MONASH University

Ecophysiological studies on freshwater microalgae:

Implications for life in a high CO_2 world

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Abstract

Humans depend on freshwater rivers and lakes for drinking water globally, with few exceptions. The dominant microscopic primary producers in these waters, specifically phytoplankton, can, in some cases, cause irritation, odour and, if toxic, lead to death of humans and livestock. To better understand the community structure of the phytoplankton populations in one such Australian system, we undertook a thorough investigation of the physiology of a number of phytoplankton species, isolated from Lake Wivenhoe, Australia. Focussing on photosynthesis, carbon acquisition and accumulation, and the impact of elevated CO_2 on these physiological parameters, three main areas were investigated. Broadly, we hypothesised that the studied species of phytoplankton would possess physiological characteristics consistent with their ecological niche in the natural environment. Further, differences in characteristics between species cultured at atmospheric partial pressures of CO_2, would aid in predictions of the algae's response to culturing at elevated CO_2 partial pressures.

Initially, the growth and photo-physiology of six strains of phytoplankton from Lake Wivenhoe (the cyanophyte *Cylindrospermopsis raciborskii*, the diatoms *Nitzschia* sp. and *Cyclotella* sp.; and the green algae *Monoraphidium* sp., *Stichococcus* sp., and *Staurastrum* sp.) were characterised. The low light-harvesting efficiency of the strain of *C. raciborskii*, combined with its low rate of photoinhibition, suggested that it is well adapted to the high light conditions present at the surface. This is consistent with its toxic bloom- forming behaviour.

Having looked at the effect of light on photosynthesis, carbon uptake and concentration were the next logical cellular characteristics to measure. Cellular affinity for dissolved inorganic carbon indicated that all species possessed active CO_2-concentrating mechanisms (CCMs). *C. raciborskii* was capable of concentrating CO_2 up to 14-fold, relative to external CO_2 concentrations, whereas

the internal CO_2 concentrations of the diatoms and green algae were much lower, reflecting their possession of more efficient Rubisco enzymes. The cellular surface area to volume ratio was shown to correlate with internal bicarbonate concentration, suggesting the scaling of this physical trait with the increasing cost of diffusive loss of CO_2.

Finally, the physiological impact of predicted future levels of CO_2 (1000 ppm) was explored. Cellular affinities for CO_2 were lower for all species with high CO_2, indicating CCM down-regulation. Photosynthetic rates and cellular surface area to volume ratio were higher for most species with CO_2. The impact of elevated CO_2 on growth rate varied between species, with both *C. raciborskii* and *Stichococcus* sp. growing at a lower rate with high CO_2. Environmental data from Lake Wivenhoe suggested that carbon availability plays an important role in defining species dominance at the surface.

This research contributes to the relatively limited physiological data on freshwater species, and will aid efforts to predict future changes to phytoplankton populations in freshwater bodies globally, as atmospheric CO_2 levels continue to rise.

Declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes one manuscript (not submitted) and two submitted publications. The core theme of the thesis is the photo-physiology and carbon acquisition characteristics of freshwater phytoplankton. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the School of biological Sciences, under the supervision of John Beardall.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of *chapters 2, chapter 3 and chapter 4*, my contribution to the work involved the following:

Thesis Chapter	**Publication Title**	**Status** *(published, in press, accepted or returned for revision, submitted)*	**Nature and % of student contribution**	**Co-author name(s) Nature and % of Co-author's contribution***	**Co-author(s), Monash student Y/N***
Chapter 2 (Paper 1)	Photosynthetic characteristics of six freshwater microalgae isolated from a subtropical reservoir	*Not submitted*	*70%. Concept and collecting data and writing first draft*	Anusuya Willis, input into manuscript 10% John Beardall, input into manuscript 20%	*No* *No*
Chapter 3 (Paper 2)	Carbon acquisition characteristics of six microalgal species isolated from a sub-tropical reservoir: potential implications for species succession	Accepted. Journal of Phycology	80% Concept, data collection analysis and first draft	John Beardall, Input into analysis and manuscript 20%	No
Chapter 4 (Paper 3)	Elevated CO_2 has differential effects on five species of microalgae from a sub-tropical freshwater lake: possible implications for phytoplankton species composition	Submitted	70% Concept, data collection analysis and first draft	Philip Orr, Data collection and manuscript 15% John Beardall, Input into analysis and manuscript 15%	No No

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student signature: [redacted] **Date: 25-10-2017**

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor signature: **Date:**

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Thank you.

Chapter 1

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Introduction

Background

Phytoplankton are small, highly efficient, solar powered, self-replicating, biological machines that are responsible for half of the world's annual carbon assimilation (Behrenfeld *et al.*, 2001), and provide the base of most aquatic food webs throughout the globe (Guo *et al.*, 2016; Hoegh-Guldberg & Bruno, 2010; Woodward *et al.*, 2010). Phytoplankton source their building blocks from their surrounding aqueous medium. Some have the capacity to actively pursue the nutrients they require, while others are reliant on currents, rains, winds or tides to bring them within reach of limiting nutrients. Marine and freshwater ecosystems, like others across the planet, are under threat from global climate change. Compared to marine systems though, relatively little research is being carried out into how global change, and particularly elevated partial pressures of CO_2, will impact these organisms present in our freshwater systems.

Lake Wivenhoe is a sub-tropical reservoir in South Eastern Queensland and is responsible for providing more than 60 % of the city of Brisbane (population ~ 2 million) with drinking water (Macova *et al.*, 2011). Like many sub-tropical bodies of freshwater, Lake Wivenhoe experiences stratified, warm spring and summer periods, interspersed with storms, and cooler winter months (Aryal *et al.*, 2016). Weather events mix Lake Wivenhoe, cycling nutrients from the depths of the lake and making them available to phytoplankton and other organisms in the surface waters, though depending on rainfall within the catchment, dilution of the nutrients can also occur (Aryal *et al.*, 2016). However, during periods of calm weather, mixing stop and nutrient concentrations in the surface waters diminish (Talling, 1966). Nitrogen, phosphorus and other elements and minerals are cycled this way, returning from the depths during weather events, and diminishing in concentration as they are utilised in biological activities during periods of stratification (MacIntyre & Melack, 1995). Along with

nitrogen and phosphorus (essential for, amongst other things, protein and nucleic acid synthesis), carbon dioxide and its hydrated inorganic carbon isoforms (carbonate CO_3^{2-}, and bicarbonate HCO_3^-) also follow this cycle (Wachniew & Różański, 1997). Respiration by microorganisms breaking down organic matter releases CO_2 at depth in the lake, while inflows from the catchment bring with them dissolved carbonates (Tranvik *et al.*, 2009). As the water's pH varies around the pK values for dissolved inorganic carbon (DIC) species (ignoring gas exchange with the atmosphere), the predominant species of DIC will change form. At 25 ºC CO_2 is predominant at pH < 7, HCO_3^- at pH 7-8, and CO_3^{2-} at pH > 9 (Holland *et al.*, 2012).

Gas exchange with the atmosphere maintains an equilibrium of CO_2 concentrations in the sub-surface boundary layer, thus at shallow depths, pH, and alkalinity affect only the concentration of bicarbonate and carbonate (Maberly, 1996), though in reality the interactions of these parameters are far more complex. The depth to which air equilibrium concentrations of CO_2 extend, is dependent on rates of mixing (i.e. by wind induced waves, and rainfall) (Gorham & Boyce, 1989) and the rate of CO_2 uptake by phytoplankton. When deep mixing occurs in freshwater lakes (via storms, and large inflows from the catchment), they often become a source of CO_2 to the atmosphere (Tranvik *et al.*, 2009). Reservoirs, due to the massive quantity of submerged organic material are almost permanently a CO_2 source (St. Louis *et al.*, 2000). Conversely, when mixing is insufficient and the waters are stable and stratified, DIC can remain held at the bottom of lakes for days, making the atmosphere the only available carbon source to surface dwelling organisms (Wachniew & Różański, 1997).

Phytoplankton, having evolved to survive in this environment, possess diverse mechanisms that achieve the processes of nutrient uptake, photosynthesis, and ultimately growth, in a wide variety of conditions. Seasonal (and often unpredictable) changes in temperature (Paerl

& Huisman, 2008), pH (Maberly, 1996), mixing rates (Huisman *et al.*, 2004), sinking (Waite *et al.*, 1997), nitrogen availability (Muhid & Burford, 2012), phosphorus availability (Grover, 1989), light intensity (Huisman *et al.*, 1999), salinity (Fong *et al.*, 1996) and predation (Jiang *et al.*, 2014) are some of the most common and important pressures that phytoplankton of freshwater lakes must cope with. No individual species has evolved to dominate under all conditions that occur during and between seasons. Thus, the species-dominance changes throughout the year, dependent on changes in the physico-chemical characteristics of the lake and the individual characteristics of the phytoplankton themselves, their cell concentration, and capacity to acclimate or adapt to the dynamic environment. Indeed some species of phytoplankton directly influence the lake (on a small scale), further enhancing conditions for their own growth over that of other competing species. An example of this is during a summer cyanobacterial bloom, where surface waters become limiting for inorganic carbon and the influx of atmospheric CO_2 is greatly consumed by a dense layer of cyanobacteria, preventing other phytoplankton at greater depths from its supply, as well as limiting their light supply (Paerl & Huisman, 2008). In a dynamic environment, a species' capacity to harvest, utilise and in some cases dissipate light energy is vital to its success. Therefore investigating the photosynthesis of a number of previously unstudied species of freshwater phytoplankton may provide insights into the population structure. This thesis is divided into three main topics, which correspond to the three manuscripts presented in this thesis. The following is a brief introduction to each of these topics and more detailed introductions are found in the individual chapters.

How photosynthesis affects algal dominance

Photosynthesis is the process by which energy, in the form of light, is used to biochemically assimilate inorganic carbon from the environment into organic compounds, thereby storing

the sun's energy in a usable chemical form. This process releases approximately one molecule of oxygen for every molecule of CO_2 fixed. Fundamental to the existence of life on earth today, cyanobacteria were the first photosynthetic organisms to produce oxygen and evolved around between 2.5 and 3.5 billion years ago (Raven *et al.*, 2012; Schopf, 2000). Today, cyanobacteria and other phytoplankton are responsible for approximately half the world's oxygen production (Behrenfeld *et al.*, 2006). Current phytoplankton populations comprise many species, all competing for the same resources. In these assemblages, photosynthesis is often limited by access to light. Thus, how a species responds to changes in light intensity, at both the high and low extremes, is vital to its success.

By measuring the response of an algal species to changes in light intensity, inferences can be made about how that species will respond to changing conditions. In a landmark paper, Richardson *et al.* (1983) compiled and discussed the responses of many species of phytoplankton to light intensity, describing how changes in the P vs. I curve could indicate differing strategies of adaptation/acclimation to high or low light habitats. From an ecological perspective, the authors discussed the formation of different light habitats as caused by seasonal variation of mixing rates and solar radiation. In late autumn, winter and early spring the water column is generally well-mixed, resulting in a habitat where nutrient availability is homogeneous and the incident photon flux density (PFD) varies from high intensities to zero (depending on depth). In this habitat, growth of diatoms should be favoured, as they tend to possess lower critical light intensities for growth and photosynthesis and can also cope with high light levels.

