Are Nutrients the Key Driver in Prompting Dominance of Toxic Cyanobacterial Blooms in a Sub-Tropical Reservoir?

Author

Posselt, Amanda J

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Are nutrients the key driver in promoting dominance of toxic cyanobacterial blooms in a sub-tropical reservoir?

Amanda J. Posselt BSc. (Hons)

Griffith School of Environment
Science, Environment, Engineering and Technology
Griffith University

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For William and your future Brothers and Sisters

Statement of originality

The work presented in this thesis, to the best of my knowledge and belief, is original and my own work except as acknowledged in the text. This material has not been submitted either in whole or in part, for a degree at this or any other University.

Amanda J Posselt BSc. (Hons)

Abstract

Toxic cyanobacteria cause major problems, both for drinking and recreation, within water systems and bulk water storages, worldwide. Many investigations have been conducted to determine how, and why, they proliferate as well as why, and when, they produce toxins. A common assumption is that cyanobacteria grow in response to an increase in water column nutrient availability, but this is an oversimplification.

In a sub-tropical reservoir (L. Samsonvale, South East Queensland, Australia), the toxic cyanobacterium *Cylindrospermopsis raciborskii* has been dominating the phytoplankton community in the summer months for many years. The reason for this is unknown. Lake Samsonvale typically has relatively low phosphorus (P) concentrations, relatively high nitrogen (N) concentrations and *C. raciborskii* can grow without significant nutrient inputs from the catchment or point sources. The relatively high N concentrations in L. Samsonvale mean that it is unlikely to be a key nutrient in promoting dominance of *C. raciborskii*. The low phosphorus (in particular the phosphate) concentrations on the other hand may suggest a reason for *C. raciborskii* dominance in L. Samsonvale. Studies of a non-toxic strain of *C. raciborskii* originally isolated from the northern hemisphere found that a rapid phosphate uptake rate and high phosphorus storage capacity was contributing to its dominance in a phosphate-limited reservoir (Istvánovics et al. 2000).

The aim of this thesis was to characterise the relationship between phosphorus and *C. raciborskii* in L. Samsonvale. To achieve this, three levels of investigation were used: 1. Physiological studies at the species level; 2. Manipulative experiments at the phytoplankton community level; 3. Characterisation of *C. raciborskii* ecology at the whole of system level.

The relationship between *C. raciborskii* and phosphorus was studied using a multilevel approach. Knowledge gained from this allowed detailed investigation of

the relationship between the dominance of this species within the phytoplankton community of L. Samsonvale.

Using continuous culture experiments, the phosphate uptake and storage capacity of two toxic Australian strains of *C. raciborskii* was determined. One of these strains was isolated from the reservoir of interest, L. Samsonvale. P dependent growth rate and toxin production were also quantified. Both strains of *C. raciborskii* had a high maximum phosphate uptake rate (450 – 600 μmol P mg C^{-1} d^{-1}) with a relative low half saturation constant (0.64 μmol P L^{-1}). This study suggests that *C. raciborskii* is capable of taking full advantage of any available phosphate that may be introduced (such as run off) or regenerated within the phytoplankton/bacterial community. The P dependent growth rates were similar for both strains of *C. raciborskii* with a maximum growth rate at the lowest concentration of P tested (0.03 μmol P L^{-1}). Growth rates were lower overall than in other strains of *C. raciborskii*. When *C. raciborskii* cells were starved of P, they produced much more toxin than when they were grown in a nutrient sufficient environment. This indicates that toxin production may be related to a stress response.

Some phytoplankton have been shown to produce alkaline phosphatase. This enzyme cleaves phosphate from organically bound forms, targeting esters, which can be taken up and used by the cell. Since *C. raciborskii* appears to proliferate in phosphate limited systems, its potential to secrete this enzyme, and whether it was capable of growth with an organically bound source of phosphate, were investigated. Alkaline phosphatase activity was detected and *C. raciborskii* was found to be capable of multiplying in a culture media containing only an organic form of P (glucose-6-phosphate, G-6-P). However, the maximum growth rate was lower (~0.13 d^{-1}) when cells were grown in G-6-P compared to phosphate (~0.22 d^{-1}) The ability of *C. raciborskii* to use: 1. Organic P; 2. Rapidly utilise phosphate; and 3. Grow at a maximum rate at relatively low phosphate concentrations, are likely to make *C. raciborskii* a dominant competitor in phosphate-limited systems.

To determine whether *C. raciborskii* has a competitive advantage over other phytoplankton in the P-limited system of L. Samsonvale, *in situ* dialysis tube bioassays were used to test the phytoplankton response to nutrient addition. The dialysis tube bioassay is a novel approach aiming to minimise the confounding problem of artificial nutrient limitation associated with traditional closed bottle bioassays. Samples of the phytoplankton population were subjected to nutrient additions at four different times over a summer period, to test whether a change in phytoplankton species composition (with particular reference to *C. raciborskii*) could be seen after four days. In phytoplankton communities where the proportion of *C. raciborskii* was equal to, or above, 50% (biovolume), a statistically significant increase in *C. raciborskii* dominance occurred when phosphate was added as a daily spike at either of two concentrations (0.32 and 16 μM P). However, *C. raciborskii* dominance decreased when phosphate was constantly added in very high concentrations or when N and P are added together. From the bioassay experiments it can be inferred that *C. raciborskii* has a competitive advantage in L. Samsonvale due to its ability to rapidly take up phosphate. But, when the phosphate concentration is constantly high (>6.4 μmol P L^{-1}), *C. raciborskii* loses this competitive advantage. Analysis of historical data has shown that there is no correlation between periodic nutrient inputs (e.g. rainfall) and an increase in *C. raciborskii* dominance. The mechanisms by which *C. raciborskii* is accessing phosphate within L. Samsonvale were therefore examined.

One theory about how *C. raciborskii* is accessing phosphate in L. Samsonvale is that it comes from nutrient injections in the bottom waters caused by mixing the reservoir using artificial destratification. The concentration of dissolved organic phosphorus (DOP) may also provide *C. raciborskii* with available phosphate. To assess these two hypotheses, the nutrient concentration and phytoplankton cell concentrations throughout the water column were measured, both before and after artificial destratification. The DOP fraction was measured over a summer. Phosphate remained

below detection limits throughout the study, therefore the role of the destratifier in injecting phosphate into the water column was difficult to determine. A difference in phytoplankton distribution was noted with *C. raciborskii* being found at higher concentrations lower in the water column post destratification. In contrast, the other toxic species of cyanobacteria *Microcystis aeruginosa* present in substantial cell concentrations significantly decreased in cell concentrations after the destratifier was turned on. DOP was found to be a significant fraction (total mean 32%) of the total P in the water column of L. Samsonvale and may therefore provide an important source of P for *C. raciborskii* under low phosphate conditions.

This study has shown *C. raciborskii* has adapted to the low concentrations of P in L. Samsonvale to gain a competitive advantage. Reservoir management, particularly in relation to nutrient loads, should take this into account, as efforts to reduce P loads may not lead to a decrease in *C. raciborskii* cell number or phytoplankton dominance.

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Poster presentations

International Conference on Toxic Cyanobacteria (August 2007) Phosphorus addition affects *C. raciborskii* dominance within the phytoplankton community of a sub-tropical reservoir. Rio de Janeiro, Brazil

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List of abbreviations

DIP	Dissolved Inorganic Phosphorus
G-6-P	Glucose- 6-phosphate
DOP	Dissolved Organic Phosphorus
N	Nitrogen
P	Phosphorus
DS LP	Daily Spike Low Phosphate (10 µg L^{-1})
DS HP	Daily Spike High Phosphate (500 µg L^{-1})
CRP	Constant Release Phosphate
N+P	Nitrogen + Phosphorus
P_{cell} µ=0	Mass of P within the cell when growth rate = 0 (µg P mg C^{-1})
P_{cell} µ=max	Mass of P within the cell when growth rate = maximum (µg P mg C^{-1})
R_{max}	Maximum P uptake rate (µg P mg L^{-1} d^{-1})
RC_{max}	Maximum P uptake rate cell normalised (µg P mg C^{-1} d^{-1})
µ	Growth rate (d^{-1})
K_R	Half saturation constant of P uptake (µM P)
P_{cell}ex	Mass of P within the cell at P saturation (16 µM P) (µg P mg C^{-1})
NH_4	Ammonia
NO_X	Nitrates and Nitrites
L	Lake

Chapter 1. Introduction

1.1 Evolution of the Research Question

Toxic cyanobacteria currently cause major problems to the water industry world-wide. A recent report produced by an international committee of scientists highlighted the need for a global research effort due to the limited understanding of the biological, physical and chemical processes that regulate toxic cyanobacterial blooms (Hundrell 2008). From this report, a poor understanding of nutrient loadings and the link with increased frequency of toxic cyanobacteria was identified as a priority of future research. The current study addresses the nutrient priority by determining the relationship between phosphorus (P), its sources and effect on toxic *Cylindrospermopsis raciborskii* blooms in a key resource, drinking water supplies.

Phosphorus was identified as an important nutrient in promoting the dominance of the toxic cyanobacterium, *C. raciborskii*, due to its ability to rapidly take up and store phosphate (Istávnovics et al. 2000). Lake Samsonvale, on the subtropical east coast of Australia, has experienced high summer *C. raciborskii* cell abundance (>50 000 cells mL^{-1}) since the 1980s and typically has low (<0.06 µM) concentrations of phosphate. The link between nitrogen (N) fixation and *C. raciborskii* dominance has been previously investigated in L. Samsonvale (Burford et al. 2006) however, the link with low phosphate availability has been largely ignored. For this reason the relationship between P only and *C. raciborskii* was investigated in the current study.

1.2 Toxic Cyanobacteria

Toxic cyanobacteria are a natural part of the phytoplankton community of many aquatic systems including both marine and freshwater. They occupy both planktonic and benthic environments and cover a broad range of genera (Rippka et al. 2002). In

Australian freshwaters there are four common toxic bloom-forming species; *Cylindrospermopsis raciborskii, Aphanizomenon ovalisporum, Microcystis aeruginosa* and *Anabaena circinalis* (McGregor and Fabbro 2000). All these cyanobacteria vary in their morphology, physiology, geographic and water column distribution.

The toxins produced by toxic cyanobacteria also vary in their structure and potency, but all have detrimental effects to mammalian tissue (Rippka et al. 2002). A challenging issue of cyanobacterial research is that not all blooms of toxic cyanobacteria produce toxins (Hundrell 2008). Genetic characteristics may be the key determining factor in toxicity of cyanobacteria (Kaebernick & Neilan 2001, Schembri et al. 2001). However, the functions of cyanotoxins and the mechanisms controlling their production are poorly understood.

One of the most studied toxic cyanobacteria in freshwaters is *Microcystis aeruginosa* and the toxin it produces (microcystins: MYST) is the only one with a World Health Organisation safe drinking water guideline (1 μg L^{-1} MYST). *M. aeruginosa* is a colonial, planktonic cyanobacteria which forms surface scums. It is found in temperate regions and in many circumstances the installation of destratification systems has resulted in its demise as a summer bloom forming organism (Littlejohn 2004). Most of our current knowledge about how to manage toxic cyanobacteria in Australia comes from studies involving *M. aeruginosa* (Garnett 2005). However, this species is different to other toxic cyanobacteria in terms of the toxin it produces, its morphology and its physiology.

1.2.1 *Cylindrospermopsis raciborskii*

One of the increasingly studied toxic cyanobacteria is the genus *Cylindrospermopsis*. Species of *Cylindrospermopsis* include: *C. raciborskii, C. philippinensis, C. africana, C. cuspis, C. catemaco* and *C curvispora*. These species (*apart from C. raciborskii*) are at present restricted to tropical regions and their toxicity is unknown (Rippka, et al. 2002). Originally *C. raciborskii* (Fig. 1.1) was also thought to be non-toxic, however, an outbreak of human hepatoenteritis on Palm Island, Australia in 1979 (Byth 1980) led to the isolation and identification of the alkaloid cytotoxin, cylindrospermopsin (CYN) from a bloom of *C. raciborskii*.

Figure 1.1 *Cylindrospermopsis raciborskii* filament showing terminal heterocyst

Cylindrospermopsis is a member of the subsection IV (formally Nostocales) of the Phylum Cyanophyceae, which includes filamentous cyanobacteria that divide exclusively by binary fission in one plane (Rippka et al. 2002). The members of this taxonomic section form heterocysts in the absence of dissolved N and are therefore able to fix atmospheric N. Some members of this section including *Cylindrospermopsis* have the ability to produce akinetes, which are resistant to environmental extremes such as cold and desiccation. This means that akinetes can allow the organism lay 'dormant' for extended periods. Morphologically, *C. raciborskii* resembles *Aphanizomenon* and *Cylindrospermum* however:

C. raciborskii has pointed terminal heterocysts (lacking in *Aphanizomenon)* and the presence of conspicuous gas vesicle clusters (never observed in *Cylindrospermum). C. raciborskii* is also known to have a small trichome (straight or coiled) diameter (0.8-4 µm) and uneven division of terminal cells (Rippka, et al. 2002).

C. raciborskii is found in many parts of the world, from the equatorial tropics (Padisak 1997) to the temperate regions of Europe (Fastner et al. 2007) and New Zealand (Wood and Stirling 2003). However, it is difficult to identify in water storages as it does not form surface scums or change water colour as some other toxic cyanobacteria. For this reason, the worldwide distribution of *C. raciborskii* is probably vastly underestimated.

The ability of *C. raciborskii* to produce toxins appears to be restricted to certain parts of the world. Strains isolated from Brazil produce paralytic shellfish poison (saxitoxin) (Lagos et al. 1999 and Molica et al. 2002) while strains isolated from Australia (Ohtani et al. 1992) and more recently from New Zealand (Wood and Stirling 2003) and Thailand (Li et al. 2001) produce cylindrospermopsins. However, strains from Europe (Fastner et al. 2007) and Florida (Yilmaz et al. 2008) appear to be non-toxic.

Cylindrospermopsin (CYN) was originally isolated from *C. raciborskii* (Ohtani et al. 1992) but is now known to be produced by *Anabaena bergii*, *Aphanizomenon ovalisporum*, *Raphidiopsis curvata* and *Umezaezakia natans* (Duy et al. 2000, Li et al. 2001 and Schembri et al. 2001) The CYN isolated by Ohtani et al. (1992) has recently been assigned the new term, 7-epicylindrospermopsin (7-epi-CYN) after a recent

experiment indicating that the CYN isolated from an Israeli strain of *Aphanizomenon ovalisporum* (which is slightly different in chemical structure) be assigned the term CYN (Heintzelman et al. 2002). An analogue of 7-epi-CYN (deoxycylindrospermopsin) has recently been reported, however, it may not contribute to the toxicity of *C. raciborskii* (Norris et al. 1999). Heintzelman et al. (2002) suggest that the CYN produced by Australian strains of *C. raciborskii* is 7-epi-CYN however: this thesis will use the term CYN only. Cylindrospermopsin largely affects the liver and can cause hepatorenteritis in humans (Chorus and Bartram 1999). Cylindrospermopsin also suppresses glutathione and protein synthesis (Runnegar 1995 and Kaebernick and Neilan 2001) and can inhibit plant protein synthesis (Metcalf 2004).

In Australia and more specifically Queensland, the incidence of documented *C. raciborskii* blooms is increasing (Baker 1996, Padisak 1997, Griffiths et al. 1998, Saker and Eaglesham 1999, Saker et. al.1999 and Saker and Griffiths 2000). In a three year study of 47 Queensland weirs and reservoirs, *C. raciborskii* was found in 70% of the storages, 50% of which were seasonally dominated by *C. raciborskii* (McGregor and Fabbro 2000). CYN was recorded in 40% of the storages containing *C. raciborskii* where the mean concentration was 3.4 μg CYN L^{-1}. The reason for *C. raciborskii* dominance and toxin production has been attributed to many factors (Fig. 1.2) including long water residence time, water column stratification, ability to move vertically throughout the water column (buoyancy regulation), high temperature, high incident irradiance, rapid akinete germination, N fixation and low ambient phosphate concentrations (Hawkins and Griffiths 1993, Dokulil and Mayer

1996, Fabbro and Duivenvoorden 1996, Padisak 1997, Istávnovics et al. 2000 and Saker and Griffiths 2002).

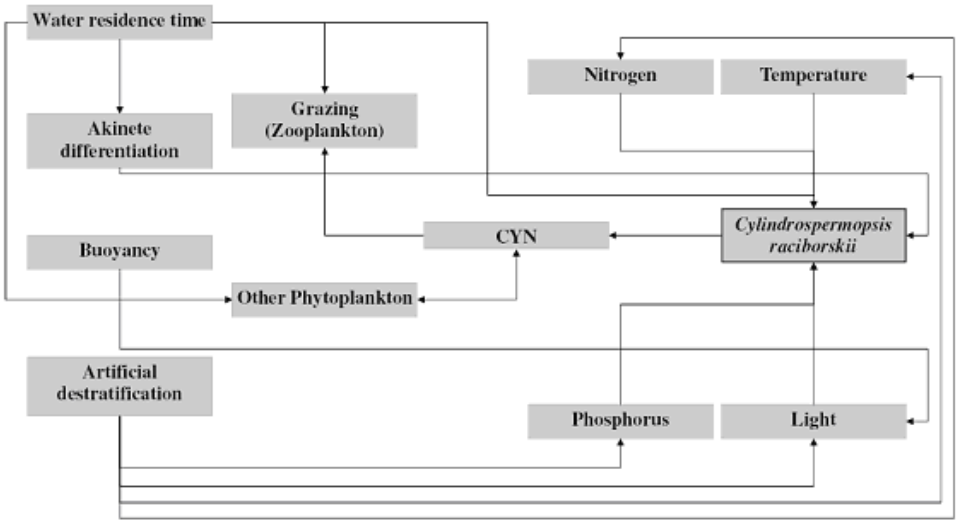

Figure 1.2 Factors that can potentially affect *C. raciborskii* dominance in L. Samsonvale.

1.3 Factors promoting growth of Cylindrospermopsis raciborskii

1.3.1 Temperature

Generally, cyanobacterial dominance in phytoplankton populations shows a positive correlation with temperature (Huszer et al. 2000), and blooms of *C. raciborskii* and *M. aeruginosa* have been linked to elevated water temperatures (Branco and Senna 1994). Previous studies have shown that *C. raciborskii* has a temperature threshold of 25ºC for bloom development (Padisak 1997) however: with the increasing abundance of this species in temperate regions, this conclusion is questionable. For example, *C. raciborskii* filaments were observed in the water column of a temperate lake (northern Germany) when the temperature rose above 15-17º C (Wiedner et. al. 2007). Also, Saker and Griffiths (2000) showed that for seven *C. raciborskii* isolates from northern Australia, six of the seven were able to grow at 20ºC however, the maximum growth rate was between 25ºC and 30ºC. The optimal temperature for growth may be

strain-specific and related to the origin of the organism (Saker and Griffiths 2000). In a study of ten strains of *C. raciborskii* (both toxic and non-toxic from countries including Brazil, Australia, Germany, Hungry, France and Senegal), all ten strains displayed positive net growth in a wide range of temperatures (from 20 to 35 ºC), with maximum growth rates at around 30 ºC (Briand et al. 2004). From these studies it appears *C. raciborskii* has a wide temperature tolerance and may grow in systems with temperatures between 15 and 35 ºC. Whilst this temperature tolerance may provide it with a competitive advantage in temperate systems, it is unlikely to be the key driver in L. Samsonvale where temperatures are relative high (>25 ºC in summer months) and optimal for a range of other phytoplankton species.

Temperature also appears to affect toxin production in *C. raciborskii*. Saker and Griffiths (2000) showed that there is a strong negative correlation between CYN content in *C. raciborskii* and temperature for isolates that produced CYN at temperatures between 25 – 30°C. However, CYN production was not recorded when cultures were grown in temperatures above 35°C, despite some isolates being close to their maximum rate of growth. Some Brazilian strains of *C. raciborskii* produce saxitoxin and production also correlates negatively with temperature (Castro et al. 2004). It is difficult to determine whether temperature directly affects toxin production or whether it induces stress within the organism, which indirectly affects toxin production. Temperatures above 35°C are high for many phytoplankton and would be likely to induce stress.

1.3.2 Light

Light is essential for the survival of all autotrophic cyanobacteria as photosynthesis is their source of energy, however, the optimal light levels for growth of a particular

species varies. Compared to some other cyanobacteria (e.g. *M. aeruginosa*), *C. raciborskii* can tolerate low light conditions. Briand et al. (2004) demonstrated that for ten different strains of *C. raciborskii*, the I_k (saturating light intensity below which growth is light limited) was between 15 and 26 µmol photon m^{-2} s^{-1}, and Shafik et al. (2001) showed an I_k value between 22 and 26 µmol photon m^{-2} s^{-1} for a strain isolated from Lake Balaton, Hungary. The low values of I_k indicate the ability of *C. raciborskii* to grow at low light intensities. Shafik et al. (2001) also showed that maximum growth occurred at an irradiance of 121 ± 13 µmol photon m^{-2} s^{-1}. This work was also supported by Saker (2000) who showed that the highest growth of *C. raciborskii* occurred at light intensities between 50 and 100 µmol m^{-2} s^{-1}. These culture based studies are also consistent with the recent finding that *C. raciborskii* in L. Samsonvale is favoured by water column mixing (artificial destratification) due to its ability to grow at a maximum rate in only moderate light intensities (O'Brien et al. 2008)

The production of toxin by *C. raciborskii* can also be affected by light intensity. In a culture of *C. raciborskii*, the highest cellular CYN concentration was obtained at light intensities well outside the range optimal for its growth (50-100 µmol $m^{-2}s^{-1}$) (Saker 2000). This study showed a negative correlation between CYN concentration (per cell, dry weight) and growth rate, as limited by light intensity. Again it is difficult to determine whether light directly or indirectly affects toxin production. The findings that the highest concentrations of CYN were measured at light intensities well outside optimal for growth may indicate that toxin is produced as a stress response. However, during exponential growth phase, *C. raciborskii* cultures grown under light intensities of 18-75 µmol m^{-2} s^{-1} exhibited a strong linear relationship between light intensity and

both intracellular and extracellular CYN concentrations (Dyble et. al. 2006). The highest recorded toxin production from this study was also at the highest light intensity (140 µmol m^{-2} s^{-1}), approximately 50% more than the light level required for optimal growth rate. In summary, the relationship between light, growth and toxin production in *C. raciborskii* is not clear.

1.3.3 Carbon

Redfield (1958) first pointed out that phytoplankton cellular content correlates with the available elements C, N and P in the molar ratio of 106:16:1, where a change in the ratio would result in growth limitation of one of the nutrients. However, carbon is unlikely to be a limiting factor because cyanobacteria can grow well in low dissolved CO_2 concentrations (Scheffer et al. 1997 and Caraco and Miller 1998). Carbon is one of three essential nutrients likely to limit phytoplankton growth. In aquatic systems dissolved CO_2 is converted to bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) and the relative concentrations of each is determined by a variety of factors such as pH, temperature and pressure. Many photosynthetic organisms, including cyanobacteria, produce the enzyme carbonic anhydrase which catalyses the reversible conversion of CO_2 to bicarbonate (Badger and Price 1994 and Sasaki et al. 1998). External carbolic anhydrase, produced by cyanobacteria, allows these organisms to use HCO_3^- if CO_2 becomes limiting in the surrounding water (Tsuzuki and Miyachi 1989). For this reason carbon is unlikely to be a limiting factor.

1.3.4 Nitrogen

The nitrogen (N) cycle is complex with many forms being biologically available to cyanobacteria (Luque and Forchhamer 2008). Firstly, atmospheric N can be acquired by N-fixing organisms (such as *C. raciborskii*) where it is assimilated into cellular components (e.g. amino acids, protein and chlorophyll). Decomposition of organic

matter (e.g. phytoplankton and fish) results in ammonia being released which is available for phytoplankton growth (Mancinelli 1996). Ammonia can also be converted to nitrate (via the intermediate nitrite) by nitrifying bacteria (e.g. *Nitrobacter* and *Nitrosomonas*) and be used by phytoplankton or it can be subsequently converted back to N_2 gas by the process of denitrification (Zumft 1997). Cyanobacteria can also use certain forms of organic N, including urea and free amino acids (Luque and Forchhamer 2008).

Although there are a number of N forms which cyanobacteria can assimilate, there is generally an order of preference. Ammonium is the preferred source of N for cyanobacteria in many waters (Sahlstein and Sorensson 1989, Dortch 1990 and Presing 1996). For example, in the phytoplankton community of L. Samsonvale, NH_4^+ was the preferred source followed by NO_3^- and then N_2 (Burford et al. 2006). The presence of terminal heterocysts suggest that *C. raciborskii* may have a competitive advantage over other cyanobacteria and micro algae because of its' ability to fix atmospheric N; however, that would only likely to occur in conditions where NH_4^+ and NO_X^- levels were depleted. Burford et al. (2006) found that the ability of *C. raciborskii* to fix N_2 was unlikely in providing it with a competitive advantage in L. Samsonvale. Also, Ferber et al. (2004) found that N fixation rarely supplied more than 2% of the N required by phytoplankton populations in a eutrophic lake (Burlington, USA) dominated by the heterocystous cyanobacteria *Aphanizomenon* and *Anabaena*. This was also confirmed by the low numbers of heterocysts (<3 per 100 cells) observed in these genera. Ammonium was found to be the preferred N source, providing 82–98% of total N.

The production of toxin can also be influenced by N source and concentration. Saker (2000), found that cultures of CYN-producing *C. raciborskii* grown in N-free medium generally produced more toxin than other cultures grown in media containing NH_4^+ and/or NO_X^-. This suggests that toxin may be produced as a stress response, when dissolved N is lacking. In contrast, within cultures of *M. aeruginosa*, cellular concentrations of the toxin microcystin (MYST), positively correlated with cellular N concentration and N uptake rates (Downing et al. 2005). The authors suggest that MYST production occurred irrespective of growth rate and was therefore directly related to N concentration.

Whilst N fixation may be important in the growth of cyanobacteria, the ability to fix N would only give *C. raciborskii* or other heterocystous cyanobacteria a competitive advantage in systems or at times when dissolved N concentrations are low (< 1 μM). The ability of *C. raciborskii* to fix N does not provide it with a competitive advantage in L. Samsonvale (Burford et al. 2006).

1.3.5 Phosphorus

All organisms require phosphorus (P) for growth and metabolism (Bostrom et al. 1988, Currie and Kalff 1984 and Howarth 1988), however, it is not always in plentiful supply. The cycling of P in aquatic environments is essentially the conversion of P from the organic state to the inorganic state and back again (Filippelli 2008). Unlike the N cycle, there are no gaseous states of P however, global aeolian P deposition (aerosols of P such as dust) has been estimated to be approximately 4.5×10^{10} mol P y^{-1} (Graham and Duce 1981) indicating a potentially significant source of P. Phosphorus in general, does not commonly undergo oxidations or reductions and

remains combined with oxygen in its pentavalent state phosphate (PO_4^{3-}) (Filippelli 2008). There are two pools of P in aquatic environments, the large, slowly cycled pool of P contained in the sediment (both runoff and bottom lake sediment) and the small but rapidly cycled pool of biologically active P in the water column (Filippelli 2008). This smaller pool exists predominately as dissolved organic and inorganic P and suspended particulate P (Jones 1998).

Most of the P in the sedimentary pool consists of inorganic P bound to iron (Fe) and this pool is particularity sensitive to redox variations in freshwater systems (Harris et al. 1999). Under oxic conditions, dissolved P readily adsorbs to insoluble Fe (III) and delivery of the P back to the water column is slow. As O_2 availability decreases in bottom waters, Fe (III) reduces to a soluble form (because Fe (III) can be used as a respiratory electron acceptor in anoxic conditions) which releases P as PO_4. This results in a large increase in PO_4 in bottom waters that can diffuse to the overlying water column. The degree and rate of P flux can vary spatially and temporally between lakes, from weeks (Hart et al. 2003), to hours or less (Webster et al. 2001) and appears to be specific to each system.

The current understanding of microbial phosphate uptake at the cellular level stems from early research on *Escherichia coli* in the 1970s. *E. coli* utilises two modes of phosphate uptake, the phosphate inorganic transporter (P_{it}) and the phosphate-specific transporter (P_{st}) (Rosenberg et al. 1977). The P_{st} is used in situations of phosphate deficiency and is a periplasmic permease (transport protein) that comprises a periplasmic substrate-binding protein and three membrane bound components (Ames 1988). This P transport system is ATP driven and controlled by external

concentrations of phosphate. In the cyanobacterium *Synechococcus*, this system is used when external concentrations of phosphate are less than 4 μM P. The P_{it} system is a proton/phosphate symporter and is energetically more favourable than the P_{st} system. It takes part in the phosphate exchange process where phosphate is continuously exchanged between the cell and the surrounding environment. This system is used in relatively high concentrations of phosphate (>20 μM P in *E.coli*).

An essential aquatic food-web pathway, often overlooked by many researchers, is the microbial turnover of DOP to phosphate (Heath 2005). However, some studies have linked DOP concentration with changes in phytoplankton cell numbers. For example, in the Mississippi River, DOP may cycle quickly through microbial turnover and lead to a source of phosphate, changing the N:P ratio and potentially causing a shift in the planktonic community (Rinker and Powell, 2006). Also, two separate studies (Monaghan and Ruttenberg 1999 and Pakulski et al. 2000) observed a positive correlation between chlorophyll *a* and DOP concentration in the water column. These studies highlight the importance of DOP as a source of bioavailable P in aquatic systems.

Some micro-organisms can directly use dissolved organic forms of P (DOP), (Cembella et al. 1984) although most studies report the presence of phosphatases or phosphatase activity (Heath and Edinger 1990, Bjoerkman and Karl, 1994, Hernández et al. 1996 and Śtrojsová et al. 2003) and not the uptake of organic P. Phosphatases are a group of enzymes that cleave the phosphate group from a substrate by hydrolysing phosphoric acid monoesters into a phosphate ion and a molecule with a free hydroxyl group (also known as dephosphorlyation). Alkaline phosphatase (AP) is

one such enzyme which is readily produced by phytoplankton in response to low environmental phosphate concentrations (Smith and Kalff 1982). Synthesis of the AP enzyme results in phosphate being released from DOP sources such as bacterial biomass, nucleotides, phosphocarbohydrates and polyphosphates that are excreted from zooplankton or released from algal cell lysis (Scanlan, 2003). The release of AP can be regulated by extracellular P concentrations in some cyanobacteria such as *M. aeruginosa* (Štrojsová et al. 2005).

1.3.5.1 Current knowledge of the relationship between phosphorus and *Cylindrospermopsis raciborskii*

Istávnovics et al. (2000) studied phosphate dependant growth and phosphate uptake kinetics for *C. raciborskii* in culture and found that this organism appears to be opportunistic with regard to P. *C. raciborskii* has a high excess phosphate storage capacity and high affinity for phosphate uptake. However, the strain used in this study did not produce toxin and was isolated from a temperate lake in the Northern hemisphere (Lake Balaton, Hungary) that is in contrast to *C. raciborskii* isolated from sub-tropical freshwater reservoirs. Therefore applying these findings to toxic strains from other regions should be used with caution.

Other factors such as adaptations to low light and ability to grow at relatively low temperatures are also possible in providing *C. raciborskii* with a competitive advantage over other phytoplankton in L. Samsonvale. The focus of the present study however, is the apparent competitive advantage *C. raciborskii* has with regard to rapid phosphate uptake and storage together with the possibility of producing AP.

1.3.6 Other physico-chemical factors

There are many factors other than temperature, light, carbon dioxide and nutrients that have the potential to affect cyanobacterial dominance (Fig 1.2), including; akinete differentiation, water residence time, zooplankton grazing, phytoplankton buoyancy and mixing, discussed below.

The formation of akinetes (reproductive spores that on germinating, can contribute to future populations) in cyanobacteria is thought to occur in response to nutrient and light limitation (Sili et al. 1994) and hence help the organism evade unfavourable growing conditions and provide a potential inoculum for future growth. There has been little investigation into akinete production of *C. raciborskii* although some studies have investigated the effect of nutrient limitation and temperature on differentiation of akinetes (Kovacs et al. 2003, Padisak 2003 and Moore et al. 2003). Maximum akinete differentiation occurs when there are multiple diurnal temperature fluctuations (from 15ºC to 25ºC), a four fold increase in light intensity (25 – 100 µmol m^{-2} s^{-1}) and high phosphate concentrations (>70 µg L^{-1}) (Moore et al. 2005).

Water residence time indicates the turnover time for a body of water, i.e. how long a particular section of water remains in the lake. In drought conditions where there is little inflow, residence time is quite high, conversely, if there is a high volume of inflow (sufficient water to overflow the dam wall) a lot of water is released and the residence time is relatively low. *C. raciborskii* prefers high water residence times and usually blooms after a lag of approximately three months following a significant inflow (Harris and Baxter 1996). This was evident in an impoundment of the Fitzroy River (Queensland) where exponential growth and bloom formation of *C. raciborskii* coincided with the arrival and retention of the first wet-season inflows into the river

impoundment. (Fabbro and Duivenvoorden 1996 and Bormans et al. 2004). Also, in a reservoir in the semi-arid region of north-eastern Brazil, *C. raciborskii* dominance was linked to low annual rainfall coinciding with no water renewal within the reservoir (Bouvy et al. 1999).

C. raciborskii, like many other phytoplankton, are subject to grazing by zooplankton, however, this relationship is poorly understood. There has also been some speculation as to whether large cyanobacteria are directly consumed by zooplankton in the absence of filter-feeding cladocerans (Knoechel & Holtby 1986). Work and Havens (2003) examined whether zooplankton directly consume large cyanobacteria in a eutrophic lake. It was shown that all macrozooplankton can graze colonial and filamentous cyanobacteria, however, they may prefer diatoms. The structure of the relatively long *C. raciborskii* filament, may result in it being too difficult to consume by zooplankton, however, rotifers and copepods have been shown to cut up and shorten the filaments to edible size for other zooplankton species (Bouvy et al. 2001). Also, the feeding rate of the large ciliate, *Paramecium cf. Caudatum Ehrenberg* increased with *C. raciborskii* cell abundances up to 1 367 cells animal^{-1} h^{-1} indicating that it is able to graze on blooms of *C. raciborskii* (Fabbro et al. 2001). In bloom situations zooplankton may have little choice but to graze on cyanobacteria. This suggests that if the toxins produced by some cyanobacteria are detrimental to zooplankton, a competitive advantage to toxic species may be evident. In a culture of *Microcystis aeruginosa*, microcystin concentrations were up to five times greater when the cells were exposed to the zooplankton *Daphnia magna* and *D. pulex* (Jang et al. 2003). Also, micocystin in a potent inhibitor of protein phosphatase activity in the zooplankton *Daphnia pulicaria* and *Diaptomus birgei* (Demott & Dhawale 1995).

However, in the Gulf of Finland, copepod grazing of blooms of the toxic cyanobacteria *Nodularia spumigena* and *Aphanizomenon flos-aquae* was relatively unimportant in dissipating the blooms (Sellner et al. 1994)

The presence of gas vacuoles gives *C. raciborskii* and many cyanobacteria the ability to regulate their buoyancy (through carbohydrate acquisition via photosynthesis) allowing it to rise and fall in the water column in relation to different light levels and availability of nutrients (Brookes et al. 1999). Light conditions and nutrients appear to be affecting the buoyancy of some cyanobacteria. For example, in *Microcystis aeruginosa*, persistent buoyancy was observed in most cells at adequate N concentrations (100 µM), while the greatest buoyancy loss was observed in the highest P concentration (10 µM) (Brookes & Ganf 2001). This study also showed that when light was in short supply, gas vacuoles tended to accumulate in *M. aeruginosa*. Also in *Anabaena circinalis*, high irradiance increased carbohydrate content which decreased the floating velocity of the filaments (Brookes et al. 1999) Buoyancy in *C. raciborskii* is poorly understood but preliminary studies suggest that *C. raciborskii* is neutrally buoyant (M. Kehoe unpublished data).

