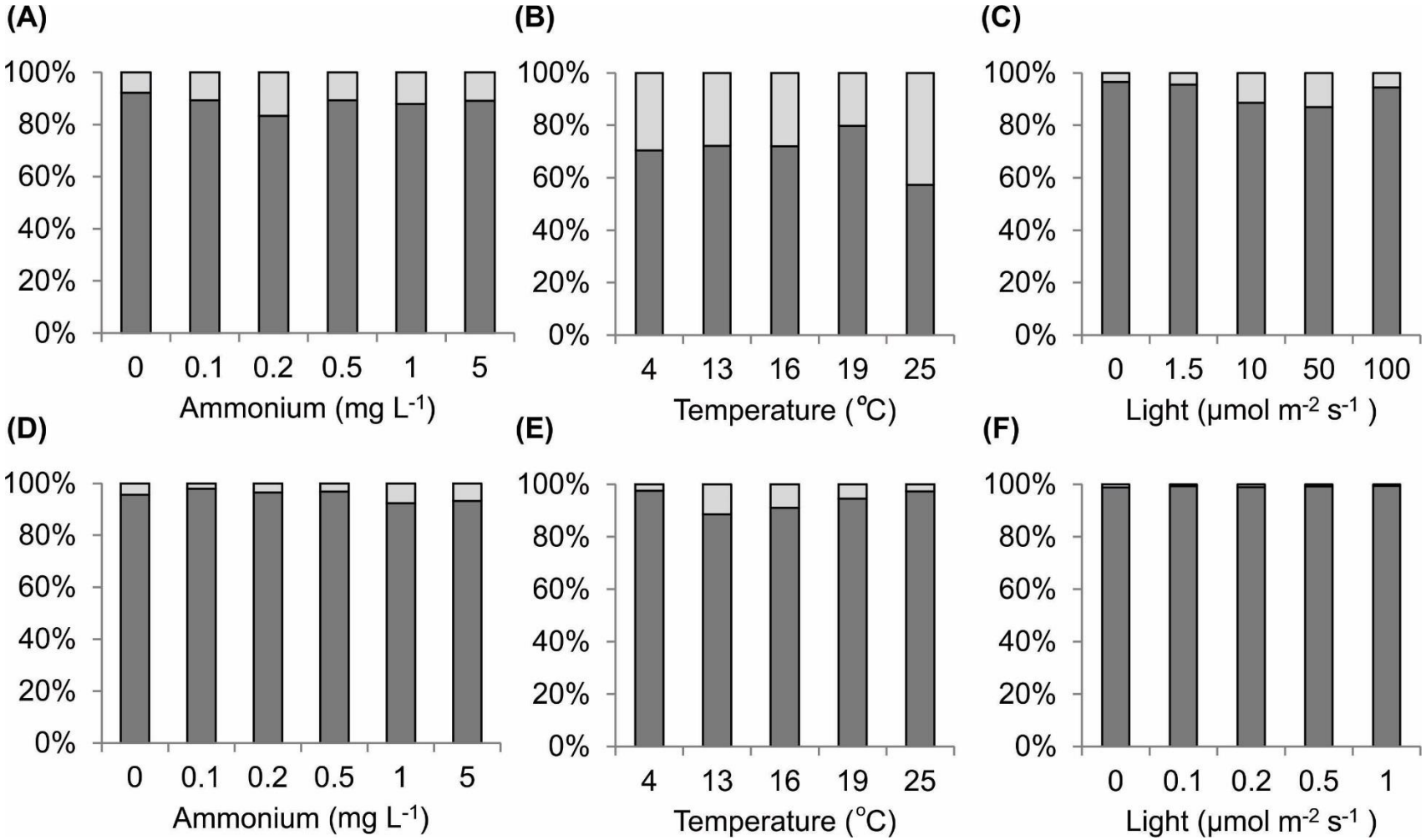

Figure 3.3: Relative abundance of *Microcystis aeruginosa* single cells (dark grey) and colonies (light grey: all colony sizes grouped together) at day 12 of the ammonium, light intensity and temperature treatments. A, B, C are near-shore and D, E, F are mid-lake samples.