As local temperatures increase, and rainfall decreases, Richardson *et al.* (1983) suggest that, with reduced rates of mixing due to stratification and limited inflows, multiple habitats develop - high light intensities and high temperatures at the surface declining to low (or zero)

light intensities, and cooler temperatures at lower depths. Consequently, any phytoplankton species, well adapted to the conditions of a specific region of the water column, may grow successfully and, depending on the initial densities present and maximum density achievable, summer blooms might also occur. More recently, both modelling (Huisman *et al.*, 1999) and empirical evidence (Huisman, 1999), have shown that due to light attenuation, a species capable of inhabiting the surface water will out-compete other species deeper in the water column, based on competition for light. This, in turn, may starve the other available habitats in the water column of energy. It is this strategy that many bloom-forming cyanobacteria utilise to great effect, having evolved mechanisms to modulate buoyancy, scavenge nutrients, produce toxins, and survive the high temperatures and light intensities at the surface during summer (Burford *et al.*, 2016; Burford & Davis, 2011; Paerl & Otten, 2013).

The physiology of the phytoplankton of Lake Wivenhoe is not well studied, though some considerable work has gone towards understanding how nutrient availability affects the toxic cyanobacterium *Cylindrospermopsis raciborskii* and its ability to form blooms (Burford *et al.*, 2016; Burford *et al.*, 2007; Hong *et al.*, 2015; Willis *et al.*, 2016). It is this deficiency that the first data chapter (paper 1) seeks to address.

Inorganic carbon acquisition by phytoplankton

Phytoplankton need CO_2 for photosynthesis, and so it follows that its supply, and the capacity of microalgae capacity to consume it, are possible critical factors controlling growth rates and species dominance in Lake Wivenhoe. In photosynthesis, the enzyme ribulose-1, 5-bisphosphate carboxylase oxygenase (Rubisco) is responsible for catalysing the carboxylation of an acceptor, ribulose-1,5-bisphosphate. Rubisco, with the primary function of a carboxylase, may also bind oxygen and carry out the competing oxygenase reaction, leading to the loss of carbon in the process known as photorespiration. The selectivity factor (S_{rel}) of Rubisco is a measure of the enzymatic preference for either CO_2 or O_2, and takes into account both affinity (Michaelis-Menten half saturation substrate concentrations) for CO_2 and O_2 [$K_{0.5}(CO_2)$ and $K_{0.5}(O_2)$ respectively], and substrate saturated rates (mol substrate × mol active sites^{-1}) of carboxylase or oxygenase activity [$k_{cat}(CO_2)$ and $k_{cat}(O_2)$ respectively].

$$S_{rel} = \frac{k_{cat}(CO_2) \times K_{0.5}(O_2)}{k_{cat}(O_2) \times K_{0.5}(CO_2)}$$

To maximise rates of carbon fixation and minimise photorespiration, CO_2 must be in saturating supply, which, in most cases due to the enzyme's poor kinetic efficiency and selectivity, is a concentration much higher than that found in ambient conditions. To concentrate CO_2 at the active site of carbon fixation, most photosynthetic cell utilise a CO_2 concentrating mechanism (CCM), responsible for the scavenging of extracellular DIC, its influx (both active and passive), and its conversion to CO_2 for Rubisco (Beardall & Raven, 2016). There are, however, a number of species shown not to possess CCMs that rely on diffusive entry of CO_2 for carbon acquisition (Maberly *et al.*, 2009; Raven & Giordano, 2017).

CCMs vary broadly in function and mechanism, including active bicarbonate and CO_2 pumps at the cell membrane and internal membranes (such as plastid envelope and thylakoid membranes, depending on the taxon), as well as intracellular and extracellular carbonic anhydrases. Cyanobacterial CCMs utilise active bicarbonate and CO_2 pumps at both the plasmalemma and thylakoid membranes, in concert with carbonic anhydrase (CA) enzymes to concentrate DIC in the form of bicarbonate within the thylakoid, minimising losses of CO_2 by leakage (Giordano *et al.*, 2005). Bicarbonate then diffuses into carboxysomes (protein structures where Rubisco is present), where other carbonic anhydrases dehydrate bicarbonate and produce high concentrations of CO_2 in close proximity to Rubisco, overcoming the oxygenase reaction for maximum rates of C fixation (Badger & Price, 2003). Eukaryotes possess similar mechanisms by which DIC can be concentrated internally. Active transport of bicarbonate and CO_2 occurs in the plasma membrane, chloroplast envelope, or both, while extracellular carbonic anhydrases may enhance the level of CO_2 at the cell surface by catalysing its formation from bicarbonate in the periplasmic space. Other pumps and CAs act inside the chloroplast at the thylakoid membrane and to saturate Rubisco, which is often located, at high concentrations, within pyrenoids (a structure hypothesised to act in a similar way to carboxysomes in cyanobacteria) (Giordano *et al.*, 2005).

Rubisco reaction kinetics vary greatly from species to species, though generalisations can be made across taxa. Green algae, with Form 1B Rubisco are half saturated with CO_2 ($K_{m\,(CO_2)}$) at concentrations of 30-60 µM (Badger *et al.*, 1998), while diatoms with Form 1D Rubisco are saturated at around 30 µM CO_2 (Young *et al.*, 2016). Cyanobacterial Rubisco (Form 1A or 1B) are the least kinetically competitive, and become half saturated at concentrations above 150 µM of CO_2 (Badger *et al.*, 1998). Considering that at air equilibrium, the concentration of CO_2 in sea water is around 12-15 µM, it is clear that almost every species'

Rubisco will be less than half saturated if their entire carbon supply were to come from diffusion of CO_2 alone.

Depending on the taxon, CCM expression is generally regulated by the availability of DIC in the external medium and also a range of other external factors (Beardall & Giordano, 2002). In a number of studied eukaryotes, it was shown that CCM activity is regulated by CO_2 concentration (Berman-Frank *et al.*, 1995; Matsuda & Colman, 1995; Matsuda *et al.*, 2001), whereas in cyanobacteria, the CCM appears to be regulated based on HCO_3^- concentrations (Mayo *et al.*, 1986). Irrespective of the form of carbon used to regulate the CCM, it is likely that its expression is proportionate to changes in the DIC concentration at sub-saturating levels. Thus, a slight decrease in DIC, within operational ranges, would result in a slight up-regulation of the CCM (Matsuda & Colman, 1996), as opposed to a complete activation or deactivation in response to a slight change in DIC concentration. As CCM regulation is dependent on external DIC concentrations, it follows that variables that affect DIC equilibrium may also lead to changes in CCM operation. Primarily, changes in pH, temperature and alkalinity may lead to an environment switching from high to low CO_2 concentrations (Maberly, 1996).

CCM operation is energy intensive, with high rates of turnover of the molecular machinery, and maintenance of concentration gradients (Raven *et al.*, 2014). Thus, along with DIC availability, most species' primary energy source, light, also plays an important role in regulating or at least facilitating CCM function. Shiraiwa and Miyachi (1983) showed that the DIC affinity of low CO_2 adapted *Chlorella vulgaris* increased with increasing light intensity. With a similar theme, Beardall (1991), and Young and Beardall (2005) were able to show *Anabaena circinalis* [currently regarded as a synonym of *Dolichospermum sigmoideum* (Nygaard) Wacklin, Hoffman and Komarek] and *Dunaliella tertiolecta* also experienced

decreases in CCM activity, though only at very low light intensities. The vital point is that carbon uptake via CCM activity is closely linked with a cell's capacity to harvest light.

It is for this reason that CCMs are necessary and common among phytoplankton. It is also for this reason that changes in atmospheric CO_2 partial pressure are predicted to have wide ranging effects on the physiology of phytoplankton world-wide. To understand how CO_2 supply might affect species competition, it is important to understand cellular affinities for, and acquisition of, inorganic carbon. Due to the massive biodiversity of phytoplankton, in combination with the limited physiological information of freshwater species, a large information gap exists and this research seeks to contribute to filling this gap through information presented in data chapter 2 (paper 2).

Potential impacts of future CO_2 levels

Due to anthropogenic action, and inaction, atmospheric levels of CO_2 are expected to increase from current levels of ~400 ppm, to up to 1000 ppm by the year 2100 (Meehl *et al.*, 2007). Atmospheric temperature increases, acidification of water bodies, and changes in weather dynamics are all predicted consequences of the increase in CO_2. For phytoplankton, and indeed for other carbon fixing organisms, changes in the partial pressure of CO_2 are expected to have significant impacts. It should be noted however, that in most productive freshwater lakes, the surface water is rarely in equilibrium with the air (Seekell & Gudasz, 2016). Due the supply of inorganic carbon by a lake's terrestrial catchment acting in opposite effect to the potentially high rates of carbon draw down by phototrophic organisms, surface waters rarely stay at air-equilibrium levels of CO_2 for long.

As already discussed, the operation of an energetically expensive CCMs is largely regulated by external DIC concentrations. It follows, that as partial pressures of CO_2 in the atmosphere rise, and air equilibrium concentrations of CO_2 increase, CCM operation will be down-regulated proportionately in those phytoplankton whose Rubisco would be unsaturated without CO_2 enrichment. This reduced expression of CCM machinery, and thus the decrease in necessary energetic expense has been expected to result in an increase in growth rates of algae, as more energy becomes available for growth processes (Raven *et al.*, 2014). Changes in growth rate should be proportionate to the energetic benefit of down regulation of the CCM; thus different species with different Rubisco kinetics may exhibit differing responses to elevated atmospheric CO_2 and this may influence competition between species and species dominance.

For instance, Spijkerman *et al.* (2005) measured the DIC uptake of a variety of desmid species from different ecological niches, and discussed the likelihood that carbon acquisition significantly contributes to species distribution. More recently, after observing differences in CO_2 affinity between two strains (one wild-type, one mutant) of the same species of *Microcystis aeruginosa* in monoculture, Van de Waal *et al.* (2011) recorded reversal in competitive dominance of mixed cultures with changes in CO_2 partial pressure. This change provides a perfect example of what might be seen on a large scale in the environment, where many thousands of species with slightly different mechanisms for carbon uptake will react differently as atmospheric CO_2 levels rise.

Super-saturated CO_2 conditions already exist in many freshwater ecosystems, thus the species that inhabit these waterways are potentially well adapted to high CO_2 conditions. There are, however, periods of CO_2 depletion in the surface waters that regularly occur during summer months. It is during these periods that species with highly active CCMs would be expected to thrive.

As CO_2 (and DIC in general) levels increase, CCM activity is down regulated as less accumulation of DIC is required to saturate Rubisco. This results in a reduction in whole cell affinity for DIC, and thus an increase in $K_{0.5\ (DIC)}$.

As well as changes to carbon uptake, other changes to cellular physiology also occur. Increased CO_2 concentrations often result in increased photosynthetic rates (Burkhardt *et al.*, 2001; Collins & Bell, 2004; Pierangelini *et al.*, 2014; Yang & Gao, 2003), as the energy that was once diverted to CCM operation becomes available and is directed towards carbon fixation (Raven *et al.*, 2014). If excess light energy is harvested, this energy must be

dissipated as heat, often through the process known as non-photochemical quenching (Lambrev *et al.*, 2012).

For a species in culture, short term changes to CO_2 partial pressure could be either beneficial or detrimental, depending on its capacity to utilise or dissipate this newly available energy, while maintaining saturated rates of carbon fixation. The energy savings provided by the increase in DIC concentration should be proportionate to the energetic cost of CCM operation, though, depending on the regulation of different CCM- related mechanisms, this change may not be linear. Over a longer period of time, more complex regulatory changes should occur to ensure the continued survival of the species. For instance, if the light harvesting complex under conditions of CCM down-regulation initially accumulates more energy than can be funnelled into carbon fixation, changes in the synthesis of light harvesting pigments may occur, or an increased capacity to fix carbon may also be a strategy employed to cope with this change. It is likely that these approaches will vary between species.