Artificial destratification involves the mechanical mixing of water to provide uniform temperatures and dissolved oxygen throughout the water column. Systems such as bubble plume diffusers and surface mechanical mixers are designed to prevent stratification and minimise algal growth. This is achieved by decreasing the temperature in the top layer of water and physically pushing the surface water downwards to where there is less light available. A long term data set was investigated in L. Samsonvale to determine the effect of artificial destratification on

phytoplankton composition (Antenucci et al. 2005). This study indicated that whilst overall chlorophyll *a* was reduced, *C. raciborskii* dominance increased after the destratification system was installed. For phytoplankton that can tolerate low light, artificial destratification appears to provide a competitive advantage and has been demonstrated in some lakes (Burgi and Stadelmann 2002 and Steinberg and Tille-Backhause 1990).

1.4 Aims and Objectives

The aims of this study are to understand the role of P in promoting growth and dominance of the toxic cyanobacteria, *Cylindrospermopsis raciborskii* in L. Samsonvale. Specifically the research hypotheses are:

1. Changes in P concentration (Total P, Dissolved Organic P or Dissolved Inorganic P) affect *C. raciborskii* abundance in L. Samsonvale
2. Increasing DIP concentrations will increase *C. raciborskii* abundance in the mixed phytoplankton community of L. Samsonvale
3. The strategy for superior P utilisation by *C. raciborskii* involves:
    a. Rapid uptake rate of DIP
    b. Ability to grow with DOP source only (DIP-free)

Chapter 2. Experimental Design – the three levelled approach

The natural environment of phytoplankton is highly variable and it is often difficult to isolate or control factors of interest. It is therefore necessary to simplify the system by isolating variables, in order to conduct manipulative experiments. For studies into phytoplankton, a hierarchy of scale can be considered (Table 2.1).

For water resource managers, the level of interest is obviously the highest level (whole system scale e.g. lakes). However, due to the difficulty in manipulating the whole systems, inferences are made from small scale experiments. There is a fine line between validity and applicability of experiments (Hecky and Kilham 1988). For example, culture studies imply validity but not necessary applicability at the whole system level. However, increasing the applicability (to the natural environment) of an experiment means decreasing the control of variables and difficulty with statistical analysis (Hurlbert 1984). The approach in this study is to use three levels of investigation centred on the aim to characterise the P response of *C. raciborskii* in L. Samsonvale. The aim is to combine the results at all three scales which can be used to answer the question of whether phosphate is the key driver in promoting blooms of *C. raciborskii* in L. Samsonvale.

Table 2.1. Hierarchy of scale for phytoplankton studies. Adapted from Garnett (2005) originally from Hecky et al. (1988)

Generic name	System components	Experiment time scale	Usefulness
Organism: eg. Culture	Culture Growth Medium One organism	Hours - days	Control all variables, measure specific species response, artificial environment, may have limited applicability in water body
Enclosure: eg. *in situ* bioassay	Phytoplankton community Additions (eg nutrients) Competition Predation Atmospheric and sediment exchange	Days-Months	Control some variables, observe community interactions, more applicable to the water body
Whole system: eg. lake	Phytoplankton community Competition Predation Atmospheric and sediment exchange Evolutionary change	Months - Years	Actual environmental conditions, unable to see species specific responses, difficult to manipulate

2.1 Whole System (Lake)

Understanding the factors driving phytoplankton growth and species composition often involves correlating field data as a way of determining cause and effect. Unfortunately, correlation does not necessarily mean causation and interactions may be too complex to identify drivers. Correlations can either give us a basis to design manipulative experiments or provide a broad context for experimental results. For example, finding a correlation between P concentrations and *C. raciborskii* does not necessarily mean P concentration affects *C. raciborskii* numbers, but when we combine it with results from field experiments and laboratory cultures we can increase our knowledge of how P affects *C. raciborskii* numbers. Field data (including cell abundances, nutrient concentrations, light intensities and temperature profiles) from

L. Samsonvale will be used to design the experiments at the culture and community level (Chapter 4 and 5).

2.1.1 Lake Samsonvale

Lake Samsonvale (Fig 2.1) was created with the construction of North Pine Dam in 1974. The primary function of the reservoir is to supply drinking water to parts of South-East Queensland, Australia including the areas of Caboolture, Brisbane, Pine Rivers and Redcliffe. It is located just north of Brisbane (27º 15' S, 152º 55' E) and lies 15 km from the coast. It has a storage capacity of 215 000 ML and lies in a 348 km^2 catchment. The mean annual rainfall for L. Samsonvale is 986 mm and the average monthly temperatures are 13.5 ºC (minimum) and 26.0 ºC (maximum) (Bureau of Meteorology, Australia). Generally, L. Samsonvale contains low concentrations of P, variable concentrations of NH_4^+, and relatively high concentrations of nitrate (Burford and O'Donohue 2006). Nitrate concentrations are typically highest in late summer through winter, particularly in surface waters, with concentrations up to 10.7 μM N. In contrast, ammonium concentrations are generally below 3.6 μM N with the exception of summer peaks (November – March) in bottom waters. Phosphate concentrations are generally near detection limits (0.06 μM) with no obvious differences seasonally or between surface and bottom zones.

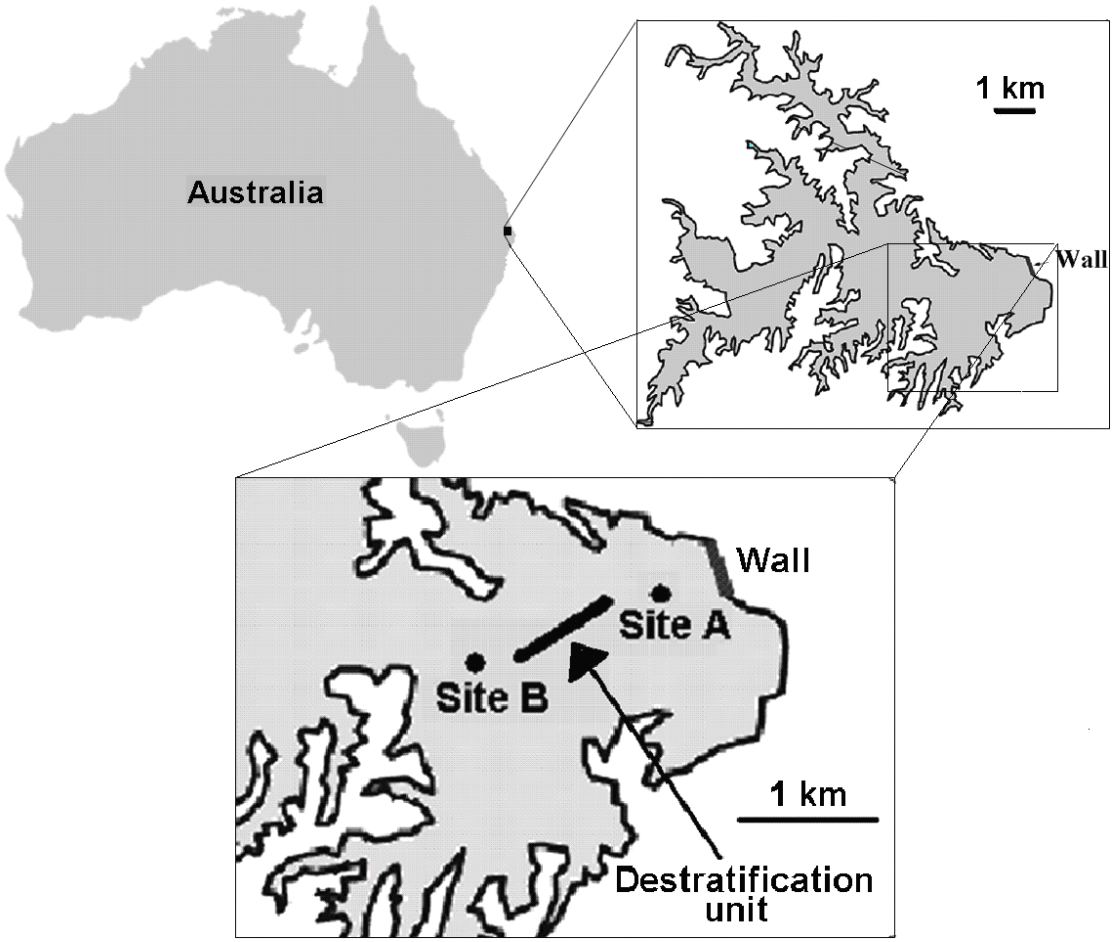

Figure 2.1 Location of L. Samsonvale within Australia and south east Queensland indicating two sampling and experimental sites A and B as well as the destratification unit.

Lake Samsonvale is classed as a warm, monomictic lake that thermally stratifies regularly in the warmer months of the year. It has experienced blooms of *C. raciborskii* in the warmer months of the year since the 1980's. In 1995 a destratification unit was installed in part to control the summer peaks of *C. raciborskii*. However since its installation, *C .raciborskii* cell abundances have increased and are more sustained (Antenucci et al. 2005). The reason for this was largely unknown.

2.2 Enclosure (in situ bioassays)

In situ bioassays were used to determine the 'response' of *C. raciborskii* to nutrient addition within the phytoplankton community. Microcosms or closed bottle bioassays have been used extensively to determine whether a phytoplankton population is nutrient limited (Elser et al. 1990, Sommer 1991, Carter et al. 2005 and Spears and Lesack 2006). A microcosm refers to a small sealable container where lake water (including a representative of the phytoplankton community) is stored therefore providing a simple means of population analysis. A common use of bioassays is to determine phytoplankton nutrient limitation within systems. This is usually by adding certain nutrients (e.g. N and P) and measuring a 'response' (e.g. change in photosynthetic activity). Some literature (Carpenter 1996 and Levin 1992) however, suggests that bioassays are not adequate to determine nutrient limitation because they may deviate in terms of the physico-chemical parameters (such as ambient nutrient concentration) from the original source water.

To combat the problem of artificial nutrient limitation, dialysis tube bioassays (Fig 2.2) were trialled in this study. Dialysis tubing bags provide an environment similar to that in the closed bottle however, the phytoplankton community has access to ambient dissolved nutrients, thus eliminating artificial nutrient limitation. The dialysis tubing (Visking, Medicell International Ltd, London) that were used contained pores (12 000 – 14 000 kDa) big enough for dissolved nutrients to pass through but small enough to retain cyanobacteria other algae and bacteria.

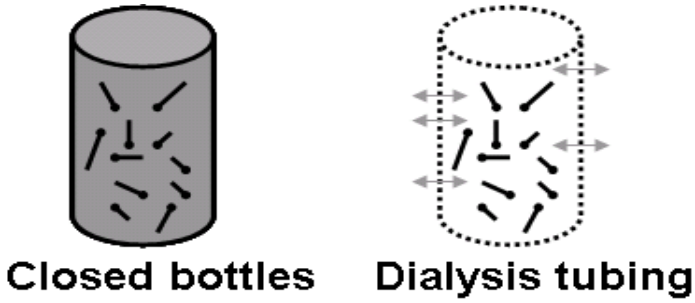

Figure 2.2 Diagram of dissolved material flux in the closed bottle and dialysis tube bags

Whilst dialysis tube bioassays are not a solution to all of the problems associated with microcosms, they may assist in minimising artificial nutrient limitation. Other factors such as biofilm growth or restricting the vertical movement of cells will still be confounding issues. However, as we have seen in Table 2.1, no means of testing is perfect and all have their limitations. Using results from bioassay experiments together with results from culture studies and in the context of system observations, we will be able to achieve solid answers and hopefully provide management advice on *C. raciborskii* control.

2.2.1 Assessment of dialysis tube bioassays as a method for determining nutrient pulse effects within the phytoplankton community

A pilot study aimed at finding a suitable bioassay material that eliminated the problem of artificial nutrient limitation was conducted. This involved comparing dialysis tubing and 600 mL plastic PET bottles that were attached to wire racks and deployed in L. Samsonvale. Each tube and bottle was filled with water from L. Samsonvale (Site A Fig. 2.2) collected from depths ranging from the surface to 7 m. A rack containing five dialysis tubing bags and five bottles was deployed at midday on day 1. The wire racks containing the tubes and bottles were covered in shade cloth (allowing 50% of ambient light through) and suspended just below the surface of the water

attached to poles rammed into the sediment. The racks were in approximately 1 m of water, 10 m from the shore line in a protected bay of L. Samsonvale.

The bioassays were incubated over five days from 30 January to 3 February 2005. Water samples for PHYTO-PAM analysis (5 mL) were taken before racks were deployed, and then at 24, 48, 72 and 96 h. Temperature (eTemperature) was logged in one of the control bottles every 30 mins for the duration of the experiment. A light profile (Licor 4-pi sensor) of the adjacent water column was measured on day one.

The photosynthetic yield response of phytoplankton in the dialysis tubing fluctuated from day to day (Fig 2.3). In contrast, there was a consistent decrease in photosynthetic activity over 96 h in the closed bottles. The continued decrease in photosynthetic activity observed in the bottles may be attributed to nutrient deficiency, whilst the fluctuating yield in the dialysis tubes was similar to the fluctuating yield in the ambient water and may reflect fluctuating nutrient concentrations. Given the difference in yield of the bottles and the tubes, closed bottle bioassays may only be useful in determining phytoplankton nutrient limitation for short incubations (maximum 40 h).

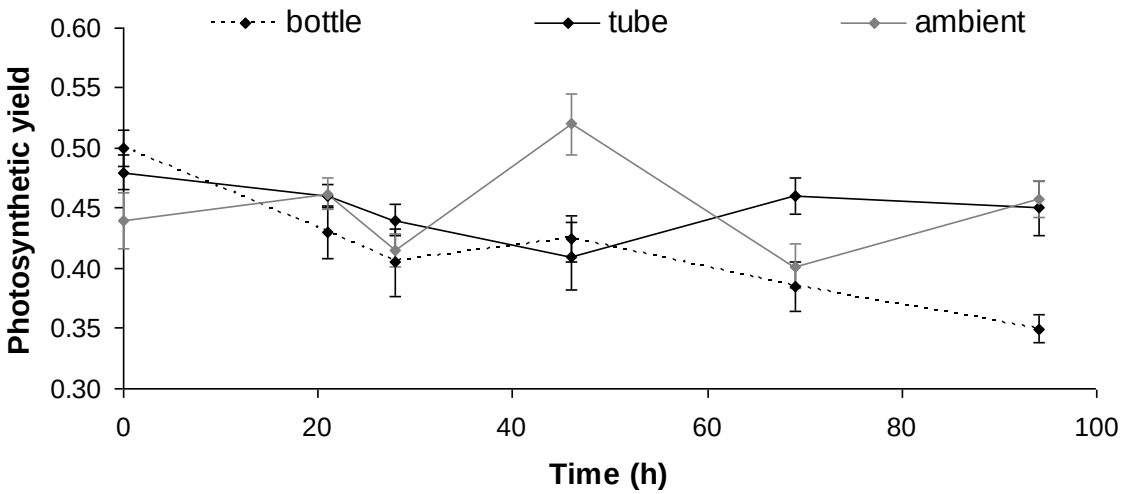

Figure 2.3 The photosynthetic activity of the phytoplankton community in L. Samsonvale under different conditions; within closed bottles (bottle), within dialysis tubing (tube) and in ambient conditions (ambient).

The results of this pilot study suggests that dialysis tube bioassays provide the enclosed phytoplankton community with nutrient concentrations closer to ambient whilst the closed bottle bioassays show a decline after 48 h. Therefore, the results suggest that if *in situ* experiments are run for longer than 48 h dialysis tube bags provide a better control than the closed bottles. The purpose of the *in situ* bioassays is to test the change in phytoplankton species distribution with regard to that of *C. raciborskii* dominance when P is added. *C. raciborskii* is thought to have a relatively low growth rate (approx 0.3 d^{-1}) which means *in situ* bioassays run for 40 h only may not be long enough to measure changes in cell abundance. For this reason dialysis tube bags were used to conduct four day *in situ* bioassay experiments (Chapter 4).

2.3 Organism (cell culture)

Using cell cultures in laboratory conditions allows us to see specific responses at the species level. By controlling all variables, specific questions can be set and cultures can be manipulated accordingly. This study will test two strains of toxic *C. raciborskii*

isolated from different regions in Australia, AWT/205 (isolated from Sydney: South East Australia) and NPD strain (isolated from L. Samsonvale). Studies have shown that Australian strains of some species of cyanobacteria are genetically quite different to the same organism isolated from other countries (Neilan et al. 2003). This emphasises the need for research using local strains.

There are two common ways to grow cyanobacterial cells in the lab; batch culture and through-flow culture, both having their advantages and disadvantages. Batch culture is where growth media is inoculated with a small number of cells, which are then incubated and allowed to follow the normal growth curve including lag, exponential, stationary and death phase. By far the greatest advantage of batch culture is its relative simplicity however, the response of cyanobacteria to treatments can vary depending on the growth phase they are in. For example cells may be smaller, uptake rates faster and metabolite biosynthesis reduced in cells found in exponential phase when compared to stationary phase. Through-flow cultures, on the other hand allow cells to be maintained in exponential phase indefinitely. Through-flow cultures involve a chamber of cells where growth media is constantly added and culture is constantly removed. By changing the rate at which media is added and the composition of incoming media, the growth rate of the cells in the chamber can be changed. Through-flow cultures differ from conventional chemostats in that the growth rate is set by the concentration of incoming media and the dilution rate instead of the dilution rate alone. Chapter 3 will discuss results from a series of batch and through-flow culture experiments where the direct effect of changing P source and concentration on *C. raciborskii* will be determined.

Culture studies on their own are not adequate to provide meaningful answers to ecological questions. They are too far removed from the 'natural' system where factors such as wind velocity and direction, temperature, light intensity and rainfall may all affect experimental results. To combat this, the culture studies will be used in conjunction with the bioassay experiments which will test 'responses' of *C. raciborskii* to specific manipulations within the phytoplankton community.

2.4 The advantage of a multi-levelled approach

The advantages of a multi-levelled approach (laboratory cultures, bioassays and whole of system) are numerous. Results from laboratory cultures (high replication and control) can be compared to bioassays (less replication and control, more closely related to the environment) to determine specific, 'semi-natural' results. This can then be analysed within the context of the whole-of-system.

Chapter 3. Profiling *Cylindrospermopsis raciborskii* in Lake Samsonvale

3.1 Introduction

Lake Samsonvale has had high *C. raciborskii* cell abundances since the mid 1980s (Harris and Baxter 1996 and Antenucii et al. 2005). The reason for this remains unknown therefore a sampling program was designed to determine whether correlations could be observed between *C. raciborskii* and a range of physico-chemical parameters including; artificial destratification, nutrients including DOP, temperature, light intensity, rainfall and lake level.

Observing correlations between physico-chemical parameters to determine ecological relationships may provide useful insight into whether a relationship is worth investigating.

3.2 Methods

3.2.1 Profiling *Cylindrospermopsis raciborskii* and nutrients before and during artificial destratification in Lake Samsonvale

The destratification unit in L. Samsonvale is typically turned on in the spring (September/October), in response to a decrease in dissolved oxygen (<2 mg L^{-1}) in the bottom waters. At the time of this experiment the artificial destratifier was activated on 10 October 2005. To determine whether the destratifier was affecting nutrient concentrations and cell abundances of *C. raciborskii*, water column profiles were collected before and after the destratifier was turned on. There were two sampling times: pre-destratification (14 and 5 d before) and post (1, 7 and 21 d after). The water depth at time of sampling was 18 ± 0.3 m. On each sampling day, three replicates of 3 L samples were collected from depth profiles in L. Samsonvale. Samples were collected using a water sampler (Van Dorn) from the surface, 2, 5, 7, 10, 15 and 18 m and processed for dissolved nutrient concentration and cell abundances of

C. raciborskii and *M. aeruginosa* (sections 3.2.1.1 and 3.2.1.2). At the time of sampling there was a bloom of *M. aeruginosa* therefore it was counted as well as *C. raciborskii*.

3.2.1.1 Analysis of total and dissolved nutrients

A number of experiments were conducted throughout the study where nutrient analyses were required. These analyses were performed by Queensland Health Scientific Services (QHSS) and Technical Services at Griffith University. Samples for dissolved inorganic nutrients (Phosphate, Nitrates/nitrites and Ammonicial nitrogen) were syringe filtered through a 0.45 µm syringe filter upon collection and stored frozen until analysis which was performed simultaneously using an automated LACHAT 8000QC flow injection system (0.032 µM). Phosphate analysis involves the ascorbic acid reduction of phosphomolybdate (Ref.4500-P). Nitrate/nitrite analysis involves the cadmium reduction of nitrate to nitrite by the diazotisation of nitrite with sulphanilamide and coupling with N-(1-nathyl)ethylenediamine dihydrochloride. (Ref.4500-NO_3-I). Ammonicial nitrogen analysis involves the production of indophenol blue colour complex (Ref.4500-NH_3H) (American Public Health Association 1995). Samples for total N (detection limit 20 µg L^{-1}) and P (2 µg L^{-1}) were collected directly into 30 mL vials and stored frozen until analysis where they were digested using simultaneous persulfate procedure as described by Hosomi and Sudo (1986). Samples for dissolved organic phosphorus (DOP) were collected as per the dissolved nutrient samples (10 mL filtered through a 0.45 µm syringe filter) and analysed as per the total nutrient samples (persulfate digestion). The samples were then analysed in the same way as the dissolved fractions described above. Particulate P and C concentrations were determined by filtering known volumes of sample (lake

water or culture media, 100-400 mL) through 0.75 µm glass fibre filters (Whatmann) under gentle vacuum. For the P samples, filter papers were digested and analysed as for total P. For the particulate carbon samples filter papers were analysed with an Elemental Analyser (Eurovector EA 3000, Milan Italy). This system uses the Durmas method where gas types produced by the combustion are time separated by gas chromatography, detected by a supersensitive Thermo Conductivity Detector and peaks are integrated by Callidus software. Standard quality assurance and quality control protocols were employed to ensure the quality of results.

3.2.1.2 Counting phytoplankton cell numbers using microscopy

C. raciborskii trichomes (later converted into cells per mL, see below) were counted on a haemocytometer (Neubauer New Improved) under x 400 magnification with phase contrast microscopy (Leica). When cell abundances were low, a Sedgewic-Rafter counting chamber (Graticules, UK) was used as it samples a much larger volume. A minimum of 23 trichomes were counted for each sample ensuring a counting error estimated with a 95% confidence limit to be less than ± 30% of the results obtained (Hotzel and Croome 1999). The precision of this method is based on the Equation 1.

Equation 1. Couting error $\% = \frac{\sqrt{2}}{(100N)}$

N= number of units counted, assuming a Poisson distribution following any sub-sampling steps (Hotzel and Croome 1999).

The number of cells per trichome was previously determined for each strain. A minimum of 100 trichomes were analysed and a mean and standard deviation was obtained. For the cultures there were 12.3 ± 0.7 cells per trichome for the NPD strain 12.6 ± 0.5 cells per trichome for the AWT/205 strain.

Counting field samples involved quantifying *C. raciborskii* as well as the other cyanobacteria including *Microcystis, Planktolyngbya, Aphanocapsa/Cyanodictyon, Aphanizomenon* and *Anabaena* as these were the most dominant in L. Samsonvale. Lake water samples, to determine cell abundances, were fixed with 1 % lugols iodine solution to ensure there was no growth/degradation during transport and storage. Green algae and diatoms were also counted however, not determined down to the genus level. To determine the biovolume of cyanobacteria the following values were multiplied by the cell abundances; *C. raciborskii* 22.4 μm^3 $cell^{-1}$, *M. aeruginosa* 71 μm^3 $cell^{-1}$, *Planktolyngbya* 2.3 μm^3 $cell^{-1}$, *Aphanizomenon* 43 μm^3 $cell^{-1}$, *Anabaena* 65 μm^3 $cell^{-1}$, and *Aphanocapsa/ Cyanodictyon* 0.5 μm^3 $cell^{-1}$ (McGregor pers com, DNRW). The biovolumes of green algae and diatoms were determined on an individual basis where cell length, width and approximate height were used to calculate volume.

3.2.2 Profiling a *Cylindrospermopsis raciborskii* summer bloom in Lake Samsonvale

Regular water sampling was done fortnightly from 25 October 2007 to 5 March 2008. Samples were collected by A. Posselt and staff from the water authority, SEQ Water. Surface and bottom water samples were collected for nutrients (dissolved inorganic, total and dissolved organic N and P), phytoplankton cell abundance, silica and iron concentrations. Silica and iron were collected to give a greater water quality context

to other previously investigated parameters (phytoplankton cell abundance and nutrients). Three replicates from both the surface and bottom waters were collected fortnightly at approximately 10 am. At a nearby weather station (site B Fig. 2.2 section 2.1.3.1) water temperature profiles and air temperature was logged every 20 to 30 min and solar radiation (in watts per m^2) was collected every 20 min and the data was converted to µmol photon s^{-1}. All data except DOP concentrations was obtained with permission from SEQ Water. Rainfall data was obtained from the Bureau of Meteorology website (www.bom.gov.au) and values from the Samford site (27.36°S 152.89°E) were used.

3.2.3 Phytoplankton phosphate uptake and regeneration in Lake Samsonvale

Phosphate uptake rate and regenerations experiments, using the radioisotope ^{33}P, were carried out on phytoplankton communities from L. Samsonvale and L. Borumba (Fig 3.1). At the time of experiments *C. raciborskii* cell abundances in L. Samsonvale were low (around 10 000 cells mL^{-1}), however, nearby L. Borumba had *C. raciborskii* cell abundances exceeding 100 000 cells mL^{-1}.

Lake Borumba (26.46ºS and 152.66ºE) is situated approximately 100 km north of L. Samsonvale and is subject to a very similar climate. The mean annual rainfall for this lake is 1176 mm compared with 986 mm for L. Samsonvale. The average monthly temperatures for L. Borumba are 13.6 ºC (minimum) and 26.7 ºC (maximum) compared with 13.5 ºC and 26.0 ºC for L. Samsonvale (Bureau of Meteorology, Australia). Lake Borumba has a capacity of 46 000 ML and lies in a 466 km^2 catchment. The phytoplankton population is also similar to L. Samsonvale which

is dominated by cyanobacteria (M. Kehoe pers comm) with the main genera being *C. raciborskii, Planktolyngbya, Cyanodictyon* and *M. aeruginosa.*

Figure 3.1 Map showing location of L. Borumba in relation to L. Samsonvale and Brisbane

Approximately 20 L of water (0-3 m integrated sample) was collected from L. Samsonvale (19 February and 5 March 2008) and L. Borumba (9 and 19 March 2008) for phosphate uptake and regeneration experiments. Sub-samples for total and dissolved nutrients and cell abundances were also collected (sections 3.2.1.1 and 3.2.1.2). The bulk water was transported back to the lab for the uptake and regeneration experiments which were conducted approximately 5 h after collection.

To determine the phosphate uptake rates of phytoplankton communities from L. Samsonvale and L. Borumba, the radioisotope ^{33}P was obtained in 0.025 µCi aliquots (Sigma-Aldrich, Castle Hill, Australia). Pulses of ^{33}P and ^{31}P in

concentrations ranging from 3.2 x 10^{-6} to 0.64 µM P L^{-1} (combined concentration) were used to determine maximum P uptake and half saturation constants (Table 3.1).

Table 3.1 Amount of ^{33}P and ^{31}P and percentage of ^{33}P in phosphate uptake

^{33}P µmol	^{31}P µmol	% ^{33}P to combined ^{31}P + ^{33}P
1.28 x 10^{-6}	0	100
1.28 x 10^{-6}	1.28 x 10^{-5}	4.0
1.28 x 10^{-6}	1.28 x 10^{-4}	0.4
1.28 x 10^{-6}	1.28 x 10^{-3}	0.04
1.28 x 10^{-6}	0.032	0.004
1.28 x 10^{-6}	0.096	0.0013
1.28 x 10^{-6}	0.16	0.0008
1.28 x 10^{-6}	0.32	0.0004
1.28 x 10^{-6}	0.64	0.0002

Lake water 500 mL was placed in PET plastic bottles and incubated at 28°C under constant illumination (80 µmol photon m^{-2} s^{-1}) for 20 minutes to allow cells to become photosynthetically active. Bottles were then spiked with ^{33}P and ^{31}P and a 3 mL sub-sample was taken at the following times: 0, 10, 20, 30 and 45 seconds, 1 1.5, 2.5, 5, 7, 10, 15 min. The 3 mL sub-samples were immediately filtered through 0.6 µm membrane filters (Nucleopore) and placed in scintillation vials with 0.5 mL deionised water. 2.5 mL scintillation fluid was then added to each vial was gently mixed. Each vial was read within the following 5 h with a liquid scintillation analyser (Tri-Carb 2100TR, PerkinElmer, U.S.A). Due to the relative short half life of ^{33}P (24 h) every 2-3 h quenching standards were run to correct conversion from counts per minute (cpm) to disintegrations per minute (dpm). The dpm was converted to a particulate P concentration which allowed uptake rates to be determined (Equation 2).

Equation 2. [P] (pmol) = $\frac{dpm}{2.2(10^6)} \times 0.25 \times \frac{1}{\%^{33}P}$

[P] = concentration of P (sample i.e. filtrate)
dpm = disintegrations per minute of sample (measure of radioactivity)
$\%^{33}P$ = Percentage of ^{33}P from Table 2.2
Conversion factors: 2.2×10^6 = number of dpm in 1 μCi (radioactivity of ^{33}P)
0.25 = pmol P in 1 μCi

Concentration of P within the cell was plotted against time for each of the P concentrations in Table 3.1 to determine uptake rate at given concentrations. This allowed maximum uptake at given concentrations to be determined (Equation 3) and a phosphate uptake rate curve for external P concentrations to be calculated (Equation 4). The radioactivity of ^{33}P from filter papers containing phytoplankton cells was measured and converted to a phosphate concentration within the cells. This was plotted against time to give a curve and the initial straight line was calculated as the maximum phosphate uptake rate.

Equation 3.

Maximum uptake rate (pmol P mL^{-1} min^{-1}) = $\frac{(\Delta [P] / \Delta T)}{V}$

Δ [P] = change in P concentration (pmol L^{-1}water)
Δ T = change in time (min)
V = Volume

Equation 4.

Uptake rate (pmol P mL^{-1} min^{-1}) = $\frac{(P_{max} \text{ x } [P])}{([P] + K_s)}$

P_{max} = maximum rate of uptake (pmol mL^{-1} min^{-1})
[P] = concentration of P (pmol P L^{-1})
K_s = half saturation constant (pmol P L^{-1})

To determine phosphate regeneration rates of phytoplankton communities of L. Samsonvale and L. Borumba ^{33}P 'chaser' experiments were performed. This involved the addition of a saturating concentration of ^{31}P after the phytoplankton had taken up all available ^{33}P and was based on the study by Nowlin et al. (2007) (originally described by Hudson and Taylor 1996). The theory suggests that as 'cold' ^{31}P is added and taken up it replaces ^{33}P within the cells, which is then released. Measuring the ^{33}P concentration within the cells over an extended period of time allows the calculation of phosphate release rates and hence regeneration time. Cold ^{31}P was added to the 'labelled' cells at a concentration of 1 000 μM P (later reduced to 100 μM P which was still believed to be saturating) and samples were taken over the following 48 h (Table 3.2). Samples (3 mL) were taken and processed in the same way as described in Section 3.2.1. The two L. Samsonvale experiments were run for approximately 24 h because it was initially thought that this was ample time to see regeneration based on the method proposed by Nowlin et al. (2007), which suggests experiments run for 8 h.

Table 3.2. Experimental design of regeneration rate experiments using phytoplankton populations from Lakes Borumba and Samsonvale. Y= sample taken. Time 0 = just before 'cold' ^{31}P was added

Sample times	Samsonvale		Borumba	
	19 February	5 March	9 March	19 March
0	Y	Y	Y	Y
15 min	Y	Y	Y	Y
1 h	Y	Y	Y	Y
2 h	Y	Y	Y	-
3 h	Y	Y	Y	-
4 h	Y	-	-	-
5 h	Y	Y	-	-
6 h	Y	-	-	-
7 h	Y	-	-	-
8 h	Y	-	-	-
12 h	Y	-	-	-
19 h	-	Y	-	-
24 h	Y	Y	Y	Y
48 h	-	-	Y	Y

The release rates of each experiment were determined from the time when ^{33}P concentration started to decrease and calculated using Equation 5. The results from these calculations were then used to determine total P regeneration rate (Equation 6).

Equation 5

$$^{33}P \text{ release rate } (\mu M\ P\ d^{-1}) = \frac{R_S \times P}{R \times T}$$

R_S = radio activity of the sample (dpm)
P = concentration of total P in sample (μM)
R = radio activity of the added P (dpm)
T = time (d)

Equation 6.

$$\text{Total P regeneration rate } (\mu M\ P\ d^{-1}) = \frac{^{33}P_{RR} \times [TP]}{^{33}P \text{ intl. act.}}$$

$^{33}P_{RR}$ = ^{33}P release rate from equation 15
[TP] = total concentration of P added to sample (μmol)
^{33}P intl. act. = initial activity of ^{33}P (dpm)

Uptake rate as a function of P concentration was also determined for L. Samsonvale. Using a combination of ^{33}P and cold ^{31}P additions and assuming non-biased uptake of both ^{31}P and ^{33}P when added simultaneously, maximum P uptake with specific P concentrations (3, 30, 300, 3000 *p*M 0.03, 0.16, 0.32 μM) were determined.

3.2.4 Analysis of general physico-chemical water quality parameters

Physicochemical parameters including concentrations of silica, manganese and iron as well as pH, conductivity, total suspended solids, fluorescence, dissolved oxygen and redox potential (ORP) were measured in conjunction with most field sampling. All sampling was collected by SEQ Water and analysed either on site or at QHSS. Silica was determined colormetrically using the molybdosilicate method (Eaton et al. 2005 pg 4-166). Manganese was determined photometrically using a persulfate digestion

method (Eaton et al. 2005 pg 3-19). Iron was determined using the flame-acetylene method (Eaton et al. 2005 pg 3-17). Other parameters (as above) were measured *in situ* in L. Samsonvale using a YSI 6920 (YSI Incorporated, U.S.A)

3.2.5 Statistics

All statistical analyses was performed using the data analysis function in Microsoft Excel (version 2007, Vista). Prior to analysis, the BIONMDIST function (McNemar's test) was used to test for homogeneity of variances. Where applicable, standard deviations bars were used in all graphical representation. Correlation coefficients were used to assess the significance of a relationship between parameters.

3.3 Results

3.3.1 Profiling *Cylindrospermopsis raciborskii* and phosphorus in relation to artificial destratification

There was an increase in *C. raciborskii* cell abundances post destratification and a decrease in *M. aeruginosa* (Fig 3.2). Prior to the destratifier being switched on, *M. aeruginosa* was in relatively high concentrations (43 000 cells mL^{-1}) and within a week had decreased by approximately 80%. After the destratifier was switched on, *C. raciborskii* cell abundances were higher than before the destratifier was turned on and highest in the 7 m sample (5 000 cells mL^{-1}).

Phosphate remained below the detection limit (0.064 µM) throughout the study. Prior to artificial destratification, ammonical N concentrations ranged from approximately 0.7 to 2.8 µM N throughout the water column and after, concentrations as high as 6.2 µM N were observed in bottom waters (Fig. 3.2). As with NH_4^+ concentrations after the destratifier was turned on, NO_X concentrations were highest at 18 m however,

there was no significant difference ($p>0.05$) between NO_X distribution before and after the destratification was activated (Fig 3.2). There was no statistical difference ($p>0.05$) between the three samples post-destratification with regard to nutrient distribution through the water column and concentration.