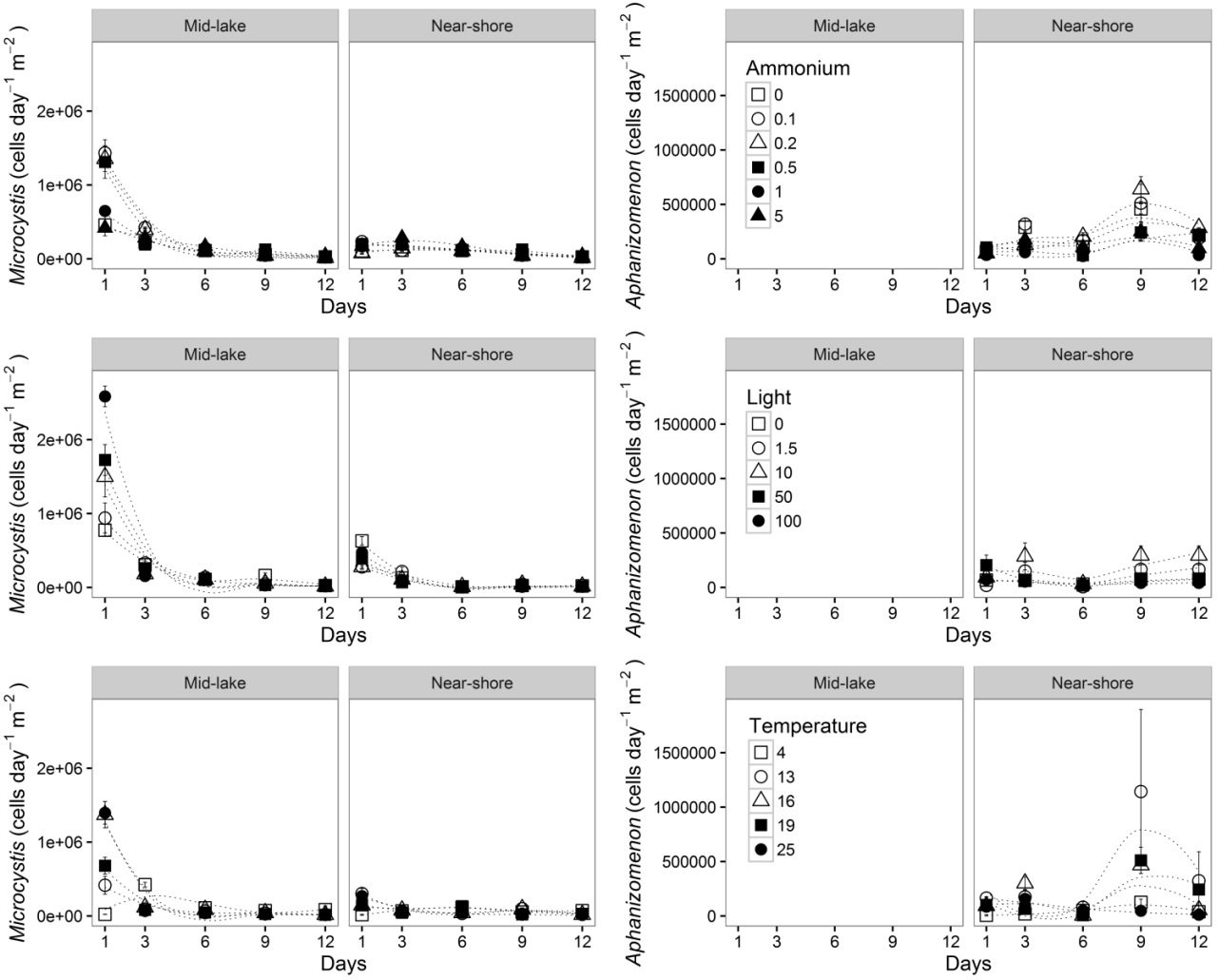

Figure 3.4: Recruitment rate (cells day-1 m-2) of *Microcystis aeruginosa* (left) *Aphanizomenon gracile* (right) in (top panel) ammonium (mg L^{-1}), (mid panel) light intensity (µmol m^{-2} s^{-1}) and (bottom panel) temperature (°C), experiments over the 12-day sampling period.

3.3.2 Changes in the ultrastructure of benthic *Microcystis aeruginosa*

Benthic *M. aeruginosa* cells isolated from the sediment and analysed by TEM showed changes in cellular biovolume and in the abundance of cellular ultrastructure components over the sampling period (Fig. 3.5). The highest biovolumes were measured in January 2015 and were significantly reduced by March 2015 ($df=2$, $F=4.2$, $p<0.02$; Table 3.1). The percentage of transverse cell section occupied by gas vesicles increased significantly from 0.02% to 11.27% over the three sampling time points ($df=2$, $F=22.51$, $p<0.001$; Fig. 3.5; Table 3.1). The percentage of area occupied by structured granules also varied significantly with cells collected in January 2015, with a lower percentage than in October 2014 or March 2015 ($df=2$, $F=10.04$, $p<0.001$; Fig.3.5; Table 3.1). The percentage area in transverse section that was occupied by polyphosphate bodies varied markedly among cells regardless of sampling period and no significant differences were observed (Fig.3.5; Table 3.1).

Table 3.1: *Microcystis aeruginosa* cell biovolumes and percentage of transverse sections of *M. aeruginosa* cells occupied by gas vesicles (GV), polyphosphate bodies (PB), and structured granules (SG) in each studied month. Data are means (n=30) ± one standard deviation. Superscripts indicate Tukey's HSD pairwise test groupings ($p<0.05$).

Date	Biovolume (μm^3/cell)	GV (%)	PB (%)	SG (%)
Oct-14	19.01 ± 6.77 ^{AB}	0.02 ± 0.12 ^{A}	4.22 ± 2.24	4.78 ± 2.06 ^{A}
Jan-15	22.34 ± 6.42 ^{B}	1.31 ± 6.62 ^{A}	2.89 ± 3.14	2.54 ± 1.69 ^{B}
Mar-15	17.74 ± 5.81 ^{A}	11.27 ± 10.37 ^{B}	4.08 ± 3.56	4.98 ± 3.05 ^{A}