As CO_2 levels rise, phytoplankton that have adapted to take advantage of surface water CO_2 depletion may begin to experience increased competition from other species that require a higher concentration of CO_2 in their habitat. Depending on the frequency and duration of low CO_2 periods, at current atmospheric conditions, changes in CO_2 partial pressure may lead to dramatic shifts in population structure if CO_2 acquisition defines the competitive dominance in this scenario. Commonly, it is thought that cyanobacteria fall into this category, though exactly what contributes to their capacity to form blooms varies, and is contingent upon multiple variables (Burford *et al.*, 2016; Hyenstrand *et al.*, 1998; Paerl *et al.*, 2001; Soares *et al.*, 2009).

How elevated CO_2 will affect species composition freshwater systems is not well understood, though valuable research into this area has begun (Low-Décarie *et al.*, 2015; Low-Decarie *et al.*, 2011; Low-Décarie *et al.*, 2013). With the research presented in paper 3, we seek to contribute to the understanding of how elevated CO_2 levels will affect species composition among phytoplankton species in freshwater systems.

Structure and themes of the thesis

This thesis is comprised of three data chapters in the form of manuscripts; the first is still undergoing final changes before submission, while the second and third have been submitted for peer review. Following the data chapters, a general conclusions chapter summarises the key results of the work and attempts to draw broad conclusions, as well as make recommendations for future research. There is no methods chapter, as all methods can be found within the manuscripts themselves.

Carbon acquisition and photosynthesis are two key physiological characteristics that will respond to elevated CO_2 partial pressure and lead to changes in phytoplankton populations. Adding to knowledge of how phytoplankton store DIC and respond to light will improve the understanding of population dynamics in current conditions, as well as increase the quality of predictions of these populations as CO_2 levels increase into the future.

In this study, isolated strains of freshwater phytoplankton from Lake Wivenhoe were studied to better understand how their physiological characteristics might contribute to species dominance. These species of algae include the toxic cyanobacterium *Cylindrospermopsis raciborskii*, the green algae *Staurastrum* sp., *Monoraphidium* sp., and *Stichococcus* sp., and the diatoms *Cyclotella* sp. and *Nitzschia* sp.. With these species we sought to add to previous, but limited, physiological studies on the photosynthetic and carbon uptake characteristics of freshwater phytoplankton, while improving the understanding of current competitive dominance within the phytoplankton of Lake Wivenhoe. Further, we aimed to investigate how elevated levels of CO_2 might impact phytoplankton population structure into the future, with a focus on determining whether toxic bloom formation by *C. raciborskii* will increase into the future. Given the differing taxa studied, and their differences in pigmentation and

other physiological measures, diversity in photosynthetic and carbon uptake characteristics, as well as response to elevated CO_2 was expected. Very little is known about these species with the exception of *C. raciborskii* which was known to bloom in surface waters during summer months.

A species of considerable focus, we examined a cylindrospermopsin-producing toxic- bloom-forming strain of the cyanophyte *C. raciborskii*. Of particular industrial and public interest, costly mitigation strategies are in place to minimise the risk of harm to waterway users from toxic algal blooms. *C. raciborskii* possesses the characteristics of many toxic cyanobacteria, such as high temperature tolerance, buoyancy, a capacity for nitrogen fixation, tolerance of high light and possession of a highly active CCM (Burford & Davis, 2011; Paerl & Otten, 2013). It regularly forms toxic blooms at the lake's surface during heavily stratified summer months (Burford *et al.*, 2016).

Stakeholders of Lake Wivenhoe are thus eager to know how their waterway will change into the future, and whether further mitigation strategies should be put in place to allow its continued use, or alternatively whether the risks of toxic cyanobacteria may subside (Paerl *et al.*, 2011; Visser *et al.*, 2016).

Aims

Overall, the aim of this thesis was to study the physiology of the phytoplankton of Lake Wivenhoe, with a focus on carbon utilisation and the impact of future levels of CO_2. Standard techniques were used to characterise photosynthesis, carbon uptake and storage. We hypothesised that the studied species of phytoplankton would possess physiological characteristics consistent with their ecological niche in the natural environment and that differences in characteristics between species cultured at atmospheric partial pressures of

CO_2, would aid in predictions of the algae's response to culturing at elevated CO_2 partial pressures.

The aim of the first data chapter was to investigate the physiological and photosynthetic characteristics of six species of phytoplankton isolated from Lake Wivenhoe, and identify whether these characteristics may influence competitive dominance. For the second data chapter we aimed to characterise and quantify each species' ability to concentrate CO_2, as well as measure cellular affinity for CO_2, also relating these characteristics to the niche in the natural environment, each species inhabit. The relationship between cell surface area to volume ratio and internal bicarbonate concentration was also discussed. Lastly, the aim of the third data chapter was to determine how elevated CO_2 might affect these species into the future, and understand some of the possible causes for these changes. We observed changes in these characteristics in response to CO_2 elevated to the levels expected by 2100, and related these to some observation of the population dynamics of the selected species in Lake Wivenhoe.

A final chapter (General Conclusions) attempts to draw together the results of the experiments described in the data chapters to show where this work contributed to scientific understanding of microalgal physiology in freshwater ecosystems, and how predicted CO_2 levels in the future will affect them. This chapter also discusses the strengths and limitations of the research carried out.

Chapter 2: Paper 1

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Photosynthetic characteristics of six freshwater microalgae isolated from a subtropical reservoir

This manuscript has not yet been submitted.

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Abstract

Freshwater phytoplankton populations in large bodies of water (such as lakes and reservoirs) often undergo sequential changes in dominance of different species. Understanding the ecophysiology of important members of the phytoplankton population might assist in efforts to model the observed changes in community composition and phytoplankton biomass. In this study six common species of freshwater phytoplankton were isolated from Lake Wivenhoe, an important water resource for South Eastern Queensland, Australia, which suffers from annual cyanobacterium blooms dominated by *Cylindrospermopsis raciborskii*. The growth and photosynthetic physiology of these isolates, including the cyanobacterium, *Cylindrospermopsis raciborskii*, two diatom species (*Nitzschia* sp., *Cyclotella* sp.), and three green algae (the chlorophytes *Monoraphidium* sp., *Stichococcus* sp. and the desmid *Staurastrum* sp.), were characterised to explore the diversity of their growth and photosynthetic characteristics when grown under replete nutrient conditions. Growth rates varied between 0.21 ± <0.01 day^{-1} for *C. raciborskii*, and 0.8±<0.01 day^{-1} for *Monoraphidium* sp.. Maximum photosynthetic rates ranged from 10.4 to 735.4 nmol O_2 $(10^7 cells)^{-1}$ min^{-1} for *C. raciborskii* and *Staurastrum* sp. respectively. Respiration as a percentage of net photosynthesis showed *Monoraphidium* sp. with the least, and *Cyclotella* sp. with the greatest, relative respiration rates of 1.57% and 16.4% respectively. Light harvesting efficiency was lowest for *C. raciborskii*, at 0.1 nmol O_2 $(10^7 cells)^{-1}$ min^{-1} (µmol photons m^{-2} $s^{-1})^{-1}$ and highest for *Staurastrum* sp. at 4.7 O_2 $(10^7 cells)^{-1}$ min^{-1} (µmol photons m^{-2} $s^{-1})^{-1}$. This study shows considerable variability in the growth rates and photosynthetic abilities of the different phytoplankton species. While the maximal photosynthetic capacity of *C. raciborskii* was too low to explain its capacity to dominate phytoplankton blooms there are other characteristics of its photosynthetic performance, such as low light harvesting

efficiency and high light tolerance, which may confer a competitive advantage in a high light environment.

Introduction

Phytoplankton populations are a mix of diverse species, morphologically single celled, colonial or filamentous, with the major groups being diatoms, dinoflagellates, green algae, and cyanobacteria. Phytoplankton play an important role in freshwater ecosystems where in many cases they provide the base of the food web, providing fixed-carbon into the system (Field *et al.,* 1998). Changing species composition and sequential dominance of phytoplankton species occur seasonally as environmental conditions alter. These changes in species composition can have a downstream effect on the food web and are an indicator of ecosystem health (Sterner & Hessen 1994; Hay & Kubanek 2002; Ptacnik *et al.,* 2008). For example, the nutritional value of cyanobacteria to *Daphnia* is less than that of diatoms, due to their lack of particular poly-unsaturated fatty acids (Martin-Creuzburg *et al.,* 2008; Guo *et al.,* 2016) and this has been shown to affect grazing zooplankton and fish populations (Oglesby 1977; Ahlgren *et al.,* 1990). The presence of toxins in many cyanobacteria may also be of importance when consumed by predators. It should be noted, however, that changes in nutrient availability and other environmental conditions can affect nutrient and toxin composition of any given species. Understanding the ecophysiology of important members of the phytoplankton population might assist in efforts to model changes in community composition and phytoplankton biomass (Van de Waal *et al.,* 2011).

Lakes and reservoirs can show wide ranges in their conditions, and phytoplankton thrive in an equally wide variety of changing environmental factors. The major factors driving phytoplankton primary production are temperature, hydrology, stratification, nutrient loads and light (Field *et al.,* 1998; Behrenfeld *et al.,* 2006). The photosynthetic capacity can differ greatly between algae (Falkowski 1980; Richardson *et al.,* 1983) and lead to changes in growth and population species composition in response to changing light conditions. In sub-

tropical and temperate freshwater bodies, changes in environmental conditions are caused by seasonal variation in temperature, water inflow and nutrient loads (Burford & Davis 2011). Seasonal mixing also affects the environmental conditions in sub-tropical systems; for instance, high mixing rates during the wet season can increase turbidity, while during the dry season minimal mixing and stratification lead to high surface water temperatures and higher light intensities (Huisman *et al.,* 1999). These variations in environmental factors can drive sequential changes in species dominance. For example, in an Amazonian flood plain lake (Lago Batata, Brazil), diatom and desmid prevalence declined as water levels dropped, with populations transitioning to filamentous cyanobacterial dominance as mixing decreased and stratification occurred (Huszar & Reynolds 1997). Similar sequential changes in community composition are observed in water bodies throughout the world (Schindler 1977; Pinckney *et al.,* 1998; Rocha *et al.,* 2002; Smith 2003; Muhid 2011). A species' ability to effectively utilise low and high light intensities, move within the water column, or acquire nutrients from dilute solutions are traits that confer competitive advantages to populations experiencing various environmental changes (Dokulil & Teubner 2000; Paerl *et al.,* 2001). Successful strains are often capable of forming blooms, given the right conditions. Cyanobacterial species in particular provide widely reported examples of bloom formation during summer months where stratification is high, resulting in conditions where their higher temperature optimum (Lurling *et al.,* 2013) and nutrient scavenging or nitrogen fixing capacity results in dense, often toxic, blooms (Downing *et al.,* 2001; Schindler *et al.,* 2008). However, recent work suggests that the capacity to fix nitrogen is unlikely to be a major factor in the dominance of *Cylindrospermopsis raciborskii* (used in the present study) in tropical and sub-tropical systems, as only low rates of growth can be sustained by nitrogen fixation in the absence of other nitrogen sources (Paerl *et al.,* 2014; Willis *et al.,* 2016a).