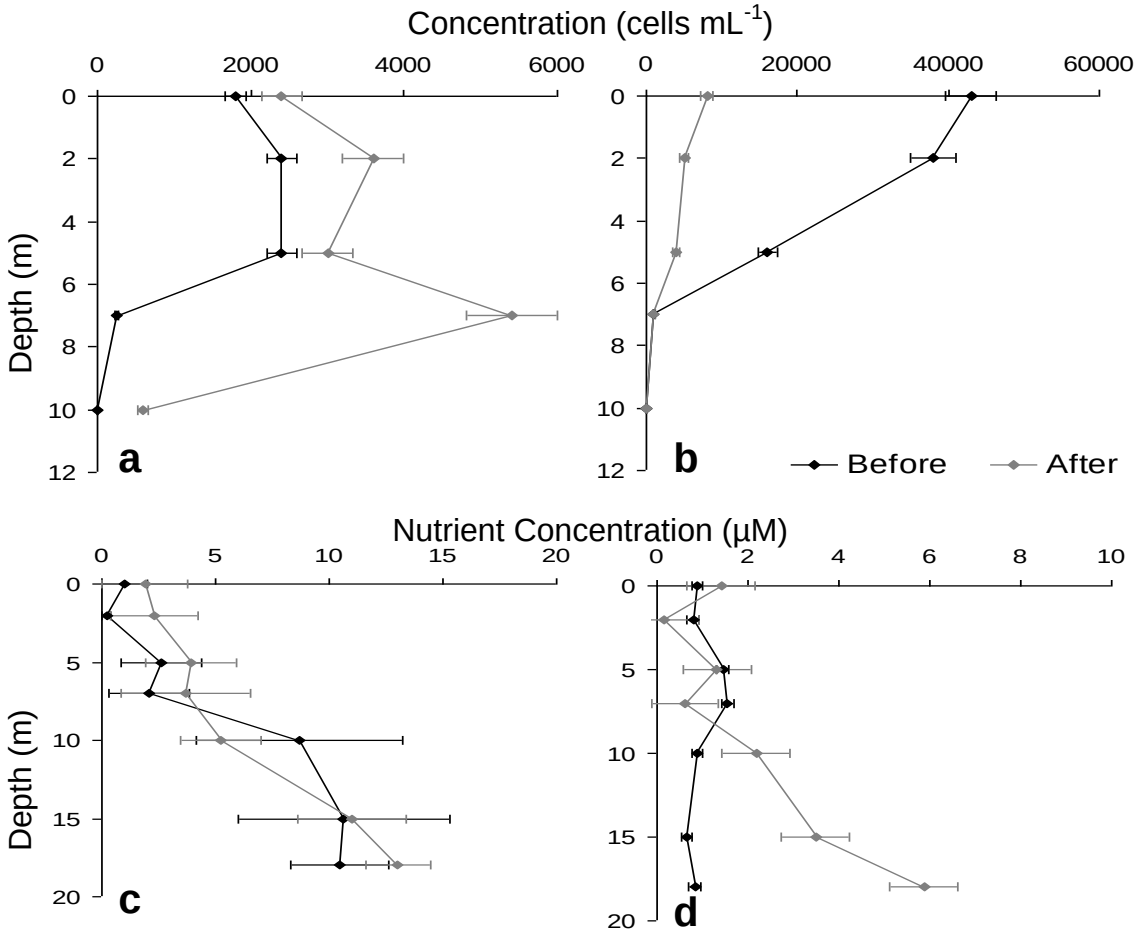

Figure 3.2 Mean (±SD)concentrations of a) *C. raciborskii* (cells mL-1), b) *M. aeruginosa* (cells mL-1), c) NO_X (µM) and d) NH_4 (µM) at site A before (5 October) and after (17 October) the destratification system was turned on in L. Samsonvale.

3.3.2 Profile of a summer *Cylindrospermopsis raciborskii* bloom

During the summer 2007/08 in L. Samsonvale, cyanobacteria comprised approximately 80% of the phytoplankton cell abundances for the majority of the summer, with diatoms comprising approximately 50% from February 2008 (Fig 3.3). Chlorophyta were not detected. There were changes in dominance of genera however, the cyanobacteria *Planktolyngbya, Aphanocapsa*, *C. raciborskii* and *Microcystis* were the most dominant in terms of cell abundance. At the beginning of the sampling, the phytoplankton community was dominated by *Microcystis (botris* and/or *aeruginosa)* which was in very high cell abundances (100 000 cells mL^{-1}). *C. raciborskii* was in relatively low concentrations (<500 cells mL^{-1}) until late November 2007 when it began to increase in both cell abundance and proportion of total cell abundances until a peak (30 000 cells mL^{-1} and approximately 75% of phytoplankton biovolume) in early January 2008. After this it decreased to approximately 25% of the biovolume where it remained at that level into February. The solitary filamentous cyanobacteria, *Planktolyngbya* also increased in a similar way to *C. raciborskii* reaching a peak in cell abundance (28 000 cells mL^{-1}) in early January 2008. The small, colonial cyanobacteria *Aphanocapsa* was in relatively high numbers throughout the summer but did not comprise much (>10%) of the total biovolume for most of the summer.

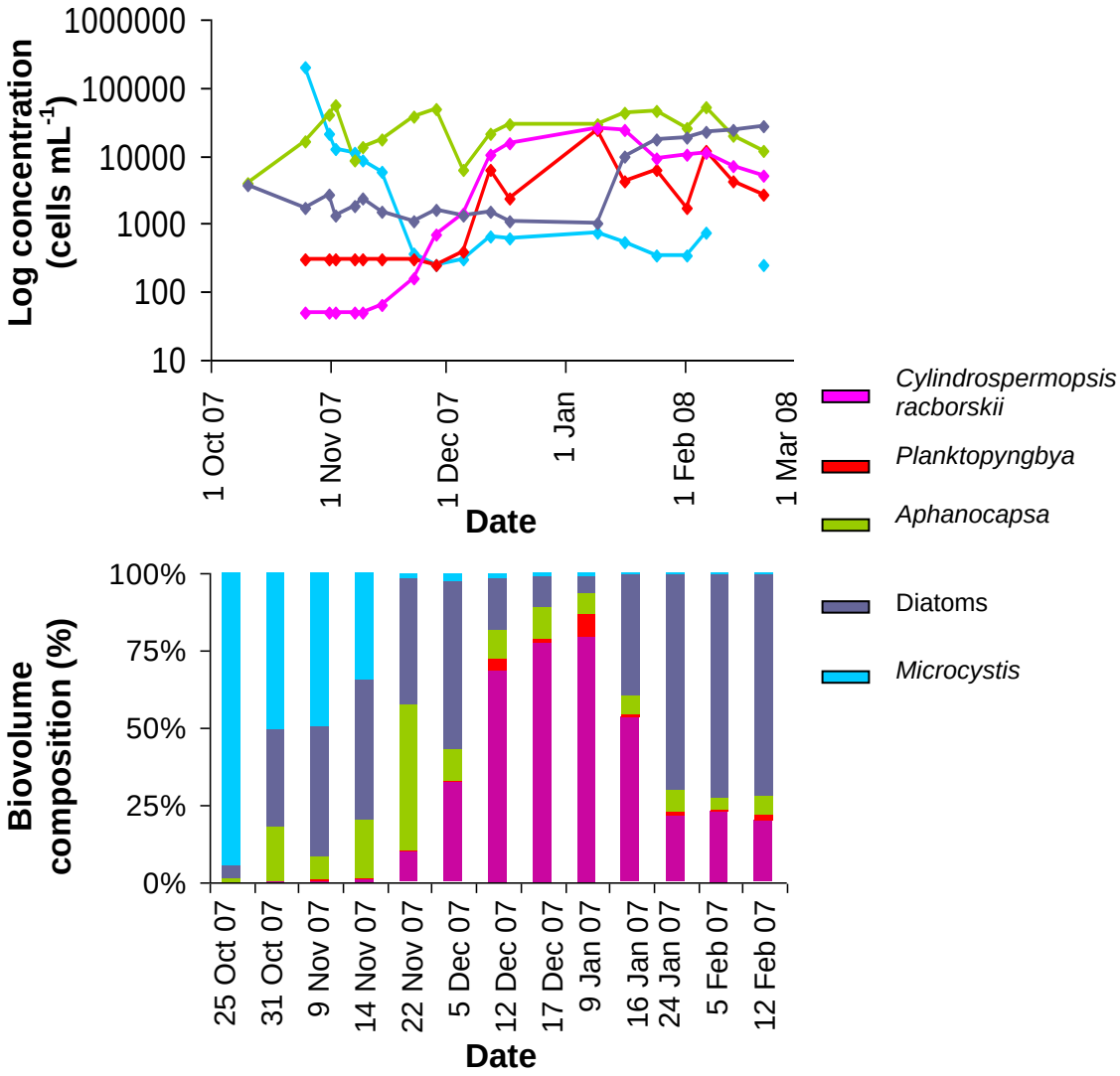

Figure 3.3 Phytoplankton composition (a) cell abundances (cells mL-1) and (b) biovolume (%) of major phytoplankton genera during the summer 2007/08 in L. Samsonvale.

From October 2007 to March 2008, DOP concentrations ranged from 0.01 to 0.32 µM at the surface (0-3 m) and bottom except for an increase on the 9 January 2008 (Fig 3.4). On this day, DOP was the significant component of total P. Phosphate remained below detection limit throughout the summer and particulate P did not vary substantially (0.32 ± 0.16 µM). The concentrations of total P in the bottom waters were generally higher than the surface waters however, this was not always statistically different (Fig 3.4).

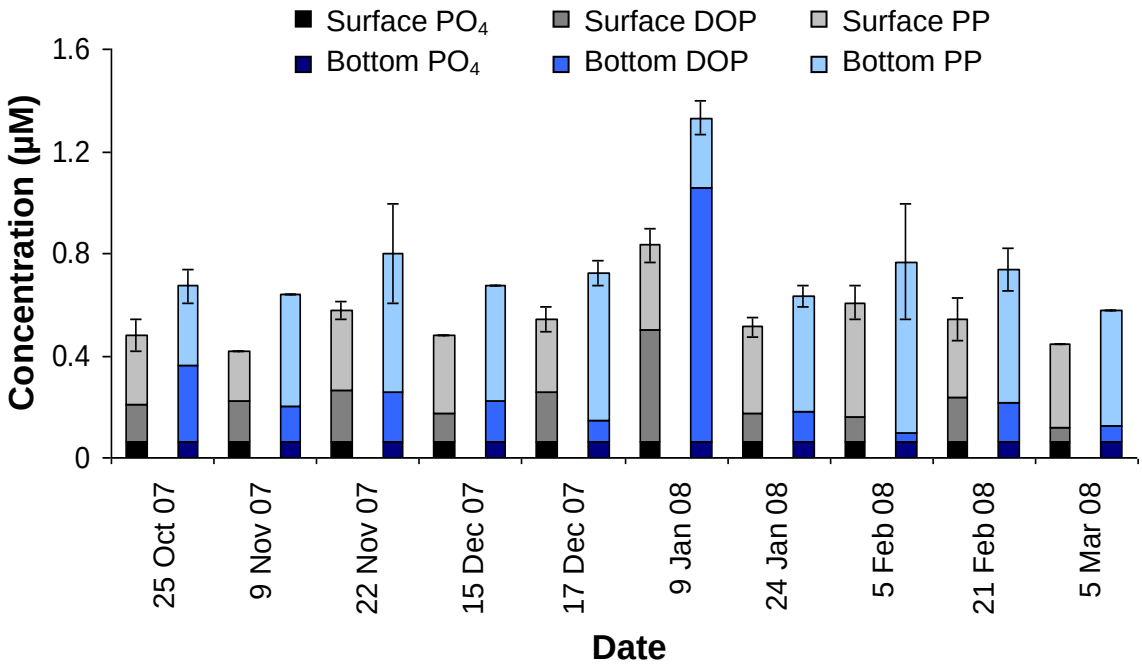

Figure 3.4 Mean (±SD) P fractions (µM) in L. Samsonvale over the summer 2007/08 PO_4: phosphate DOP: Dissolved Organic phosphorus, PP: particulate phosphorus (Total P minus DOP and PO_4). Method detection limit 0.064 µM.

Generally there was no change in the total N concentrations at both the surface and bottom (Fig. 3.5) during the summer 2007/08. Total N concentrations were 45 ± 10 µM at the surface and 50 ± 8 µM at the bottom except for the 9 January when surface concentrations were 70 ± 4 µM and bottom concentrations were 75 ± 1 µM. The DON fraction was always the largest (>75%) proportion of total N and the DIN did not significantly change over the summer (28 ± 3 µM) except for November and December at the surface where it decreased to near detection limit on the 5 December. The molar N:P ratio was always above 50 and was generally similar in the surface and bottom waters (Fig 3.6).

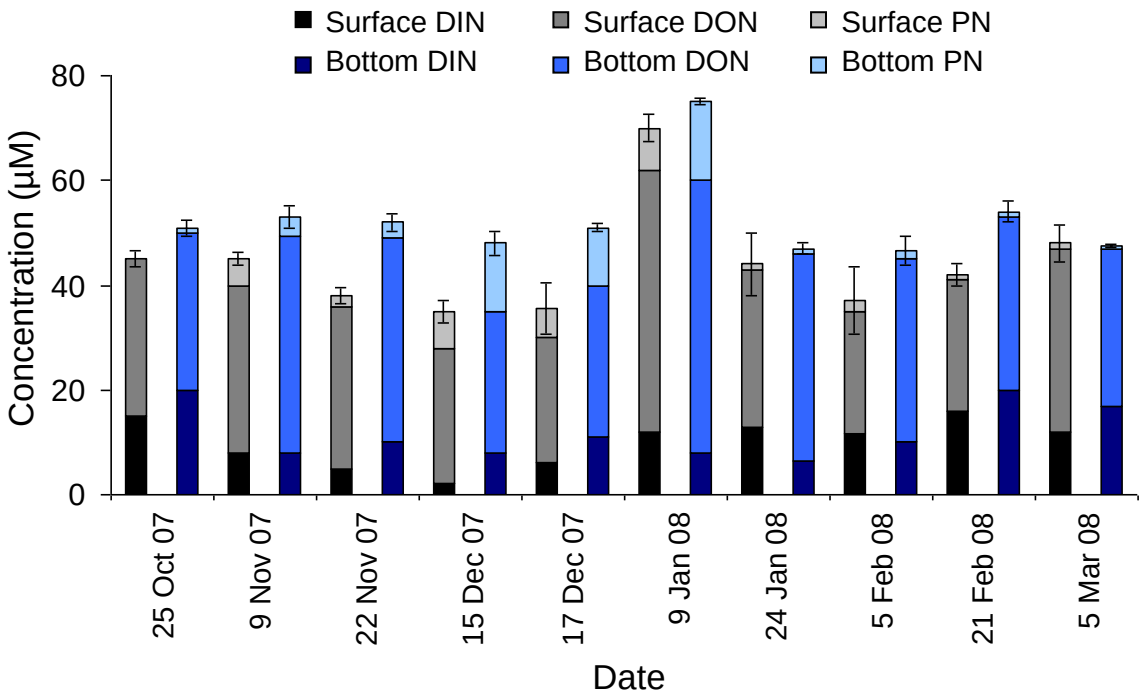

Figure 3.5 Composition of the N fraction (µM) in L. Samsonvale over the summer 2007/08. DIN: Dissolved Inorganic Nitrogen, DON: Dissolved Organic Nitrogen, PN: particulate nitrogen (Total N minus DON and DIN). Method detection limit 0.064 µM.

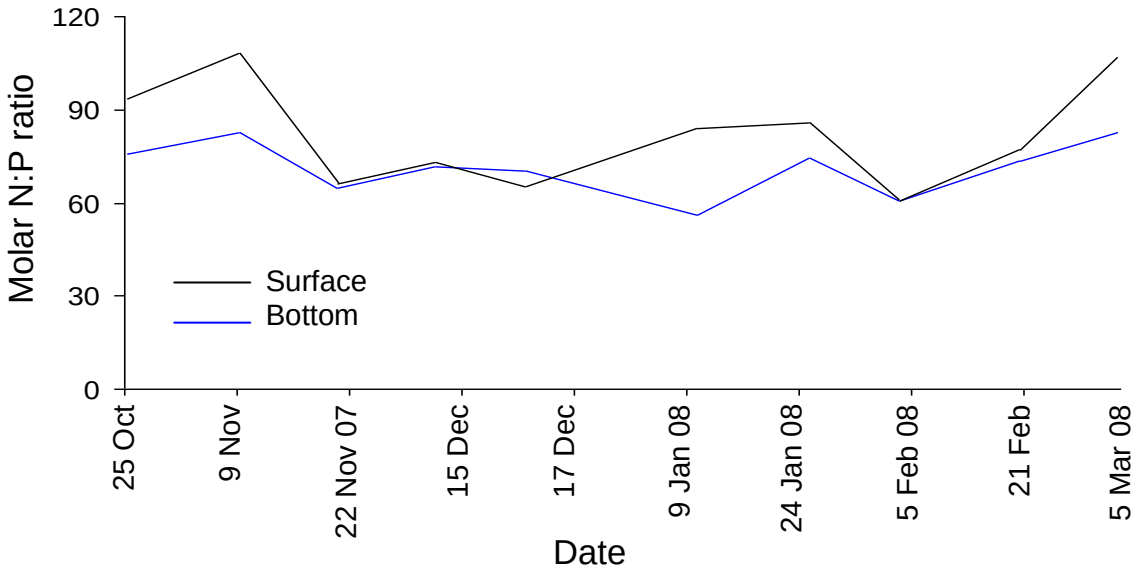

Figure 3.6 Molar N:P ratio for surface (0-3 m) and bottom waters in L. Samsonvale over the summer 2007/08. N:P ratio calculated from total N and P concentrations.

Lake Samsonvale experienced a mild, dry summer with only a few days of extreme temperatures (maximum >35 ºC) and generally little rainfall. The water temperature (from the surface to 8 m) was approximately 25 ºC throughout the summer (Fig. 3.7). There were six occasions where the daily rainfall in the area was above 20 mm and only two significant inflows in early January and early February 2008 (Fig. 3.8). The rainfall data was reflected in the inflow data as shown by an increase in lake level from 4 January 2008 (Fig 3.8). There was no seasonal trend for manganese and iron (8 ± 4 and 7 ± 3 μM respectively) and silica increased from early January 2008 (32 to 250 μM at the beginning of March) (Fig 3.9). Mean light intensity was highly variable from day to day however, there was no significant trend over the summer (Fig 3.10). Dissolved oxygen (DO) concentrations were relatively unchanged in the surface water (0-8 m) (Fig 3.11). The DO concentrations in the bottom waters were low and highly variable until the beginning of November through to the end of January 2008. Generally there was no significant changes with most of the water quality parameter over the summer with pH, conductivity, total suspended solids, ORP and fluorescence being statistically unchanged from October to March (Table 3.3).

Correlation coefficients were used to determine whether a correlation exists between *C. raciborskii* and the parameters measured (Table 3.4). The strongest positive correlation was observed between *C. raciborskii* and *Planktolyngbya* (0.74). Surface total P, bottom DOP and turbidity also showed a strong correlation with *C. raciborskii.*

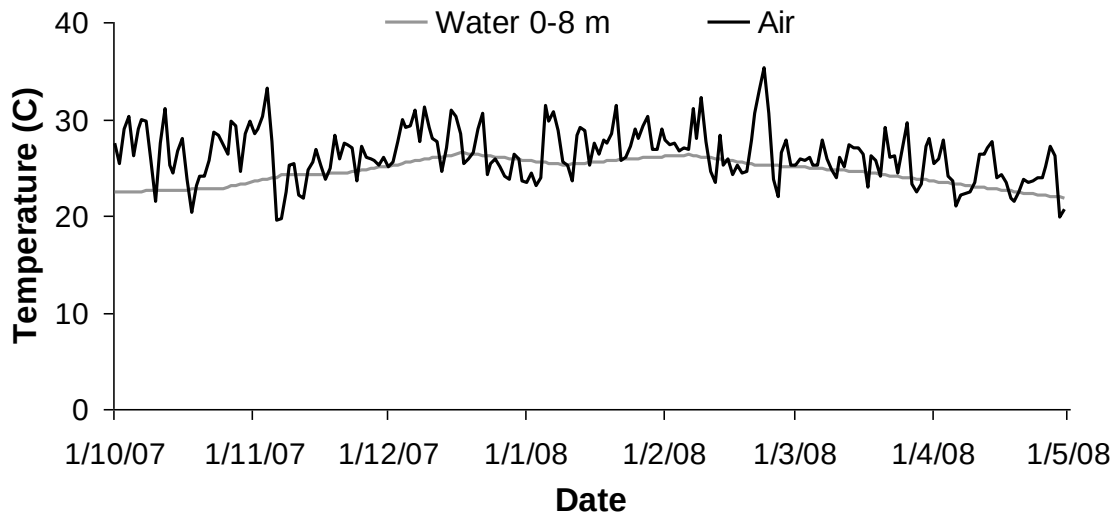

Figure 3.7 Mean daily (24 h from midnight to midnight) temperatures (ºC) at L. Samsonvale during the summer 2007/08.

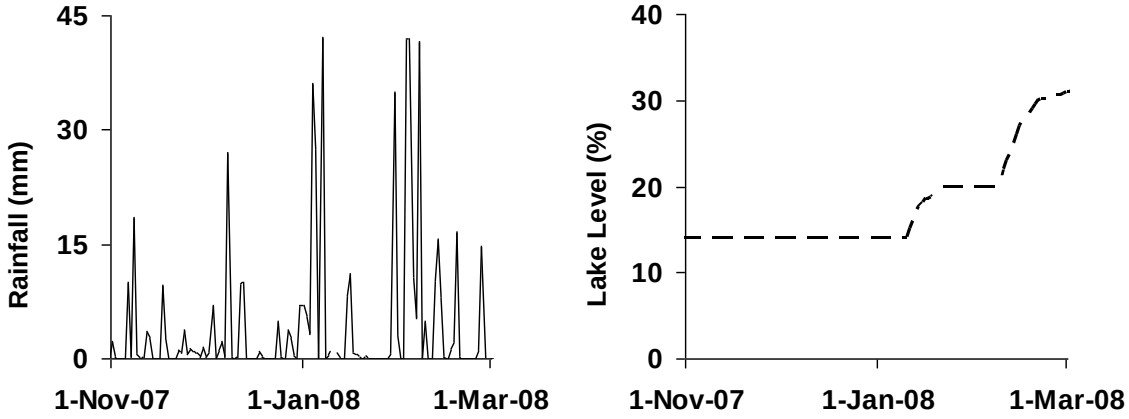

Figure 3.8 Daily rainfall (mm) and lake level (% full capacity) of L. Samsonvale during the summer 2007/08.

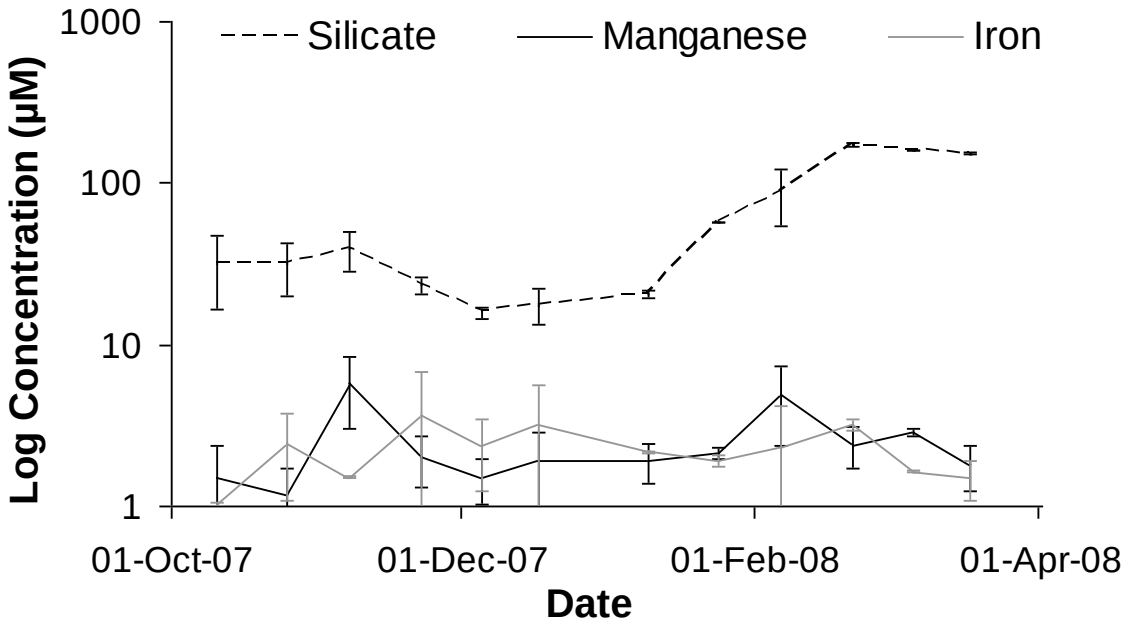

Figure 3.9 Concentrations of silicate, manganese and iron in L. Samsonvale during the summer 2007/08. Samples taken from site A (Fig 2.2).

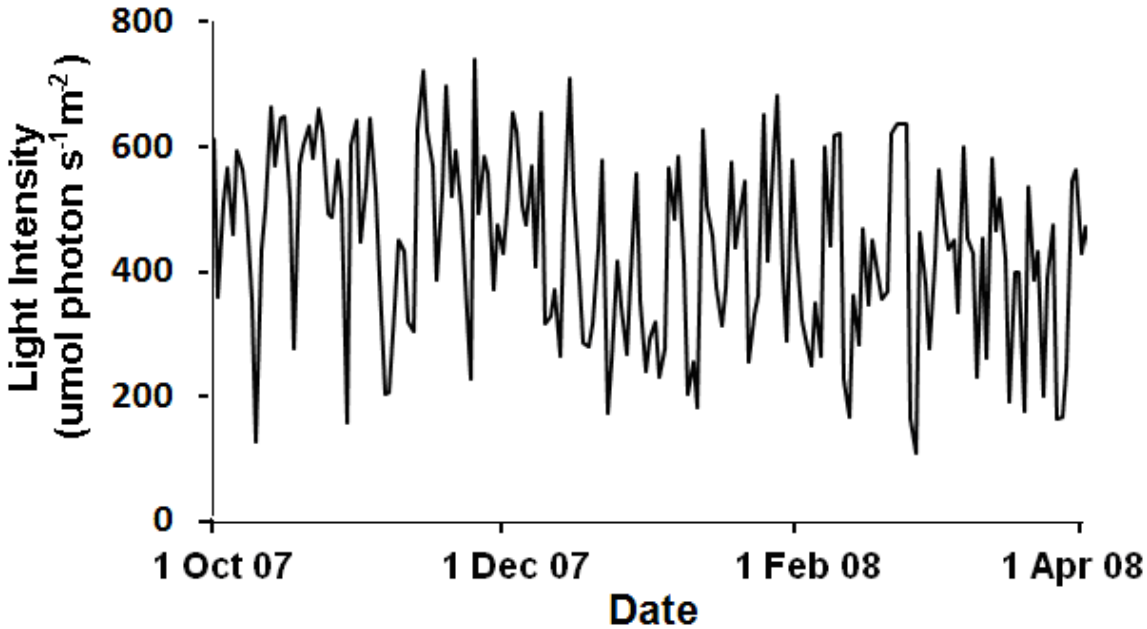

Figure 3.10 Mean daily solar radiation at L. Samsonvale during the summer 2007/08. Mean taken from sunrise to sunset. The dot (9 January 2008) indicates when *C. raciborskii* cell abundances were highest.

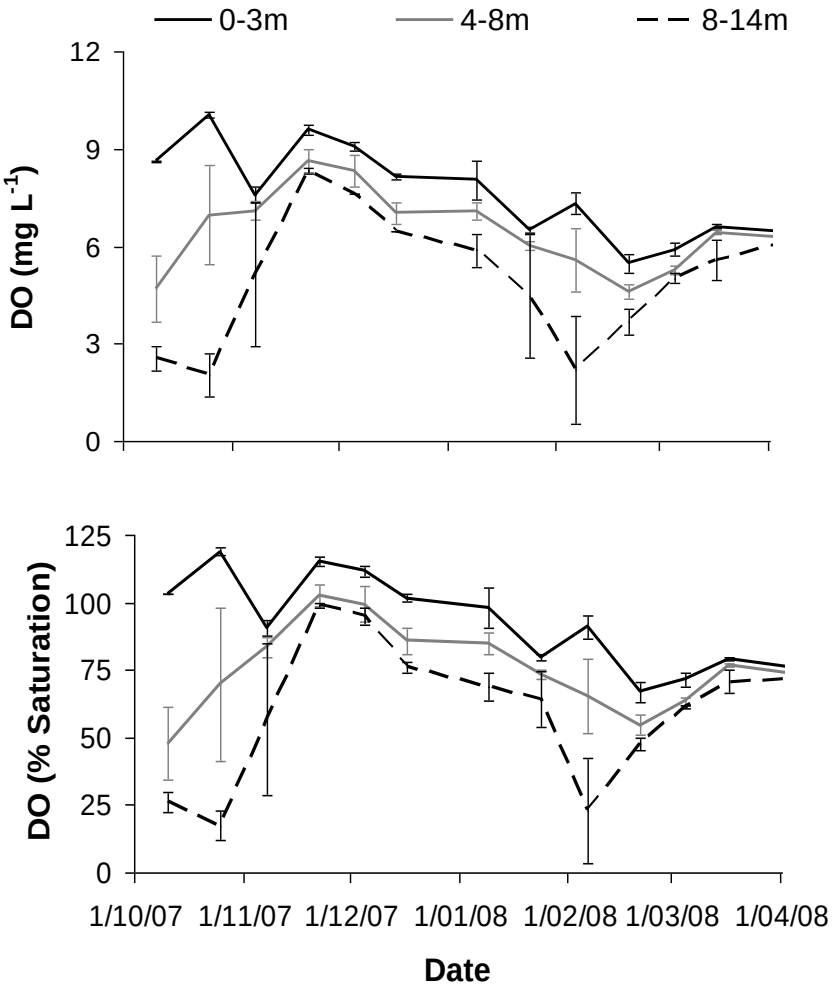

Figure 3.11 Mean (± SD) dissolved oxygen concentrations (% saturation and mg L-1) at approximately 10am in L. Samsonvale during the summer 2007/08. Samples taken from site A (Fig 2.2).

Table 3.3 Water quality parameters integrated from surface to 8 m in L. Samsonvale during the summer 2007/08

Parameter	Mean	SD
pH	8.0	0.48
Specific Conductivity at 25ºC (µC cm^{-1})	310	20
Total suspended solids mg L^{-1}	3.5	1.6
ORP (Redox potential: mV)	90	20
Sechi Depth	1.2	0.20
Fluorescence %FS	1.5	0.56

Table 3.4 Correlation coefficients for parameters potentially affecting *C. raciborskii* biovolume dominance in Lake Samsonvale

Parameter	*C. raciborskii*
Planktolyngbya	**0.74**
Bottom Dissolved Organic Phosphorus	**0.62**
Surface Total Phosphorus	**0.57**
Surface Water Temperature	0.39
Water Temperature 7 m	0.39
Water Temperature 3 m	0.38
Water Temperature 5 m	0.32
Chlorophyll a	0.33
Percentage cloud cover	0.32
Diatoms	0.22
Aphanocapsa	0.21
Surface ammonium	0.05
Bottom Dissolved Organic Nitrogen	0.01
Silcate	-0.08
Iron	-0.08
Manganese	-0.10
Surface Dissolved Organic Phosphorus	-0.14
Particulate Carbon	-0.17
Dissolved Oxygen 4 to 7 m	-0.23
Microcystis	-0.26
Dissolved Oxygen surface to 3m	-0.27
pH	-0.34
Surface Dissolved Organic Phosphorus	-0.35
Surface nitrates/nitrites	-0.41
Surface total nitrogen	-0.46
Turbidity	**-0.88**

Numbers in bold show statistically significant correlations

3.3.3 Assessing phytoplankton phosphate uptake and regeneration rates in two sub-tropical lakes

3.3.3.1 Lake Samsonvale and Lake Borumba

There was no statistical difference ($p>0.05$) in the total nutrient concentration or water temperature between lakes Samsonvale and Borumba in February and March 2008 (Table 3.5). However there was a significant difference in the *C. raciborskii* cell abundances. Lake Borumba had cell abundances exceeding 100 000 cells mL^{-1} for most of the summer (G. McGreggor pers. comm.) including the sample dates whilst L. Samsonvale was experiencing a declining *C. raciborskii* population from 30 000 cells mL^{-1} to approximately 7 000 cells mL^{-1} at the time of sampling (19 February –

19 March 2008). Therefore at the time of sampling L. Borumba was dominated by *C. raciborskii* (>80% biovolume) whilst L. Samsonvale was not (~30% biovolume). There was no statistical difference ($p>0.05$) in phosphate, DOP, total P and total N between the two reservoirs. However, L. Borumba had a higher concentration of particulate C (7 ± 2.8 compared to 4 ± 0.75 mg L^{-1}) whilst L. Samsonvale had a higher concentration of ammoniacial N (9.8 ± 4.8 compared to 2.1 ± 0.32 µM).

Table 3.5. Comparison of mean water quality data and phytoplankton cell abundance from L. Samsonvale and L. Borumba.

		Borumba		Samsonvale	
		µM	**SD**	**µM**	**SD**
Surface nutrients	PO_4	<0.064		<0.064	
	DOP	0.16	0.064	0.224	0.096
	TP	0.64	0.16	0.416	0.096
	TN	58.1	7.70	67.9	5.60
	DON	45.5	5.60	36.4	8.40
	NH_3	2.10	0.70	9.80	0.56
	NO_X^-	8.40	2.80	25.2	0.70
Particulate Carbon	mg L^{-1}	7.00	2.80	4.00	0.75
Tempreature	ºC	24.5	1.0	23.7	0.5

Cell abundance	**Cells mL^{-1}**	**SD**	**Cells mL^{-1}**	**SD**
C .raciborskii	105 000	10 000	7 000	1 000
Planktolyngbya	22 000	8 888	3 000	1 000
Aphanocapsa/Aphanothece	31 000	6 000	10 000	3 500
Total cyanobacteria	160 000	25 000	25 000	5 000
Total chlorophytes	20 000	2 000	nd	
Total chrysophytes and diatoms	nd		nd	

Biovolume abundance	**µm^3**	**SD**	**µm^3**	**SD**
C .raciborskii	2 352 000	224 000	156 800	22 400
Planktolyngbya	50 600	20 442	6 900	2 300
Aphanocapsa/Aphanothece	15 500	3 000	5 000	1 750

nd: not detected

3.3.3.2 Phosphate uptake rates

For the L. Samsonvale community maximum phosphate uptake rate was approximately 0.105 μM P min^{-1} with a half saturation constant of 0.05 μM P (Fig. 3.12). The maximum phosphate uptake rate was achieved with a phosphate concentration of 0.32 μM P.