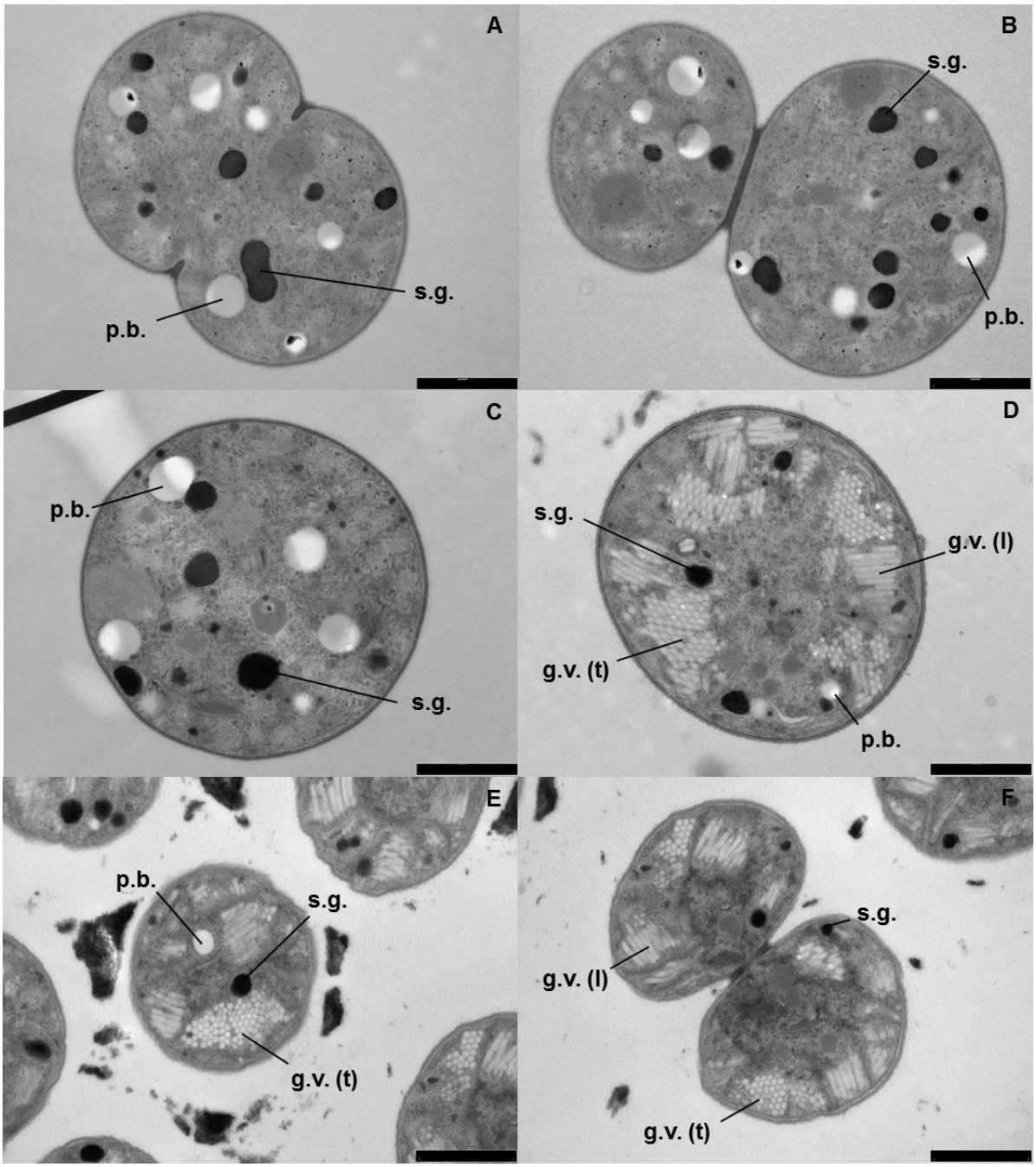

Figure 3.5: A selection of the images analysed of the ultrastructure of benthic *Microcystis aeruginosa* cells sampled in October 2014 (A and B), January 2015 (C and D) and March 2015 (E and F) from Lake Rotorua, New Zealand. Scale bar (bottom right) = 1µm; s.g. = structured granules; p.b. = polyphosphate bodies; g.v. (t) = gas vesicles (transversal section); g.v. (l) = gas vesicles (longitudinal section). The complete set of images is provided in Appendix Fig. 3.4-6.

Between October 2014 and March 2015, *M. aeruginosa* concentrations increased in the surface water from 0 to 36,730 cells mL^{-1} (Table 3.2). This increase of *M. aeruginosa* in

the surface water corresponded with the increase in the abundance of gas vesicles in the sediment-bound *M. aeruginosa* observed by TEM (Fig. 3.5; Table 3.1).

Table 3.2: Cyanobacterial concentrations (cells mL^{-1}) in surface water samples from Lake Rotorua collected on the same day as sediment samples (Table 3.1).

Date	*Dolichospermum* sp.	*Aphanizomenon gracile*	*Aphanocapsa* sp.	*Microcystis aeruginosa*
Oct-14	1,210	102,150	0	0
Jan-15	2,260	25,090	4,782,000	16,460
Mar-15	19,720	11,690	544,200	36,730

3.4 Discussion

Previous studies using in-lake migration traps have shown differential recruitment associated with shallow versus deep regions of lakes (Rengefors *et al.*, 2004). Most studies suggest deep regions are of greater importance as they receive a larger portion of cells that sediment out of the water column (Verspagen *et al.*, 2005). However, Brunberg and Blomqvist (2003) observed high recruitment rates in the shallow bays of Lake Limmaren (Sweden). They attributed this to high concentrations of sinking cells following wind-accumulated blooms, and suggested that these sites are generally exposed earlier than deep sites to environmental triggers, e.g., light and temperature, that promote recruitment. Only a limited number of studies have compared recruitment rates for samples collected from different lake habitats in laboratory-based experiments where environmental parameters can be controlled (Rengefors *et al.*, 2004). In the present study, *M. aeruginosa* recruitment was significantly higher in samples collected from the mid-lake sites compared to near-shore sites. The initial *M. aeruginosa* inoculum in the sediment samples was similar, eliminating inoculum size as a causative factor. Unlike in-lake studies where environmental conditions such as light intensity and

temperature can vary between near-shore and mid-lake habitats, in our experiments both habitat types were exposed to identical conditions. Although there was no difference in the total number of *M. aeruginosa* cells in the starting inoculums, the near shore sample contained a higher portion of single cells. It is plausible that greater turbulence at this site prior to settlement or faster breakdown rates in the sediment caused this discrepancy. This difference is not the reason for the observed variability in recruitment rates as there was significantly higher recruitment of single cells in treatments inoculated with mid-lake samples.

The most striking difference between the two sample types was the presence of high concentrations of *A. gracile* in the near-shore sediment samples. *Aphanizomenon gracile* was not observed in the mid-lake sediment samples microscopically, although very low recruitment rates were recorded which suggests that it was present at very low concentrations (Fig. 3.2). It is not apparent why the *A. gracile* cell density differed between the near-shore and the mid-lake sediment, but it could be the result of *A. gracile* cells settling after a wind-accumulated bloom along the lake edge.

To explain the decreased *M. aeruginosa* recruitment in the presence of *A. gracile* we suggest two possible scenarios; (1) *A. gracile* out-competes *M. aeruginosa* for resources, thereby constraining recruitment, or (2) *A. gracile* has an allopathic effect on *M. aeruginosa*, inhibiting processes involved in recruitment. Under scenario one, while resources such as light and nutrients are adequate there is a decrease in the suppression of *M. aeruginosa*. However, no significant differences in recruitment rates were observed within treatments (ammonium concentration, light intensity or temperature) in the near-shore samples. Extracts of *Aphanizomenon flos-aquae* have been shown to have allopathic effects on *Rhodomonas* sp. by reducing its cellular chlorophyll-a content

and CO_2 uptake (Suikkanen *et al.*, 2006, 2004). Another example of possibly allopathic effects is that "non-toxic" cyclic peptides (e.g. anabaenopeptin B and anabaenopeptin F) can provoke lysis of *Microcystis aeruginosa* cells, as observed in the study of Sedmak et al. (2008). In the reverse situation to that proposed here, *Microcystis* extracts have been shown to inhibit *A. flos-aquae* growth (Ma *et al.*, 2015). Further investigations are required to confirm the potential allopathic effects of *A. gracile* on *Microcystis* recruitment.

Another possible explanation for the differences in *M. aeruginosa* recruitment between the near-shore and mid-lake samples is that the physiological state of the cells could differ among habitats. Variations in physiological state may occur, for example, if cells were exposed to differences associated with exposure to anoxia, temperature or decomposition processes. Whilst we did not assess the physiological state of cells prior to commencing the experiments, microscopic analysis of cells from the sediment samples provided no evidence to support this theory, and unlike other studies the difference in depth (2 m) and distance (2-300 m) between the near-shore and mid-lake sampling sites would be less likely to support major environmental gradients between the two sites.