Lake Wivenhoe is a reservoir in subtropical South Eastern Queensland that provides potable water to the city of Brisbane. Given that this system (in common with other water bodies around the world) is subject to seasonal changes in environmental factors, as well as periodic blooms of potentially toxic cyanobacteria [including *Cylindrospermopsis raciborskii* used in this study, and in the study by Willis *et al.* (2016a)], with major implications for water quality and public health, understanding how individual species, characteristic of the water body, react to environmental factors, such as light intensity, is important for lake or reservoir management. Further, the effect of future environmental change is unpredictable; however, understanding the growth and photosynthetic characteristics of locally relevant freshwater phytoplankton in its present state, is important for explaining future changes in ecology of a given system. This research may potentially be used to develop mitigation strategies to deal with toxic bloom formation.

We here report growth and photosynthetic characteristics, focussing on response to light, of six species of freshwater phytoplankton, to explore the diversity of physiology of phytoplankton species within Lake Wivenhoe, and to determine if these characteristics might be related to the observed dominance of *Cylindrospermopsis* in summer and diatoms and green algae at other times of the year.

Materials and Methods

Six strains of phytoplankton were isolated from a surface water sample collected in January 2013 from Lake Wivenhoe, South Eastern Queensland (27° 16' S, 152° 32' E). The water sample was enriched with Jaworski's +Si medium (see below) and strains were isolated by micropipette. All strains have been added to the Monash University culture collection.

Unialgal cultures were grown and identified to the genus level by light microscopy. The isolated strains were *Cyclotella* sp. (Class: Bacillariophyceae), *Cylindrospermopsis raciborskii* (Wołoszyńska) Seenaya & Subba Raju [Class: Cyanophyceae; strain WSO7, capable of producing cylindrospermopsin as described in Willis *et al.* (2016b)], *Monoraphidium* sp. (Class: Chlorophyceae), *Nitzschia* sp. (Class: Bacillariophyceae), *Staurastrum* sp. (Class: Zygnematophyceae, order: Desmidiales), and *Stichococcus* sp. (Class: Chlorophyceae). The strains were all grown at 25ºC in modified Jaworski's medium (Andersen 2011) supplemented with 300 µM Na_2SiO_3 (JM+Si), under a photon flux of 60 µmol photons m^{-2} s^{-1}, with a light: dark cycle of 12h: 12h, except for *C. raciborskii*, which was grown under a photon flux of 20 µmol photons m^{-2} s^{-1} [preliminary experiments showed this was a low-light preferring strain (Willis *et al.* 2016b)]. Growth light intensity was selected to be close to saturating, but without stressing the algae and was within the spectral region, 400 nm to 700 nm, measured with a LiCor Li188B and a cosine corrected quantum sensor. Cultures were agitated to prevent settling; agitation was optimised for each species, with *Nitzschia* sp., *Staurastrum* sp., *Stichococcus* sp. and *Monoraphidium* sp. mixed with magnetic stirrer bars, while *Cyclotella* sp. and *C. raciborskii* grew better when hand-shaken for 30 seconds, twice daily, to homogenise the culture. These species were selected to represent the most frequent different classes (cyanobacteria, green algae and diatoms) of phytoplankton in the surface water.

For each experiment, 250 mL Erlenmeyer flasks containing fresh medium (100 mL) were inoculated from a culture growing in exponential phase. Growth curves based on daily cell counts (see below) were carried out to determine maximum growth rate, and the timing of exponential phase. For later experiments, cells for each strain were collected in mid-

exponential phase, after four to five days of growth (depending on the strain), for physiological analysis.

Cell concentration was determined using an improved Neubauer haemocytometer (Boeco, Germany). Due to the trichomic morphology of *Cylindrospermopsis*, enumeration of individual cells using this method was not possible with the available equipment. Instead the average number of cells per trichome was estimated using a Zeiss Axioskop optical microscope (Zeiss, Göttingen, Germany) for at least 40 trichomes as previously described by (Hötzel & Croome 1999). This value was then multiplied by the number of trichomes per millilitre as determined with a haemocytometer, to obtain a final cell density. The value of cells per trichome did not change greatly throughout the course of the experiments (data not shown), however separate values were determined for each experiment. Cellular biovolume was determined for each strain via measurements taken using a light microscope and calculated using the formulae of Hillebrand *et al.* (1999) and Vadrucci *et al.* (2013).

The relationship between photosynthesis and light was estimated by performing photosynthesis vs. irradiance (P vs. I) curves using a temperature-regulated Clark-type oxygen electrode (Hansatech, Norfolk UK) to measure oxygen production by the algal cells. Strains were grown to late exponential phase before being concentrated via centrifugation for 8-10 min at 2500 x g. Pelleted algae were immediately resuspended in 2.2 ml buffered (20 mM HEPES, pH 7.35) medium containing 2 mM $NaHCO_3$ (saturating based on preliminary experiments). A sample was taken for cell counting before 2 ml of the suspension was transferred to the oxygen electrode chamber. Cells were dark acclimated for at least ten minutes, which allowed measurement of the steady state dark respiration rate (R_d). Cells were then exposed to light intensities between 10 and 2400 µmol photons m^{-2} s^{-1} for approximately 30 seconds, until rates of O_2 evolution were stable. Nitrogen gas (N_2) was used to keep

oxygen levels equal to or less than air equilibrium, thus avoiding the inhibitory effects of high O_2 levels (van Wijk & Krause 1991). Equation (1) as described by (Eilers & Peeters 1988), was used to model the photosynthetic response (oxygen evolution) to increasing light intensity:

$$P = \frac{I - I_c}{a(I - I_c)^2 + b(I - I_c) + c} \qquad (1)$$

where P is the photosynthetic rate (in nmol O_2 $(10^7 \text{ cells})^{-1}$ min^{-1}), I is the incident light intensity (in µmol photons m^{-2} s^{-1}) and I_c is the compensation light intensity (in µmol photons m^{-2} s^{-1}), and a, b and c are constants. The least sum of square differences method was used to fit the curve to the data points by solving for a, b and c using the software Graphpad Prism7. I_c is derived using the oxygen evolution rate in the absence of light (dark respiration rate, I=0 µmol photons m^2 s^{-1}) and the lowest tested light intensity (I = 10 µmol photons m^{-2} s^{-1}) to identify the theoretical light intensity at which the oxygen evolving process (photosynthesis) balances the oxygen consuming process (respiration). After fitting the curve, equations (2), (3) and (4) were used to calculate the maximum photosynthetic rate (P_{max}), light saturation onset (I_k) and light harvesting efficiency (α). Rates of photoinhibition (β) (the decline in oxygen production rate at high light intensities), if present, were calculated as the slope of the fitted curve, at light intensities from 1200 and 2400 µmol photons m^{-2} s^{-1}.

$$P_{max} = b + 2\sqrt{ac} \qquad (2)$$

$$I_k = \frac{c}{b + 2\sqrt{ac}} \qquad (3)$$

$$\alpha = \frac{1}{c} \qquad (4)$$

One-way ANOVA and Tukey's multiple comparison test were used to determine significance between species, and were carried out with Graphpad Prism 7 software. The accepted level of

significance was $P \leq 0.05$. All data are reported as means ± standard error from 3 independent cultures.

Results

Growth rates of species were determined to provide a broad assessment of the competitive dominance of the studied species by comparing monoculture physiological characteristics. Growth rates of all strains varied from 0.21 to 0.8 d^{-1} (Table 1). The two smallest green algae, *Stichococcus* sp. and *Monoraphidium* sp., had the highest specific growth rates of 0.73 ± 0.06 d^{-1} and 0.80 ± 0.01 d^{-1} respectively. *Cylindrospermopsis raciborskii* had the lowest growth rate of 0.21 ± 0.002 d^{-1}. Differences in species can be attributed to many characteristics, so to account for size differences their cellular biovolume was estimated microscopically. Cell volumes ranged from 2278 μm^3 $cell^{-1}$ for the large desmid *Staurastrum* sp., to 60 μm^3 $cell^{-1}$ for *C. raciborskii* (Table 1).

To further elucidate the observed differences between species' growth rates, photosynthetic and respiration rates were measured and are presented on both a 'per cell' and 'per cubic millimetre' basis. The maximum net photosynthetic rate for *Staurastrum* sp. (Fig. 1) when calculated per cell, was 735.4 ± 53.8 nmol O_2 $(10^7 cells)^{-1}$ min^{-1}, more than five times higher than the next highest rate of 64.9 ± 22.5 nmol O_2 $(10^7 cells)^{-1}$ min^{-1}, observed for *Cyclotella* sp.. *Cylindrospermopsis raciborskii* had the lowest photosynthetic rate per cell at 10.24 ± 1.41 nmol O_2 $(10^7 cells)^{-1}$ min^{-1}. After normalisation to cellular volume (Fig. 2) the differences between strains were smaller. *Monoraphidium* sp., *Staurastrum* sp. and *Nitzschia* sp. had the highest rates of P_{max} per mm^3 biovolume while the value for *Cyclotella* sp. was significantly lower than those of all strains other than *C. raciborskii*. The highest rates of dark respiration per cell were found in *Cyclotella* sp. and *Staurastrum* sp. (10.72 and 27.84 nmol O_2 $(10^7 cells)^{-1}$ min^{-1} respectively). Per unit cell volume, highest rates were in *Cyclotella* sp. and *Nitzschia* sp. (1.73 and 2.2 nmol O_2 mm^{-3} min^{-1} respectively) (Figs. 3,4).

Cylindrospermopsis raciborskii had the lowest rates of oxygen consumption on both bases,

namely 0.18 nmol O_2 $(10^7 cells)^{-1}$ min^{-1} and 0. 3 nmol O_2 mm^{-3} min^{-1}. To add perspective to these rates, respiration as a percentage of net photosynthetic rate was calculated (Fig. 7), and varied between one and five percent for most species. Both diatoms had higher relative R_d: P_{max} ratios, with *Cyclotella* sp. having values significantly ($P \leq 0.025$) higher, at 16.5 ± 5.5%, than in all other strains.

To broadly observe the mechanisms that contribute to the effectiveness by which the algae and cyanobacterium studied harvest light, rates of light harvesting efficiency, α, were calculated from P vs. I curves. Values for α were significantly ($P \leq 0.002$) higher for *Staurastrum* sp. and *Cyclotella* sp. (4.7 and 1.4 nmol O_2 $(10^7 cells)^{-1}$ min^{-1} (µmol photons m^{-2} $s^{-1})^{-1}$ respectively) than in all other strains (Fig. 5). The lowest measured α value (0.1±0.01 nmol O_2 $(10^7 cells)^{-1}$ min^{-1} (µmol photons m^{-2} $s^{-1})^{-1}$) was exhibited by *C. raciborskii*.

At high light intensities, photoinhibition (β) can negatively affect the performance of phytoplankton, and may help explain the dominance of one species over another. Rates of photoinhibition (Fig. 6), were lowest for *C. raciborskii* (0.005 nmol O_2 $(10^7 cells)^{-1}$ min^{-1} (µmol photons m^{-2} $s^{-1})^{-1}$) and highest for *Staurastrum* sp. (0.067 nmol O_2 $(10^7 cells)^{-1}$ min^{-1} (µmol photons m^{-2} $s^{-1})^{-1}$), which was significantly higher value than in all other strains ($p<0.001$). The light saturation onset measure, I_k, as can be seen in Table 2, also provides insight into the intensity of light a species is acclimated to (Richardson *et al.*, 1983). This was lowest for *Stichococcus* sp. and *Nitzschia* sp. (42 and 62 µmol photons m^{-2} s^{-1} respectively), and highest for *Staurastrum* sp. (153 µmol photons m^{-2} s^{-1}). The compensation light intensity, I_c (Table 2), is a measure of the light intensity at which photosynthetic oxygen production is balanced by respiratory oxygen consumption, and ranged from 1.6 ± 0.6 µmol photons m^{-2} s^{-1} (*Monoraphidium* sp.) to 5.9 ± 2.4 µmol photons m^{-2} s^{-1} (*Cyclotella* sp.). For a species with a

low compensation light intensity, net carbon fixation will occur at lower light levels, also providing a competitive edge in certain situations.