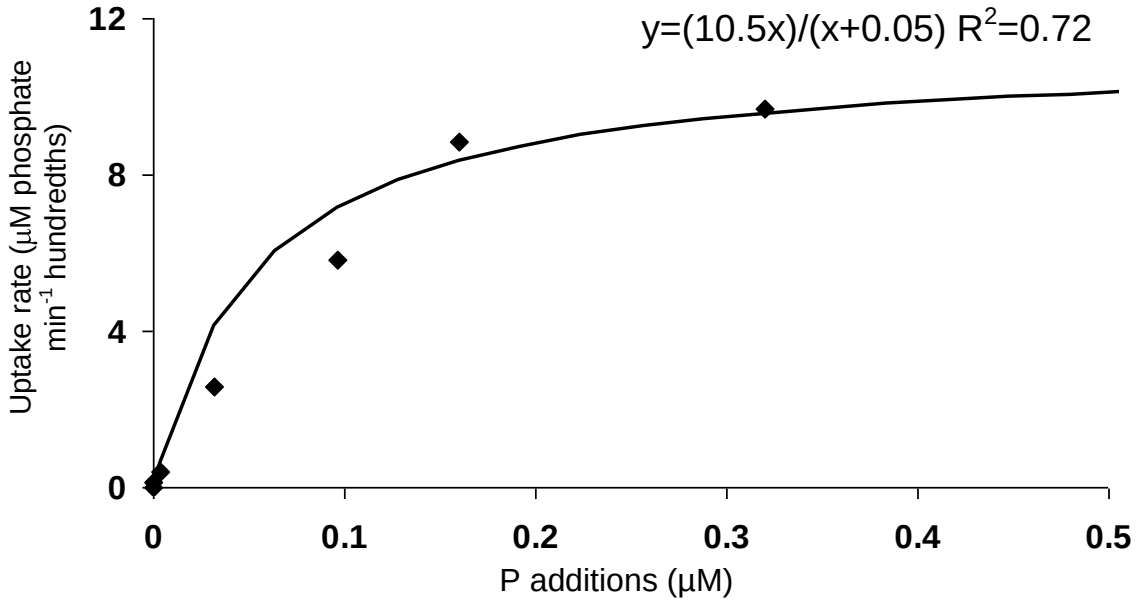

Figure 3.12 Phosphate uptake rates of L. Samsonvale phytoplankton community calculated from 19 February and 5 March 2008 data.

Phytoplankton phosphate uptake rates were compared for L. Samsonvale (19 February and 5 March 2008) and L. Borumba (9 and 19 March 2008) reservoirs. The phosphate uptake rates were similar for the two sampling occasions in L. Samsonvale and the two for L. Borumba but were different between the two reservoirs (Fig 3.13 and 3.14). The phosphate uptake rates for L. Samsonvale (0.046 and 0.043 μM phosphate min^{-1}) were approximately half that of L. Borumba (0.086 and 0.078 μM phosphate min^{-1}).

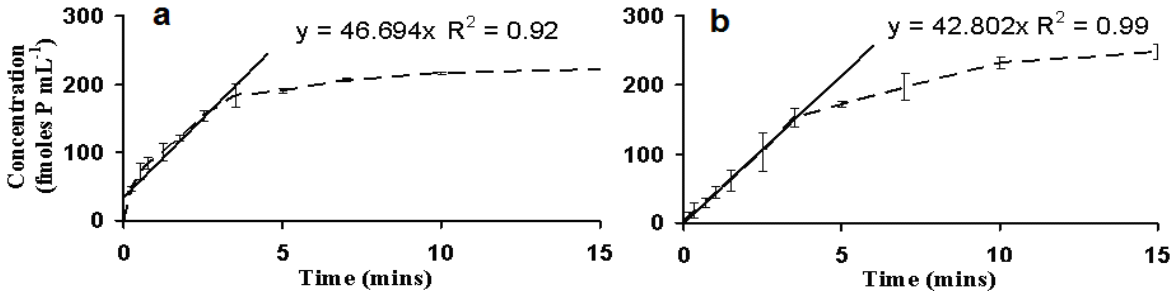

Figure 3.13 Mean concentrations (±SD) of ^{33}P within the cells of L. Samsonvale lake water after a spike of ^{33}P (1.3 pM). a) Sample collected on 19 February 2008, b) Sample collected on 5 March 2008. Linear curves fitted to the initial increase in phosphate concentration.

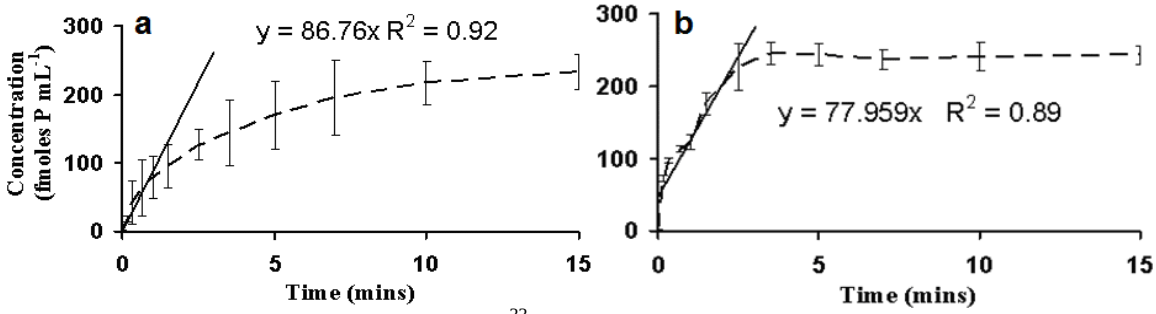

Figure 3.14 Mean concentrations (±SD) of ^{33}P within the cells of L. Borumba water after a spike of ^{33}P (1.3 pM). a) Sample collected on 9 March 2008, b) Sample collected on 19 March 2008 Linear curves fitted to the initial increase in phosphate concentration.

When phosphate uptake rates were converted to units of phytoplankton (mg C) rather than volume of water (mL), there was also no statistical difference ($p>0.05$) between the two lakes (Table 3.6). This was despite L. Borumba having a higher cell abundance of *C. raciborskii* (~90 000 cells more than L. Samsonvale) which has been previously shown to have a rapid phosphate uptake rate.

Table 3.6 Phosphate uptake rates of phytoplankton communities in Lake Samsonvale and Lake Borumba

	Borumba	Samsonvale
µmol P mg C^{-1} d^{-1}	16.5 x 10^{-6}	10.7 x 10^{-6}

3.3.3.3 Phosphate regeneration rates

Phosphate regeneration rates were determined on two occasions for the Borumba phytoplankton populations (9 and 19 March) and once for L. Samsonvale (5 March) (Fig 3.15 and 3.16). On the 19 February the ^{33}P content within the L. Samsonvale population did not decrease after 24 h, therefore a regeneration rate could not be determined. In all four experiments however, there was an increase in cellular ^{33}P content immediately after cold ^{31}P was added.

The L. Samsonvale phytoplankton community was found to have a regeneration rate of 2.88 x 10^{-4} μM phosphate min^{-1} on the 5 March 2008. This was calculated by the decrease in ^{33}P cellular content from 5-20 h after the experiment began. The two experiments from L. Borumba (Fig 3.16) showed a much slower onset of phosphate regeneration (after 20 h) than the L. Samsonvale experiment (5 March, Fig 3.15) which was after 5 h. The regeneration rates for the two L. Borumba samples were calculated from the decrease in cellular ^{33}P content after 24 h. The regeneration rates on 9 March were 1.2 x 10^{-4} μM phosphate min^{-1} and on the 19 March they were 2.4 x 10^{-4} μM phosphate min^{-1}.

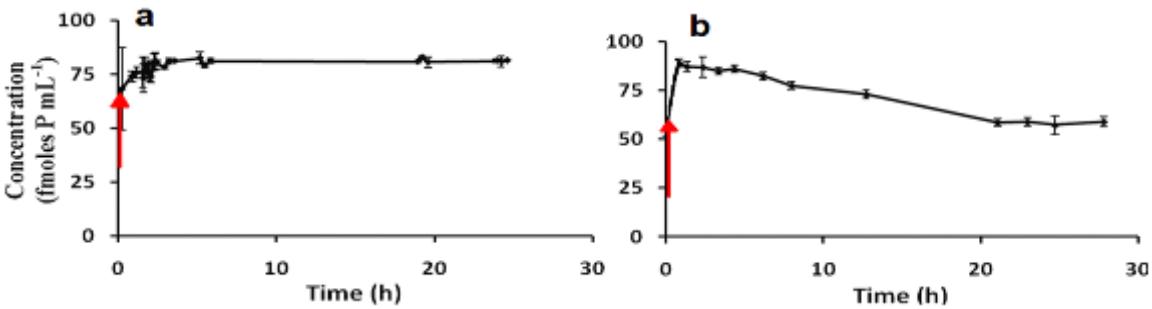

Figure 3.15 Concentrations of ^{33}P within the cells of L. Samsonvale lake water after a spike of ^{31}P (3.2 μM) as indicated by the arrow. a) Sample collected on 19 February 2008, b) Sample collected on 5 March 2008

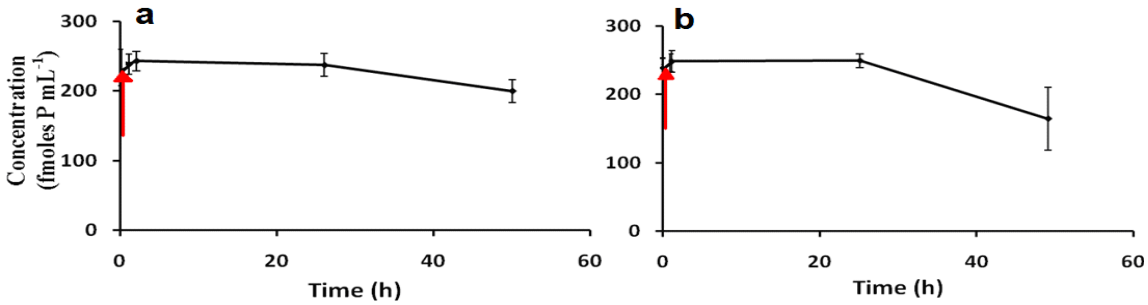

Figure 3.16 Concentrations of ^{33}P within the cells of L. Borumba water after a spike of ^{31}P (3.2 µM) as indicated by the arrow. a) Sample collected on 9 March 2008, b) Sample collected on 19 March 2008.

3.4 Discussion

3.4.1 Linking Artificial Destratification with *Cylindropsermopsis raciborskii* dominance

The preliminary investigation showed that the artificial destratifier in L. Samsonvale was affecting the phytoplankton community by decreasing the concentration of *M. aeruginosa* cells and increasing the concentration of *C. raciborskii* cells. This is consistent with previous studies in L. Samsonvale (Littlejohn 2004 and Antenucci et al. 2005). Also, when comparing L. Samsonvale to two other nearby reservoirs which are subject to relatively high *C. raciborskii* cell abundances (L. Wivenhoe and L. Sommerset, neither of which have an artificial destratifier), the *C. raciborskii* cell abundances in L. Samsonvale increased earlier in the year (in conjunction with the artificial destratifier) and were more sustained (Burford and O'Donohue 2005). However, analysis of two tropical reservoirs, one artificially destratified and the other not, showed that *C. raciborskii* dominated the lake without destratifiers and decreased in numbers in a reservoir with a destratifier (Hawkins 1993). The contrast between these studies (Hawkins 1993, Littlejohn 2004 and Antenucci et al. 2005) indicates that the relationship between *C. raciborskii* dominance and artificial destratification may be specific to each system. Lake Samsonvale typically has low phosphate

concentrations which may be increased by artificial destratification and in turn effect phytoplankton distributions and *C. raciborskii* dominance.

This study confirmed that the destratifier in L. Samsonvale is not completely mixing the water column and results in higher NH_4 concentrations in the bottom waters post-destratification which was consistent with Littlejohn (2004). Phosphate was always below detection limit which makes it difficult to draw conclusions about whether the destratification unit was affecting phosphate availability in L. Samsonvale. The increase in NH_4 concentrations in the bottom waters post destratification however, may indicate an increase in phosphate availability as well. Further studies examining cellular P content in phytoplankton and phosphate sediment fluxes pre and post-destratification would give greater insight into the effect of destratification on nutrient distribution in L. Samsonvale.

Whilst the relationship between artificial destratification and sediment phosphate remineralisation is yet to be quantified, recent research has revealed that light availability plays a more significant role in *C. raciborskii* growth and potential dominance in L. Samsonvale (O'Brien et al. 2008). This study shows that a decrease in light availability, as a result of deeper mixing after the destratification unit was switched on, gave *C. raciborskii* a competitive advantage, since it has a wide tolerance to light intensities. This is consistent with the finding that *C. raciborskii* numbers were higher at a depth of 7m (where light availability was lower than closer to the surface) post destratification. The increase in overall *C. raciborskii* numbers post-destratification is also consistent with overall increase in chlorophyll *a* after the destratification unit was installed in L. Samsonvale (Antenucci et al. 2005). However,

the recent finding that *C. raciborskii* growth is higher under low light availability (O'brien et al. 2008) suggests that the effect of mixing on increasing nutrient availability is unlikely to be the major contributing factor in *C. raciborskii* dominance. Therefore the destratification unit was not investigated further in this study.

3.4.2 Linking physico-chemical parameters with *Cylindrospermopsis raciborskii* dominance

3.4.2.1 Linking Dissolved Organic Phosphorus with *Cylindrospermopsis raciborskii* cell number

For the first time DOP concentrations in L. Samsonvale were measured over a summer period in conjunction with *C. raciborskii* cell abundance and other parameters. Whilst the number of studies linking phytoplankton growth and DOP are increasing, speciation of DOP is poorly understood in freshwaters. This is largely due to the relatively low sensitivity in current analytical techniques (Cooper et al. 1999). However, one study has shown that less than 5% of the DOP entering a river after rainfall was found to be in the high molecular weight fraction (>50 kDa) (Rinker & Powell 2006). Also, in the oligotrophic everglades (Florida, U.S.A) 90% of the DOP entering the system was between 350 and 600 Da (Cooper et al. 1999).

Dissolved Organic Phosphorus speciation within marine systems has been extensively quantified (Ridal and Moore 1990, Karl & Yanagi, 1997, Clark et al. 1998, Suzumura et al. 1998 and Monbet et al. 2008) and may provide clues for speciation in freshwaters. Dissolved Organic Phosphorus is comprised of approximately 50-80% low molecular weight (LMW <10 k Da) and approximately 15% high molecular weight (HMW >50 kDa) with less than 5% being comprised of compounds between 10 and 50 kDa (Benitez-Nelson 2000). A further breakdown of the DOP fraction and

relative abundance of specific molecules indicates that as much as 50% may be polyphosphates (Solorzano 1978). Other forms of DOP may include monophosphate esters, nucleotides, nucleic acids, phospholipids and phosphonates (Ridal and Moore 1990, Karl and Yanagi 1997, Clark et al. 1998 and Suzumura et al. 1998). Speciation of DOP was not determined in this study however, it can be assumed (based on the studies above) that much of the DOP could be hydrolysed by alkaline phosphatase, but further work is required to validate this.

The presence of DOP in all samples over the summer indicates that there is a pool of potentially bioavailable P for the phytoplankton even when phosphate concentrations are low. *C. raciborskii* and other phytoplankton may be capable of producing the enzyme alkaline phosphatase (AP) which cleaves phosphate from organically bound forms. This makes it possible for such organisms to satisfy their phosphate requirements in phosphate free conditions. Both heterotrophic and autotrophic microbial communities in Florida Bay (U.S.A) are moderated by bioavailability of dissolved organic matter (Boyer et al. 2006). Alkaline phosphatase activity increased in bioassays with the addition of two times background dissolved organic matter and resulted in an increase in microbial numbers. Alkaline phosphatase activity was not measured in the field sampling of L. Samsonvale however, it would be useful in determining whether AP production leads to a significant source of phosphate in L. Samsonvale when phosphate concentrations are low.

Although much of the research concerning the use of DOP by bacteria and phytoplankton has focused around the production of enzymes for utilising DOP, some bacteria have been shown to directly take up DOP without prior hydrolysis (Larson et

al. 1982, Wanner 1994). Heterotrophic bacteria may actively take up DOP one of two ways; either by ATP dependant protein transport when phosphate concentrations are low (Wanner 1994) or by the glycerol transport system (where glycerol may be glycerol-3-phosphate) which is unaffected by phosphate concentration (Larson et al. 1982). The presence of heterotrophic bacteria associated with phytoplankton may indicate a potential phosphate pathway irrespective of external phosphate concentrations. The presence of and ability for heterotrophic bacteria to take up DOP was not investigated in the current study however: it would be useful in determining the bioavailability of phosphate in L. Samsonvale.

With the increase in DOP on the 9 January 2008, and the assumption that most of this was bioavailable, L. Samsonvale phytoplankton were unlikely to be P-limited at this time. Samples were not collected in the days before and after 9 January therefore it cannot be determine whether there was a sustained increase (over a number of days) in P concentration.

In the summer 2007/08 there was an increase in diatom cell abundance after the increase in DOP concentration. The increase in diatom dominance coincided with a decrease in *C. raciborskii* dominance. It is possible that the increase in diatoms and decrease in *C. raciborskii* may be linked to a sustained and relatively high increase in phosphate concentration (that was immediately taken up therefore not seen as part of the phosphate results). However, the two week sampling regime was not frequent enough to capture whether or not there was a sustained (over a number of days) increase in DOP.

3.4.2.2 Linking other physico-chemical parameters with *Cylindrospermopsis raciborskii* cell number

C. raciborskii cell abundances peaked on the 9 January 2008. At this time there was also an increase in silicate and diatom cell abundances. This is also the time when DOP concentrations were the highest. Diatoms have been shown to dominate a phytoplankton community above a silicate threshold concentration of 2 µM (Egge and Asknes 1992). The silicate in L. Samsonvale was above 2 µM throughout the summer 2007/08 however, P concentrations were generally low, except for the increase on 9 January 2008. It is likely that the diatoms were P limited until 9 January 2008 when they began to increase, correlating to an increase in P. The data from this chapter indicates that a sustained increase in P concentrations may influence phytoplankton composition by encouraging the growth of diatoms and therefore reducing *C. raciborskii* dominance.

The focus of this study was on the link between *C. raciborskii* and DOP however: it is likely that the increase in *C. raciborskii* was also linked to other factors (Fig 1.2 page 6). There was no change in nutrient concentration (including DOP) in mid November, when *C. raciborskii* cell abundance started to increase and it is likely that factors other than nutrient concentration initiated the bloom. There was no significant link between temperature and *C. raciborskii* cell abundances as average daily water temperature were relatively unchanged throughout the summer. In contrast to temperature, the initial increase in *C. raciborskii* cell abundance was linked to an increase in bottom water DO, which is consistent with mixing. Mixing has previously been linked to an increase in *C. raciborskii* cell abundances in this reservoir (Littlejohn 2004 and Antenucci et al. 2005 and O'Brien et al. 2008) and it is likely to have affected the increase in *C. raciborskii* in November 2007.

3.4.3 Phytoplankton phosphate uptake and regeneration rates

Phosphate uptake rates were measured for two phytoplankton populations (L. Borumba and L. Samsonvale) dominated by *C. raciborskii* on two occasions. The L. Borumba phytoplankton community had a slightly slower uptake rate compared to the L. Samsonvale community, however, both had much lower uptake rates (per unit C) than two strains of *C. raciborskii* (AWT/205 and NPD strains) in culture. However, the uptake rates observed in the phytoplankton communities of L. Borumba and L. Samsonvale (which were dominated by *C. raciborskii*) were more similar to cultures of the desmid *Cosmarium abbrevistum* (3.44 µmol P mg C^{-1} d^{-1}) and diatom *Asterionella formosa* (3.14 µmol P mg C^{-1} d^{-1}) than cultures of *C. raciborskii* (Istávnovics et al. 2000). In addition, phytoplankton phosphate uptake rates are generally controlled by the small plankton class size (<3 µm) as this size is generally responsible for a large proportion (~80 %) of phosphate uptake (Mazumder et al. 1988, Vadstein et al. 1988, Taylor and Lean 1991 and Nowlin et al. 2007). This suggests that the phosphate uptake rate of the phytoplankton community may not be dictated by the most dominant species but rather the most dominant species < 3 µm. In L. Samsonvale the most dominant species under < 3 µm are *Planktolyngbya* and *Aphanocapsa/Cyanodictyon.*

The maximum phosphate uptake rate of the L. Samsonvale community was used to construct a nutrient budget based on the model proposed by Burford et al. (2006) (Fig. 3.17). This budget measured NH_4^+ and NO_3 uptake rates to determine phytoplankton nutrient uptake rate and used the Redfield (1958) ratio to assume phosphate uptake (1296 t PO_4 y^{-1}). However the maximum phosphate uptake rates measured in this study (8939 t PO_4 y^{-1}) indicate a much faster potential phosphate uptake rate that does not conform to the Redfield (1958) ratio or that of Sterner et. al. (2008) when

measured N values are used. In contrast, the molar N:P ratio of particulate N and P was 19:2, similar to that of Redfield (1958) and Sterner et. Al. (2008) which are 16:1 and 21:1 respectively. This indicates that luxury P uptake may be occurring within the phytoplankton community of L. Samsonvale.

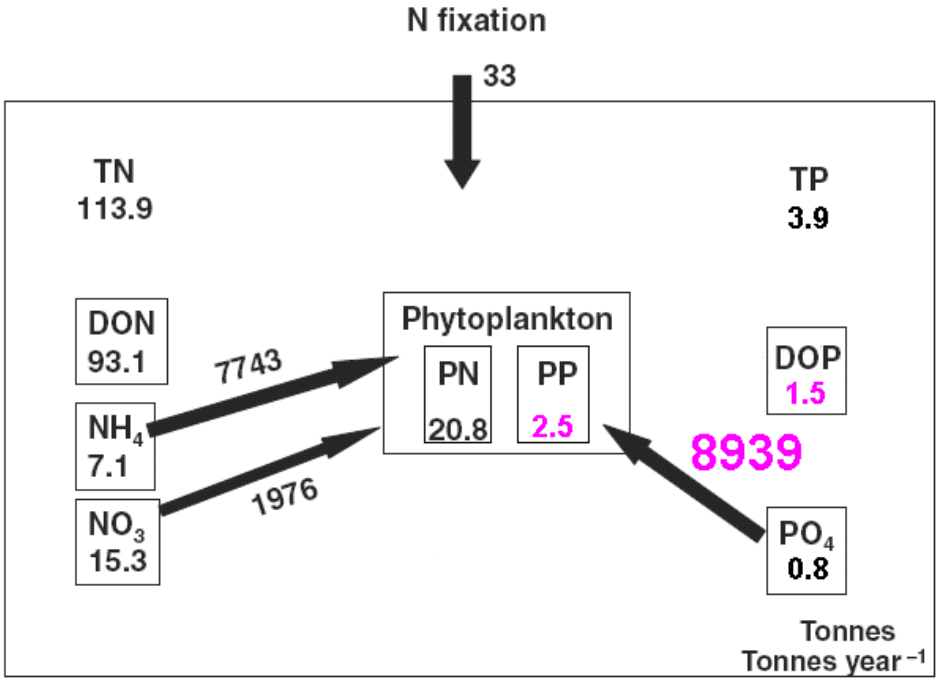

Figure 3.17 Modified from Burford et al. (2006). N and P pools (T) and phytoplankton demand (T year-1) in the water column in L. Samsonvale, based on measurements at the reservoir wall site. Pink numbers represent calculations from current study, black numbers represent calculations from Burford et al. 2006. NH_4, ammonium; NO_3, nitrate; PN, particulate nitrogen; DON, dissolved organic nitrogen; TN, total nitrogen; TP, total phosphorus; PO^4, phosphate.

The nutrient budget by Burford et al. (2006) assumes maximum uptake rate is the same as regeneration rate however, this was not seen in the current study. The regeneration rate of the L. Samsonvale community was much lower than the maximum phosphate uptake rate (Fig 3.18), which further suggests luxury P uptake. The phosphate regeneration rate was also relatively rapid (243 t y^{-1}) in the context of the pool of available phosphate (0.8 t). Furthermore catchment inflows are 20 times lower than the P coming from sediment remineralisation. This suggests that significant P cycling is occurring in L. Samsonvale due to small scale microbial turnover (such as algal cell leakage and zooplankton excretion) and P decomposition

and remineriliation. However, more details are required (such as the alkaline phosphatase activity, fractionation of sediment P and inflow P) to fully understand the sources of P influencing phytoplankton in L. Samsonvale.

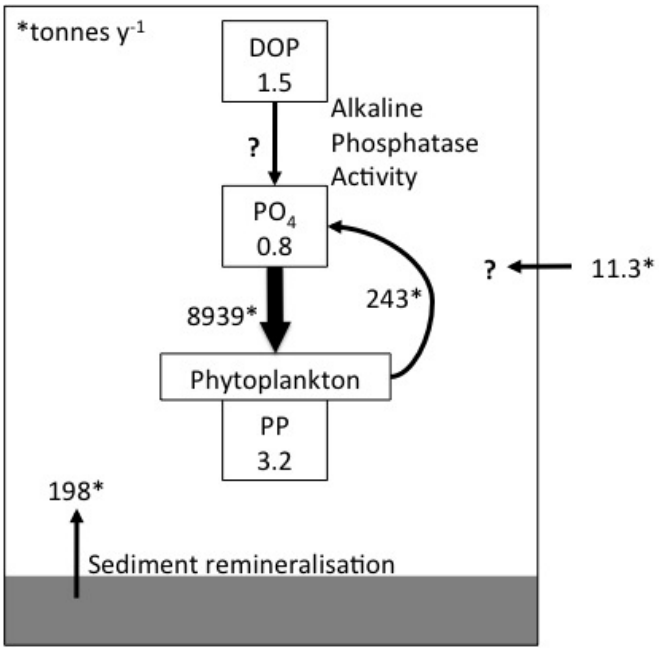

Figure 3.18 Sources of P in L. Samsonvale showing measured phosphate uptake and regeneration rates and water column P concentrations with potential concentrations from catchment inflow (Harris and Baxter 1996) and sediment (Longmore 2001) DOP: Dissolved Organic Phosphorus, PO_4: Phosphate, PP: Particulate Phosphorus

Microbial nutrient cycling is a fundamental process that maintains most of the primary production in aquatic environments. The regeneration rates calculated in this study were rapid enough to cycle the TP pool (which includes the phosphate pool) every 0.3 d^{-1} in L. Samsonvale and every 0.6 to 1.2 d^{-1} in L. Borumba. This is consistent with eight lakes of British Columbia, Canada, where regeneration rates of 0.7-2.1 d^{-1} were recorded (Nowlin et al. 2007). The majority (>50%) of phosphate turnover within these lakes occurred in the <20 µm fraction of phytoplankton and this is consistent with other studies (Mazumder et al. 1988, Vadstein et al. 1988, Taylor and Lean 1991). Size fractionation of P regeneration was not determined in the

current study however: from the studies above we can assume the majority is occurring in the pico- and nanoplankton communities. A rapid P cycling rate in the smaller plankton community is indicative of P-limitation where smaller organisms with a relatively large surface area to volume ratio are competitively superior at phosphate uptake compared to larger organisms (DeBruyn et al. 2004, Sterner et al. 2004).

In both lakes (Borumba and Samsonvale), P regeneration did not occur in the first 24 h after phosphate was added in a saturating concentration (3.2 µM). Furthermore there was an increase in the cellular ^{33}P after the saturating addition of ^{31}P. This is not consistent with any of the literature reviewed (Mazumder et al. 1988, Vadstein et al. 1988, Taylor and Lean 1991, Hudson and Taylor, 1996, Benitez-Nelsen and Buesseler 1999, Hudson et al. 1999, DeBruyn et al. 2004, Sterner et al. 2004, Nowlin et al. 2007). For example in Nowlin et al. (2007) there is no mention of an increase in cellular ^{33}P and the author assumes ^{31}P displaced ^{33}P continually over approximately 8 h. The delay in phosphate regeneration in the current study may suggest a relatively high storage capacity of the phytoplankton community in L. Samsonvale and L. Borumba. Also the results may indicate that phosphate may have been loosely bound to the phytoplankton cells but not incorporated in particulate P concentration.

Phosphomonesterase have been shown to be in sheaths and mucilaginous material of some cyanobacteria (Weckesser et al. 1988 and Grainger et al. 1989). This means that P may be ‘bound’ to the cell but not actually part of the cellular concentration. *C. raciborskii* has been shown to be relatively ‘fragile’ with regard to filtering (Burford pers. comm.) which means that filtering may remove loosely bound

molecules within sheaths and mucilaginous material. The increase in ^{33}P within the material retained on the filter paper (cellular material) immediately following the addition of saturating ^{31}P may be explained by a selective uptake of surface bound ^{33}P (which prior to the ^{31}P addition was external to the cell and removed with filtering). The increase in ^{33}P after the addition of ^{31}P was seen in all experiments.

The current study has shown that the phytoplankton community dominated by *C. raciborskii* (of L. Samsonvale and L. Borumba) has a rapid phosphate uptake rate and possible high P storage capacity. Furthermore the current study is consistent with the functional grouping hypothesis of Reynolds et al. (2002) that suggests that *C. raciborskii* (functional group S_N) dominates in lakes with nutrient deficiency.

3.5 Conclusion

The phosphate regeneration rate of the phytoplankton communities in L. Samsonvale and Borumba are consistent with phosphate regeneration rates of other P-limited communities (Nowlin et al. 2007). However the ability of the phytoplankton communities to store phosphate for extended periods, as was seen in L. Samsonvale and Borumba was not seen in other studies. This was a confounding problem with the method and warrants further investigation. To determine accurate regeneration rates, the experiment should be repeated with higher concentrations (>3.2 µM) of ^{31}P. However, care should be taken to avoid an oversupply of ^{31}P which may lead to cell stress.

The presence of DOP in the water column of L. Samsonvale indicates a pool of bioavailable phosphate not previously identified. The ability of *C. raciborskii* to produce alkaline phosphatase (discussed in chapter 5), would possibly give it a

competitive advantage over other phytoplankton in L. Samsonvale which may explain the dominance of this species in this reservoir. The concentration of DOP within L. Samsonvale was consistently above detection limit at both the surface and bottom waters. This suggests that DOP is part of the total P pool throughout the summer and indicates a possible pool of accessible phosphate (through alkaline phosphatase) for *C. raciborskii* when phosphate is below detection limit. To fully quantify whether DOP is part of the total P pool year round a twelve month study should be conducted and this should be repeated over a number of years. The high cell abundances of *C. raciborskii*, together with its ability to produce alkaline phosphatase and the constant fraction of DOP, warrant incorporation of DOP analysis in regular sampling of this reservoir.

It was difficult to determine a direct link between fluctuating *C. raciborskii* cell numbers in L. Samsonvale and a range of physico-chemical parameters despite some parameters showing significant correlations. This highlights the uncertainty in evaluating long term monitoring data without the context of manipulative experiments both in the field and laboratory. Future investigation into reasons why *C. raciborskii* and other toxic species may dominate the phytoplankton community should focus on laboratory and field experiments rather than simply monitoring physico-chemical parameters. The following chapters include both laboratory and field experiments to attempt to understand the relationship between P and *C. raciborskii* in L. Samsonvale.

Chapter 4. The role of phosphate in promoting dominance of *Cylindrospermopsis raciborskii* within the phytoplankton community

4.1 Introduction

Interspecific competition for nutrients by phytoplankton play an important role in the species composition and seasonal succession of phytoplankton in lakes and reservoirs (Stewart 1973, Taylor 1975, Eloranta 1993, Chesson 2000, Chase and Lelbold 2003, Rubin et. al. 2007, Litchman and Klausmeier 2008). Previous studies in L. Samsonvale have revealed that thermal stratification and/or lack of available dissolved N are not likely to be causing *C. raciborskii* dominance (Antenucci et al. 2005 and Burford et al. 2006). It has also been suggested that low surface phosphate concentrations (<0.06 μM phosphate) may be selective for the growth of phytoplankton such as *C. raciborskii* (Burford and O'Donohue 2006). Istávnovics et al. (2000) showed that *C. raciborskii* has the ability to rapidly take up and store phosphate in culture and suggests that it is opportunistic with regard to phosphate. It has also been suggested that *C. raciborskii* dominance may be related to this rapid phosphate uptake in natural environments (Padisak 1997, Shafik et al. 2001).

In a study involving the testing for phytoplankton nutrient limitation in the Baltic Sea, three week mesocosm experiments revealed the consequence of removing N and P from 'the system' (Kangro et al. 2007). The authors were able to determine the effect of nutrient reduction on the phytoplankton. Reducing N resulted in an overall decrease in phytoplankton abundance but a shift in dominance to N-fixing, toxic filamentous cyanobacteria (e.g. *Aphanizomenon sp.* and *Nodularia spumegina*). However, reducing P supply resulted in an overall increase in phytoplankton numbers which the authors suggest indicates a high level of P storage. The authors conclude that a

reduction in N and P is necessary, but, this study also highlights that reducing nutrient loads may not have the desired outcome of a reduction in toxic cyanobacteria numbers.

This present study involves testing the effect of phosphate additions on the phytoplankton community (dominated by *C. raciborskii*) of L. Samsonvale. *In situ* dialysis tube bioassays with added pulses of ammoniacal N and/or phosphate were used. Traditional closed bottle bioassays or microcosms have been plagued by issues related to their applicability in natural systems and therefore interpretation of results (Gerhart and Likens 1975, Stephenson et. al 1984, Bloesch et al. 1988 and Carpenter and Kitchel 1988). One of the major arguments against closed bottle bioassays is that they have the potential to cause artificial nutrient limitation (Carpenter 1996). This study used dialysis tube bioassays, allowing through flow of ambient nutrients, to ensure the phytoplankton community was always exposed to ambient nutrient concentrations.

4.2 Methods

Incubators for bioassays were constructed using dialysis tubing (Visking, MWC 12 000 – 14 000, Medicell international, London, UK) with a diameter of 75 mm. The tubing was cut into 400 mm lengths and prepared using the manufacturers' instructions. The ends were folded three times and clamped with plastic seals. The bags were filled with lake water (approximately 500 mL) collected from a depth integrated sample of the top 7 m, close to the dam wall in L. Samsonvale. The bags were secured to racks using zip ties, deployed near the dam wall (Fig. 2.1 Section 2.1.1) and suspended 2.5 m below the surface (Fig. 4.1). The bioassays were fixed at this depth to ensure no photoinhibition of phytoplankton with approximately 50 %

ambient light reaching the bioassays. The depth of the water column ranged from 11 to 12 m over the course of the study.

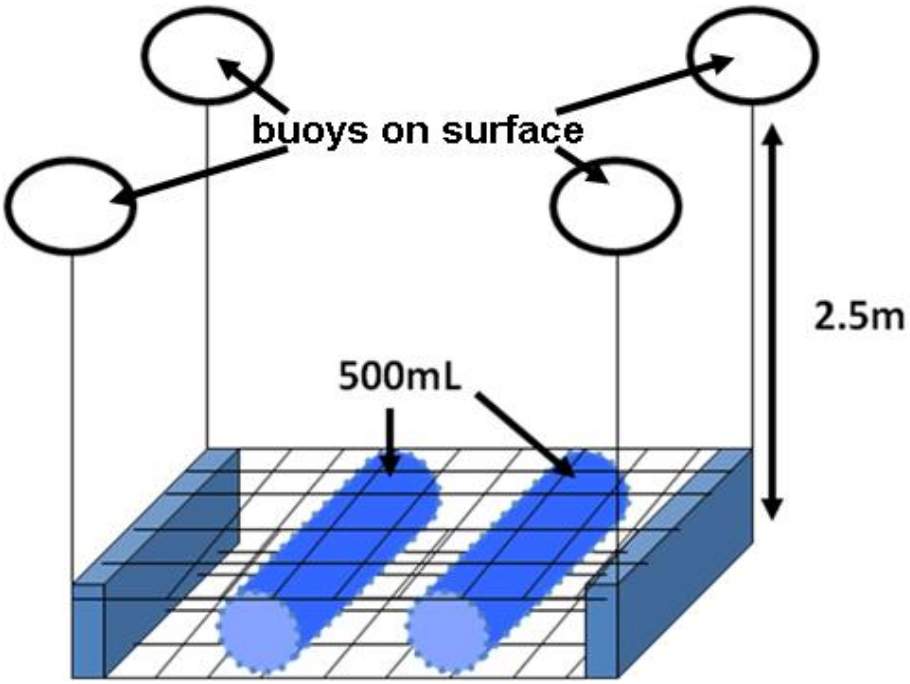

Figure 4.1 Method for securing the dialysis tube bioassays 2.5 m below the surface in L. Samsonvale

Bioassay experiments were conducted in the summer of 2006/07 and the same experiment was repeated four times (December 11-15 2006, January 15-20, February 12-17 and March 12-17 2007). The dialysis tube bags were sampled by bringing the racks to just below the surface and suspending them from the edge of a boat. Each bag was unfolded from the top and sampled using a 10 mL syringe. After a sample was taken, nutrients were added and the bag was reclamped (Fig 4.2). After all bags on a rack were sampled (ca. 10 min) the rack was returned to 2.5 m below the surface. There were four treatments for each month and three replicates of each treatment (Table 4.1). Samples for dissolved nutrient concentrations were taken at 0, 3, 24, 48, 72 and 96 h, and cell count samples were collected at 0 and after 96 h.