A substantial difference between the results of this study and those reported previously was the presence of high concentrations of single *Microcystis* cells. To our knowledge all previous recruitment studies only report on the abundance of *Microcystis* colonies. It is unclear whether single cells were not present in these studies or overlooked due to methodological approaches. For example, some studies report of the use of sonication to dissociate colonies and aid in microscopic enumeration (Misson *et al.*, 2011), others filter samples with a mesh size(63 µm) that would not retain single cells or small

colonies (Tsujimura *et al.*, 2000), and many have used magnifications of less than 400× for enumerations, which would likely prevent identification of single cells (Misson and Latour, 2012). In our study, single *M. aeruginosa* cells accounted for greater than 55% of total cell concentrations in all experiments and treatments, and we recommend that future studies ensure methods are adequate to enable their detection. Previous studies have shown that single cells can aggregate relatively rapidly (Yang *et al.*, 2006), thus enabling colony formation and activation of some of the advantages it confers post recruitment (e.g., buoyancy regulation and predation defence; Oliver, 1994, Wu and Song, 2008, Yang and Kong, 2012). During our study, single cells were recruited from the sediment faster than colonies. By contrast, cells within colonies may be exposed to environmental gradients, where cells in the middle of a colony receive reduced light and access to nutrients compared to those on the outside(Mulling *et al.*, 2014, Paerl, 1983). Light, nutrients and other possible environmental triggers may play a role in initiating gas vesicle synthesis (Walsby, 1994). Theoretically, if gas vesicle synthesis is delayed in central cells of a colony these may collectively 'weigh down' the colony and delay buoyancy. Evidence to support our theory comes from several studies which have shown a higher abundance of small colony sizes (ca. 2-8 cells) in newly established planktonic populations (Kurmayer *et al.*, 2003, Tao *et al.*, 2005).

Recruitment of *M. aeruginosa* in the laboratory experiments was rapid, with the highest rates occurring within 24 h (Fig. 3.4). Similar recruitment rates have been observed in laboratory studies even when colonies have been buried in sediment for almost three years (Misson and Latour, 2012). The TEM analysis (discussed below) suggested that when cells were collected for the benthic recruitment experiments, the percentage containing gas vesicles was low (ca. 0.02%). Lehmann and Jost (1971) demonstrated that gas vesicles synthesis can occur rapidly (within 12 h). Whilst we did not assess gas

vesicle formation during our experiments, the high recruitment rates after 24 h support the proposition of rapid gas vesicle synthesis.

The absence of *A. gracile* from the mid-lake samples enabled us to assess the relative importance and effects of varying ammonium concentrations, light intensity and temperature on *M. aeruginosa* recruitment. Recruitment occurred in all experiments and treatments, even in conditions we had anticipated would not facilitate it, such as complete darkness. These data indicate that multiple factors may regulate gas vesicle synthesis in Lake Rotorua. Within each experiment significant differences among treatments were observed. In the ammonium experiment recruitment was highest in the mid-range of concentrations (0.1, 0.2 and 0.5 mg L^{-1}). Ståhl-Delbanco *et al.* (2003) observed a similar response in enclosure experiments, with the highest *Microcystis* recruitment recorded at intermediate concentrations of dissolved inorganic nitrogen concentrations (ca. 0.1 and 0.5 mg L^{-1}). Likewise, Chu *et al.* (2007b) observed significant decreases in gas vesicle abundance when there was nitrogen limitation, which highlights a likely role for this nutrient in their synthesis.

Temperatures above 15-20 °C have repeatedly been implicated in promoting *Microcystis* recruitment (Tao *et al.*, 2005, Tsujimura *et al.*, 2000, Yamamoto, 2009). Some studies have shown discrete thresholds below which no recruitment occurs; e.g., <9 ℃ Tang *et al.* (2010), <14 °C Tao *et al.* (2005), and <15 °C (Li *et al.*, 2003). The results of our study, which showed significantly greater recruitment at 16 °C and 25 °C, are congruent with enhanced recruitment at higher temperatures. However, in contrast to some previous studies, recruitment was still observed at low temperatures (4 °C). In these samples recruitment rates were markedly lower and delayed. It appeared that the

low light used in this experiment, regardless of temperature, was sufficient to stimulate recruitment, albeit with a delay and at reduced levels.

In-lake studies have demonstrated the importance of light penetration to bottom sediment as a factor for stimulating recruitment (Brunberg and Blomqvist, 2003, Reynolds *et al.*, 1981). In our study, as *M. aeruginosa* recruitment was significantly increased at the two highest intensities tested (50 and 100 µmol m^{-2} s^{-1}). Further, of the three environmental parameters tested, high light resulted in the greatest total recruitment. Deacon and Walsby (1990) showed that the optimal photon irradiance for the production of new gas vesicles in *Microcystis* is 35 µmol m^{-2} s^{-1} with a marked decrease at higher intensities. This was in contrast to the observations of our study, although we did not test the specific intensity (the closest were 10 and 50 µmol m^{-2} s^{-1}) and this optimum may therefore have been missed. Although lower than the other light intensities tested, recruitment was still observed in the samples maintained in darkness. Deacon and Walsby (1990) demonstrated that some gas vesicle production can occur in darkness, but only if the cells have accumulated energy reserves. If the cells used in our experiment had maintained some photochemical activity on the sediment surface during overwintering (Verspegan et al. 2004) then this may explain the ability of these cells to recruit to the water column. Thomas and Walsby (1986) also showed that non-buoyant colonies regained buoyancy in the dark, and that this was enhanced when temperatures were 20 °C.

Significant differences were observed in the cellular structure of the *M. aeruginosa* cells isolated from the sediment during spring, early and late summer. Most notable was the low number of gas vesicles in samples collected in spring. Similar observations have been made by Šmarda and Maršálek (2008) and Šmarda (2009) who observed no gas

vesicles in overwintering populations. In contrast Reynolds *et al.* (1981) collected colonies from sediment over a three-year period and found that every cell examined contained gas vesicles. Such discrepancies between studies highlight that the processes involved in benthic *Microcystis* recruitment likely vary between lakes, with factors such as competition (e.g., with other cyanobacterial or algal species), the presence of organisms involved in bioturbation (e.g., fish), water temperature, depth and nutrient status synergistically influencing recruitment. The data presented in this study only captures *M. aeruginosa* ultrastructural changes from a single year covering the spring to summer period, and results from a longer period are required to increase knowledge temporal dynamics in gas vesicles production in this lake.