Discussion

This study has demonstrated the variation in photosynthetic capacity that exists within representatives of some of the most common classes of freshwater phytoplankton found in Lake Wivenhoe. Six species were characterised, including two diatoms (*Cyclotella* sp. and *Nitzschia* sp.), three green algae (*Staurastrum* sp., *Monoraphidium* sp. and *Stichococcus* sp.) and a cyanobacterium (*Cylindrospermopsis raciborskii*). These species are all found within the phytoplankton of the photic zone but differed greatly in cell size and growth rate. Summer blooms of *C. raciborskii* occur annually while the phytoplankton population is dominated by diatoms during the winter (Muhid 2011). These seasonal changes in dominance between *C. raciborskii* and diatoms was, to some extent, reflected in their photosynthetic characteristics (at least when grown, as in this work, at near optimum conditions). Acclimation of photosynthetic and metabolic characteristics to a defined light environment occurs both between different species and between strains of a given species (Richardson *et al.,* 1983) and species can also show genetic adaptation to light availability. *C. raciborskii* is commonly described as a low-light adapted species and light conditions are one of the main parameters regulating its occurrence in the environment (Padisák 1997; Briand *et al.,* 2002; 2004; Vidal & Kruk 2008). This is counterintuitive, as *C. raciborskii* also frequently forms surface blooms during summer months, where temperatures and light intensities are at their highest. Species-specific light adaptation gives us insight into how different light conditions (both quantity and quality) can trigger different competition or dominance in natural ecosystems. In this study, the maximum rate of photosynthesis (P_{max}), light harvesting efficiency α, rate of

photoinhibition (β), light saturation onset parameter I_k and light compensation point I_c were measured, together with dark respiration (R_d), growth rate and cell biovolume to characterise the photosynthetic ability of each species. Of the six species measured, significant differences between the species were observed. Due to its relatively large size, *Staurastrum* sp. had the highest absolute rates of photosynthesis and respiration. Conversely, consistent with its smaller cell size, *C. raciborskii* had the lowest absolute rate of photosynthesis per cell.

A high maximum photosynthetic rate confers the ability to fix carbon at a high rate when light is saturating and carbon is not limiting. Normalising for cellular volume allows us to better compare relative net carbon fixation rates between cells of different size. *Cyclotella* sp., the second largest species with the second highest rate of photosynthesis per cell, has a significantly lower rate of photosynthesis than all other strains apart from *C. raciborskii* on a P_{max} vol^{-1} basis, whereas the remainder of the species all have similar rates.

As important as photosynthetic rate, the light harvesting efficiency, α, defines the rate of response of cells to changes in light intensity. A high α enables efficient utilisation of available light in dynamic conditions where light intensity fluctuates, while a lower α may provide a defence against photo-damage and photoinhibition at high light intensities, as cells harvest a lower proportion of incoming photons (Richardson *et al.*, 1983). The species *Staurastrum* sp. and *Cyclotella* sp. had the highest light harvesting efficiencies, while *C. raciborskii* and *Monoraphidium* sp. had the lowest. This finding suggests that *Staurastrum* sp. and *Cyclotella* sp. evolved to utilise variable light levels, while *Monoraphidium* sp. and *C. raciborskii* are more tolerant of high light. This is consistent with existing literature showing that *C. raciborskii*, though it prefers low light for growth, is tolerant of high light intensities because of its low light harvesting efficiency and low rates of photoinhibition (Briand *et al.*,

2004; Burford *et al.*, 2016; Xiao *et al.*, 2017). Data presented here support this previous work, with *C. raciborskii* having the lowest rate of photoinhibition (β).

Monoraphidium sp., like *C. raciborskii*, has relatively low light harvesting efficiency and low rates of photoinhibition, yet does not frequently bloom in the lake. It is likely that given the right light conditions and nutrient loading, small sized and high-light tolerant strains of green algae such as *Monoraphidium* sp., studied here, might be capable of bloom formation due to their high rates of growth and photosynthesis (per unit volume).

It is important to note that algal photosynthetic performance is impacted by pigmentation which defines the wavelengths of light that may be harvested. Phycobiliproteins (present in cyanobacteria), are pigments that are capable of harvesting wavelengths of light not readily utilised by other algae (though fucoxanthin, an accessory pigment in diatoms, is capable of absorbing in the 'green window' to a lesser extent) (Mimuro & Akimoto 2004). In a turbid and populated system, most red and blue light will be absorbed at the surface, hence the capacity to harvest green light and as broad a range of wavelengths as possible at low light intensities is an advantage.

Motility and buoyancy enable species to control their exposure to light, which reduces the need for photosynthetic adaptation to changing conditions. *Nitzschia* sp., either through exopolysaccharide propulsion or other means, is capable of vertical migration, (Round & Eaton 1966; Cohn & Weitzell 1996; Smith & Underwood 1998), providing it with the capacity to escape potentially photoinhibiting light intensities. Conversely, *Cyclotella* sp. lacks motility and is neutrally buoyant, though may be capable of changing its sinking rate (Humphries & Lyne 1988; Raven and Waite 2004), thus *Cyclotella* sp. is largely dependent on vertical mixing for access to light and possesses a significantly higher light harvesting efficiency than *Nitzschia* sp.. This attribute would allow *Cyclotella* sp. to photosynthesise

more efficiently when conditions are suitable (high mixing rates), though it will be vulnerable to photoinhibition under high light intensities. Winter blooms of diatoms in Lake Wivenhoe are an example of how high α values prove a competitive advantage in dynamic conditions. *C. raciborskii* is generally considered neutrally buoyant (Dokulil & Teubner 2000; Burford & Davis 2011) and thus summer blooms in stratified waters of *C. raciborskii* may reflect the benefits of a low α and β.

Rates of photosynthesis cannot be considered without also taking respiration into account. High rates of respiration observed in the two diatoms may be associated with the high metabolic costs associated with exopolysaccharide (EPS) production, though we did not make observations on these parameters. Conversely *C. raciborskii*, the only prokaryote studied here, has the lowest respiration rate, consistent with its relatively simple internal structure and organisation. The respiration to photosynthesis ratio has been shown not to be size dependent (Banse 1976) which is also true in this study. Rates of photosynthesis and respiration are often examined to better explain observed rates of growth, because they can act as a proxy for the energy balance of the cell.

The specific growth rates of the species examined here ranged from 0.2 to 0.8 day^{-1} and are comparable to reported growth rates of strains within the same genus (Dauta *et al.,* 1990; Coesel & Wardenaar 1994; Litchman *et al.,* 2003; Butterwick *et al.,* 2005; Mitrovic *et al.,* 2010; Holland *et al.,* 2012; Moazami-Goudarzi & Colman 2012; Lurling *et al.,* 2013; Pierangelini *et al.,* 2014). As previously mentioned, large blooms of *C. raciborskii* are frequent in Lake Wivenhoe, thus its inability to outgrow any of the other strains in our batch monoculture experiments is somewhat surprising if not taken in context. Replete levels of nitrogen and phosphorus sources in the medium remove any observable effect on growth that differences in nutrient scavenging or assimilation may have. Thus these differences in growth

rate, while interesting, do not necessarily reflect relative growth rates in the species' habitat. A further factor to consider, that may explain differences between observed monoculture growth rates, and bloom formation is that of grazing pressure. Though not studied here, it is likely that the trichomes of *C. raciborskii* are consumed by predators at lower rates than smaller, single celled species, thus predator resistant species may accumulate at a higher rate in the waterway, irrespective of growth rates.

The potential of a species to acclimate growth to changing light intensities was not investigated in this study. However, it is logical that in a dynamic system, a high adaptive capacity should confer some competitive advantage to the strain. The photosynthetic characteristics presented here provide a snapshot of how the studied species behave at relatively low (but close to saturating) light intensities with replete nutrient concentrations; however, variations in any environmental condition would likely affect species and strain characteristics. When comparing photosynthetic characteristics, *C. raciborskii* appears more robust than its growth rate might suggest. A light saturation onset value (I_k) of 181 µmol photons m^{-2} s^{-1} and a low light compensation point for *C. raciborskii* indicate that the strain is capable of fixing carbon within a wide variety of light levels (Xiao *et al.,* 2017), despite being grown at much lower intensities. *Staurastrum* sp. was the only strain to have a higher I_k value, at 210 µmol photons m^{-2} s^{-1}. *Nitzschia* sp. *Cyclotella* sp. and *Stichococcus* sp. all had light acclimation constants close to the growth light intensity of 60 µmol photons m^{-2} s^{-1}. This may be due to a flexible light harvesting apparatus, or they may simply be low light preferring species.

It follows that a species' ability to occupy various niches in the water column is largely defined by its capacity to harvest light and utilise available nutrients. Changes in nutrient or light availability may therefore lead to changes in competitive dominance, having

implications for all other organisms which are dependent on the water body, including humans. Lake Wivenhoe is an important water resource for South Eastern Queensland, understanding the growth and photosynthetic characteristics of a cross-section of its phytoplankton inhabitants may prove valuable as CO_2 levels rise and climate changes continue. This study found that the photosynthetic characteristics of *C. raciborskii* may confer a competitive advantage that contributes to its ability to dominate in high light conditions. This data, in combination with its ability to thrive under higher temperatures, as well as its ability to carry out nitrogen fixation add further to the understanding of the dominance of *C. raciborskii* over competing species in Lake Wivenhoe.