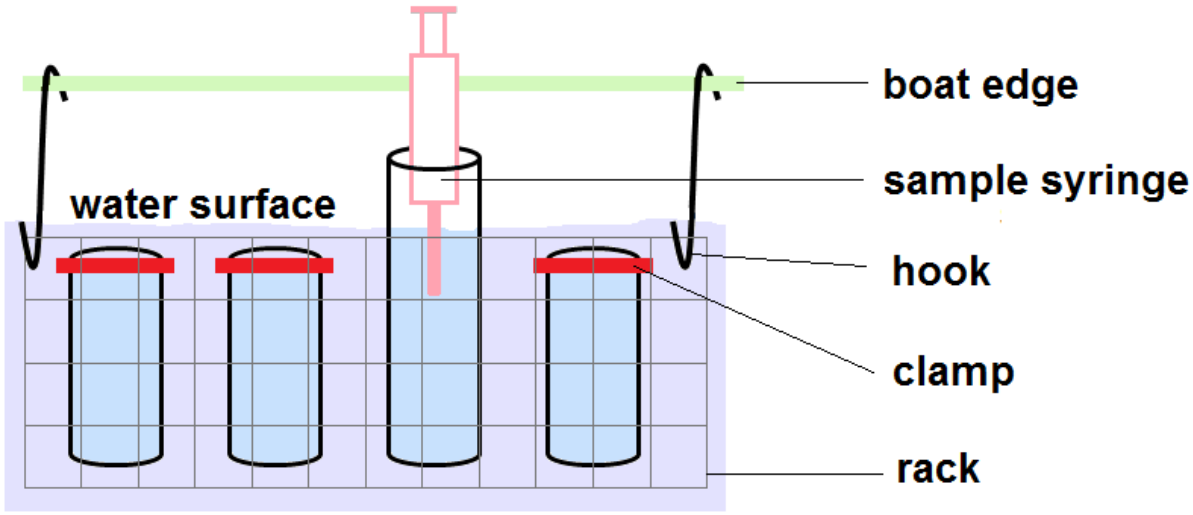

Figure 4.2 Sampling the dialysis tube bags

Table 4.1 Nutrient enrichment in dialysis tube bioassays

Name	Treatment	n
Control	none	3
DS LP	Daily Spike of Low Phosphate (0.32 µM)	3
DS HP	Daily Spike of High Phosphate (16 µM)	3
N+P	Daily spike of N (7.1 µM) combined with Phosphate (0.32 µM)	3
CRP	Constant Release Phosphate resulting in a constant high concentration of phosphate	3

Physicochemical parameters were also measured at the time of each bioassay. These included ambient dissolved nutrient concentration, temperature and irradiance (details in Chapter 3). Surface mixed layer (SML) was determined by the depth at which temperature was 0.5 °C below surface (Levitus 1982) and euphotic depth (Zeu) was determined by the depth at which photosynthetic available radiation (PAR) was 1% of the surface (Lee et al. 2007). Temperature was logged at the bioassay depth (2.5 m) every 30 minutes (E Temperature, On solutions). The surface mixed layer was determined using temperature profiles at a thermistor chain 20 m away. Euphotic depth was collected from a light profile using PAR 4-pi sensor (Licor, Lincoln, Nebraska, U.S.A) (200 mm increments from surface to 4 m) at the time of each sampling. Solar radiation was measured at the thermistor chain (5 m from the

bioassays) in watts m^2 every 10 minutes and converted to μmol photons m^{-1} s^{-2}. Phytoplankton cell abundance including *C. raciborskii* were also collected fortnightly from December 2007 to March 2008. Water was collected from a 0-3 m integrated sample from site A (Fig 2.1 Section 2.1.1) in L. Samsonvale.

In a separate experiment, the concentration of N and P within the dialysis tube bag after a spike (DS LP, DS HP, N+P) was added, was determined over 2.5 h. Dialysis tube bags were spiked with either or both N and P and samples were collected for dissolved nutrient analysis at 0, 4, 23, 38, 84, 126 and 150 mins.

To test pulsed versus constant nutrient supply, pellets of phosphate (CRP) were used. The concentration of CRP was unknown so laboratory experiments were performed to determine the likely concentration leeching from the CRP pellets. Three x 20 L drums of deionised water with a dialysis tube bag containing a 30 mg CRP pellet were analysed over 50 h. Samples for dissolved nutrient analysis were collected from within the dialysis tube bags and the ambient water within the drum at 0, 6, 30, 60, 120, 180 and 3000 mins (3000 minutes is approximately 2 d).

4.3 Results

Water temperatures (at 2.5 m) over the summer in which the experiments were performed ranged from 24.5 – 28.1 °C and the water depth (11-12 m) was consistent (Table 4.2). Ambient ammoniacal N and NO_X^- concentrations varied within each experiment and over the summer, whilst phosphate concentrations were always below detection limit (0.06 μM). NO_X^- concentrations were always higher than ammoniacal N except for day one in February when ammoniacal N was 2.4 μM and nitrate was 1.6 μM. The ambient molar DIN:phosphate ratios (assuming phosphate = 0.06 μM) were

always substantially higher (> 43) than the Redfield ratio (16:1). The addition of nutrients resulted in a DIN:phosphate ratio in the N+P treatment above 26 and the DS LP, DS HP and CRP were equal to or below 16 (Table 4.3).

Table 4.2. Ambient physico-chemical parameters and photosynthetic yield for the four experiment days during December 2006, January, February and March 2007 (mean ± SD).

	December 2006	January 2007	February 2007	March 2007
[a]**Temperature (°C)**	25.4 (0.4)	26.3 (0.5)	27.1 (0.3)	27.1 (0.4)
[b]**Zeu (m)**	6.26 (0.4)	5.92 (0.8)	4.8 (0.3)	5.48 (0.4)
[c]**SML (m)**	5-7	5-7	5-7	5-7
PO_4 (µM)	<0.06	<0.06	<0.06	<0.06
NH_4^+ **(µM)**	1.00 (0.3)	1.27 (0.75)	1.66 (0.5)	0.96 (0.11)
NO_X^- (µM)	3.51 (0.5)	3.73 (0.68)	2.21 (0.73	2.64 (0.4)
Ambient Light (mol d^{-1})	53.24 (4)	49.1 (6)	32.8 (10)	40.96 (5)
Yield (control)	0.356 (0.05)	0.362 (0.03)	0.374 (0.07)	0.334 (0.07)

[a]MEan Daily Temperature at 2.5m below the surface (depth of the bioassays)
[b]Euphotic depth (where light is 1% of surface)
[c]Surface Mixed Layer

Table 4.3. Dissolved inorganic nitrogen:phosphate (DIN:phosphate) molar ratios (in treatments) after pulses of nutrients were added to the bioassays (Mean ± SD, after 4 d). Values represent measured concentrations plus theoretical added pulse at time of addition.

	[a]Control	N+P	DS LP	DS HP	[b]CRP
December '06	59.8 (±8.7)	31.8 (±1.3)	12.6 (±1.5)	0.28 (±0.03)	0.4 (±0.13)
January '07	63.4 (±12.2)	33.2 (±4.1)	13.6 (±3.6)	0.31 (±0.08)	0.5 (±0.17)
February '07	38.6 (±11.6)	30.0 (±1.2)	10.6 (±1.5)	0.24 (±0.02)	0. 3 (±0.21)
March '07	45.0 (±6.8)	30.0 (±2.4)	10.6 (±2.5)	0.23 (±0.05)	0.6 (±0.29)

[a]Control assumes 0.06 µM for phosphate
[b]CRP concentrations taken from final day of sampling, therefore ratio was always lower in previous days

The concentration of phosphate, which the phytoplankton were exposed to in the dialysis tubes containing the constant release phosphate, was highly variable in the laboratory experiments. The concentration of phosphate released from the pellets was close to maximum after 3 h and reached 16 – 29 μM P after 50 h (Fig 4.3). The release of phosphate from the pellet fitted the curve $y = 0.1292 \text{ Ln}(x) + 0.02518$ with an R^2 value of 0.9 where x = time in hours. In contrast to the laboratory experiments which did not involve phytoplankton, the concentration measured in the dialysis tube bags was initially high (22.4 – 24.3 μM P) after 3 h and then decreased rapidly to 6.4 ± 6.4 μM P after 24h. This data fitted the curve $y = 1.3912x^{-0.767}$ with an R^2 value of 0.8 where x is time in hours. The specific phytoplankton uptake of phosphate in the L. Samsonvale dialysis tube experiment was calculated from the measured concentration in the laboratory and the measured concentration in the dialysis tubes (Fig. 4.4). Phytoplankton uptake rates were determined using Equation 7.

Equation 7.

$$\text{Uptake rate} = (R_1 P_f e^{-R1t}) + (R_2 P_o e^{-R2t})$$

R_1 and R_2 = Rate of increase and decrease in P concentration (from Fig 4.)
P_f and P_o = Concentration of P final and original
t = Time in hours

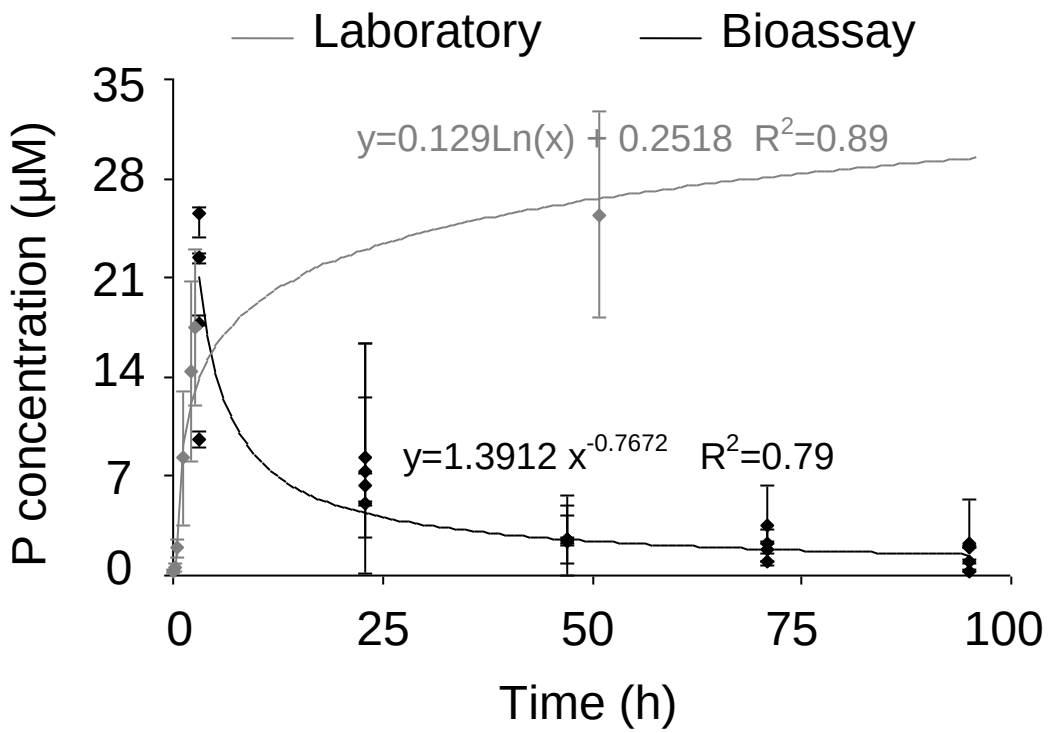

Figure 4.3. Constant release phosphate concentrations in both laboratory and field experiments. Laboratory = release rate from the constant release pellet (concentration within the dialysis tube) in DI water under laboratory conditions. Bioassay = release rate from the constant release pellet (concentration within the dialysis tube) in lake water under field conditions. Bioassay line shows diffusion out of dialysis tubes under field conditions.

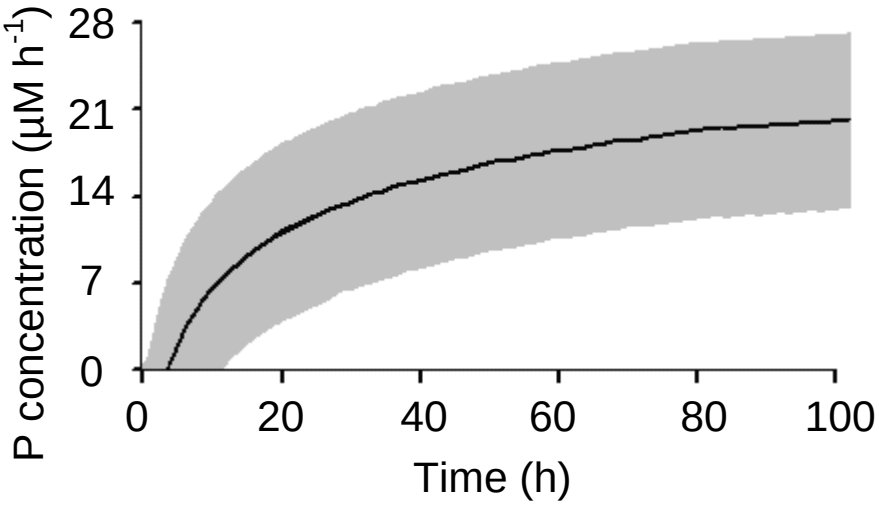

Figure 4.4. Concentration of phosphate taken up within the dialysis tube bags. Black line indicates mean and grey shading indicates 1 SD from the mean.

The phosphate added in a daily spike 16 µM (DS HP) was below detection limit after 2 h whilst the DS LP treatment was below detection limit after 4 minutes (Fig 4.5). This included the 0.32 µM concentration of phosphate which was added with the 7.14 µM N. In contrast, approximately 60 % of the N remained in the dialysis tube bags after 2.5 h.

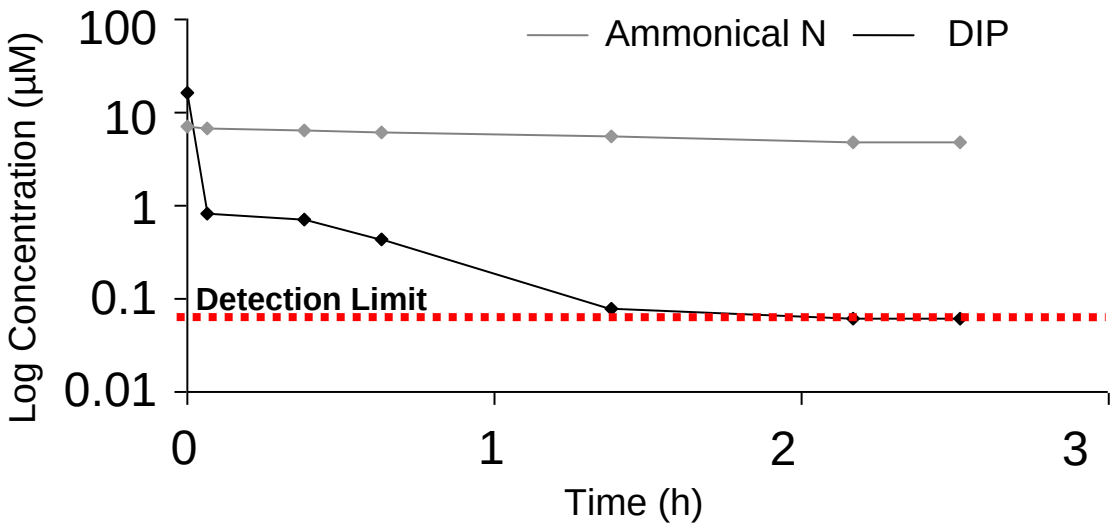

Figure 4.5. Concentration of the daily nutrient pulse (µM) in situ in dialysis tube bioassays over time showing relative uptake and regeneration of N and P. The 0.32 µM phosphate concentration was below detection limit after 4 min.

The composition of the phytoplankton community and relative cell abundances changed over the four experiment months (Fig. 4.6). However, cyanobacteria were always the dominant phytoplankton group and of them *C. raciborskii* was most dominant (both in terms of cell abundances and biovolume). The other main groups of phytoplankton at the time of the experiments were *Aphanocapsa/Cyanodictyon* (cyanobacteria), *Planktolyngbya* (cyanobacteria) and diatoms. *Aphanocapsa* and *Cyanodictyon* were grouped together because they could not be differentiated.

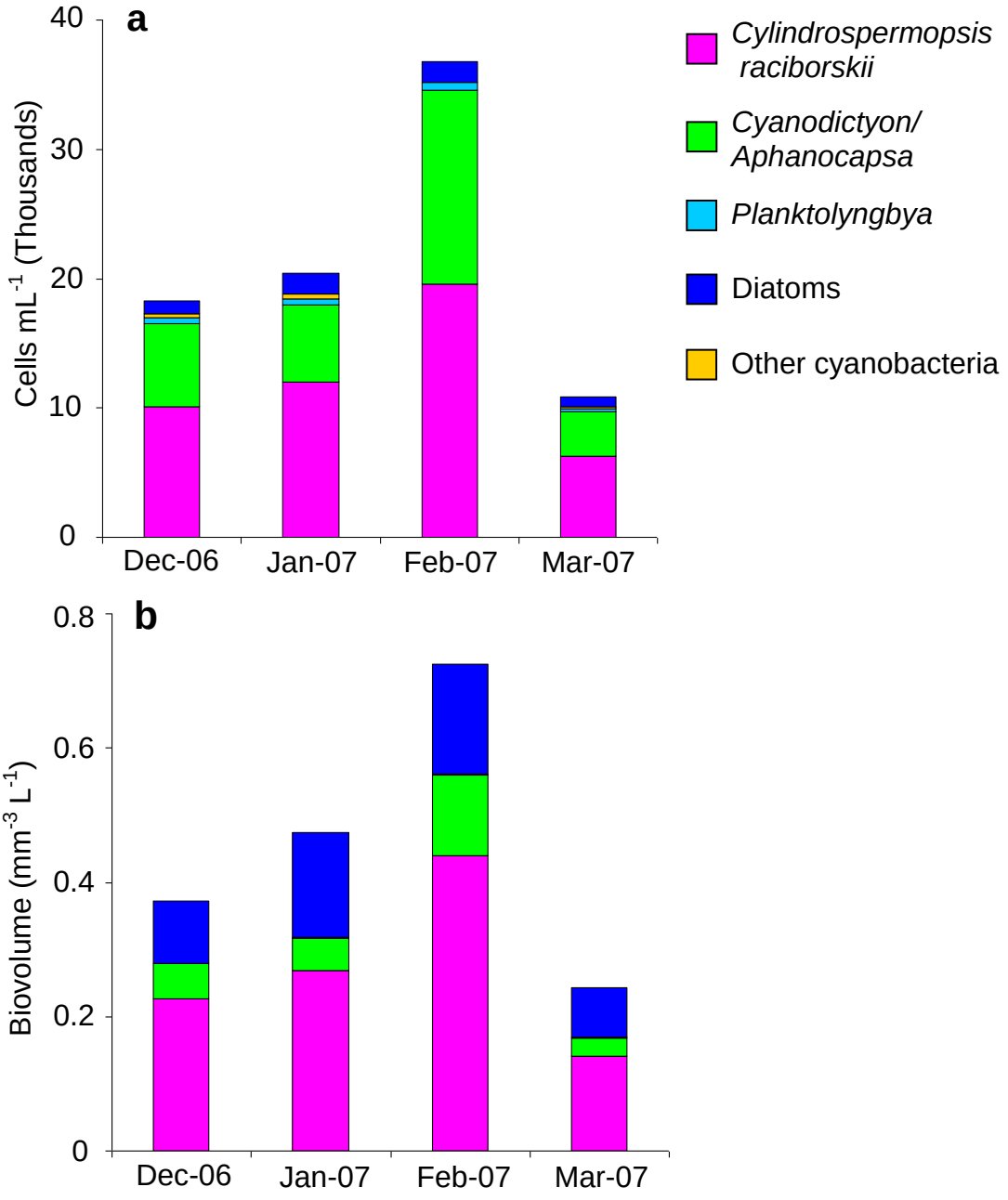

Figure 4.6. Composition of the a. cell abundances (cells mL^{-1}) and b. biovolume (mm^3L^{-1}) of the phytoplankton community in L. Samsonvale at time zero of each experiment month.

The dominance of *C. raciborskii* (as a percentage of total phytoplankton biovolume) within the community increased after 4 d with the daily spikes of phosphate (DS LP and DS HP) (Fig 4.7). On average the increase was 25% (± 6%) in all months except February. This was in contrast to a decrease in dominance of *C. raciborskii* when both N and P were added in three of the four experiments. When phosphate was added at a

constant high concentration (CRP), *C. raciborskii* dominance decreased. In December, January and March the percentage of *C. raciborskii* within the control did not change after 4 d. In the February experiment however, there was a significant increase ($p<0.05$) in *C. raciborskii* dominance in the control. In this experiment there was also an increase in *C. raciborskii* dominance with the N+P and CRP treatments, which was not seen in the other three experiments.

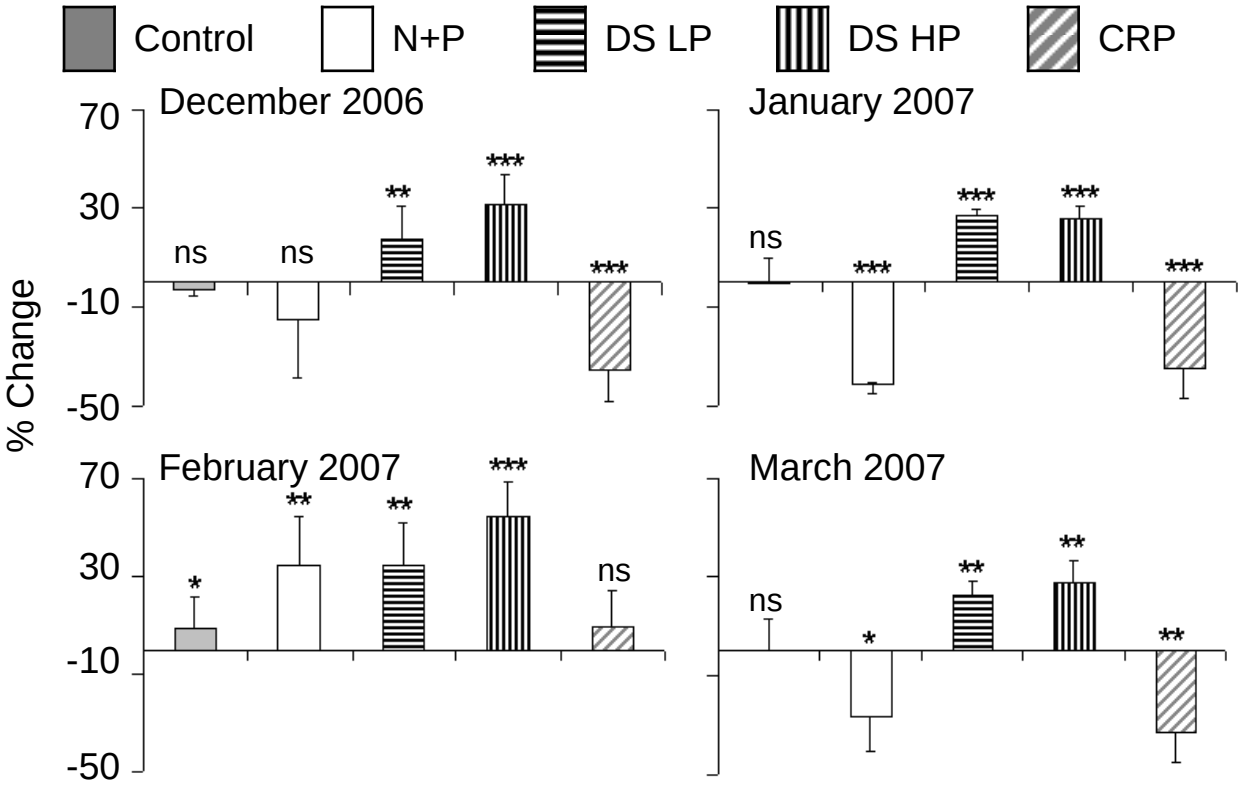

Figure 4.7. Percent change in *C. raciborskii* dominance (±SD) (percentage of the total phytoplankton biovolume) relative to time zero (T0). Results show change after 4 d in *C. raciborskii* dominance within nutrient bioassays in L. Samsonvale. C: control, N+P: daily pulse of ammoniacal N (7.1µM N) and phosphate (0.3µM P), DS LP: daily pulse of low phosphate (0.3µM P), DS HP: daily pulse of high phosphate (16µM P), CRP: constant release phosphate (> 6.4 µM) ns: not significant, *$p<0.05$, **$p<0.01$, ***$p<0.005$. Statistics represent change in *C. raciborskii* dominance after 96 h from time 0.

There was an increase in total biovolume with all phosphate-only treatments (DS LP, DS HP and CRP) in all experiments except February (Fig 4.8). This was in contrast to no significant increase in total biovolume ($p>0.05$) when phosphate was added with N (N+P treatment). *C. raciborskii* biovolume increased in the DS LP and DS HP treatments in three of the four experiments and decreased in the CRP treatment. In December the highest total biovolume was observed with a daily spike of 16 µM P (DS HP), while in January the highest total biovolume was seen with a daily spike of 0.32 µM P (DS LP). In March both the DS LP and DS HP treatments showed the highest total biovolume after 4 d. In February, total biovolumes for all nutrient treatments and the control decreased or remained constant after 4 d.

At the start of each experiment, total phytoplankton biovolumes were similar in December and January (0.45 ± 0.05 mm^3 L^{-1}), highest in February (0.75 ± 0.08 mm^3 L^{-1}) and lowest in March (0.24 ± 0.02 mm^3 L^{-1}) (Fig 4.8). This was reflected in the fortnightly cell abundance of *C. raciborskii* (Fig 4.9). During the summer in which the experiments were conducted, *C. raciborskii* cell abundances were highest in February and lowest in March 2008. L. Samsonvale experienced a 'bloom' of *C. raciborskii* in late November 2007 (30 000 cells mL^{-1}) after which the counts dropped to approximately 10 000 cells mL^{-1} except for the increase in February to 20 000 cells mL^{-1}.

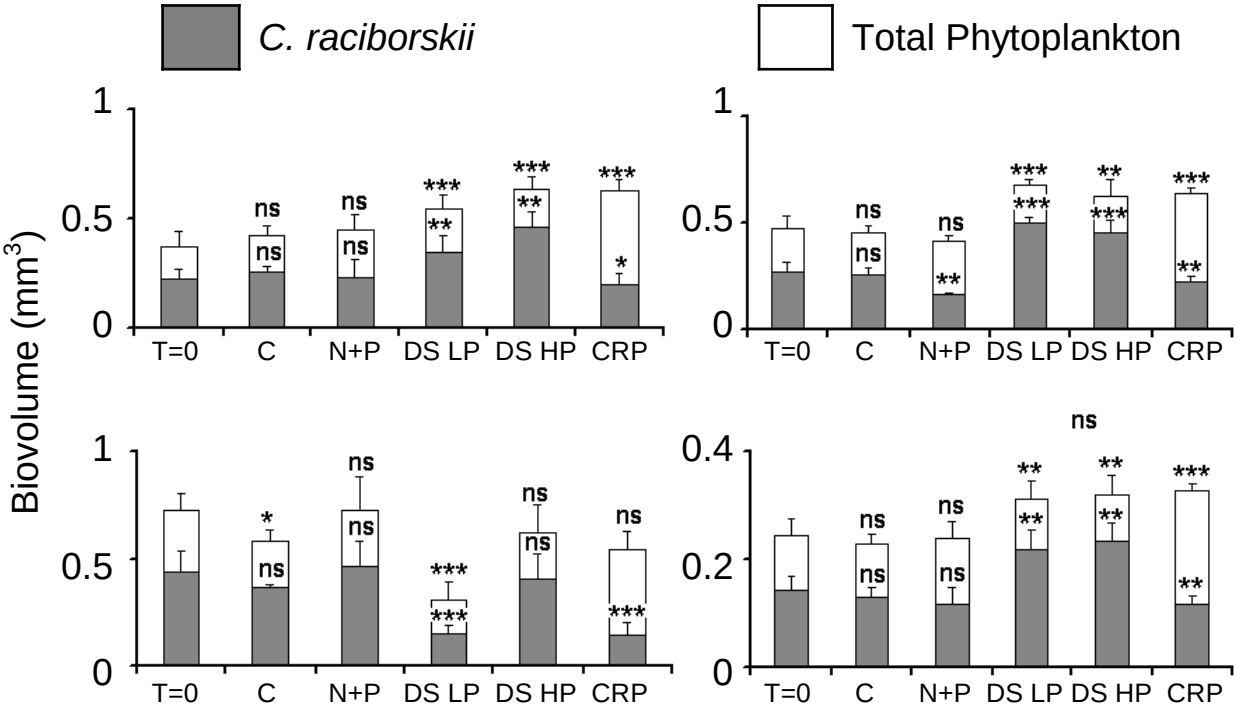

Figure 4.8. Change in *C. raciborskii* and other phytoplankton biovolume (mm3 L-1 ±SD) in in-situ bioassays with added nutrients at time zero (T0) and after 96 h. C: control, N+P: daily pulse of 7.1µM ammoniacal N and 0.3µM P, DS LP: daily pulse of low phosphorus (0.3µM P), DS HP: daily pulse of high phosphorus (16µM P), CRP: constant release phosphate (> 6.4 µM) ns: not significant, *:p<0.05, **:p<0.01, ***:p<0.005. Statistics represent change in biovolume after 96h from time 0.

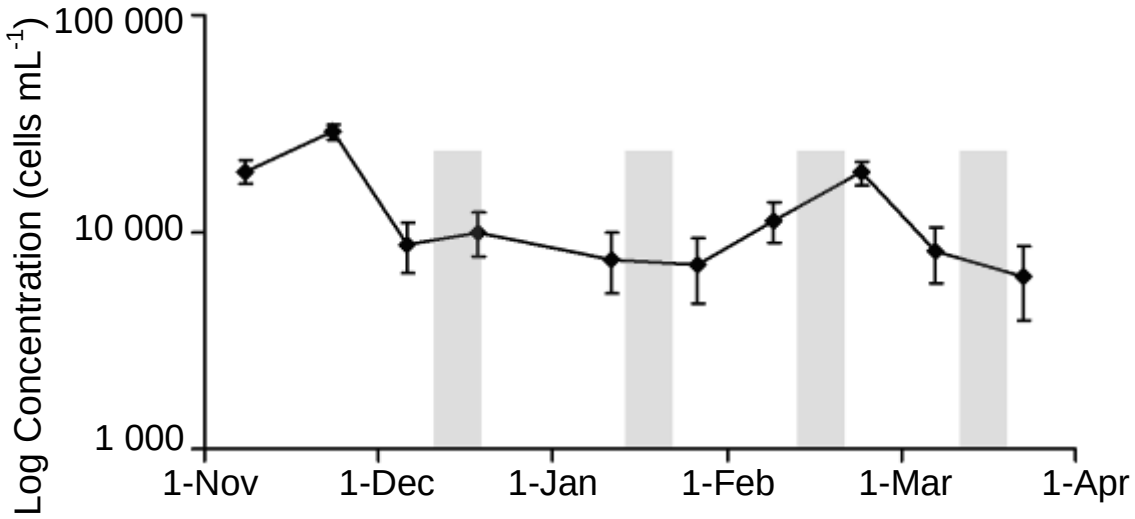

Figure 4.9. Cell abundance (Log cells mL^{-1} ±SD) for *C. raciborskii* in L. Samsonvale at the Dam wall site (near the bioassays) over the 2006/07 Summer. Shaded bars represent time and duration of bioassay.

4.4 Discussion

This study showed that daily pulses of phosphate caused not only an increase in *C. raciborskii* cell abundances but also an increase in dominance of *C. raciborskii* in L. Samsonvale. Furthermore, when phosphate was constant in high concentrations *C. raciborskii* dominance decreased. The results from this chapter indicate that *C. raciborskii* gained a competitive advantage within the phytoplankton community probably by rapidly taking up phosphate when added daily although appears to lose this advantage when phosphate is in constant high supply.

C. raciborskii phytoplankton dominance increased with pulsed phosphate supply. It is thought that this is due to it's ability to rapidly take up and store phosphate (Istávnovics et al. 2000), therefore gaining a competitive advantage over other phytoplankton. The composition of a phytoplankton community can be changed by the addition of one or more nutrients (Holz and Hoagland 1996, Pinckney et al. 1999, Worm et. al. 2000, Mitrovic et al. 2001, Manoylove and Stevenson 2001 and Flinders and Hart 2009). For example, in a eutrophic estuary (Neuse River Estuary, North Carolina), changes in phytoplankton composition were manipulated by pulsed nutrient supply and the authors suggest that this is due to the adaptive growth rate of some species (Pinckney et al. 1999). Also, in culture experiments the cyanobacterium *Microcystis novacekii* suppressed the growth of the green alga *Scenedesmus quadricauda* when ammonia was supplied in a pulse (Yoshinda et al. 2005). However, when ammonia concentrations were constantly high, *S. quadricauda* outgrew *M. novacekii*. The competition for phosphate between a toxic dinoflagellate species, *Alexandrium minutum*, and a non-toxic species, *Heterocapsa triquetra* was investigated (Labry et al. 2008). Using phosphate starvation and single pulsed P supply, the authors demonstrated that *A. minutum* was able to out-compete during

phosphate starvation (with P storage), whilst *H. triquetra* was able to out-compete immediately after pulsed phosphate supply due to its ability to rapidly increase growth rate in the presence of phosphate.

There was a decrease in *C. raciborskii* dominance with a constant high concentration of phosphate. There was difficulty in determining the concentrations of phosphate to which the phytoplankton were exposed due to a high variability in leeching between replications and a high variability between experiments. However, the change in structure of the phytoplankton assemblage may depend on the mode of nutrient supply (Militza et al. 2006) and not the concentration. For example in culture experiments of a 'natural phytoplankton assemblage' phosphate was added in the same concentration in different rates (pulse every 2 d, every 6 d, total starvation or constantly low) and resulted in changes in the phytoplankton composition (Ghosh et al. 1999). *Navicula cryptocephala* became dominant in cultures starved of phosphate, *Phormidium mucosum* and *Hormidium sp.* were dominant in cultures pulsed with phosphate and *Synedra ulna* was dominant in cultures with continual phosphate supply. Their study demonstrates that the mode of phosphate supply is more likely to affect the phytoplankton composition than the concentration of phosphate.

The decrease in *C. raciborskii* dominance in the constant concentrations of constant release phosphate (CRP) bioassay was due to an increase in diatoms. Diatoms in general are rapidly growing organisms when in non-nutrient-limiting conditions and a decrease in diatom dominance has been linked to a decrease in phosphate concentration (Egge 1998).

The concentrations of pulsed P used in this study were comparable to the range of concentrations previously measured in L. Samsonvale (Burford et al. 2005). The 0.32 μM phosphate treatment corresponds to approximately five times background concentrations (0.06 μM phosphate) and represents a relatively low concentration pulse whilst the 16 μM phosphate pulse is similar to concentrations recorded during inflows (Harris & Baxter 1996). Studies on streams flowing into L. Samsonvale found phosphate concentrations of 1.3-2.9 μM in low flow conditions and up to 20 μM phosphate during extreme rainfall events (Harris and Baxter 1996). This indicates that a range of phosphate concentrations likely to enter L. Samsonvale which may result in an increase in *C. raciborskii* dominance.