In conclusion, this study has highlighted the highly interactive nature of the variables which regulate *M. aeruginosa* recruitment, and that the relative importance of these can differ among lakes. We have demonstrated that even in small shallow lakes there is variability in the rate of recruitment of benthic *M. aeruginosa* collected from different habitats. Recruitment was significantly reduced in the near-shore samples compared to the mid-lake samples, and the main difference between habitat types was the presence of high densities of *A. gracile*. Species from this genus are known to elicit allopathic effects on other algae and the possibility that *A. gracile* inhibits *Microcystis* recruitment requires further investigation. A major difference in this study compared to those reported previously was the high percentage of recruited single cells, as opposed to colonies. Single cyanobacterial cells may have more immediate access to growth promoting factors than colonies and may therefore regain buoyancy more rapidly. This aspect of benthic *Microcystis* recruitment points to the need to better understand and differentiate intercellular variations of colonies (Mulling et al. 2014). In the mid-lake sample,s ammonium concentration, temperature and light intensity all had effects on

recruitment, suggesting that all these parameters individually or synergistically regulate recruitment, with exposure to high light intensities promoting the greatest recruitment. The very low abundance of cells with gas vesicles in the sediment in spring (the inoculum used for laboratory experiments) suggested that synthesis of these cellular structures is necessary for benthic *M. aeruginosa* recruitment in Lake Rotorua.

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Chapter 4

Conclusions and recommendations

4.1 Conclusions

Lack of good quality freshwater is one of the most serious global issues, and cyanobacteria that proliferate and produce toxins are a key factor in reducing surface water palatability in many countries. Under favourable environmental conditions, cyanobacteria can form dense and expansive blooms, which are aesthetically unpleasant, can have serious environmental impacts and pose a health risk to humans and animals (Azevedo *et al*., 2002). Because of these issues there is an on-going need to study cyanobacteria and increase knowledge of factors that promote cyanobacterial blooms, to assist in risk assessment and management, and to develop appropriate mitigation actions. Numerous researchers have provided evidence that cyanobacterial blooms will increase in severity and duration under predict climate change scenarios (Brookes and Carey, 2011; Carey *et al*., 2012; Kosten *et al*., 2012; Paerl and Huisman, 2008; Paerl and Huisman, 2009; Paerl and Paul, 2012).

The findings of chapter two of my thesis demonstrated that when trying to understand seasonal changes in biomass and species composition, cyanobacteria should not be treated as a single group and consideration should be given to the varying physiological attributes of each species. An improved understanding of the impact and interactions amongst environmental factors, physiological attributes of species and the successional sequence of species is required to better

understand the triggers of bloom formation. My study took place over two summers with contrasting extreme whether events (floods and drought), providing new insights into the possible effects of climate change on cyanobacteria species composition. For example, during flood events in summer 2014, an increase in nitrate concentrations was observed in the lake water, and consequently an increase in *Microcystis aeruginosa* occurred under ideal temperature conditions, while in 2015 there was lower rainfall and higher biomass of nitrogen fixing cyanobacteria taxa was observed.

The findings of this chapter also showed how environmental factors influence the dynamics of toxic and non-toxic *Microcystis aeruginosa* genotypes and microcystin concentrations. The key findings were that microcystin quota showed a significant relationship with water surface temperature and there were no significant relationships between the environmental variables explored and the percentage of toxic *Microcystis* cells. A review of other studies suggests that drivers of genotype succession are very divergent among the studies. For example, Rinta-Kanto *et al*. (2009) demonstrated that total phosphorus, water temperature, and dissolved inorganic carbon were key variables influencing toxic *Microcystis* dynamics and microcystin distributions in space and time, while Conradie and Barnard (2012) found that only surface water temperature influenced toxic *Microcystis* dynamics and MC distributions.

Chapter three of my study highlighted the highly interactive nature of the variables which regulate benthic *Microcystis aeruginosa* recruitment, and showed that the relative importance of these can vary among lakes. Even in small shallow lakes such as Rotorua, there is high variability in the rate of recruitment of benthic

Microcystis collected from different habitats. Benthic recruitment was significantly reduced in the near-shore samples compared to the mid-lake samples, and the main difference between these habitat types was the presence of high densities of *A. gracile*. Species from this genus are known to elicit allopathic effects on other algae. Further work is required to investigate whether *A. gracile* inhibits *Microcystis* recruitment. A major difference in this study compared with others reported previously (Misson *et al.*, 2011; Tsujimura *et al.*, 2000) was the high percentage of single cells, as opposed to colonies, recruited to the water column from the sediments. Single cyanobacterial cells may have more immediate access than colonies to growth promoting factors, such as light and nutrients, due to larger surface area to volume ratios, and may therefore regain buoyancy more rapidly and be recruited earlier.

4.2 Recommendations

The results on this thesis have shown that consideration should be given to developing a management strategy for Lake Rotorua to prevent occurrences of major cyanobacterial blooms. Based on the findings of chapter 2 of this thesis it is likely that cyanobacterial blooms will continue to increase in severity and duration, especially under a warming climate (Carey *et al*., 2012). As a next step I suggest that a lake and catchment model, such as an existing coupled hydrodynamic-ecological model (DYRESM-CAEDYM) and a catchment model (e.g., CLUES) could be applied to complement long term monitoring data from the lake. Once developed, the models would enable different management scenarios to be tested, such as reduction in external and internal nutrient loadings, and to examine how changes in other environmental parameters (e.g. temperature,

wind speed, rain) would influence bloom formation. It would also be useful to modify the models so that they incorporate benthic cyanobacteria recruitment, as the results of chapter three showed that these can contribute substantially to cyanobacterial blooms in this lake.

A recommendation for future studies is to examine the chemistry of Lake Rotorua sediment as well as the water column dissolved oxygen dynamics. Biological activity can stimulate the seasonal development of sediment anoxia and lead to the release of phosphate and ammonium to the overlying water by reduction (and dissolution) of iron oxide-hydroxide minerals that sorb phosphorus and from organic material decomposition by heterotrophic bacteria, respectively (Rozan *et al*., 2002; Bostrom *et al.*, 1988). The results of this study provide some preliminary evidence, i.e. the prolonged stratification in 2015 and occasional high nutrient concentrations in surface waters during summer, to suggest that this could play an important role in stimulating cyanobacterial blooms. If this hypothesis is confirmed, strategies such as sediment capping might reduce phosphorus release and possibly minimise cyanobacteria blooms and benthic recruitment from Lake Rotorua sediment. Crosa *et al.* (2013), for example, showed that applications of lanthanum-modified bentonite clay, a sediment capping technique able to bind phosphorus, produced a sharp reduction of more than 80% of the phosphorus concentrations in the water column. These types of measures may be required to avert the impacts of more extreme blooms of cyanobacteria predicted to occur with human-induced global change.