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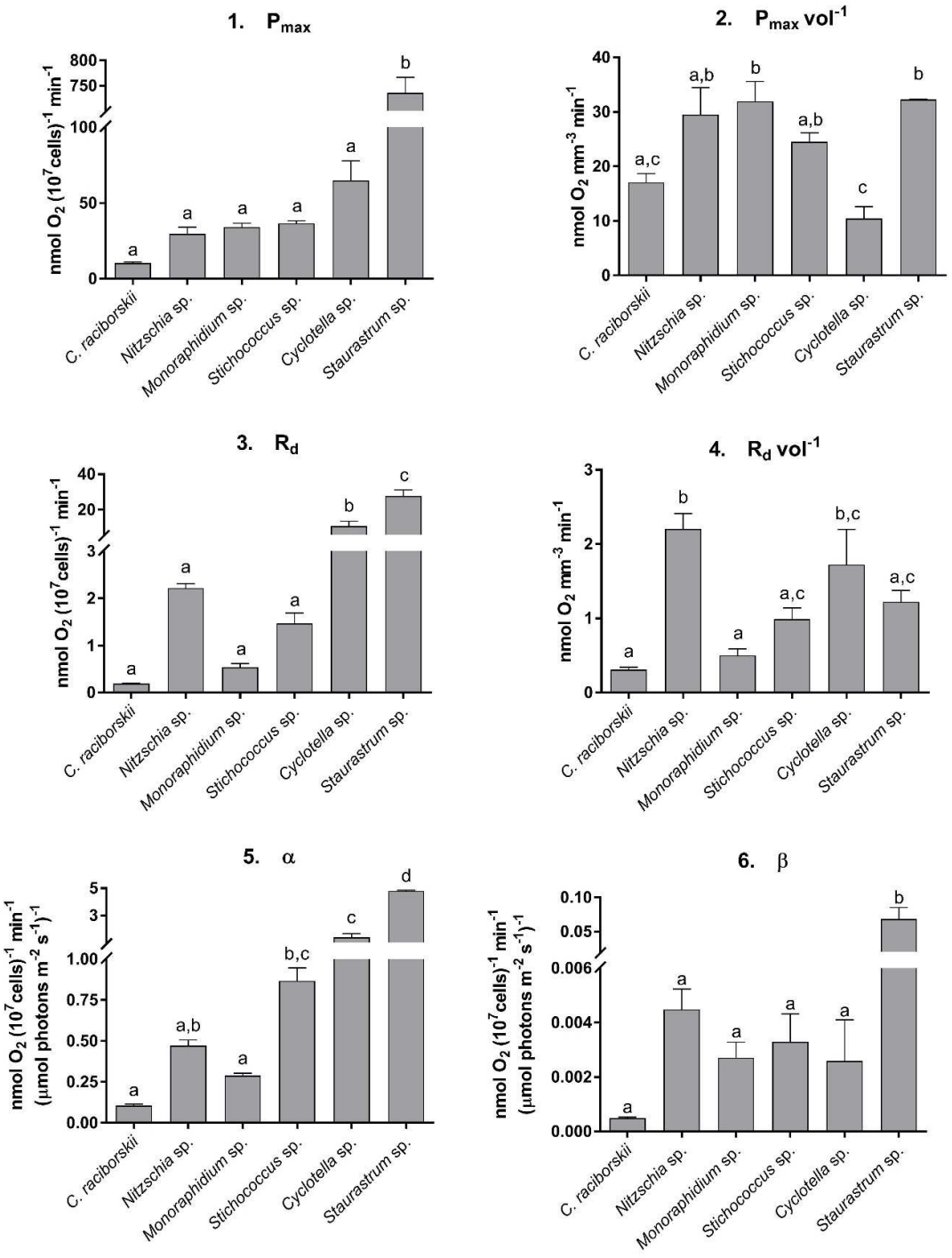
Figures and tables – Paper 1

Table 1. Specific growth rates (n = 3, units: d^{-1}) and cellular volumes ($n \geq 18$, units: μm^3) of the six microalgal strains grown in JM+Si medium at 25 ºC and the light intensities described in the methods. Data are means ± standard error (given in parentheses). Different superscript letters indicate statistically significant differences, $p \leq 0.05$ (based on ANOVA and post-hoc Tukey's test).

Species	**Growth rate** d^{-1}	**Measured cell volume** μm^3
Cylindrospermopsis	0.21^{a} (<0.01)	60^{a} (2.9)
Nitzschia sp.	0.45^{b} (0.05)	100.7^{a} (8.4)
Monoraphidium sp.	0.81^{c} (0.01)	106.9^{a} (9.2)
Stichococcus sp.	0.74^{c} (0.01)	148.9^{a} (6.9)
Cyclotella sp.	$0.28^{a,b}$ (0.05)	621.3^{b} (31.7)
Staurastrum sp.	$0.37^{a,b}$ (0.06)	2278^{c} (99)

Table 2. Light saturation constant (I_k) and compensation light intensity (I_c) derived from P vs. I curves. Data are means ± standard error (given in parentheses), n = 3, units: µmol photons m^{-2} s^{-1}. Different superscript letters indicate statistically significant differences, $p \leq 0.05$ (based on ANOVA and post-hoc Tukey's test).

Species	**I_k** **µmol photons m^{-2} s^{-1}**	**I_c** **µmol photons m^{-2} s^{-1}**
C. raciborskii	99.0[a] (14.3)	2.5[a] (0.1)
Nitzschia sp.	62.2[b] (4.4)	3.8[a,b] (0.3)
Monoraphidium sp.	118.7[a,c] (10.8)	1.6[a] (0.3)
Stichococcus sp.	42.8[b] (4.50)	2.0[a] (0.2)
Cyclotella sp.	50.8[a,b] (14.2)	6.0[b] (1.4)
Staurastrum sp.	153.6[c] (5.53)	4.1[a,b] (0.4)



Figs. 1-6: Photosynthetic and respiratory characteristics of the six species investigated. Measurements were made at 25 ºC, pH 7.35, with 20 mM HEPES and 2 mM DIC added to the medium. Data are means ± standard error, *n* = 3. Different letters above the columns

indicate statistically significant differences, $p \leq 0.05$ (based on ANOVA and Tukey's multiple comparisons test).

Fig. 1. Maximum net photosynthetic rate per cell (P_{max}).

Fig. 2. Maximum net photosynthetic rate per unit biovolume (P_{max} vol^{-1}).

Fig. 3. Dark respiration rate per cell (R_d).

Fig. 4. Dark respiration rate per unit biovolume (R_d vol^{-1}).

Fig. 5. Light harvesting efficiency (α)

Fig. 6. Photoinhibition (β)

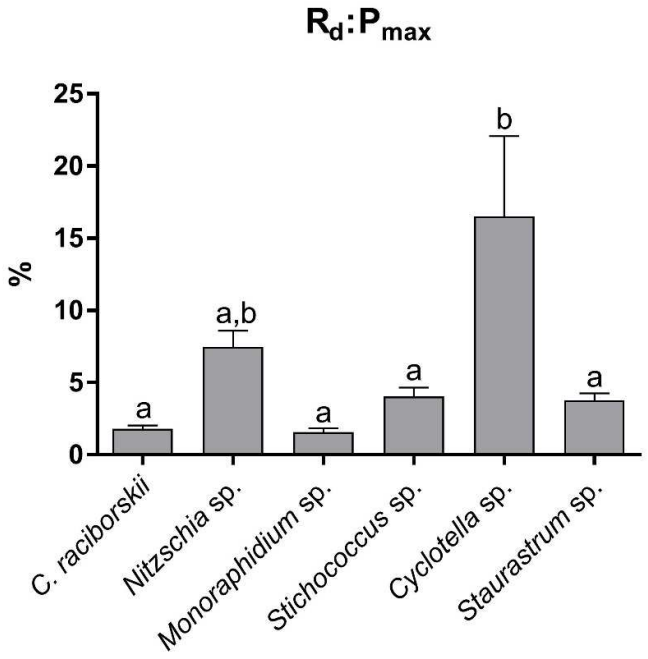


Fig. 7. Respiration rate expressed as a percentage of net photosynthetic rate. Data are means ± standard error, *n* = 3. Propagation of errors was taken into consideration and calculated accordingly. Different letters above the columns indicate statistically significant differences, $p \leq 0.05$ (based on ANOVA and post-hoc Tukey's test).

Chapter 3: Paper 2

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Carbon acquisition characteristics of six microalgal species isolated from a sub-tropical reservoir: Potential implications for species succession

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Abstract

CO_2 levels in freshwater systems can fluctuate widely, potentially influencing photosynthetic rates and growth of phytoplankton. This in turn can lead to bloom formation and affect water quality. This study investigated the acquisition of dissolved inorganic carbon by six species of microalgae, *Cylindrospermopsis raciborskii*, *Cyclotella* sp., *Nitzschia* sp., *Stichococcus* sp., *Staurastrum* sp., and *Monoraphidium* sp., isolated from a sub-tropical reservoir in Australia. Carbon acquisition characteristics, specifically the affinity for DIC, internal pH and internal DIC concentrations were measured. Affinities for CO_2 ($K_{0.5(CO_2)}$) ranged between 0.7 and 6 µM CO_2. This was considerably lower than air-equilibrated surface water CO_2 concentrations, and below reported affinities for CO_2 of Rubisco, suggesting operation of active carbon dioxide concentrating mechanisms (CCMs) in all species. At 180 µM DIC (approximate air-equilibrium DIC concentration), the rate of spontaneous CO_2 supply from bicarbonate in air-equilibrated suspensions was much less than drawdown during photosynthesis, suggesting involvement of bicarbonate use in CCM operation. Internal pH was lowest for *Cyclotella* sp. at 7.19, and highest for *Staurastrum* sp., at 7.71. At 180 µM external DIC, ratios of internal:external CO_2 ranged from 2.5 for *Nitzschia* sp. to 14 in *C. raciborskii*. Internal HCO_3^- concentration showed a linear relationship with surface area to biovolume ratio (SA:Vol). We hypothesised that species with a higher SA:Vol suffer more from diffusive escape of CO_2, thus storage of DIC as bicarbonate is favoured in these strains. For *C. raciborskii*, under stratified summer conditions, its strong CCM, and resilient photosynthetic characteristics may contribute to its bloom forming capacity.

Key index words: carbon dioxide-concentrating mechanism, internal inorganic carbon pool, carbon acquisition, competitive dominance, freshwater.

Abbreviations: CCM, carbon dioxide-concentrating mechanism; DIC, dissolved inorganic carbon; C_i, inorganic carbon; CO_2, carbon dioxide; HCO_3^-, bicarbonate; SA:Vol, surface area to biovolume ratio; sp., species; RuBP, ribulose-1,5-bisphosphate; P_{max}, maximum photosynthetic rate;

Introduction

Carbon acquisition is central to phytoplankton growth, thus the variability in dissolved inorganic carbon (DIC) supply is a possible driver for species succession. Most phytoplankton species require CO_2 at the active site of the enzyme ribulose-1,5-bisphosphate (RuBP) carboxylase oxygenase (Rubisco) at concentrations well above ambient levels to achieve effective rates of net carbon fixation (Giordano *et al.*, 2005). In photosynthesis, Rubisco catalyses the carboxylation of RuBP using CO_2 with the net formation of one molecule of glyceraldehyde-3-phosphate for every three CO_2 assimilated via the Calvin-Benson-Bassham cycle (otherwise known as the Photosynthetic Carbon Reduction Cycle, PCRC) (Beardall & Raven, 2016). In addition to the carboxylase function, Rubisco also facilitates the binding of RuBP to oxygen, leading to inefficiencies in net C fixation through the competitive oxygenase reaction, leading to photorespiration (Raven *et al.*, 2014). The reaction kinetics of Rubisco vary broadly between species (Badger *et al.*, 1998). Those possessing Rubisco with a low affinity for CO_2 (shown by a high $K_{m(CO_2)}$), require a carbon dioxide concentrating mechanism (CCM) to actively concentrate CO_2 at the active site, maximising rates of carbon fixation at external CO_2 concentrations close to ambient levels. For almost all species whose CCMs and Rubisco are characterised, at air equilibrium the rate of CO_2 influx by diffusion without the use of carbon concentration mechanisms would be rate limiting (in dinoflagellates, because of the kinetics of their form of Rubisco, no net CO_2 fixation would occur without CCM function) (Badger *et al.*, 1998, Rost *et al.*, 2006, Young *et al.*, 2016). Thus most species studied to date possesses some form of CCM (Giordano *et al.*, 2005, Raven & Giordano, 2017). The molecular machinery of CCMs varies between species, however they possess a common goal, to saturate Rubisco with CO_2, maximizing the rate of carbon fixation and thus photosynthesis.

Cyanobacteria utilise a range of HCO_3^- and CO_2 transporters to concentrate bicarbonate in the cytosol for transport into the carboxysomes where Rubisco is present (Price *et al.*, 1998). A carboxysome-located carbonic anhydrase catalyses the conversion of HCO_3^- to CO_2 for fixation by Rubisco. In eukaryotes, active HCO_3^- transporters or CO_2 diffusion supply DIC across the plasmalemma to the cytosol, from where it diffuses or is actively transported into the chloroplast for fixation. Species may also actively transport HCO_3^- into the thylakoid lumen (Giordano *et al.*, 2005, Meyer & Griffiths, 2013). In all eukaryote species, a range of carbonic anhydrases located throughout the cell are regulated by DIC availability (Smith-Harding *et al.*, 2017), and have roles that include supplying Rubisco with CO_2, preventing CO_2 leakage by converting CO_2 to HCO_3^-, or enhancing rates of CO_2 diffusion into the cell (Giordano *et al.*, 2005).