The rapid disappearance of phosphate within hours in the bioassays might suggest P limitation in the phytoplankton community of L. Samsonvale. The 16 μM phosphate pulse was below detection limit after approximately 1 h and the 0.32 μM phosphate pulse was below detection limit after 4 min. The 7.1 μM N pulse was still detectable (4.7 μM) after 2.5 h. *C. raciborskii* cell abundance have been correlated with low P concentrations in this system (Burford et al. 2006) and around the world (Padisak 1997). The detection of approximately 60% of the N after 2.5 h indicates that the spikes of nutrient remained within the dialysis tube bags for at least 2.5 h. This indicates that the relative disappearance of P is due to phytoplankton uptake and not leeching from the bags.

When 0.32 μM phosphate plus 7.1 μM ammoniacial N was added in a daily pulse there was no increase in *C. raciborskii* dominance in December, January and March. This nutrient addition kept the N:P ratio above 27:1. When only phosphate was added,

the ratio was always equal to or below the Redfield ratio (16:1). The increase in *C. raciborskii* dominance with pulses of phosphate but not both ammoniacal N plus phosphate suggest that the DIN:phosphate ratio affect *C. raciborskii* dominance. Studies have shown that a low cellular N:P ratio, is more likely to control growth than P concentration for a N-fixing cyanobacterium (*Aphanizomenon flos-aquae* (DeNobel et al. 1997). Also, in a shallow Mediterranean lake (L. Kastoria, Greece) *C. raciborskii* dominated the phytoplankton when the N:P ratio dropped below 16:1 and not necessarily when phosphate concentration changed (Moustaka-Gouni et al. 2007). Thus, the effect of P concentrations on cyanobacteria dominance may be complicated by the source and concentration of available N as well as the N:P ratio. The overall biovolume in the N+P treatment was unchanged ($p>0.05$) after four days but this was due to a relatively large variation between replicates in all months except January. An analysis of the cell abundances showed an increase in the non-toxic, relatively small (approximately 40 times smaller in biovolume than *C. raciborskii*) *Cyanodictyon/Aphanocapsa* (non-N fixing) with the N+P addition. It is possible that the increase in N in the N+P treatment gave these non-N-fixing genera an advantage over *C. raciborskii*.

The change in dominance of *C. raciborskii* and total cell abundances in February were not consistent with changes in the other months. In February, there was a statistically significant decrease in total phytoplankton biovolume after four days. One possibility is that the phytoplankton community was in a senescent phase. The results in February could have been affected by a number of factors including: low light, presence of zooplankton grazers or viral lysis. Also, a decrease in phytoplankton

biovolume after nutrient addition sometimes occurs despite known nutrient limitation (Schelske 1984).

Given the consistency in results from the experiment months December, January and March it is plausible to suggest that periodic injections of phosphate may increase the dominance of *C. raciborskii* and very high sustained concentrations of phosphate may lead to a decrease in *C. raciborskii* dominance.

4.5 Conclusions

This study showed that phosphate additions to the phytoplankton community of L. Samsonvale changed the species composition. *C. raciborskii* increased in dominance with daily spikes of phosphate (0.32 and 16 µM) and decreased in dominance with phosphate when it was continuously available (>6.4 µM). Whilst the constant-release phosphate released at unrealistically high concentrations, inferences can be made about how *C. raciborskii* would respond in an environment of constantly high phosphate. To fully understand and quantify whether *C. raciborskii* dominance decreases when phosphate is readily available, lower concentrations (>6.4 µM) and a more uniform delivery should be trailed.

C. raciborskii dominance increased with daily P inputs and decreased with constant high concentrations and with N additions. This indicates that *C. raciborskii* may have a competitive advantage within the P-depleted phytoplankton community of L. Samsonvale due to it potential ability to rapidly take up phosphate (discussed in Chapter 5). However, in order to understand the relationship between *C. raciborskii* from L. Samsonvale and P, culture studies should be completed.

Chapter 5. Understanding the relationship between *Cylindrospermopsis raciborskii* and phosphorus in culture

5.1 Introduction

Phosphorus availability can be a key factor controlling cyanobacterial growth (Armstrong 1999). However, the relationship between P and *C. raciborskii* is poorly understood. For the toxic Australian strains, phosphate uptake kinetics and the subsequent effect on toxin production have not been determined. Furthermore the role of dissolved organic phosphorus (DOP) in sustaining *C. raciborskii* (or any toxic cyanobacterial) blooms has been largely ignored in the literature.

Phosphate is an essential element for cyanobacterial growth but in Australian reservoirs and lakes, is generally found in limiting supply (Davis & Koop 2006). Phosphorus limitation in cyanobacteria may lead to a reduction in the cellular content of P (Droop 1973) carbohydrate accumulation (Oliver 1994), an increase in phosphate uptake rates (Gotham & Rhee 1981) and/or storage of non-limiting nutrients such as N (Riegmann & Mur 1984). It has been suggested that cyanobacteria in general have a higher storage capacity for phosphate than other phytoplankton, potentially giving them a competitive advantage in P-limited systems (Sommer 1985).

Acquisition, storage and metabolism of phosphate are regulated by a variety of mechanisms (Reynolds 2006). For example, the expression of genes (which code for proteins involved in phosphate uptake) may be induced or up-regulated in phosphate-limiting conditions. The marine cyanobacteria, *Trichodesmium*, was found to have genes predicted to encode for proteins associated with high affinity phosphate transport and hydrolysis of phosphonates (Dyhrman 2005). The authors believe that

with the absence of these genes in other marine cyanobacteria, *Trichodesium* may be uniquely adapted to thrive in P-limited environments. The effect of phosphate limitation on gene expression in *Synechocystis* was investigated using microarrays and a phosphate-depleted culture (Suzuki et al. 2004). Phosphate limitation in a *Synechocystis* culture ultimately induced the expression of genes responsible for phosphate transport (P_{st1} and P_{st2}), alkaline phosphatase (*pho*A) and extracellular nucleases (*nuc*H). It also repressed the expression of urtA (gene responsible for the binding protein of urea ABC-type transporter) despite being located at a different site on the genome. Both the *pho*A and *nuc*H genes are involved in increasing the availability of phosphate in the extra-cellular environment (Suzuki et al. 2004). The study of gene expression in *C. raciborskii* is outside the scope of this thesis and will not be discussed further.

In environments with very low phosphate concentrations cyanobacteria may adopt more efficient ways to use P or employ different uptake pathways (Wagner and Falkner 2001). For example, the marine cyanobacteria, *Crocosphaera watsonii,* has a robust capacity for scavenging P that differs from other cyanobacteria, such as *Prochlorococcus* (Dyhrman and Haley 2005) Some cyanobacteria may have the ability to utilise organic P in addition to phosphate. Despite the potential importance of DOP as a phosphate source in phosphate-limited systems, there is little data comparing phosphate and DOP utilisation by cyanobacteria. For marine *Synechococcus*, growth rates and particulate P concentration were the same when grown using either DOP (ATP) or phosphate as a sole P source (Fu et al. 2006). The authors suggest that DOP is likely to be a primary source of phosphate in oligotrophic waters since the DOP in oligotrophic waters comprises a large fraction of the

dissolved P (Karl et al. 2002). One strain of *Aphanizomenon* has been shown to prefer phytin (the calcium salt of myo-inositol hexakisphosphate) to inorganic phosphate (Reichardt 1971) and in a study involving 50 strains of cyanobacteria, growth was reported in all strains when subjected to phosphate monoesters as a sole source of P (Whitton et al. 1991). The growth rate of all 50 strains was close to or faster than with the presence of phosphate however, relatively high concentrations of P were used; 1 mg P L^{-1} (~70 μM) (Whitton et al. 1991) and 100 μM P (Rippka et al. 2000).

Of the many species of cyanobacteria only some produce toxins and of those that do, only some strains within species produce toxins. For example *C. raciborskii* is found in many parts of the world but only Australian, New Zealand and Thailand strains produce CYN and strains in Brazil produce PSP (Ohtani et al. 1992, Lagos et al. 1999, Li et al. 2001, Molica et al. 2002 and Wood and Stirling, 2003). Also, within these strains toxin production is poorly correlated with cell abundances indicating that there are conditions that may not be favourable for growth but favourable for toxin production. Factors affecting toxin production are largely unknown. Some literature suggests that toxic strains of cyanobacteria have a higher nutrient requirement compared to non-toxic strains (Downing 2005 and Vézie et al. 2002), whilst others show that high toxin concentrations are correlated with low dissolved nutrient concentration (Saker 2000). One hypothesis is that toxin production is energetically unfavourable for the cell and therefore only occurs when energy (nutrients) is abundant, however, this hypothesis is largely untested. Some suggest toxin is produced as a defence mechanism (to avoid predation) or when the cell is under stress (nutrient limitation) (Jang et al. 2003) whilst others suggest that toxins are secondary metabolites that just happen to be toxic to humans (Apeldoorn et al. 2007).

Phosphorus has the potential to affect CYN production in *C. raciborskii*. *M. aeruginosa* growth and toxin production under different P concentrations was investigated in batch culture using both toxic and non toxic strains (Vézie et al. 2002). This study suggests that low P, whilst not favouring growth, may favour toxin production. The highest toxin (microcystin-LR) concentration was recorded at high N and P concentrations and also at low P with moderate N (Vézie et al. 2002). P concentration may also affect the location of CYN (intra or extra-cellular) within *C. raciborskii*. The external environments in which *C. raciborskii* maintains CYN within the cell is poorly understood although some studies suggest the toxin is actively released from the cell during times of stress. For example, Saker and Griffiths (2000) observed that when *C. raciborskii* was in the exponential growth phase less than 10% of the total measured CYN was released from the cell, compared to when *C. raciborskii* was in stationary phase where >50% of the total CYN was extra-cellular. In conjunction, Saker (2000) showed a strong negative correlation between DIN and CYN in cultures of *C. raciborskii* which indicates CYN may be produced as a stress response (ie when DIN concentrations are low).

This chapter will examine the effect of P, in organic and inorganic form, on growth rate, toxin production, P uptake and storage and alkaline phosphatase activity of two toxic (CYN producing) strains of *C. raciborskii*.

5.2 Methods

5.2.1 Batch Culture Experiments

Batch cultures of two non-axenic *Cylindrospermopsis raciborskii* strains (AWT/205 and NPD Strain) were used to determine growth rates under varied P conditions

including pre-condition, P source and P concentration (Table 5.1). Cells were pre-conditioned in two ways where they were either starved in P-free Jaworskii's Media (JM, Appendix 1) or allowed to grow in exponential phase in regular JM for 5 days prior to the start of batch culturing. Aliquots of each strain were then added to flasks containing one of two P sources (G-6-P and DIP) in one of five concentrations (0, 0.032, 0.16, 0.64, 16 µM). Following pre-conditioning, samples were collected to determine $time_0$ cell abundance and particulate P concentration (see sections 3.2.1.1 and 3.2.1.2). Particulate P concentrations were determined by filtering known volumes of sample through 0.75 µm glass fibre filters (Whatmann) and then analysing the filtrate for P concentration as per section 3.2.1.2

Each flask was analysed daily for optical density at 750nm using a Varian DMS 90 spectrophotometer. This method has successfully been used to determine cell abundances for *C. raciborskii* (Saker et al. 1999). To ensure accurate correlation between OD and cell abundance for the strains AWT/205 and NPD, 60 samples from each strain were analysed for cell abundance (section 3.2.3). This data was used to construct a standard curve allowing cell abundance to be calculated from OD_{750} for each strain (Fig 5.1 and 5.2). The change in cell abundance over time was used to determine growth rate using equation 8.

Equation 8.

$$\text{Growth rate} = \text{Ln} \frac{(\text{cell counts time}_2 / \text{cell counts time}_1)}{\text{time (days)}}$$

Table 5.1. Summary of the batch culture experiments

Strain	P source	Pre-condition	P concentration (µM)	n
AWT/205	G-6-P	Starved	0	3
			0.032	3
			0.16	3
			0.64	3
			16	3
		Non-starved	0	3
			0.032	3
			0.16	3
			0.64	3
			16	3
	DIP	Starved	0	3
			0.032	3
			0.16	3
			0.64	3
			16	3
		Non-starved	0	3
			0.032	3
			0.16	3
			0.64	3
			16	3
NPD	G-6-P	Starved	0	3
			0.032	3
			0.16	3
			0.64	3
			16	3
		Non-starved	0	3
			0.032	3
			0.16	3
			0.64	3
			16	3
	DIP	Starved	0	3
			0.032	3
			0.16	3
			0.64	3
			16	3
		Non-starved	0	3
			0.032	3
			0.16	3
			0.64	3
			16	3

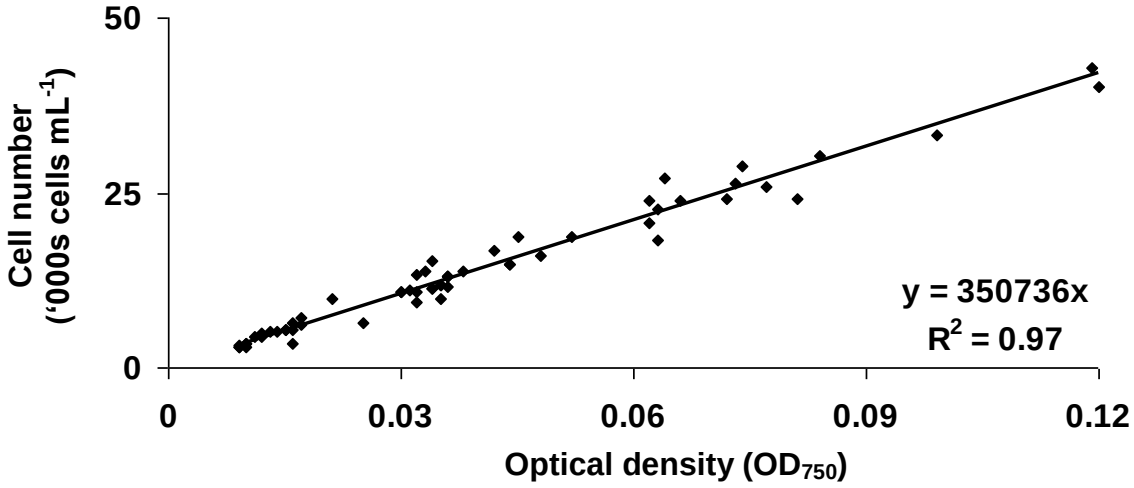

Figure 5.1. Standard curve for AWT/205 used to calculate cell abundances from optical density.

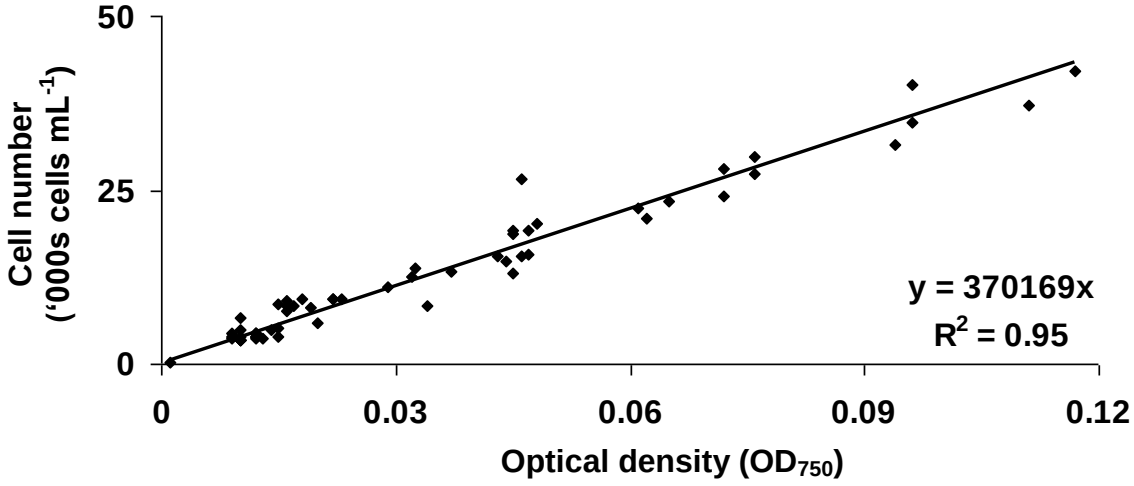

Figure 5.2. Standard curve for NPD used to calculate cell abundances from optical density.

5.2.1.1 Calculating phosphate uptake

Specific ^{33}P phosphate uptake rates were determined using batch cultures of AWT/205 and NPD strain. Cells from exponential phase batch cultures (approximately 50 000 cells mL^{-1}) were harvested and grown in P-free JM for 10 days before ^{33}P uptake experiments were performed. Pulses of ^{33}P and ^{31}P in concentrations ranging from 0.04 ng to 20 µg P L^{-1} (combined concentration) were used to determine maximum P uptake and half saturation constants (section 3.2.3).

5.2.2 Through-flow Culture Experiments

This study used through-flow cultures of *C. raciborskii* to determine *C. raciborskii* growth, phosphate uptake and toxin production under a range of phosphate and DOP concentrations. Four separate through-flow cultures were run continuously and uninterrupted for approximately three months. Two of the through-flow cultures contained JM with increasing concentrations of phosphate (potassium dihydrogen phosphate) as the sole P source and two contained JM with increasing concentrations of G-6-P (glucose-6-phsophate) as the sole P source. Two strains of *C. raciborskii* (AWT/205 and NPD) were grown, making a total of four through-flow cultures (Table 5.2).

Table 5.2 Experimental design

Through-flow culture	Strain of *C. raciborskii*	Source of P
A	NPD	phosphate
B	NPD	G-6-P
C	AWT/205	phosphate
D	AWT/205	G-6-P

The through-flow cultures consisted of 2 L vacuum flasks with an overflow tube to the side (Fig 5.3). Media was delivered to each flask via a peristaltic pump and 6 mm (internal diameter) tubing at a constant rate. The volume in the through-flow culture was kept constant with the overflow tube. Air was supplied to the cultures via sterilised compressed air through a tube in the rubber seal. Gentle mixing (to minimise biofilm formation) was maintained using magnetic stir bars. Light (80 µmol photon m^{-2} s^{-1}) was provided by fluorescent bulbs on a 12/12 h daily cycle and the temperature ranged from 27 to 29ºC.

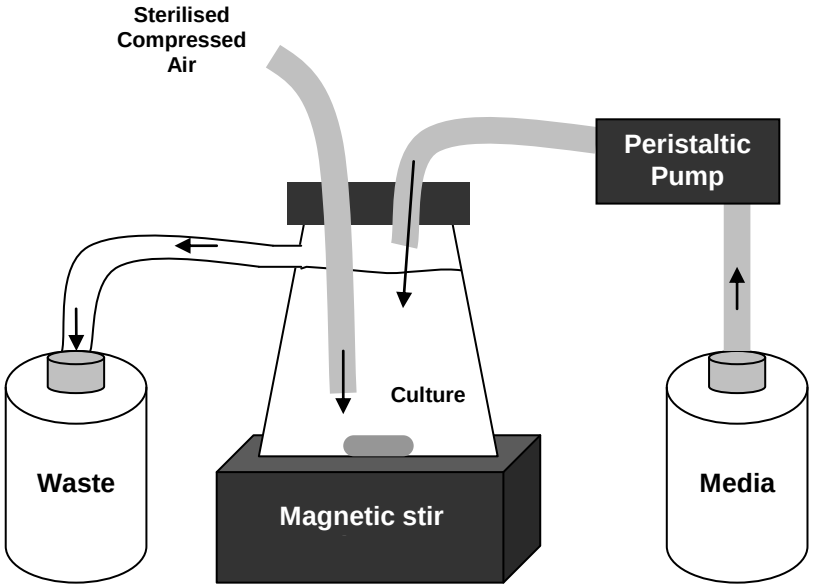

Figure 5.3 Schematic diagram of through-flow culture set up

The initial concentration of cells was 5.0 x 10^4 (±1.0 x 10^3) cells mL^{-1} in all through-flow cultures. The concentrations of P tested were 0, 0.03, 0.16, 0.64 and 16 μM (for both phosphate and G-6-P). The duration of each experiment differed slightly to allow two complete media changes before cells were sampled for a given concentration. The dilution rate was lower for the G-6-P (0.15 d^{-1}) through-flow cultures than the phosphate through-flow cultures (0.2 d^{-1}) because cells grown in a media containing only G-6-P had a slower growth rate and were diluted out when grown at the dilution rate of the phosphate through-flow cultures. Cells were starved of P by growing in P-free JM for a period of 14 d (NPD Strain) or 9 d (AWT/205). At each P concentration the through-flow cultures were sampled every day for three consecutive days and after sampling on the third day, the P concentration was changed (Table 5.3). Sub-samples (approximately 300 mL) were collected from the outflow and immediately chilled (4 ± 2 ºC). Samples were taken for cell abundances, photosynthetic yield, extra-cellular and total toxin, particulate P, total P and phosphate concentration. With the two G-6-P through-flow cultures (B and D), alkaline phosphatase activity was also determined.

Table 5.3 Sampling schedule in days from commencement and P concentration (µM) added to the four through-flow cultures (A, B, C, and D). Y = a sample was taken

Through-flow culture day				P concentration (µM)	Sample
A	B	C	D		
0	0	0	0	0	-
12	12	7	7	0	Y
13	13	8	8	0	Y
14	14	9	9	0	Y
14	14	9	9	0.03	-
29	31	20	29	0.03	Y
30	32	21	30	0.03	Y
31	33	22	31	0.03	Y
31	33	22	31	0.16	-
41	54	42	50	0.16	Y
42	55	43	51	0.16	Y
43	56	44	52	0.16	Y
43	56	44	52	0.64	-
56	75	61	71	0.64	Y
57	76	62	72	0.64	Y
58	77	63	73	0.64	Y
58	77	63	73	16	-
66	94	85	92	16	Y
67	95	86	93	16	Y
68	96	87	94	16	Y

5.2.2.1 Analysis of toxin

This study quantified cylindrospermopsin (CYN) and deoxy-cylindrospermopsin (deoxy-CYN) assuming CYN was 7-epi-CYN (see Section 1.1.1). Both total and extra cellular CYN and deoxy-CYN concentrations were determined using HPLC/MS/MS (PE-Sciex), (Eaglesham et al. 1999). Total CYN and deoxy-CYN concentration was determined from a freeze-thawed (3 times) sample, filtered (0.22 µM membrane filter - Nilex) and then analysed using HPLC/MS/MS. Extra-cellular toxin concentration was determined by gravity filtering 10mL through a 0.75 µM glass fibre filter, and a 0.22 µm membrane filter then analysed using HPLC/MS/MS. Intra-cellular toxin was calculated by subtracting the extra-cellular concentration from the total concentration.

The HPLC/MS/MS used was a LC-200 series pump, series 200 auto sampler and API 300 Ms/Ms with turbo ion spray interface (Perkin Elmer Sciex Instruments, Thornhill, Ontario, Canada). The transition from the $(M + H)^+$ ion (416 m/z) to the fragment ion at 194 m/z (CYN) or 400 m/z to 194 m/z (deoxy-CYN) was used for quantitation using the multiple reactant monitoring mode. The detection limit of the method was 0.2 µg L^{-1} and the precision of analysis is approximately 12%. (Eaglesham et al.1999).

5.2.2.2 Analysis of alkaline phosphatase activity

There are a variety of methods used to determine alkaline phosphatase activity and one of the simplest is the fluorometric method using methylumbelliferyl phosphate (Hoppe 1983) (Fig 5.4). Alkaline phosphatase was measured using the 4-methylumbelliferyl phosphate substrate solution 4-MUP (Sigma Co., St Luois, MO, USA) and Shrimp Alkaline phosphatase SAP (Sigma Co) as the enzyme standard modified from Hoppe (1983). The 4-MUP substrate solution came in a ready-to-use solution containing buffer and the SAP had a specific activity of 1 unit μL^{-1} where 1 unit is equal to the amount of enzyme causing the hydrolysis of 1 µmol of 4-MUP per minute at 25ºC. Fluorescence was determined with a fluorescence spectrophotometer (Varian Cary Eclipse, California, U.S.A) at 360 and 440 nm.

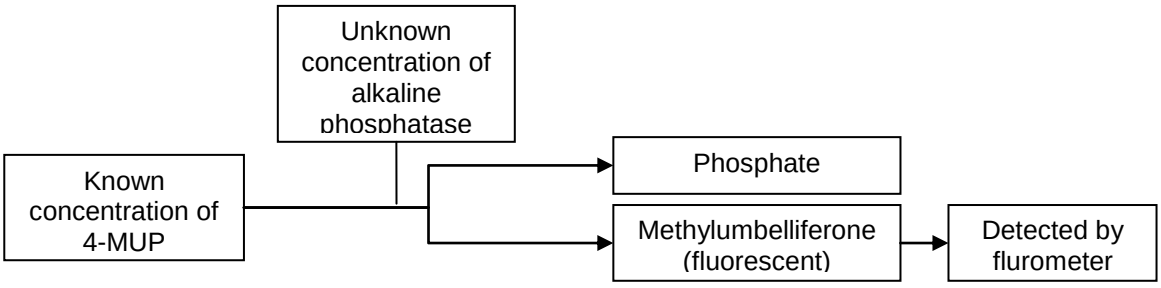

Figure 5.4 Schematic diagram of the 4-methylumbelliferone method for alkaline phosphatase quantification

A standard curve of fluorescence readings in varying concentrations of SAP was determined and used to convert to phosphate production rates (Equation 7 and 8) (Fig. 5.5). To achieve this, different concentrations of SAP were added to 1 mL 4-MUP substrate solution and 2 mL deionised H_20. These were then incubated in the dark for 6 h at 25 ºC to achieve maximum enzyme hydrolysis. To determine alkaline phosphatase activity of *C. raciborskii*, 20 mL samples from the through-flow cultures were syringe filtered (0.45 µm) and kept at 25 ºC whilst substrate solution was being added. Two mL from the 20 mL filtered samples were added to 1 mL of 4-MUP substrate solution and incubated in the dark at 25 ºC. The sample was analysed using the fluorometer as above over a 6 h period at the following times 30 s, 0.5, 1, 2, 3, 4, 5 and 6 h.

Equation 7.

Equation from standard curve slope in Fig. 5.10.

$$\text{SAP (units)} = \frac{\text{fluorescence} - 7.8991}{1006.5}$$

Equation 8.

Phosphate production rate (µmol phosphate min^{-1}) = Units of SAP x 1 µmol phosphate min^{-1}

1 unit of SAP is equal to the amount of enzyme causing the hydrolysis of 1 µmol 4-MUP (or creating 1 µmol phosphate) per minute at 25ºC.

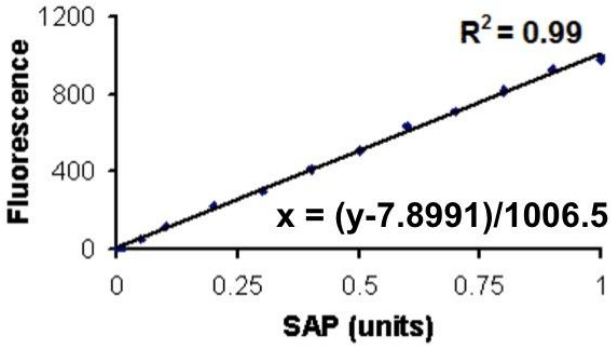

Figure 5.5 Standard curve of measured fluorescence of Shrimp Alkaline Phosphatase (SAP) units in 4-MUP solution after 6 h incubations at 25°C

5.3 Results

5.3.1 Batch Culture Experiments

The concentration of *C. raciborskii* (AWT/205) increased over time with all tested concentrations of DIP and G-6-P (Fig 5.3 and 5.4). When there was no P added, cell abundances remained stable or decreased slightly. Also, in both strains, there was considerably less growth with the two strains grown in G-6-P compared to DIP. The strains that were pre starved began to grow immediately when P was added however, there was a slight lag phase with the strains that were not pre-starved. In both strains grown in DIP, the highest cell abundance after 11 d was with the highest concentration of DIP tested (16 µM) and the lowest final cell abundance was with the lowest concentration (0.032 µM). There was no difference ($p<0.05$) in the final cell abundance for the both strains at all concentrations of G-6-P.

The maximum growth rate for both strains was achieved with the lowest concentrations of DIP and G-6-P (0.032 and 0.16 µM respectively) (Fig. 5.8). The starved pre condition resulted in higher maximum growth rates for both strains in DIP ($p>0.05$). The NPD strain achieved faster maximum growth rate than the AWT/205 strain with all concentrations, sources and pre-conditioning. The maximum growth rate for both strains grown in DIP was consistently higher than the maximum growth rate achieved in G-6-P at all concentrations.

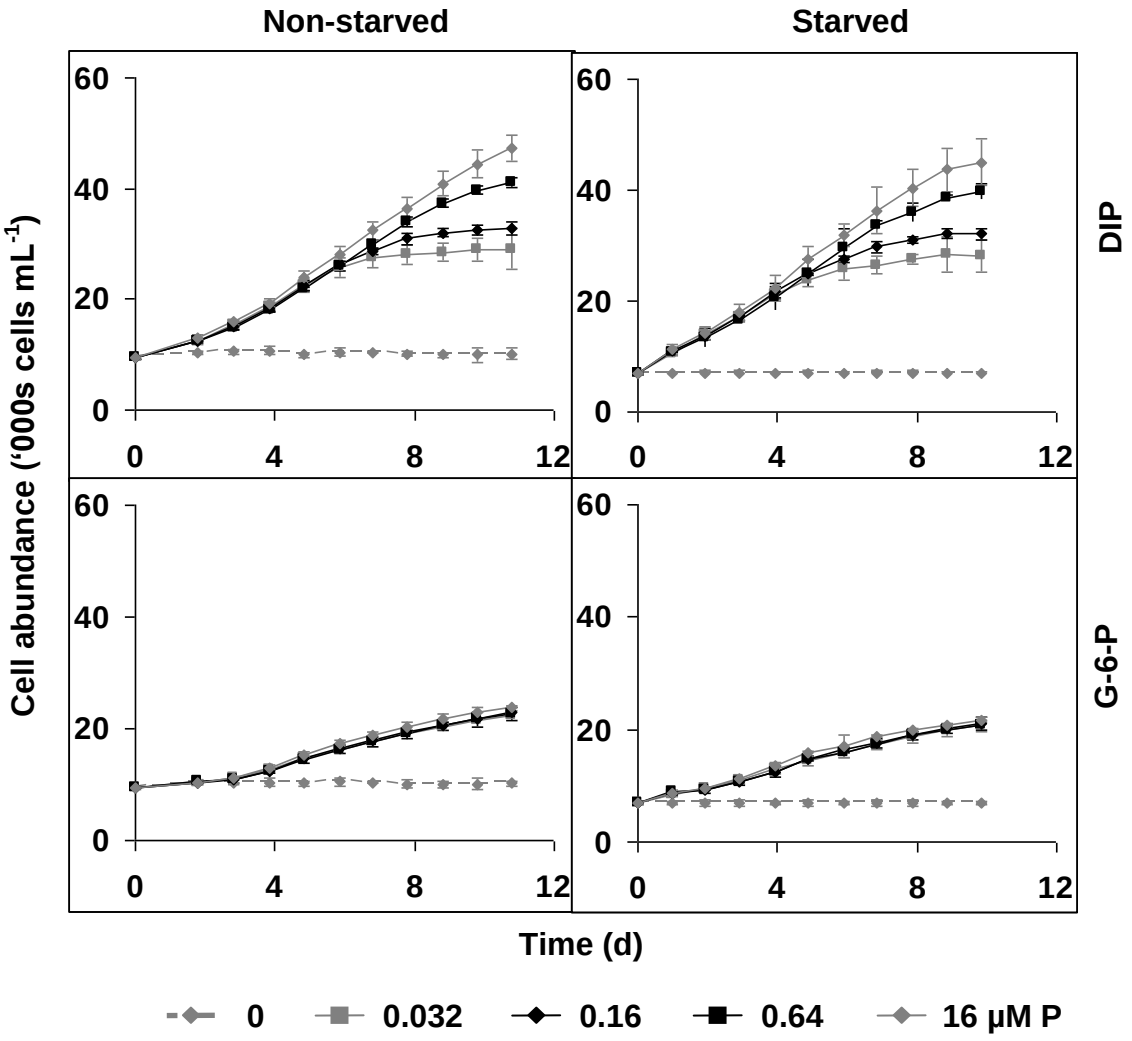

Figure 5.6. AWT/205 batch culture growth experiments showing the effect of P source (G-6-P and DIP), P concentration (0, 0,032, 0.16, 0.64 and 16 µM) and pre-condition (P-starved and non-starved) on mean (± SD) cell concentrations over time.

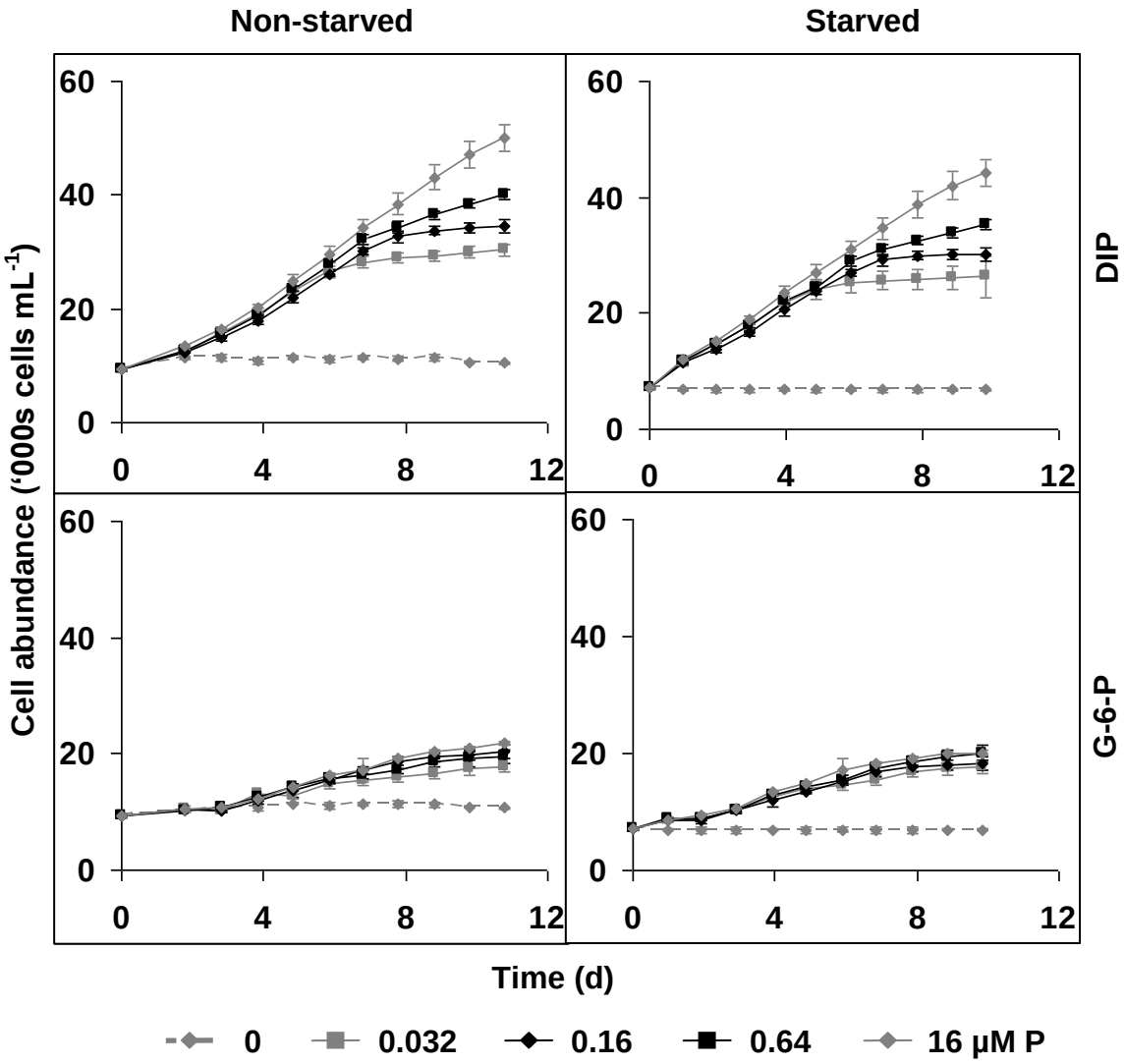

Figure 5.7. NPD batch culture growth experiments testing P source (G-6-P and DIP), P concentration (0, 0,032, 0.16, 0.64 and 16 µM) and pre-condition (P-starved and non-starved). on mean (± SD) cell concentrations over time.