4.3 References

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Chapter 5

Appendix

5.1 Appendix

Appendix Table 3.1: Concentration of *Microcystis* and *Aphanizomenon gracile* (cells mL^{-1}) in near-shore and mid-lake sediment samples.

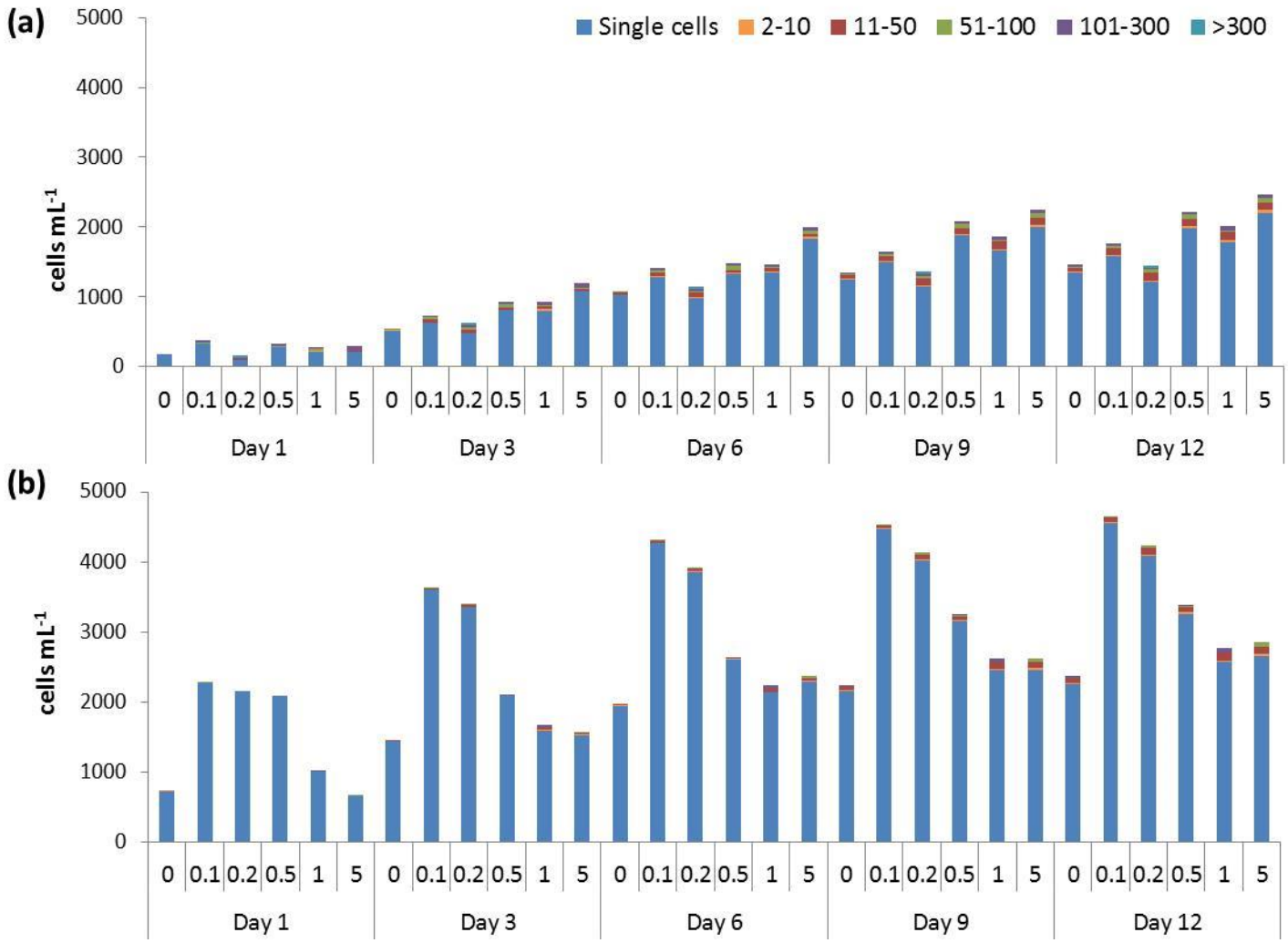
Size class	Single cells	CS1	CS2	CS3	CS4	CS5	Total *Microcystis*	*A. gracile*
No. cell in class	1	2-10	11-50	51-100	101-300	>300		
Near-shore	2153	63	527	608	400	0	3750	24800
Mid-lake	650	20	1287	1140	500	0	3600	0

Appendix Table 3.2: Averaged percentage of recruited *Microcystis* cells in the ammonium (mg L^{-1}), temperature (°C) and light intensity (µmol m^{-2} s^{-1}) experiments at each treatment on day 12 (n=3).