The variety of carbon dioxide concentrating mechanisms and the way in which they are utilised, make the physiological and ecological impact of a change in atmospheric CO_2 levels unpredictable. In the Lake District (United Kingdom), Chrysophytes became dominant when aqueous CO_2 concentrations were significantly above air equilibrium (Maberly, 2009). Later it was shown that none of the studied species of Chrysophyte possessed a CCM, and thus growth of these algae is generally limited by the availability of free CO_2 in the water column and its diffusive entry into cells (Maberly *et al.*, 2009). Other algal species are also capable of functioning well with only diffusive CO_2 entry. These tend to be restricted to environments where CO_2 levels are high - such as is the case for the freshwater red algae belonging to the Batrachospermales (Raven *et al.*, 1982) and the coccoid symbiotic green alga *Coccomyxa* using CO_2 from soil or from respiration of the host in the case of the symbiotic cells (Raven & Colmer, 2016) . CCMs are energy demanding, thus species such as marine red algae, growing where low light levels constrain photosynthesis, have DIC needs that are saturated

by diffusive entry of CO_2 (Kübler & Raven, 1994, Kübler & Raven, 1995). Beardall (1991) also showed both a decreased activity and down-regulation of CCMs in *Anabaena variabili*s at very low light intensities, showing that operation and regulation of CCMs is light dependent.

By reducing either the substrate (CO_2) limitation of Rubisco or the metabolic costs of operating a CCM, changes in atmospheric CO_2 concentration have the capacity to alter competition between species and thus change the biodiversity of primary producers in aquatic ecosystems (Sterner & Hessen, 1994, Guo *et al.*, 2016). Species with more efficient Rubiscos may need to expend fewer resources maintaining and running CCMs, making them potentially better competitors (Raven *et al.*, 2012). Understanding the present day physiology of relevant species of phytoplankton is therefore a vital tool for predicting how these ecosystems may change with the increases in CO_2 predicted in the future.

As part of a broader study investigating factors contributing to the competitive dominance of phytoplankton species in freshwater, this study aims to characterise, under replete nutrient levels and current atmospheric CO_2 conditions, the characteristics of inorganic carbon utilization of six species of algae from four classes; Chlorophyceae (green algae), Charophyceae (desmids), Bacillariophyceae (diatoms) and Cyanophyceae (cyanobacteria). These strains originate from Lake Wivenhoe, a typical sub-tropical reservoir in South East Queensland that experiences regular diatom blooms during winter months, and toxic blooms of *Cylindrospermopsis raciborskii* during summer months (Jones & Poplawski, 1998). The photosynthetic responses of these species to DIC availability, and their capacity to concentrate carbon intracellularly were measured and these characteristics were examined in relation to potential competition between species.

Materials and Methods

Strains and culturing. Microalgal cultures were established by single cell isolations from water samples from Lake Wivenhoe in south eastern Queensland (27° 16' S, 152° 32' E). The cultures used in this study were a cyanobacterium *Cylindrospermopsis raciborskii* (Wołoszyńska) Seenaya & Subba Raju [Class: Cyanophyceae; cylindrospermopsin producing strain WSO7, described in Willis *et al.*, (2016b)], two diatoms, namely *Cyclotella* sp. (Kützing) Brébisson (Class: Coscinodiscophyceae) and *Nitzschia* sp. Hassall (Class: Bacillariophyceae), and three green algae *Monoraphidium* sp. Komárková-Legnerová (Class: Chlorophyceae), *Staurastrum* sp. (Class: Zygnemophyceae, order: Desmidiales), and *Stichococcus* sp. Nägeli (Class: Chlorophyceae). All strains are available from the Monash University Culture collection, and were grown in Jaworski's medium (JM, Thompson *et al.*, 1988) supplemented with 300 µM Na_2SiO_3 (JM + Si) at 25ºC, with a light intensity of 60 µmol photons $\cdot m^{-2} \cdot s^{-1}$, and a light: dark cycle of 12h:12h. Cultures of *C. raciborskii* and *Cyclotella sp.* were resuspended by shaking cultures by hand daily, while cultures of *Nitzschia*, *Monoraphidium*, *Stichococcus* and *Staurastrum* were stirred via magnetic stirrer at approximately 100 rpm to prevent settling.

From a culture growing in exponential phase, Erlenmeyer flasks (250 mL) of fresh medium (100 mL) were inoculated for each experiment. Physiological analysis was carried out at mid- to late-exponential phase of growth of these experimental cultures with three independent cultures for each species.

Cell concentration determination. Cell concentration was determined using an improved Neubauer haemocytometer. Due to *C. raciborskii*'s morphology, enumeration of individual cells using only this method was not practical. Instead, the average number of cells per

trichome was estimated by counting the number of cells per trichome for 40 trichomes using a Zeiss Axioskop optical microscope (Zeiss, Göttingen, Germany). This value was then multiplied by the number of trichomes per millilitre (via haemocytometer) to get a final cell density. This value did not change greatly throughout the course of the experiments (data not shown), but values for average cells per trichome were determined for each experiment.

Cellular surface area and volume. Measurements of cells were carried out via microscope and applied to the geometric formulae described in Hillebrand *et al.* (1999) and Vadrucci, Mazziotti & Fiocca (2013). The formula for two truncated cones was applied to *Nitzschia* sp., the formula for a prolate spheroid was applied to *Stichococcus* sp., the formula for a cylinder was applied to both *Cyclotella* sp. and *C. raciborskii*, a monoraphidioid formula was applied to *Monoraphidium* sp., while the formula for of two truncated cones, with four attached cylinders was used for *Staurastrum* sp.. Where possible, the dimensions of individual cells were measured, and population means taken. For all species except *C. raciborskii*, volumes were taken for each cell and the resulting values averaged. In the case of *C. raciborskii*, due to its size and structure, cell widths and lengths were taken ($n > 50$), and averages of these dimensions used to determine cellular volume.

Oxygen evolution. The dependence of photosynthesis on inorganic carbon concentration was measured in photosynthesis vs. DIC (P vs. DIC) experiments, using a temperature controlled Clark-type oxygen electrode (Hansatech, Norfolk UK) to measure oxygen production by the algal cells. Strains were grown to late-exponential phase before being concentrated by centrifugation for 8-10 min at 2500 x g, and washed three times with DIC-free, buffered (20 mM HEPES, pH 7.35) JM+Si medium, before transfer to the oxygen electrode chamber. Cells were then illuminated under a saturating light intensity [as described in previous work (Lines *et al*., unpublished, see chapter 2 of this thesis)] to allow consumption of any

remaining DIC present in the chamber, before additions of DIC began and the photosynthetic response measured. Sodium bicarbonate was added to produce DIC concentrations of between 5 and 1360 µM. Average cell concentrations for both P vs. DIC experiments and CO_2 supply rate calculations (below) were 10^7 cells · mL^{-1} for *C. raciborskii*, 10^6 cells · mL^{-1} for *Cyclotella* sp., 10^7 cells · mL^{-1} for *Nitzschia* sp., 8 x 10^6 cells· mL^{-1} for *Stichococcus* sp., 5 x 10^5 cells · mL^{-1} for *Staurastrum* sp., and 10^7 cells · mL^{-1} for *Monoraphidium* sp..

Uncatalysed supply rates of CO_2 from bicarbonate were calculated and compared to uptake rates by cells, as described previously by Burns and Beardall (1987). It is assumed that the molar rate of oxygen production is equal to the molar rate of carbon fixation.

Internal pH and carbon pool. The silicon oil centrifugation method was used to measure the internal pH and internal carbon pool of the strains as described by Badger *et al.*, (1980).

In brief, layers of killing solution (50µl, 1M glycine, 1% sodium dodecyl sulphate), silicon oil (50µl), and 200 µl resuspended cells in buffered media (20 mM HEPES, pH 7.35) were introduced into a 500 µl microfuge tube. 1 µCi additions of dimethyloxazolidine-2,4-dione-5,5-[2-^{14}C], (DMO, American Radiolabelled Chemicals, used for internal pH determination), ^3H_2O (tritiated water, Perkin Elmer, to measure total volume transferred from the algal suspension into the pellet), ^{3}H-dextran (American Radiolabelled Chemicals; to determine extracellular water) or $H^{14}CO_3^-$ (Perkin Elmer, $NaH^{14}CO_3$, for internal DIC pool measurements) were made to the upper layer of buffered cells before they were exposed to light (60 µmol photons · m^{-2} · s^{-1}) for 10 min, 5 min, 20 s and 10 s respectively before the tubes were centrifuged for 60 s, driving the cells from the medium, through the oil, into the killing solution below. The appropriate ratio of dinonyl phthalate to silicon oil, used as the boundary between cells and killing solution, for each species can be found in the

supplementary information (Table S1). Radioactivity in the pellets and supernatant was determined using a Tri-Carb 2810 TR, Perkin Elmer scintillation counter. This method was not found to work with *Monoraphidium* sp., likely due to its small cell size and morphology. An oil viscosity could not be identified which would permit the passage of the *Monoraphidium* sp. cell, while preventing inversion of the oil and supernatant layers.

Statistical analysis A series of one-way ANOVAs with Tukey's post-hoc analyses were conducted to examine the differences, between species, in whole cell bicarbonate and CO_2 affinity, maximum photosynthetic rate, internal pH and internal:external DIC and CO_2 ratio. Secondly, a linear regression was performed to examine the relationship between internal bicarbonate concentration and a species' SA:Vol ratio.

Results

The $K_{0.5(CO_2)}$ values, which are a proxy measurement of CCM activity, are presented in Table 1. They ranged between 0.7-1.3 µM CO_2 for the diatoms and the cyanobacterium, which is significantly lower than the $K_{0.5(CO_2)}$ for the three species of green algae, which ranged between 3-6 µM CO_2 (one-way ANOVA, $F_{5,18} = 29.42$, $P < 0.0001$).

Maximum (light and DIC-saturated) photosynthetic rates (Table 1) varied considerably between species; from 5.5 nmol $O_2 \cdot (10^7$ cells $\cdot$ min$)^{-1}$ for *C. raciborskii*, to 850 nmol O_2. $(10^7$ cells $\cdot$ min$)^{-1}$ for *Staurastrum* sp.. The P_{max} of *Staurastrum* sp. was significantly higher than that in all other species (one-way ANOVA, $F_{5,18} = 53.34$, $P < 0.0001$), which was due to its large cell size in comparison to the other species (Lines *et al*., unpublished). After normalisation to cellular volume, photosynthetic rates were comparable across all species.

To further examine CCM activity, rates of drawdown of inorganic carbon by carbon fixation were compared to the uncatalysed rate of CO_2 supply via the dehydration of $NaHCO_3$ (Fig. 1). At the air equilibrated DIC concentration of 180 µM (CO_2 concentration ~16 µM), growth temperature of 25 ºC, and pH of 7.35, the rate of uncatalysed CO_2 supply was calculated to be 0.32 nmol $CO_2 \cdot mL^{-1} \cdot min^{-1}$. Carbon fixation rates for all species, calculated from net photosynthetic oxygen evolution at 180 µM DIC concentration, were considerably greater than the uncatalysed supply rate of CO_2 from bicarbonate, and ranged from 6.8 nmol $CO_2 \cdot mL^{-1} \cdot min^{-1}$ for *Cyclotella* sp. to 36 nmol $CO_2 \cdot mL^{-1} \cdot min^{-1}$ for *Staurastrum* sp.. Culture pH increased from 7.35 over time, to (mean values ± SE at late exponential phase) 9.4 ± 0.7 for *C. raciborskii*, 8.7 ± 0.4 for *Cyclotella* sp., 9.2 ± 0.4 for *Nitzschia* sp., 10.1 ± 0.1 for *Stichococcus* sp., 9.8 ± 0.1 for *Staurastrum* sp. and 10.0 ± 0.0 for *Monoraphidium* sp..