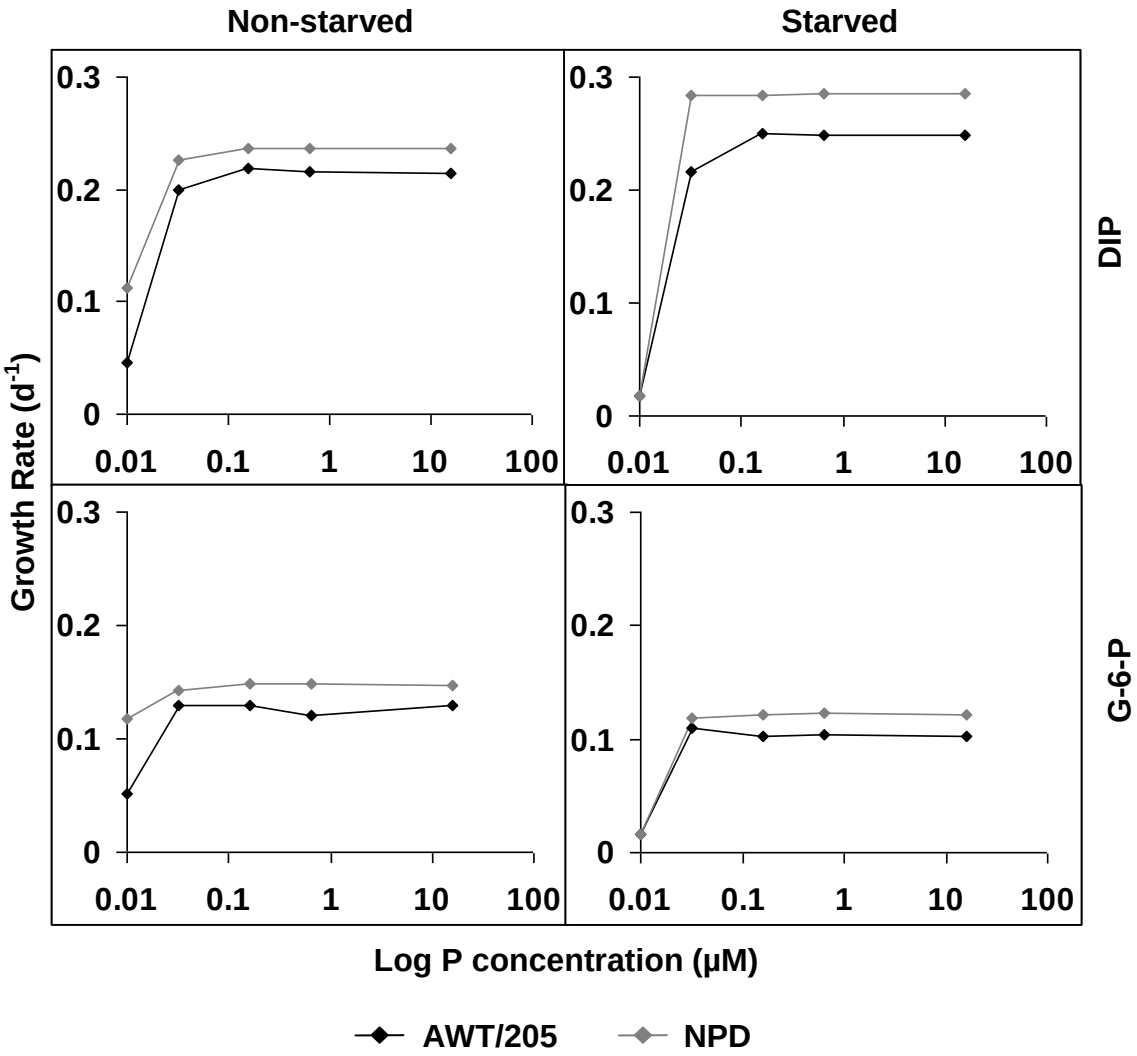

Figure 5.8. Growth rates of AWT/205 and NPD strains with different P concentrations (0, 0,032, 0.16, 0.64 and 16 µM), sources (G-6-P and DIP) and pre-condition (P-starved and non P-starved).

5.3.1.1 Phosphorus uptake rates

Phosphate uptake rate was calculated using the radio isotope ^{33}P and batch cultures of the two strains of *C. raciborskii*, NPD and AWT/205 (Fig 5.9 and 5.10). The NPD strain had a faster maximum uptake rate (2.5 pmol cell^{-1} min^{-1}) than the AWT/205 (2.0 pmol cell^{-1} min^{-1}). However their KS values were also different, with the NPD strain (0.09 pmol P L^{-1}) being less than that of the AWT/205 (0.12 pmol P L^{-1}).

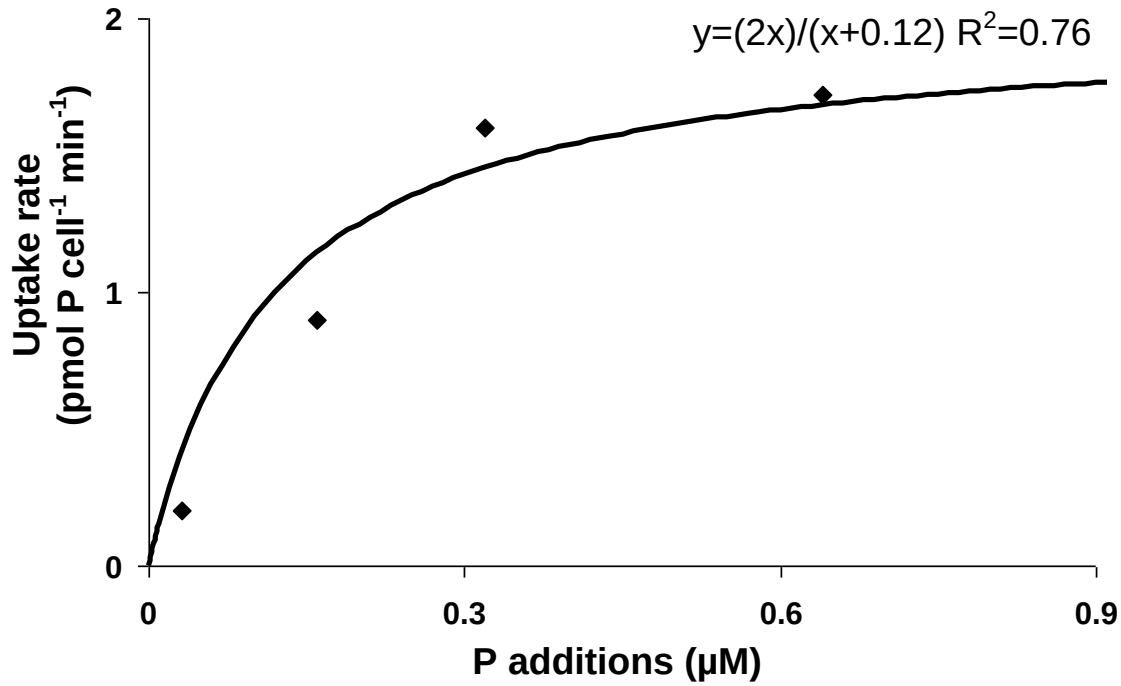

Figure 5.9. Phosphate uptake rate (pmol P cell^{-1} min^{-1}) using radioisotope ^{33}P, of *C. raciborskii* (AWT/205 Strain)

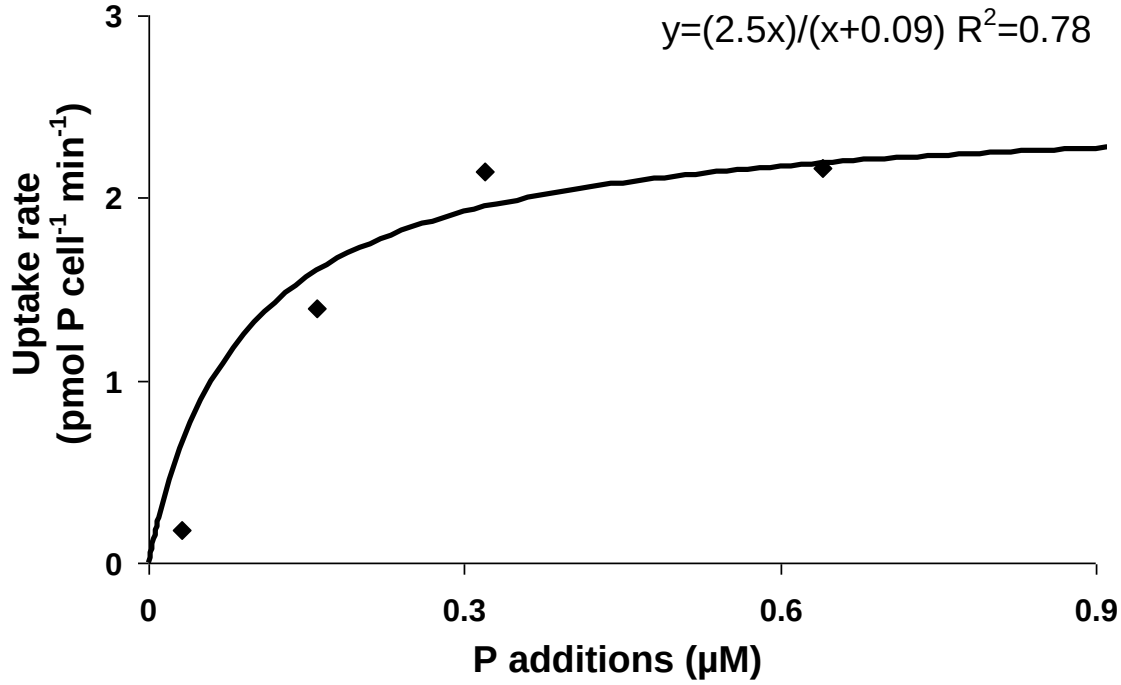

Figure 5.10. Phosphate uptake rate (pmol P cell^{-1} min^{-1}) using radioisotope ^{33}P, of *C. raciborskii* (NPD Strain)

5.3.2 Through-flow Culture Experiments

5.3.2.1 Phosphorus/Phosphate starvation

To determine the maximum time where cells could be grown in P-free media whilst still remaining viable after P was reintroduced, cultures grown in P-free media for different time periods, were tested. Neither ATW/205 nor NPD strains were viable after 15 d. At 14 d the NPD strain was still viable and the AWT/205 strain was viable after a maximum of 9 d. Since the strains were starved for different periods of time, results from the day 9 sample were used to compare the two strains in terms of P starvation response. The AWT/205 strain decreased in cell abundances at a slightly more rapid rate (13.7 x 10^3 cells mL^{-1} d^{-1}) than the NPD strain (10.1 x 10^3 cells mL^{-1} d^{-1}) (Fig. 5.11 a). Photosynthetic yield decreased linearly in both strains at a similar rate (Fig. 5.11 b). The exception was no change in the yield in the NPD strain over the first 5 d. For both the AWT/205 and NPD strain there was a decrease in filament length with phosphate starvation although the cell size did not appear to change when P was reintroduced to the cultures. After P starvation, the NPD strain contained approximately four times more carbon per cell compared to the AWT/205 strain despite having a similar overall carbon concentration (7.3 ±0.3 and 7.8 ±0.2 mg C L^{-1}). The NPD cells also contained more P and N than the AWT/205 cells and AWT/205 had a higher molar N:P cellular ratio. The NPD strain was more toxic than the AWT/205 strain ($p<0.01$) (Table 5.4).

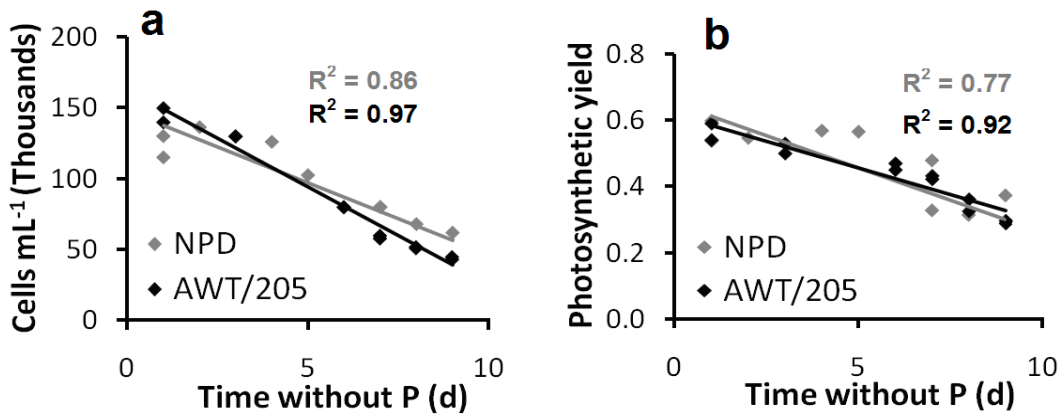

Figure 5.11 Change in (a) cell abundances of *C. raciborskii* and (b) Photosynthetic yield during P starvation. On day 0 the P concentration entering the through-flow culture went from ca. 48 to 0 µM.

Table 5.4. Mean (±SD) Parameters of *C. raciborskii* during P starvation. 9 days after inflow was changed from excess phosphate concentration (48 µM) to 0. Dilution rate = approximately 0.2 d^{-1} (n=6). Significance levels - **P<0.05 **P>0.01 ***P>0.001*

	NPD	AWT/205	Sign. level
Growth rate d^{-1}	-0.55	-0.49	
Carbon concentration (mg L^{-1})	7.3 (0.3)	7.8 (0.2)	ns
Carbon content (pg C $cell^{-1}$)	562 (8)	150 (4)	***
Cellular N:P ratio	4.5 (2)	7.4 (0.5)	*
P content (µmol P mg C^{-1})	0.20 (0.02)	0.06 (0)	**
Toxin content (mg CYN mg C^{-1})	0.77(0.40)	0.24 (0.03)	**
N content (µmol N mg C^{-1})	1.1 (0.2)	0.5 (0.1)	***

5.3.2.2 Growth rate with increasing concentrations of phosphorus

As phosphorus was removed from the culture *C. raciborskii* cells began to die off however, once phosphate was reintroduced, *C. raciborskii* cells began to grow (Fig. 5.12 and 5.13). There was considerably slower growth in both of the strains when grown in G-6-P compared to DIP at the same concentrations.

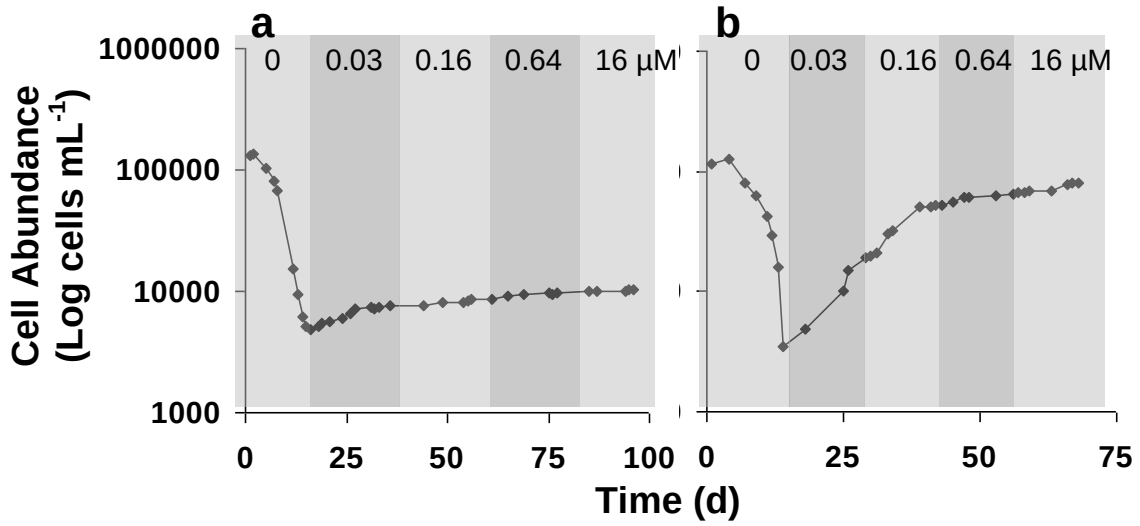

Figure 5.12. The *C. raciborskii* (strain NPD) cell abundance with changing concentrations of a) G-6-P and b) DIP in the inflow of a through flow culture. Shaded bars represent concentration of P (as DIP or G-6-P) in the inflow of the through-flow culture.

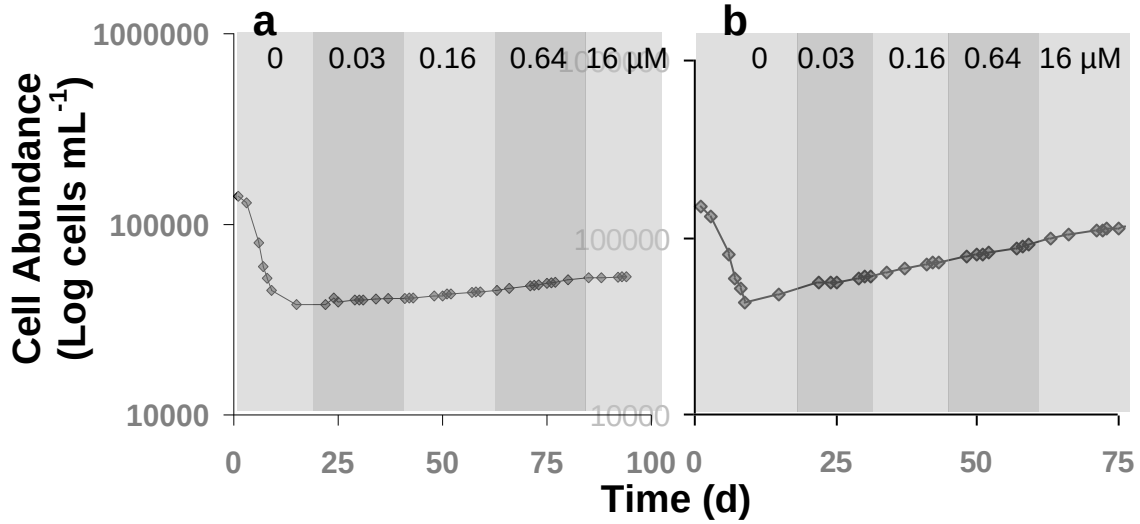

Figure 5.13. The *C. raciborskii* (strain AWT/205) cell abundance with changing concentrations of a) G-6-P and b) DIP in the inflow of a through flow culture. Shaded bars represent concentration of P (as DIP or G-6-P) in the inflow of the through-flow culture.

For the NPD strain, the concentration of P within the cell (µmol P mmol C^{-1}) was relatively high after 14 d in P-free JM (0.84 µmol P mmol C^{-1}) and decreased when phosphate was added (Fig 5.14). However, there was an increase in particulate P concentration when external phosphate increased from 0.64 to 16 µM. The AWT/025 strain contained relatively low concentrations of P within the cell after P starvation

and this decreased further (0.46 to 0.27 µmol P mmol C^{-1}) after phosphate was added (0.03 µM) (Fig 5.14). After the initial phosphate addition, particulate P concentration increased with external phosphate concentration up to 0.73 µmol P mmol C^{-1} with 16 µM phosphate.

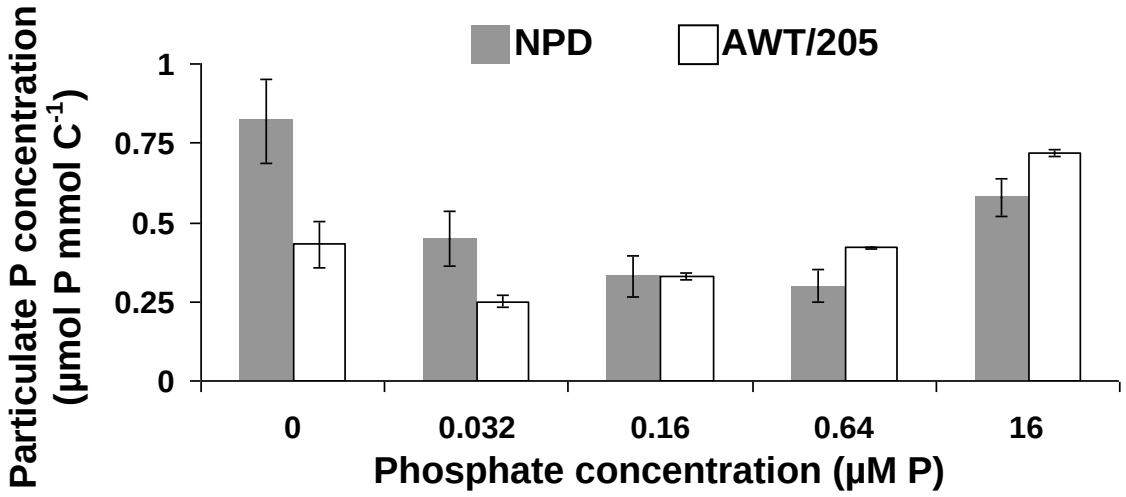

Figure 5.14 The concentration of particulate P with changing concentrations of inflow P within a through-flow culture.

Both the AWT/205 and the NPD cultures produced alkaline phosphatase. With a saturating concentration of phosphate (48 µM P) the AWT/205 culture produced more AP than the NPD strain, 0.5 and 0.3 fM phosphate h^{-1} respectively. After P starvation however, the AP increased in both strains with the NPD strain showing a higher initial activity (0.95 fM phosphate h-1) than the AWT/205 strain (0.8 fM phosphate h-1) at 0 µM P (Fig 5.15). The AP activity in the NPD strain decreased with the first two concentrations of G-6-P (0.03 and 0.16 µM) then remained constant with increasing G-6-P concentration. The alkaline phosphatase activity of the AWT/205 culture remained constant at increasing concentrations of G-6-P.

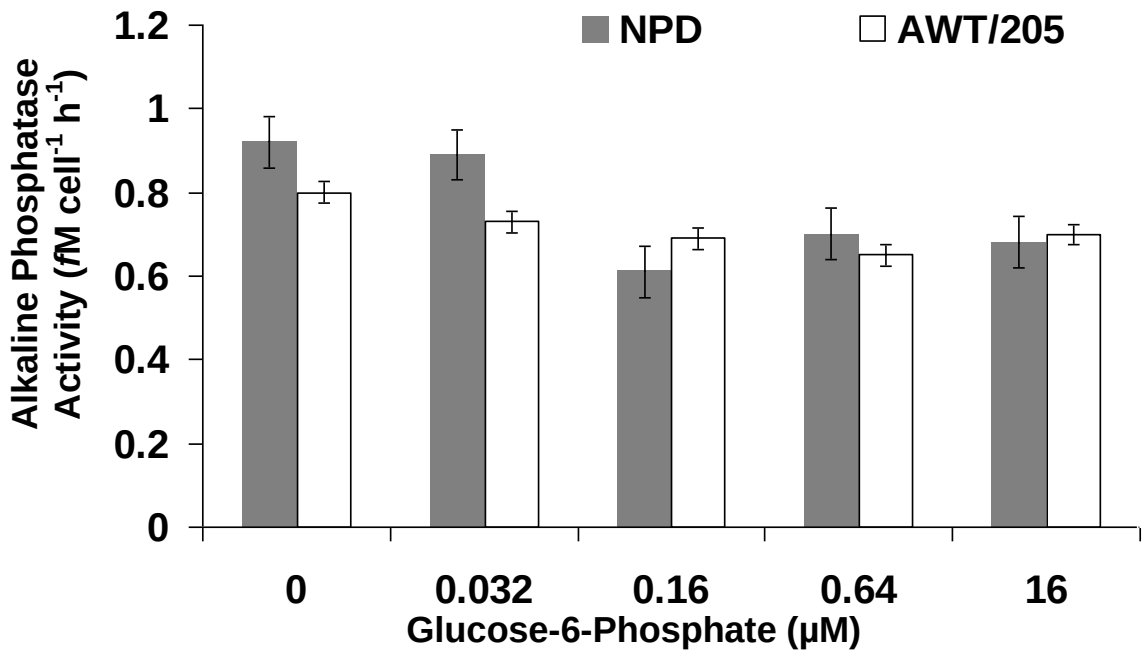

Figure 5.15 Specific alkaline phosphatase activity (fM phosphate cell^{-1} h^{-1}) with increasing concentrations of Glucose-6-Phosphate for two strains for *C. raciborskii* (NPD and AWT/205).

5.3.2.3 Toxin production

The NPD strain produced more toxin than the AWT/205 strain throughout the experiments. When the NPD strain was grown in phosphate, toxin production decreased when phosphate was added (Fig. 5.16). After phosphate starvation the NPD strain toxin concentration was 0.6 ± 0.1 mg CYN mg C^{-1} and when phosphate was added (0.03 µM P) toxin concentration decreased to 0.45 ± 0.05 mg CYN mg C^{-1} and then to 0.1 ± 0.01 mg CYN mg C^{-1} with 0.16 µM P. Toxin concentration remained low (0.1 mg CYN mg C^{-1}) with further increases in phosphate concentration (0.64 and 16 µM P). The NPD strain produced slightly more deoxy-CYN than CYN at all phosphate concentrations.

The AWT/205 strain grown in phosphate produced the same amount of deoxy-CYN as CYN at the three highest concentrations of phosphate (Fig 5.16). CYN

concentration did not change with phosphate concentration in this strain however, the concentration of deoxy-CYN was higher after phosphate starvation (0.25 ± 0.01 mg CYN mg C^{-1}) compared to when phosphate was above 0.16 µM P (0.1 ± 0.01 mg CYN mg C^{-1}).

There was a higher proportion of intra-cellular CYN mg C^{-1} in the NPD strain compared to the AWT/205 strain although this was not always statistically different ($p>0.01$) (Fig 5.17). Generally intracellular CYN mg C^{-1} increased with increasing concentrations of phosphate, however, again this was not always statistically different. There was a high variability associated with the intra-cellular fraction of CYN mg C^{-1}.

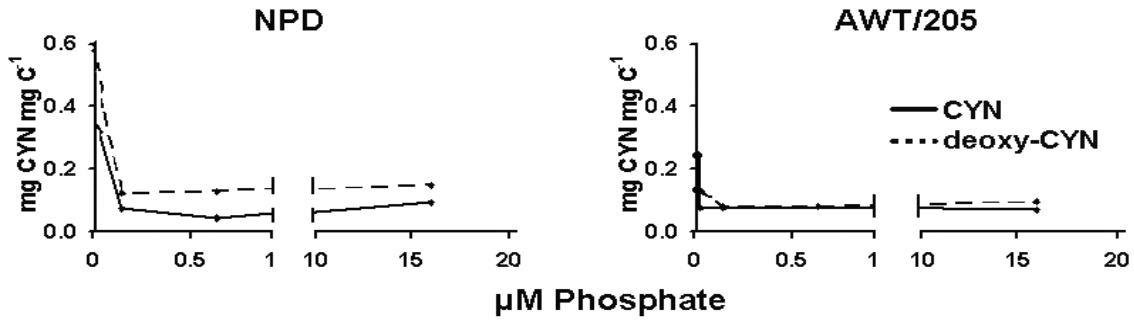

Figure 5.16. Total toxin (CYN and deoxy-CYN) concentrations (mg CYN mg C^{-1}) with increasing P concentrations (phosphate µM) in two strains of *C. raciborskii* (NPD and AWT/205)

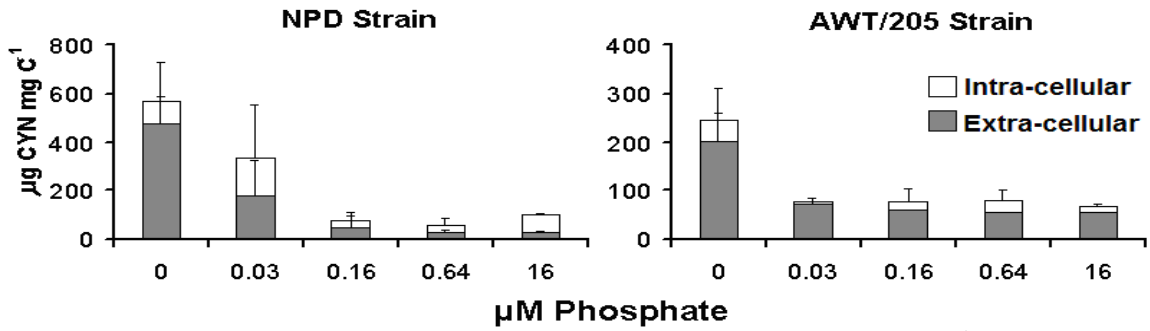

Figure 5.17. Intra-cellular and extra-cellular CYN concentrations (µg CYN mg C^{-1}) in two strains of *C. raciborskii* at increasing concentrations of P (phosphate µM).

The NPD strain grown in G-6-P produced more deoxy-CYN than CYN at all concentrations of G-6-P (Fig 5.18). After P starvation, the NPD strain produced 0.6 ± 0.05 mg deoxy-CYN mg C^{-1} and this increased to 1.7 ± 0.2 mg deoxy-CYN mg C^{-1} with the two lowest concentrations of G-6-P. With a further increase in G-6-P to 0.64 µM P, the concentration of deoxy-CYN decreased to 0.7 ± 0.02 mg deoxy-CYN mg C^{-1} and remained at that level with the highest concentration of G-6-P (16 µM P). The concentration of CYN was unchanged with the two increases in G-6-P (0.8 ± 0.2 mg CYN mg C^{-1}) and then decreased when G-6-P was 0.64 and 16 µM P (0.3 ± 0.2 mg CYN mg C^{-1}).

There was no statistical difference ($p>0.05$) between the dexy-CYN and CYN concentrations produced by AWT/205 at all concentrations of G-6-P (Fig 5.18). After P starvation both CYN and deoxy-CYN were 0.25 ± 0.02 mg CYN mg C^{-1} and then decreased to 0.05 ±0.02 mg CYN mg C^{-1} with all concentrations of G-6-P. Very little of the toxin was intra-cellular for both strains at all concentrations of G-6-P (Fig 5.19).

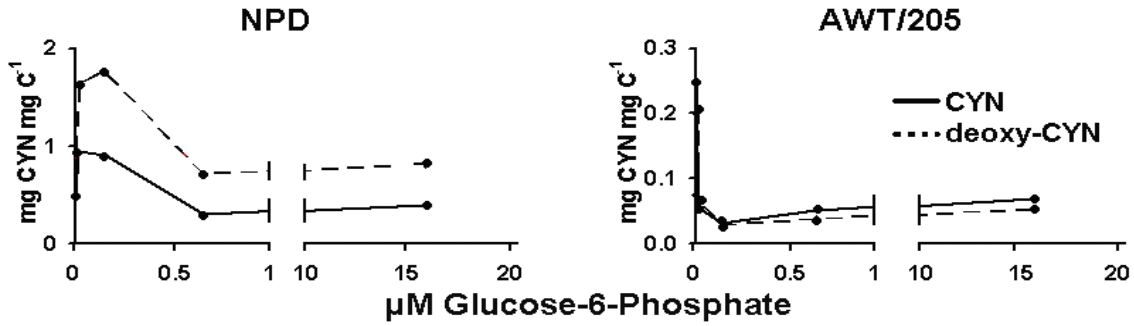

Figure 5.18. Total toxin (CYN and deoxy-CYN) concentrations (mg CYN mg C-1) with increasing P concentrations (G-6-P µM) in two strains of *C. raciborskii* (NPD and AWT/205)

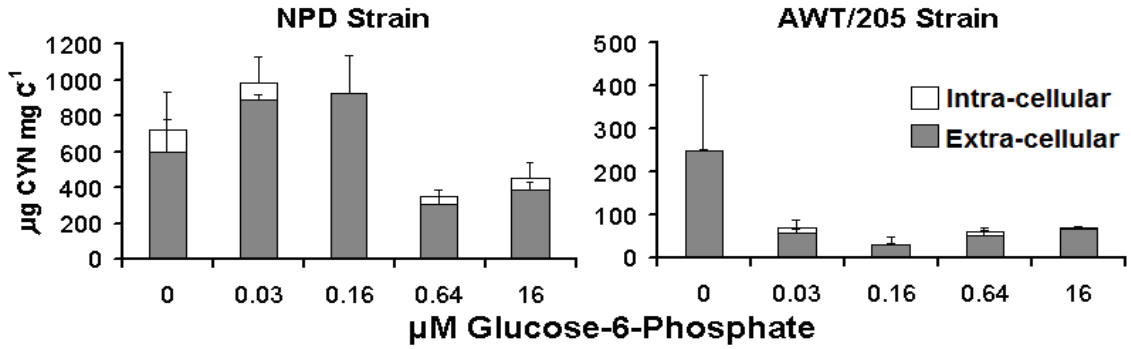

Figure 5.19. Intra-cellular and extra-cellular CYN concentrations (µg CYN mg C-1) in two strains of *C. raciborskii* at increasing concentrations of phosphorus (G-6-P µM).

When the source of P was compared to the total toxin (not per cell or mg C) there were similarities between phosphate and G-6-P. The NPD strain grown in G-6-P produced more toxin than when it was grown in phosphate at the same concentrations (Fig. 5.20). When cells were starved of P (<0.03 µM P), the amount of toxin produced by the NPD strain was 12 ± 2 mg CYN L^{-1}. After phosphate was reintroduced (0.03 µM P), there was a significant decrease ($p<0.05$) in CYN to 5 ± 0.5 mg CYN L^{-1} and the concentration of CYN remained below this level with a phosphate increase up to 16 µM. However, when G-6-P was introduced to the NPD strain after phosphate starvation the concentration increased from 12 ± 2 mg CYN L^{-1} to 17 ± 1 mg CYN L^{-1} (with 0.16 µM P). When the concentration of G-6-P was increased to 0.64 µM P, the level of toxin decreased to 6 ± 1.5 mg CYN L^{-1} and remained at that level with the highest concentration of G-6-P (16 µM)

The AWT/205 strain was much less toxic than the NPD strain (Fig. 5.20). After phosphate starvation, the level of toxin produced by AWT/205 was 3.5 ± 0.4 mg CYN L^{-1}. Unlike the NPD strain, the toxin produced by the AWT/205 strain decreased when either G-6-P or phosphate was introduced (0.03 µM P). With G-6-P the toxin level decreased to 0.5 ± 0.1 mg CYN L^{-1} and with phosphate it dropped to 1.4 ± 0.8

mg CYN L^{-1}. With both sources of P the concentration of toxin did not change with an increase in P concentration from 0.03 to 16 µM P.