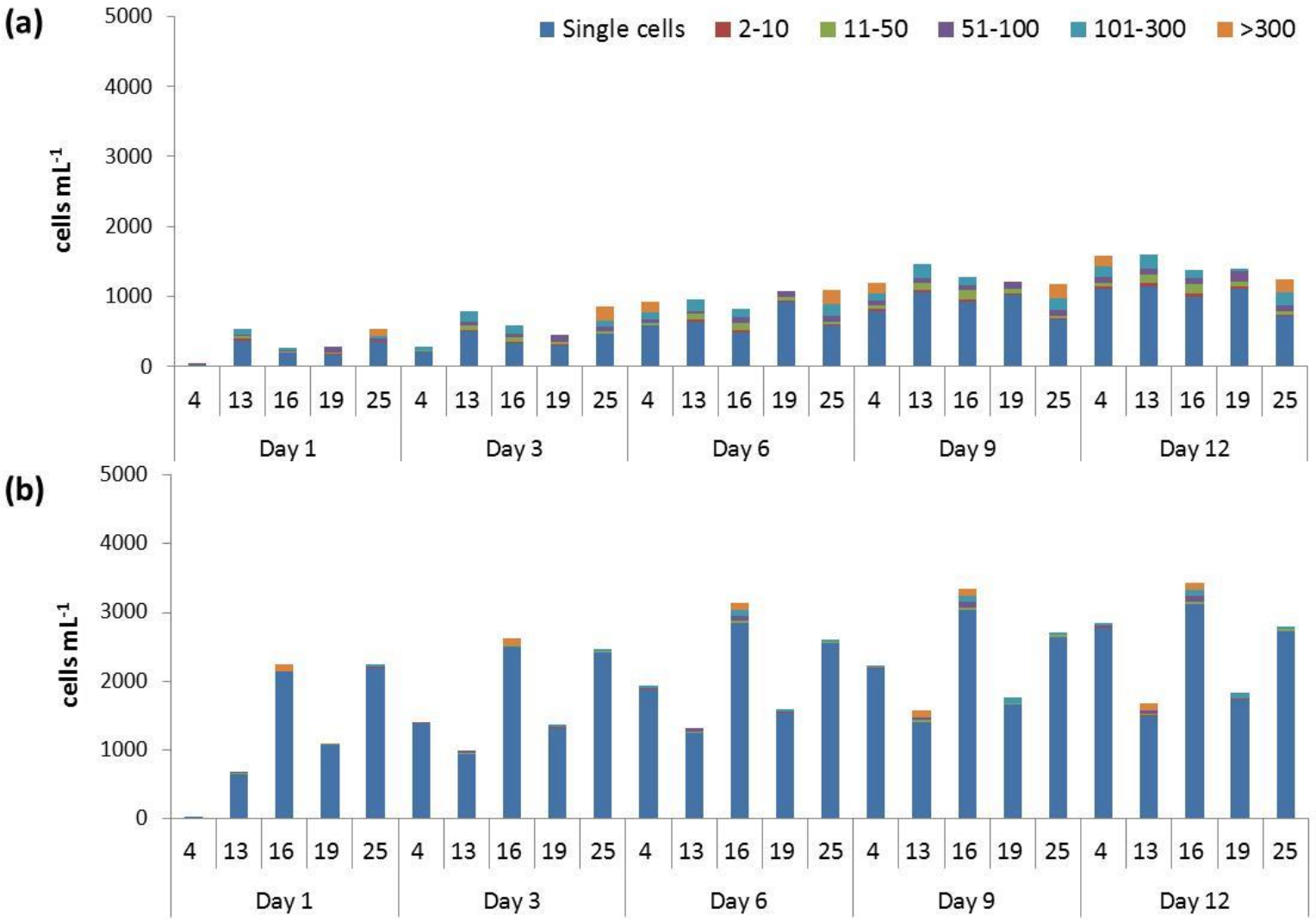
	Near-shore	Mid-lake
Ammonium (mg L^{-1})		
0	19.5 ± 4.0	32.9 ± 7.5
0.1	23.5 ± 1.6	64.7 ± 4.3
0.2	18.6 ± 2.5	58.6 ± 7.6
0.5	29.5 ± 3.1	55.3 ± 10.8
1	29.9 ± 5.4	38.6 ± 4.5
5	32.7 ± 1.9	39.6 ± 4.3
Temperature (°C)		
4	18.8 ± 2.5	37.1 ± 0.2
13	18.9 ± 3.2	21.4 ± 4.9
16	16.4 ± 0.6	43.8 ± 6.0
19	16.7 ± 2.6	24.1 ± 2.8
25	13.5 ± 2.0	37.4 ± 2.5
Light Intensity (µmol m^{-2} s^{-1})		
0	22.2 ± 3.0	49.6 ± 9.6
1.5	19.8 ± 1.1	48.1 ± 7.2
10	14.7 ± 3.6	51.6 ± 14.3
50	14.2 ± 2.1	59.2 ± 6.8
100	16.2 ± 2.4	72.5 ± 7.2

Appendix Table 3.3: Post-hoc pair-wise test (Chi-square test) results for the mid-lake habitat samples in the ammonium, temperature and light intensity experiments. Significant results (p<0.01) are shown.

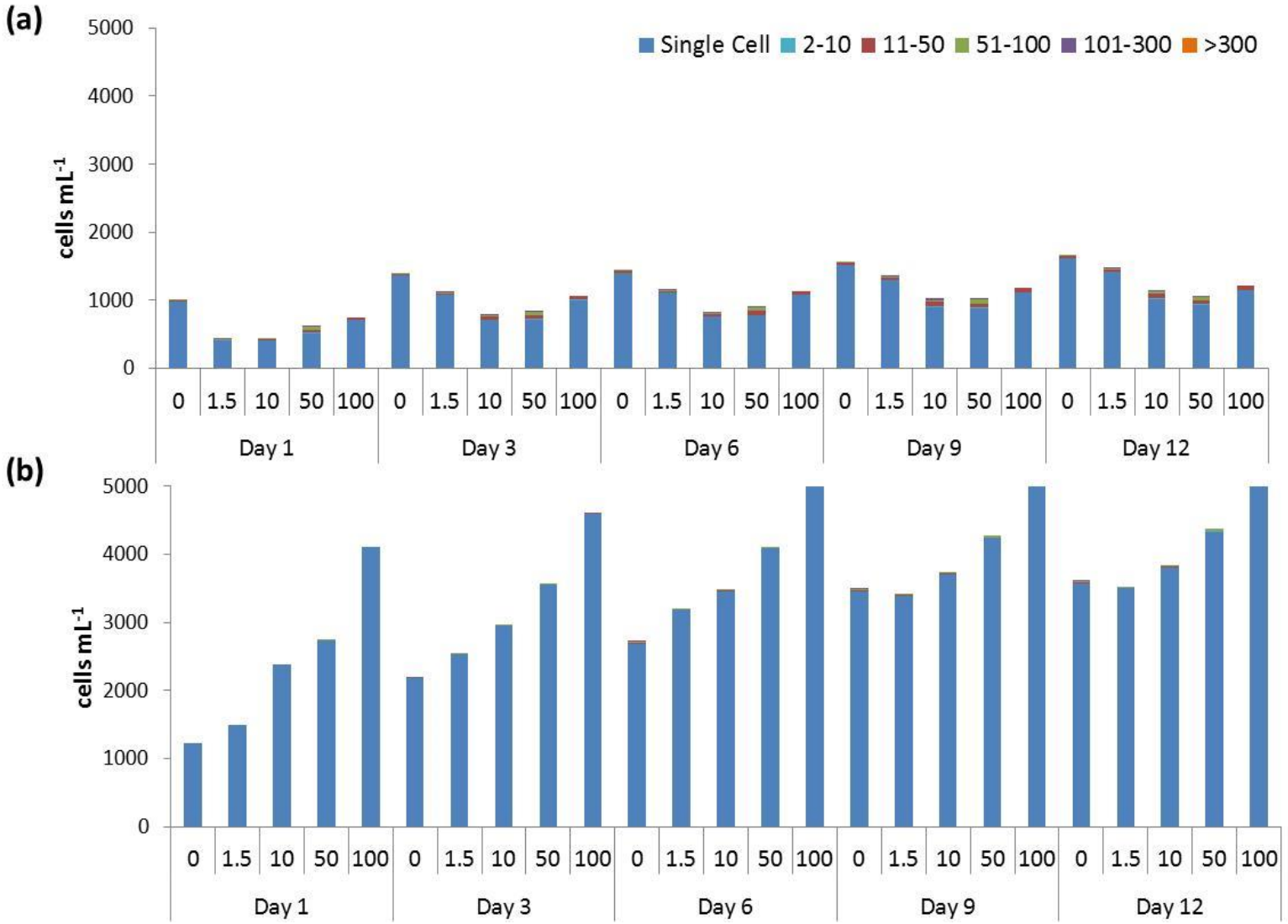
Ammonium treatments (mg L^{-1})	0	0.1	0.2	0.5	1	5
0		<0.001	<0.001	<0.001	-	-
0.1			-	-	<0.001	<0.001
0.2				-	<0.001	<0.001
0.5					<0.01	<0.001
1						-
5						
Temperature treatments (°C)	4	13	16	19	25	
4		-	<0.001	-	<0.001	
13			<0.001	-	<0.001	
16				<0.001	-	
19					<0.001	
25						
Light treatments (µmol m^{-2} s^{-1})	0	1.5	10	50	100	
0		-	-	<0.001	<0.001	
1.5			-	<0.01	<0.001	
10				-	<0.001	
50					<0.01	
100						



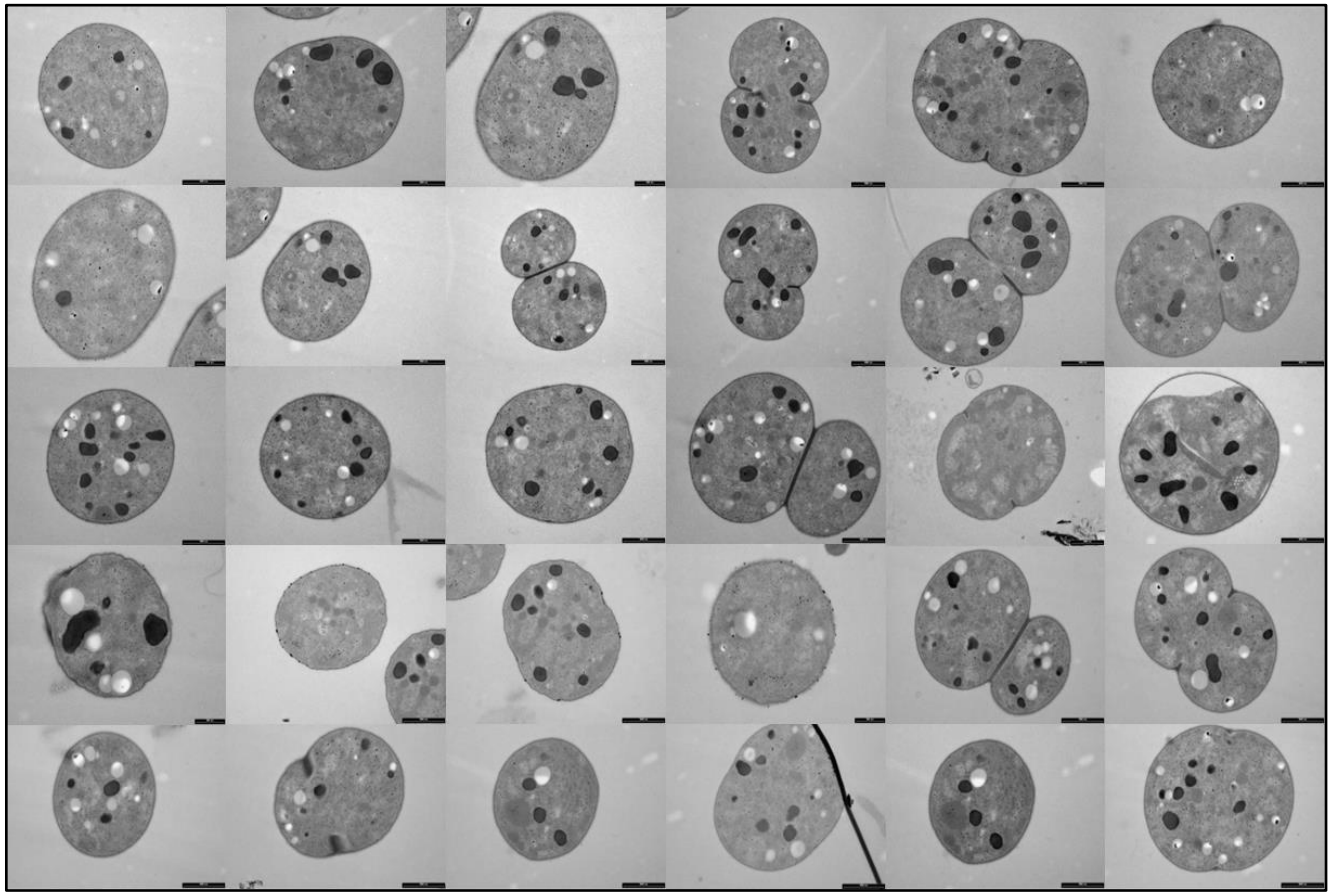
Appendix Figure 3.1: Accumulated average *Microcystis* cell concentrations (n=3), separated into different colony classes, for each ammonium concentration (mg L^{-1}) at each sampling point: (a) near-shore (b) mid-lake.



Appendix Figure 3.2: Accumulated average *Microcystis* cell concentrations (n=3), separated into different colony classes, for each temperature (°C) at each sampling point: (a) near-shore (b) mid-lake.



Appendix Figure 3.3: Accumulated average *Microcystis* cells concentrations (n=3), separated into different colony classes, for each light intensity (µmol m^{-2} s^{-1}) at each sampling point: (a) near-shore (b) mid-lake.



Appendix Figure 3.4: Transmission electron microscopy images of benthic *Microcystis* cells sampled on 10 October 2014 from Lake Rotorua, New Zealand.

Appendix Figure 3.5: Transmission electron microscopy images of benthic *Microcystis* cells sampled on benthic *Microcystis* cells sampled in 26 January 2015 from Lake Rotorua, New Zealand.

Appendix Figure 3.6: Transmission electron microscopy images of benthic *Microcystis* cells sampled on 29 March 2015 from Lake Rotorua, New Zealand.