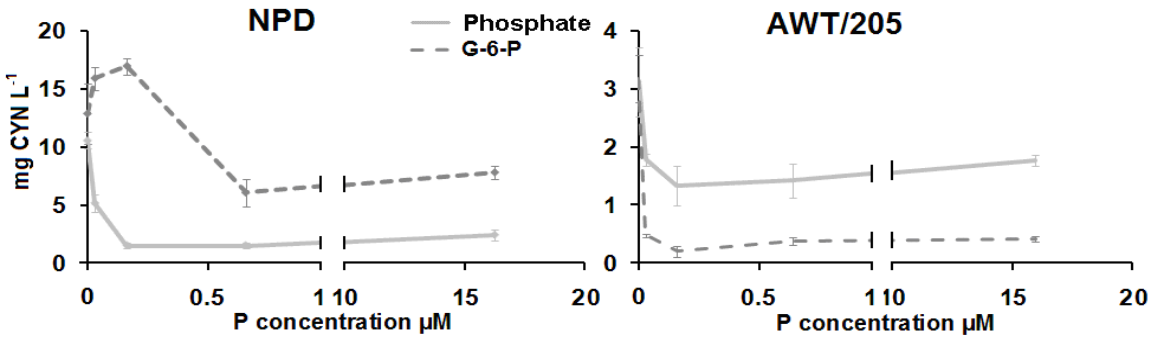

Figure 5.20. Total toxin (CYN + deoxy-CYN) concentrations (±SD) produced by *C. raciborskii* with increasing concentrations of phosphate and Glucose-6-Phosphate.

The proportion of toxin (sum of CYN and deoxy-CYN) within the cell, was generally higher in the NPD strain than the AWT/205 strain although there was a high variability in replications (Fig 5.21). For the NPD strain the culture grown in phosphate retained almost twice as much toxin within the cell when compared to the culture grown in G-6-P. For the AWT/205 strain there was no statistical difference ($p<0.05$) between intra-cellular toxin concentrations when the cells were grown in phosphate or G-6-P. There was also no correlation between toxin concentration and P concentration which was possibly due to the high variability in the replicates.

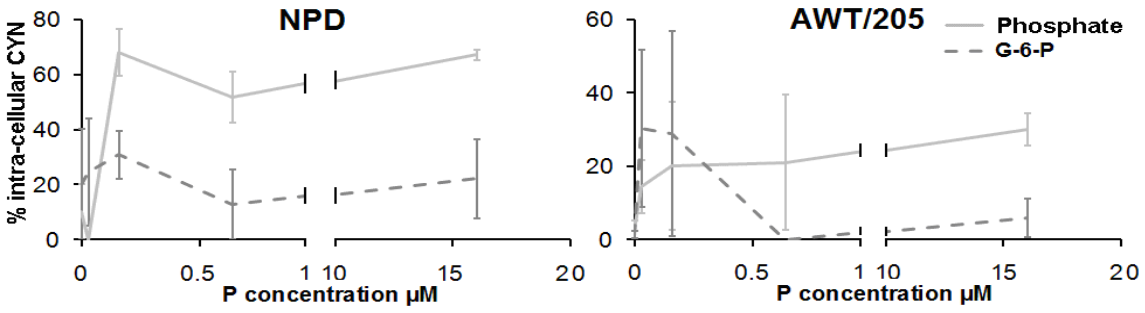

Figure 5.21. Percentage of CYN (±SD) within a *C. raciborskii* cell compared to extra-cellular concentrations at increasing phosphate and Glucose-6-Phosphate.

5.4 Discussion

5.4.1 Phosphorus/Phosphate dependant growth rate

There was a difference in the maximum growth rate of the two *C. raciborskii* strains (AWT/205 and NPD). However, the maximum growth rate of both strains (NPD : 0.28 d^{-1} and AWT/205: 0.25 d^{-1}) was comparable to some other cyanobacteria (Table 5.5) although it was significantly lower ($p<0.01$) than the maximum growth rate of another strain of *C. raciborskii* (ACT 9502) (Istvánovics et al. 2000). Similar maximum growth rates (0.6 - 1.4 d^{-1}) of *C. raciborskii* in culture have also been published (Saker et al. 1999). In contrast, *C. raciborskii* growth rates in L. Julius (*in situ*) were 0.5-0.7 d^{-1} (Saker and Griffiths 2001) and in the Fitzroy River (Queensland, Australia) were approximately 0.31 d^{-1} (Bormans et al. 2004). However, in L. Samsonvale, *C. raciborskii* growth rates have been shown to be approximately 0.28 d^{-1} (Chapter 4).

Different maximum growth rates have been observed for other cyanobacteria grown at the same concentration of limiting nutrient. Two strains of *Trichodesmium* have been shown to have different phosphate dependant growth rates despite both reaching a maximum growth rate at the same phosphate concentration (3 µM) (Fu et al. 2005). *Trichodesmium* GBRTRLI101 (isolated from the Great Barrier Reef, Australia) has a maximum growth rate of 0.17 d^{-1} whilst *Trichodesmium* IMS101 (isolated from the North Atlantic Ocean) has a maximum growth rate of 0.27 d^{-1}. Also, strains of *M. aeruginosa* isolated from two different geographic regions (Bloemfentein, South Africa and Sydney, Australia) had different maximum growth rates (~1.3 and 2.4 d^{-1} respectively) when grown in the same concentrations of phosphate (~76 µM) (Downing et al. 2005b).

Table 5.5 Maximum growth rates of cyanobacteria in culture in this study compared to other studies. μmax d^{-1}:maximum growth rate per day. M/F: Marine or Freshwater Species

Cyanobacteria	M/F	μ_{max} d^{-1}	Reference
Anabaena sp.	F	0.86-1.4	Mur and Lingeman (1992)
Microcystis sp.	F	0.60-1.2	Mur and Lingeman (1992)
C. raciborskii ACT 9502	F	0.84-0.88	Shafik et al. (2001)
Oscillatoria sp.		0.50-0.95	Mur and Lingeman (1992)
M. aeruginosa	F	0.73	Olsen (1989)
Lyngbya majuscula	M	0.32	Elmetri and Bell (2004)
C. raciborskii NPD	F	0.28	Present study
Trichodesmium IMS101	M	0.27	Downing et al. (2005 b)
C. raciborskii AWT/205	F	0.25	Present study
Trichodesmium GBRTRLI101	M	0.17	Downing et al. (2005 b)

The difference in maximum growth rates between two strains of *Trichodesmium*, *M. aeruginosa* and *C. raciborskii* highlights that maximum growth rates of cyanobacteria can be strain specific and possibly reflect the ambient nutrient status of the water body from which they were originally isolated. The NPD strain was isolated from sub-tropical L. Samsonvale approximately 8 y ago (G. McGregor pers. comm.). As previously discussed, L. Samsonvale has very low concentrations of phosphate (generally <0.03 μM P) and the phytoplankton is subsequently P limited most of the time (Burford et al. 2006). The AWT/205 strain was isolated from a temperate location (Sydney, N.S.W. Peter Hawkins). However it is not known what nutrient conditions existed in the water body from which it was isolated. The ambient nutrient concentrations from the water in which AWT/205 and NPD strains were isolated may also affect their relatively low growth rates. When trying to isolate the cyanobacterium *Synechococcus*, Ernst et al. (2005) found that one strain tolerated high concentrations of phosphate but was highly sensitive towards nitrate, while another strain tolerated high nitrate if phosphate was low. The authors suggested that

the difference between the two strains is indicative of the environment from which they were isolated.

5.4.2 Phosphate uptake

Both the AWT/205 and NPD strains had a similar maximum phosphate uptake rate and these were higher than any other phytoplankton found in the literature including another strain of *C. raciborskii* (Table 5.6). Also, *C. raciborskii* strains AWT/205 and NPD have the lowest half saturation constant (0.02 µM). The cyanobacteria *Planktothrix agardhii* and *Microcystis aeruginosa* had relatively low half saturation constants (0.3 and 0.2 µM) (Van Liere 1979 and Holm and Armstrong 1981) but these were still an order of magnitude higher than AWT/205 and NPD strains of *C. raciborskii*. A low K_S suggests that both the AWT/205 and NPD strains are adapted to low P environments.

Table 5.6 Phosphate uptake rates of phytoplankton. Adapted from Reynolds 2007 and Istávnovics et al. 2000. *assumes C content is 50% dry weight. ^ assumes 431fg C cell^{-1}

Species	RC_{max} (µmol P mg C^{-1} d^{-1})	K_s (µmol P L^{-1})	Reference
Chlorophyta (Green algae)			
Staurastrum luetkemuellerii	1.16*	-	Olsen (1989)
Staurastrum pingue	8.52*	-	Spijkerman (1996)
Scenedesmus quadricauda	21.0	1.2-4.0	Nalewajko and Lean (1978)
Chlorella pyrenoidosa	155	0.68	Nyholm (1977)
Others			
Dinobryon sociale	1.3	0.39	Lehman (1976)
Asterionella formosa	3.14	1.9-2.8	Tilman and Kilhman (1976)
Cosmarium abbreviatum	3.44*	-	Mur and Lingeman (1992)
Cyanophyta (Cyanobacteria)			
Synechococcus (CCMP 1334)	0.400^	0.80	Fu et al. (2006)
Gloetrichia echinulata	0.640*	-	Istávnovics et al. (1993)
Microcystis aeruginosa	12.0	0.30	Holm and Armstrong (1981)
Oscillatoria spp.	31.6`	-	Mur and Lingeman (1992)
Planktothrix agardhii	33.4	0.2-0.3	Van Liere (1979)
Microcystis aeruginosa	52.8	-	Olsen (1989)
Anabaena flos-aquae	64.8	1.8-2.5	Nalewajko and Lean (1978)
C. raciborskii (ACT 9502)	70-232	0.2-1.0	Istvánovics et al. (2000)
C. raciborskii (AWT/205)	454	0.02	Current study
C. raciborskii (NPD Strain)	631	0.02	Current study

The high maximum uptake rate and relative low K_S indicate that *C. raciborskii* may dominate in systems with a relatively low phosophate concentration. The uptake rate and K_S value coupled with the ability to grow at a maximum rate with relatively low phosphate (0.03 µM) suggests that *C. raciborskii* may outcompete other phytoplankton when phosphate availability becomes low. The effect of phosphate concentration on *C. raciborskii* phytoplankton dominance had been discussed in Chapter 4.

5.4.3 Phosphorus and toxin

Cylindrospermopsin production by *C. raciborskii* changes with P source and concentration. Toxin production is poorly understood in *C. raciborskii* and an understanding of the mechanisms by which toxin production is increased or decreased has great potential to improve the management of toxic cyanobacterial blooms.

The AWT/205 strain of *C. raciborskii* was, on average, much less toxic than the NPD strain and produced more toxin when grown in phosphate compared to G-6-P. The NPD strain on the other hand produced more toxin when grown in G-6-P compared to phosphate. Also, the NPD consistently produced more deoxy-CYN than CYN where as the AWT/205 strain produced similar amounts of both at the same P concentrations. The differences in toxin production between the two strains of *C. raiborskii* in this study highlight the strain specificity of this organism. It is yet another example of how the physiology of this organism may vary with its geographic location.

For both strains of *C. raciborskii* (AWT/205 and NPD), there was a decrease in toxin (deoxy-CYN and CYN) concentration with an increase in phosphate concentration. This was despite maximum growth rate being reached with the lowest concentration

of phosphate (0.03 µM). This is in contrast to microcystin production in *Microcystis* which is controlled by nutrient concentration affecting the rate of cell division, not through any direct effect on the metabolic pathways of toxin production (Orr and Jones 1998). However, a direct relationship between nutrient concentration and toxin production in *M. aeruginosa* was later investigated and suggests that low P, whilst not necessarily favouring growth, may favour toxin production (Vézie et al. 2002). Phoshorus limitation has been associated with an increase in toxin production by *Prymnesium parium* and *Chrysochromulina polylepis,* despite the cells growing at a maximum rate (Egge 1998).

Only a small proportion of toxin was retained within the cells of both strains at low P concentrations. However, the strains grown in G-6-P had a higher intra-cellular proportion of toxin even though it was relatively low and highly variable. This is in contrast to Saker and Griffiths (2000) who showed that during exponential phase, less than 10% of the CYN was released from *C. raciborskii* cells of seven Australian isolates. Saker and Griffith (2000) also showed that during stationary phase more than 50% of the CYN was extra-cellular and suggest that CYN may be released during cell stress or death. Many parameters have the potential to induce cell stress in cyanobacteria such as very low or very high light intensity and temperature, nutrient limitation and low carbon dioxide availability. However, cell stress as a trigger for CYN production is poorly understood. As an example, the light intensity at which *C. raciborskii* produces the highest concentration of CYN was well outside the light intensity required for maximum growth rate (Saker and Neilan 2001, Griffiths and Saker 2003). However, in a separate study, light intensity was found to have no effect on the CYN production in *C. raciborskii* (Garnett 2005).

The current study indicates that toxin in *C. raciborskii* may be produced under situations of stress (P-limitation) and this has been suggested for other cyanobacteria as well. For example the potential role of MYST production in *M. aeruginosa* may include cellular metal transport or heterotrophic bacteria attractants (Kaebernick and Neilan 2001). Other organisms produce secondary metabolites as a defence mechanism and increase production in times of stress. For example in nutrient stress situations in terrestrial plants, excess photosynthates are allocated to anti-herbivore defences (Van Alstyne and Pelletreau 2000).

The measured decrease in toxin concentration with increasing concentrations of phosphate may be the result of bacterial degradation. The cultures of *C. raciborskii* were not axenic and may have contained bacteria capable of CYN degradation. Some bacteria produce hydrolytic enzymes capable of breaking down cyanotoxins such as microcystin and nodularin (Imanishi et al. 2005). The bacterium *Sphingomonas* sp. strain ACM-3962 (MJ-PV), has been applied to a pilot scale for treatment of water containing microcystin (Bourne et al. 2006). With the increase in phosphate concentration it is possible that bacteria within the cultures of *C. raciborskii* increase in concentration. However *C. raciborskii* cells were checked (using phase contrast microscopy x 400) every 2-3 d and there was no observed bacterial growth throughout the experiments.

5.4.4. Dissolved Organic Phosphorus and Alkaline Phosphatase

A culture of *C. raciborskii* can produce alkaline phosphatase and grow in media containing G-6-P as the sole P form. This is an important finding given

C. raciborskii's ability to dominate P-limited reservoirs (Padisak 1997) as it suggests a mechanism by which *C. raciborskii* can grow in phosphate limited systems.

The AP activity in both strains increased when they were starved of phosphate but did not increase further with increasing concentrations of G-6-P. When two strains of *Microcystis* in batch culture were transferred from P-free media to a relatively low phosphate concentration (0.2 mg L^{-1}), AP was up to 25 µM P µg chl a^{-1} h^{-1}, compared to < 10 µM P µg chl a^{-1} h^{-1} when they were transferred to higher concentrations of phosphate (64 and 173 µM) (Shen & Song 2007).

The growth rate of the two *C. raciborskii* strains (NPD and AWT/205) was lower when the cells were grown in G-6-P compared to phosphate. In contrast to this, 50 strains of freshwater cyanobacteria (10 genera) grew equally well in Na-ß-glycerophosphate and p-nitrophenyl phosphate as they did in phosphate (Whitton et al. 1991). Also, for the marine cyanobacterium, *Synechococcus* and *Nodularia spumigena*, growth rates were the same regardless of the P source (phosphate or ATP) (Fu et al. 2006 and Vahtera et al. 2007). Whilst it is possible that these two cyanobacteria (*Synechcoccus* and *Nodularia spumigena*) were capable of directly using DOP as well as phosphate and are hence unaffected by P source, the low growth rate of AWT/205 and NPD may be explained by the level of AP that was produced.

AP activity in the two strains of *C. raciborskii* (AWT/205 and NPD) was generally low, producing approximately 0.8 x 10^{-5} pM $cell^{-1}$ h^{-1} (~ 8 000 -80 000 pM mL^{-1} h^{-1}). When put into context of the maximum phosphate uptake rate of *C. raciborskii* (1-2.5 pg P $cell^{-1}$ h^{-1}) it can be said that the amount of phosphate being produced (as a result

of AP activity) is much less than required for maximum uptake. Also, the AP activity of AWT/205 and NPD was much lower than other strains of cyanobacteria that had similar growth rates when grown in DOP compared to phosphate. For non-axenic cultures of *Nostoc* and *Gloeotrichia*, AP activity was 1.17 and 6.46 μM mg dw^{-1} h^{-1} respectively (Whitton et al. 1991). For *Trichodesmium* colonies in the northern Red Sea, AP activity ranged from 0.2–11.7 μmol *p*-nitrophenylphosphate hydrolysed μg chl $a^{-1}h^{-1}$ (Stihl et al. 2002). The inconsistencies in reporting units make comparisons between cyanobacterial AP activities difficult, but both these studies (Whitton et al. 1991 and Stihl et al. 2002) show values approximately one order of magnitude higher than the AP activity measured for *C. raciborskii* (AWT/205 and NPD strains) indicating that *C. raciborskii* may have a low AP activity in general. However, a link between cyanobacterial toxin production and phosphatase inhibition has been established in *Microcystis* and may account for the low level of measurable AP activity in these experiments (Wirsing et al. 1998 and Campus et al. 2005).

Phosphatase inhibition assays have been used to determine the concentration of the cyanotoxin microcystin in water samples and *Microcystis* cultures (Wirsing et al. 1998 and Campus et al. 2005). The protein-phosphatase inhibition assay uses the ability of microcystins to specifically inhibit the catalytic subunits of protein phosphatases (MacKintosh et al. 1990). Whilst this method has not yet been used to quantify cylindrospermopsin, it is possible that cylindrospermopsin may inhibit phosphatase. The confounding effect of toxin production on measuring AP activity suggests that the AP levels measured in this study may be underestimated. However the measured AP activity does reflect the amount of phosphate being produced as a result of DOP hydrolysis.

The present study investigated G-6-P as an example of how *C. raciborskii* may respond to DOP, however, other sources of DOP may induce different responses in growth rate or levels of AP activity. In aquatic systems DOP can be comprised of a variety of molecules such as monophosphate esters, nucleotides, nucleic acids, phospholipids and phosphonates (Strickland and Soloranzo, 1966, Taft et al. 1977, Soloranzo 1978, Matsuda 1985, Chrost et al. 1986, Ridal and Moore 1990, Karl and Yanagi, 1997, Clark et al 1998 and Suzumura et al. 1998). These sources of DOP differ in structure and molecular weight and may therefore vary in the way they affect cyanobacteria.

5.5 Conclusions

This study indicates that *C. raciborskii* has mechanisms for surviving in P-limited conditions, however, there were strain differences. Both the AWT/205 and NPD strain are capable of rapid P uptake similar to that seen in ACT 9502 (Istvánovics et al. 2000). Also both strains produce alkaline phosphatase and are capable of growing when dissolved phosphate concentrations are limiting.

The strains of *C. raciborskii* used in this study appear to be suited to very low phosphate concentrations. The maximum growth rate of both strains was achieved with 0.032 µM P which is below the current method detection limit (0.064 µM) used by many water authorities. Also with most parameters tested there was little difference between results for the 0.64 and 16 µM concentrations. This indicates that reducing phosphate concentrations to 0.64 µM P would still result in *C. raciborskii* problems. Also, toxin concentration was greatest with phosphate starvation indicating that toxin may be produced as a result of stress.

The results from this chapter provide laboratory culture experiments that support the findings from Chapters 3 and 4 showing that *C. raciborskii* has rapid phosphate uptake ability which gives it a competitive advantage in L. Samsonvale. However, to fully quantify phosphate utilisation by *C. raciborskii* phosphate regeneration rate should be calculated for *C. raciborskii* in culture. This would provide information on how long *C. raciborskii* was able to store phosphate. Investigations into whether *C. raciborskii* can utilise sources of DOP other than G-6-P (possibly higher molecular weight) would better characterise whether this provides *C. raciborskii* with a competitive advantage in systems containing limited phosphate but adequate DOP. Also, the potential for CYN to inhibit AP production should be investigated

Chapter 6 Conclusions – Is phosphorus a key driver in promoting blooms of *Cylindrospermopsis raciborskii* in Lake Samsonvale?

6.1 The relationship between Cylindrospermopsis raciborskii and phosphorus – summary of the main findings

An important focus for cyanobacterial research is gaining a better understanding of nutrient loadings and the link with increased frequency of toxic cyanobacteria (Hundrell 2008). This lead to the current study which investigated the relationship between the toxic cyanobacterium *C. raciborskii* and phosphorus (P) in a sub-tropical drinking water reservoir (L. Samsonvale). Phosphorus concentration and form (i.e. phosphate and DOP) have the potential to affect phytoplankton dynamics and potentially increase the dominance of *C. raciborskii* within the phytoplankton community.

The experimental approach used in this study moved beyond previous descriptive studies of water quality and *C. raciborskii* in water reservoirs. By combining laboratory culture experiments with manipulative *in situ* bioassays in L. Samsonvale greater insights were gained. This allowed robust findings at the species level able to be applied at the phytoplankton community and whole system level. In the current study the laboratory culture experiments complemented the *in situ* bioassays and were then used in an attempt to explain results obtained from whole of system monitoring.

In the laboratory culture experiments, two toxic strains of *C. raciborskii* were shown to have a rapid phosphate uptake rate, have maximum growth rate with very low phosphate concentrations (0.03 µM), and in the absence of phosphate, produce

alkaline phosphatase. This suggests that *C. raciborskii* is highly effective at P utilisation at low P concentrations and may explain its dominance in L. Samsonvale.

In the L. Samsonvale bioassay experiments, an increase in *C. raciborskii* was seen when phosphate was added in a daily spike (0.32 and 16 µM) further supporting the suggestion that rapid phosphate uptake is a key reason for *C. raciborskii* dominance in L. Samsonvale. However, there was a decrease in *C. raciborskii* dominance (as a result of an increase in diatom numbers) when phosphate was added in a constant high concentration or when P and N were added together. Two phytoplankton communities dominated by *C. raciborskii* (L. Borumba and L. Samsonvale) were shown to have a very high affinity for phosphate storage which may also be an important part of its strategy in P-limited systems.

The findings from the laboratory and field experiments were difficult to apply to the whole of system monitoring. As data was only available for one summer, it was difficult to determine a correlation between L. Samsonvale parameters and *C. raciborskii* dominance or cell abundance. However, the finding that *C. raciborskii* cultures produce alkaline phosphatase coupled with the presence of DOP in the water column of L. Samsonvale throughout the summer 2007/08 suggest a potential pathway for phosphate availability leading to *C. raciborskii* dominance, but more work is needed to quantify this.

6.1.1 The relationship between *Cylindrospermopsis raciborskii* and phosphorus

C. raciborskii is opportunistic with regard to phosphate. Two strains of toxic *C. raciborskii* grew at a maximum rate in 0.03 µM phosphate and there was an

increase in *C. raciborskii* phytoplankton dominance when phosphate was added in a daily spike to *in situ* bioassays. The opportunistic nature of *C. raciborskii* to low phosphate is further demonstrated by the lack of change when phosphate concentration was increased. In both the laboratory culture experiments and the *in situ* bioassays, there was little difference in *C. raciborskii* response when phosphate was added in relatively high concentrations (6.4 µM in culture and 15 µM *in situ*) compared to the lowest concentration added (0.03 µM in culture and 0.32 µM *in situ*). This shows that *C. raciborskii* grows just as well in low (as low as 0.3 µM) phosphate concentrations as it does in higher concentrations, thus allowing it to outcompete other phytoplankton in P-limited systems. The culture experiments showed that *C. raciborskii* was not only capable of growth in very low phosphate concentrations (0.03 µM), but also in the absence of phosphate coupled with very low DOP concentrations (0.03 µM). This was linked with the finding that *C. raciborskii* cultures produce alkaline phosphatase. The ability of *C. raciborskii* to sustain growth with no available dissolved phosphate and grow at a maximum rate with very low phosphate means that reservoirs with very low measurable phosphate (below the detection limit of 0.06 µM in many circumstances) will support growth of *C. raciborskii* blooms.

Toxin production by *C. raciborskii* in this study appears to be related to P-limitation. The overall toxin concentration was highest when phosphate and G-6-P were low (>0.03 µM) and the portion of intercellular toxin increased when phosphate and G-6-P increased (< 0.03 µM). This suggests that toxin is produced and released from the cell when conditions are not favourable for maximum growth (i.e. under a form of cellular stress). Toxin production is poorly understood in cyanobacteria and this study

suggests a potential reason for toxin production, nutrient cell stress. The range of effects of P source and concentration on *C. raciborskii* is summarised in Table 6.1.

Table 6.1 The effect of phosphorus source and concentration on *C. raciborskii* growth characteristics and toxin production

		Phosphate concentration (µM)		
		<0.03	**0.3-0.64**	**>0.64**
G-6-P concentration (µM)	**<0.03**	High total toxin Low CYN_{cell} No growth High AP activity	↓ Total toxin ↑ CYN_{cell} Max. µ ≈ AP activity	≈ Total toxin ≈ CYN_{cell} ≈ µ ≈ AP activity
	0.3-0.64	↑ CYN_{cell} ↑↓ Total toxin half max. µ ↓AP activity		
	>0.64	≈ CYN_{cell} ≈ Total toxin ≈ µ ≈ AP activity		

↑	Increase from previous
↓	Decrease from previous
≈	No change from previous
↑↓	Increase then decrease
Toxin	Sum of deoxy-CYN and CYN irrespective of cell abundance
CYN_{cell}	Fraction of toxin within the cell
yield	Photosynthetic yield
µ	Growth rate

6.2 Potential management implications

In terms of the reservoir management implications, perhaps the most significant finding of this thesis is the ability of *C. raciborskii* to grow and produce toxin whilst utilising alkaline phosphatase to satisfy its P requirements. This means that there is the potential for public health risks (high toxin levels in recreational and/or drinking water storages) when nutrient concentrations are very low. The current WHO (2003) and NHMRC (2008) guidelines suggest that if direct cyanobacteria counts are not possible in water quality analysis, assumptions can be made on the concentrations of ammoniacial N and phosphate. The guidelines suggest that the higher the nutrient

concentrations the more risk of a toxic cyanobacterial 'bloom'. The findings in the present study suggest that elevated nutrient concentrations are a poor indicator of cyanobacteria and associated toxin concentrations, especially when *C. raciborskii* is dominant.

In the present study, cell abundance correlated poorly with toxin concentration. This is consistent with a number of studies (Kaebernick & Neilan 2001, Laamanen et al. 2001 and Vèzie et al 2002) and has implications for water managers with regard to the cyanotoxin treatment process. Whilst cyanotoxins can be effectively removed from source water, the treatment process is not always implemented. A common industry standard is to test for cell abundances and then once they reach a certain level, test for the toxin and treat accordingly. However in the present study it was shown that the highest concentration of toxin was at the lowest cell abundance, when the cells were starved of phosphate (or under induced stress). This indicates that toxin concentrations may be elevated in times when there is no testing which is a potential public health risk. A regular monitoring program incorporating toxin concentration would be beneficial, especially in reservoirs where *C. raciborskii* is dominant. This would be significantly aided by a decrease in the cost of toxin analysis or a viable alternative such as real time PCR.

6.3 Relevance for local and international reservoirs

The ability of *C. raciborskii* to produce AP, rapidly take up phosphate and grow at a maximum growth rate in low phosphate concentrations are likely to be a significant factor driving its dominance in L. Samsonvale. However, these factors may also be providing *C. raciborskii* with an advantage in other Queensland reservoirs. In a study of 23 Queensland reservoirs, the highest *C. raciborskii* cell abundance (>600 000 cells

mL^{-1}) were generally associated with low total P concentrations (0.4 – 4.9 µM) (phosphate concentrations not determined) (McGregor and Fabbro 2000). This was consistent with a study involving seven South East Queensland reservoirs (including L. Samsonvale) which found the highest *C. raciborskii* cell abundances with total P concentrations between 0.5 and 1 µM (Burford et al. 2007).

The findings in the thesis have relevance not only for reservoirs within Queensland and Australia but also worldwide. For example in a Florida reservoir (L. Jesup, U.S.A) *C. raciborskii* has been increasing in maximum summer cell abundances since 1997 and the authors hypothesise that its dominance is due (in part) to its efficient use of P (Dobberfhul 2003). Also, *C. raciborskii* dominance has been linked to a low nutrient concentration (among other physico-chemical parameters) in a shallow pond in France (Briand 2002). The initial study (Istvánovics et al. 2000) indicating that *C. raciborskii* dominance may be linked to a high phosphate affinity in a reservoir (L. Balton, Hungary) with low phosphate availability is supported by the current study. However, a greater understanding of how phosphate concentration affects toxic strains of *C. raciborskii* growth and dominance has been achieved with the current study. An investigation into the AP production and DOP concentrations within reservoirs dominated by *C. raciborskii* together with an understanding of the phosphate uptake and regeneration rates would be useful in determining the extent of phosphate related dominance of *C. raciborskii*.

6.4 Suggested further work

The specific aims of this thesis, set out in Chapter 1, have been addressed (Table 6.1) and provide a framework for future study. The finding that *C. raciborskii* monocultures produce AP and grow with G-6-P as the sole P source warrants further

investigations into *C. raciborskii* and other sources of DOP. Also studies to speciate DOP from L. Samsonvale will add to the understanding of the *C. raciborskii*-DOP relationship and provide a better indication of potential management actions. Also, alkaline phosphatase activity was not determined in the *in situ* bioassay section of this study, but would be beneficial in understanding the complete P-*C. raciborskii* relationship.

The link between low phosphate and high toxin production provides support for the hypothesis that toxins are produced as a stress response. However there is much more work required (such as investigating alternate forms of cell stress) to better understand why cyanotoxins are produced. The link between rapid phosphate uptake and storage as well as AP production were useful in explaining *C. raciborskii* dominance in the short term however, long term investigations (longer bioassay experiments, more data (years) on DOP) would provide a better understanding of the reasons why *C. raciborskii* dominates in L. Samsonvale. Nutrient concentrations remain a key focus of cyanobacterial research and this thesis supports the need for further work regarding their role (particularly P) in species specific dominance.

Table 6.2 Summary of the conclusions of the study

Question	Answer
Does *C. raciborskii* produce alkaline phosphatase?	LIKELY – *C. raciborskii* monoculture (containing possible symbiotic bacteria) produces alkaline phosphatase
Can *C. raciborskii* grow in phosphate-free media containing only a dissolved organic form of P?	YES (only G-6-P tested)
How does phosphate concentration affect:	
Growth rate?	Maximum growth rate at low phosphate concentration (0.03 µM)
Phosphate uptake?	Rapid uptake with relatively low phosphate.
Toxin production?	Highest toxin production with phosphate starvation
Does the dominance of *C. raciborskii* within the phytoplankton community change with increasing concentrations of phosphate?	YES but only demonstrated when phosphate is added in a daily pulse for 4 d
Does continuous vs. pulsed phosphate affect dominance of *C. raciborskii* within the phytoplankton community?	YES, constant high concentrations decrease dominance but more work needed to confirm this
Is the destratifier in L. Samsonvale allowing injections of phosphate from the nutrient bottom waters in the water column therefore making it available to the phytoplankton?	UNLIKELY, *C. raciborskii* link with destratifier more likely to be related to light rather than P
Is the combination of periodically available phosphate and DOP an important factor affecting *C. raciborskii* dominance in L. Samsonvale?	POSSIBLY. Sufficient to allow *C. raciborskii* access to P through AP production

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Appendix A

Jaworskii's Media

Nutrients

	Grams per 400mL stock soln.
$Ca(NO_3)_2 . 4 H_2O$	8.0
$MgSO_4 . 7 H_2O$	20.0
$NaHCO_3$	6.36
EDTAFeNa	0.9
$EDTANa_2$	0.9
H_3BO_3	0.992
$MnCl_2 .4 H_2O$	0.556
$(NH_4)_6Mo_7O_{24} . 4 H_2O$	0.4
$NaNO_3$	32.0
KH_2PO_4	1.24
$Na_2HPO_4 . 12 H2O$	3.6

Add 1 mL stock to make 1000 mL final media

Vitamins

	Grams per 200mL stock soln.
Cyanocobalamin	0.016
Thiamine HCl	0.016
Biotin	0.016

Add 500 μL stock to make 1000 mL final media

Appendix B Correlation coefficients of physico-chemical parameters from Lake Samsonvale over the Summer 2007/08

	Chlorophyll a	DO 0-3m	DO 4-7m	S NH3	% cloud cover	S NOX	S TN	S TP	Silcate	Mn	Iron	Carbon	pH	S Temperature	Turbidity	Fluorescence	Radiation	*Planktolyngbya*	*Aphanocapsa*	Diatoms	*Microcystis*	*C. raciborskii*
Chlorophyll a	1.0																					
DO 0-3m	-0.2	1.0																				
DO 4-7m	0.3	**0.7**	1.0																			
S NH3	0.0	-0.1	-0.1	1.0																		
% cloud cover	-0.4	0.2	0.0	**0.6**	1.0																	
S NOX	**-0.6**	-0.2	**-0.7**	0.0	0.1	1.0																
S TN	-0.3	0.0	**-0.5**	0.0	0.1	**0.8**	1.0															
S TP	0.6	0.2	0.4	0.0	-0.5	-0.4	0.0	1.0														
Silcate	-0.1	**-0.8**	**-0.6**	0.0	-0.5	0.4	0.3	-0.2	1.0													
Manganese	0.5	-0.3	-0.1	**0.9**	0.4	-0.1	0.1	0.2	0.2	1.0												
Iron	0.6	0.2	0.4	-0.2	**-0.8**	-0.5	-0.5	**0.9**	-0.1	-0.2	1.0											
Carbon	0.0	-1.0	**-1.0**	-0.1	0.3	**0.7**	-0.2	**-0.5**	1.0	**1.0**	-0.8	1.0										
pH	0.0	**0.9**	**0.7**	-0.2	0.1	-0.2	-0.1	0.2	**-0.9**	-0.2	0.2	**-0.8**	1.0									
S Temperature	**0.7**	0.0	0.1	0.2	-0.2	**-0.7**	-0.8	0.5	-0.1	0.0	0.4	-0.5	0.0	1.0								
Turbidity	0.2	0.0	0.1	0.1	-0.2	0.2	0.3	0.1	0.1	0.3	0.0	**0.9**	0.1	-0.3	1.0							
Fluorescence	0.4	0.3	0.3	**-0.7**	**-0.9**	-0.5	-0.4	**0.7**	-0.5	-0.4	**0.9**	-0.6	**0.6**	0.5	-0.2	1.0						
Radiation	0.4	0.0	0.2	-0.4	**-0.9**	-0.2	-0.1	**0.5**	0.2	-0.3	**0.7**	0.2	0.0	0.2	0.3	0.6	1.0					
Planktolyngbya	-0.1	-0.2	-0.2	0.4	**0.5**	-0.3	-0.4	0.1	0.0	0.0	-0.3	-0.1	-0.4	0.3	-0.7	-0.3	**-0.6**	1.0				
Aphanocapsa	0.4	-0.1	-0.2	0.1	0.3	-0.4	-0.1	0.3	0.0	0.1	0.1	-0.2	-0.2	0.3	-0.2	0.3	-0.3	0.2	1.0			
Diatoms	0.2	-0.8	**-0.9**	0.2	-0.2	0.4	-0.1	-0.1	**0.9**	0.3	0.0	**0.8**	**-0.9**	0.3	0.2	-0.3	0.1	0.2	0.1	1.0		
Microcystis	**-0.5**	**0.5**	0.1	-0.1	0.0	**0.6**	0.5	-0.1	-0.2	-0.3	-0.1	0.2	0.3	-0.6	0.0	-0.1	0.2	-0.2	-0.2	-0.2	1.0	
C. raciborskii	0.3	-0.3	-0.2	0.1	0.3	-0.4	-0.5	**0.6**	-0.1	-0.1	-0.1	-0.2	-0.3	**0.5**	**-0.9**	0.1	**-0.5**	**0.7**	0.2	0.2	-0.3	1.0

Fe	Iron		C	Carbon		% cloud cover	Percentage cloud cover
DO 0-3m	Dissolved Oxygen surface to 3 m		S Temperature	Surface water temperature		S NOX	Surface Nitrates/Nitrite
DO 4-7m	Dissolved Oxygen 4 to 7 m		S TP	Surface Total Phosphorus		S TN	Surface Total Nitrogen
S NH3	Surface ammonium						