The internal pH, internal DIC concentration, internal CO_2 concentration, and internal to external CO_2 ratios are presented in Table 2. Internal pH of *Staurastrum* sp. (7.71) was significantly higher than that of all other species (one-way ANOVA, $F_{4, 10} = 33.22$, $P < 0.001$). Mean internal pH for *Cyclotella* sp. (7.19) was significantly lower than for all other species, with the exception of *C. raciborskii*. Internal pH was used in combination with the internal DIC concentration to determine the internal CO_2 concentration. External concentrations of DIC varied between 187 and 194 µM, which equated to external CO_2 concentrations between 12 and 17 µM, depending on the precise pH of the cultures. The species' capacity to concentrate DIC was, at its highest 12 fold for *C. raciborskii*, and at its lowest 3.4 fold for *Nitzschia* sp.. These concentrations are equivalent to a ratio of internal to external CO_2 of 14 and 2.5 fold for *C. raciborskii* and *Nitzschia* sp. respectively. *Cyclotella* sp. displayed similar characteristics to *Nitzschia* sp. with similar internal DIC concentrations and internal:external DIC ratios. However, the internal CO_2 pool was higher in *Cyclotella* sp. than in *Nitzschia* sp. due to *Cyclotella* sp.'s lower internal pH. *Staurastrum* sp. concentrated DIC to levels similar to that of *C. raciborskii*, though, due to its relatively high internal pH, it had a similar internal CO_2 concentration to that of *Stichococcus* sp., which had a lower internal pH and DIC concentration.

Observations of internal bicarbonate concentration and cellular volume suggested there may be a relationship between these two variables. Therefore, a linear regression was performed to test the significance of the trend observed. Data for *Nitzschia* sp. was excluded from the analysis because it was found to be an outlier, on the basis that it is commonly a benthic species of diatom (see the Discussion for a detailed explanation). Fig. 2 displays internal HCO_3^- concentration against the measured surface area to biovolume ratio of the species. The

linear trend line was plotted with confidence intervals and was found to be statistically significant (linear regression, $R^2 = 0.48$, $F_{1,10} = 9.37$, $P = 0.012$).

Discussion

At present atmospheric CO_2 concentrations, the role of DIC in freshwater systems remains poorly understood. A solid foundational understanding of the capacity of species to utilise and internally concentrate inorganic carbon under present environmental conditions is both necessary and valuable, particularly given the predicted rise in future atmospheric CO_2 concentration. This study sought to examine and better understand the carbon utilisation characteristics of a selection of species from a prominent lake in north eastern Australia. The results presented add to the understanding of the diversity of DIC uptake and accumulation by species present in Lake Wivenhoe.

Whole-cell half saturation constants for inorganic carbon dependent oxygen evolution were considerably lower than previously reported Rubisco half saturation constants for CO_2 ($K_{m(CO_2)}$). For example, authors have reported ~ 30 µM for diatoms (Young *et al.*, 2016), ~ 30-60 µM for green algae (Badger *et al.*, 1998), and from 150 µM for cyanobacteria (Badger *et al.*, 1998). These reported $K_{m(CO_2)}$values for Rubisco are also considerably higher than observed ambient CO_2 concentrations in Lake Wivenhoe during the summer months (0 – 12 µM) (see chapter 4 of this thesis). This finding suggests that the use of a CCM is necessary, as diffusive supply of CO_2 to Rubisco would be insufficient at these external CO_2 levels to support significant rates of net photosynthesis. The activity of CCMs in each strain is confirmed by the low $K_{0.5(CO_2)}$ values reported here, implying that the photosynthetic rate of each species would be DIC-saturated at air equilibrium levels of external CO_2. This finding is further supported by the observed rates of CO_2 fixation when compared to the rate of CO_2 supply. These suggest that bicarbonate use, either by direct uptake or via an external carbonic anhydrase (which mediates the generation of CO_2 from bicarbonate in the periplasmic space),

may be responsible for the discrepancy between CO_2 supply and fixation. The elevated pH of all cultures during exponential phase also supports the use of bicarbonate by all species studied here (Maberly & Spence, 1983, Maberly, 1990, Spijkerman *et al.*, 2005). All cultures had high pH, from 8.7-10.1, indicating the capacity of each species to grow in an environment containing very little CO_2 and a high proportion of HCO_3^-. Holland *et al.* (2012) also suggested that *C. raciborskii* can utilise bicarbonate, while the utilization of bicarbonate by diatoms and green algae is well documented (Colman & Rotatore, 1995, Giordano *et al.*, 2005, Spijkerman *et al.*, 2005). However, the presence of external carbonic anhydrase (CA) cannot be discounted, and its presence (without quantification) would not allow the conclusion that bicarbonate is directly used to be drawn.

C. raciborskii, the species shown by this research to have one of the highest CO_2 affinities, is known to bloom regularly in the summer months when the reservoir is stratified and temperatures are highest (McGregor & Fabbro, 2000). During stratified periods in productive lakes, available inorganic carbon is consumed faster than it is resupplied (Maberly, 1996). Consequently, available CO_2 in surface water declines to values less than air equilibrium, and pH increases (Heaney *et al.*, 1986). During these periods, in the absence of river inflows, sources of C_i are stored at depths beyond the reach of surface-dwelling phytoplankton, and do not enter surface waters without some form of mixing (Heaney *et al.*, 1986) which would also increase rates of gas exchange with the atmosphere. As the findings indicated, *C. raciborskii*, likely due to its kinetically inefficient Rubisco, is equipped with a strong capacity to concentrate DIC internally. *C. raciborskii* also has a higher temperature optimum than most eukaryotic species (Briand *et al.*, 2004, Burford *et al.*, 2016), and has been shown to grow faster at higher pH (Holland *et al.*, 2012), and frequently dominates the surface waters in these conditions (Visser *et al.*, 2016).

It has been suggested that during a bloom, the high C_i concentration gradient is enhanced by the capacity of cyanobacteria to internally accumulate inorganic carbon while in surface waters, which therefore limits the availability of C_i to phytoplankton deeper in the water column (Ibelings & Maberly, 1998). It is unlikely, however, that the *C. raciborskii* CCM has evolved to starve competing strains of DIC. The poor kinetics of the cyanobacterial Rubisco requires concentrations of CO_2 many hundred fold above that which may be supplied by diffusion alone. Perhaps the competitive advantage of high rates of DIC accumulation, a necessity due to the low CO_2 affinity of the cyanobacterial Rubisco, explains its lack of evolutionary kinetic improvement during the millions of years since oxygen has been present in the atmosphere, at least in strains capable of bloom formation in stratified waters.

During a bloom in stratified waters, the high internal DIC concentration observed in this study by *C. raciborskii*, may deny species of C_i at greater depths via its depletion at the surface. This is also supported by the previous work of Shapiro (1997), which showed that, while supplementation of a freshwater body with CO_2 did not prevent cyanobacterial bloom formation, the kinetics of carbon uptake were likely responsible for maintaining cyanobacterial dominance. Other work by Van de Waal *et al.* (2011) also supports this theory, where two strains of *Microcystis aeruginosa* with differing affinities for DIC were shown to sequentially dominate a culture based on the available CO_2. Alternatively, empirically proven models, developed to predict the impact of surface bloom formation on the biomass production of deeper species, show that light attenuation alone is enough to prevent significant growth of non-bloom forming species (Huisman, 1999, Huisman *et al.*, 1999). Of course, it is likely that in nature a combination of these factors is involved in driving cyanobacterial dominance.

As can be seen in this study, *C. raciborskii* is well adapted to its particular high temperature (Briand *et al.*, 2004), low CO_2 niche. Species such as *Cyclotella* sp., *Monoraphidium* sp., *Staurastrum* sp. and *Stichococcus* sp. possess other traits, such as lower temperature optima and lower light requirements, allowing them to build up biomass at other times of the year. During winter and spring, higher rates of mixing via high wind speeds, and the influx of DIC saturated catchment water, reduce the CO_2 concentration gradient in surface waters and increase the availability of DIC and other nutrients throughout the water column (Maberly, 1996). This, in combination with sources of inorganic carbon, such as decaying plant material, result in supersaturation of Lake Wivenhoe with DIC for much of the year, which can then become a source of CO_2 to the atmosphere (Raymond *et al.*, 2013). In these conditions, CCM function is almost entirely redundant.

Previous work (Lines *et al*., unpublished, chapter 2 of this thesis) found that the photosynthetic characteristics of these species contribute to explanations of species dominance in Lake Wivenhoe's dynamic environment. The low rate of photoinhibition of *C. raciborskii* benefits its growth at the surface of stratified waters, while a high light harvesting efficiency enables *Cyclotella* sp., *Nitzschia* sp. and the green algae to grow rapidly in more mixed conditions. *Staurastrum* sp. concentrates DIC internally to relatively high levels, almost equal to that of *C. raciborskii*. Both these species are light saturated at high intensities, with respective I_k values of 100 and 153 $\mu mol\ photons \cdot m^{-2} \cdot s^{-1}$(Lines *et al*., unpublished). It is conceivable that this reflects the higher energetic requirement associated with high levels of CCM activity (Beardall, 1991, Beardall & Giordano, 2002).

Internal bicarbonate accumulation seems to correlate with surface area to volume ratio, with the exception of *Nitzschia* sp. (Fig. 2. It is noted that the regression is strongly influenced by *C. raciborskii*). Due to the inefficient reaction kinetics of most species' Rubisco, CCMs must

be employed, and as can be seen by the $K_{0.5(CO_2)}$, as well as the ratio of carbon supply: carbon fixation rates; this is true for all species studied here. The CCM of each strain thus accumulates C_i internally, far above ambient levels (in these experiments), hence escape of CO_2 via diffusion is potentially of much more significance than is the uptake of CO_2 via the same mechanism. Hence, a cell with a large surface area to volume ratio must contend with this escape of diffusible C_i, more than a cell with a smaller SA:Vol. The findings of this study suggest it does this by increasing the proportion of internal C_i stored as bicarbonate, which does not diffuse across membranes as readily as CO_2. It should be noted that the presence, and permeability of pyrenoids (eukaryotes) or carboxysomes (prokaryotes) also play important roles in minimising diffusive loss of CO_2 (Raven & Beardall, 2003, Meyer & Griffiths, 2013), as does the permeability of the plasma membrane (Mangan *et al.*, 2016). Future work into this theory should take into consideration these CCM components.

Nitzschia sp. was excluded from the linear regression because it behaved as an outlier, with very low internal bicarbonate relative to its high SA:Vol. The plasma membrane of diatoms is relatively permeable to CO_2 and they rely heavily on the concentration of CO_2 in the pyrenoid to saturate Rubisco (Hopkinson *et al.*, 2011). *Nitzschia* sp. is commonly a benthic strain of diatom, whereas the other studied species are all pelagic and thus often present in surface waters. DIC levels at the bottom of freshwater lakes are often high due to the respiration of microbes, and the ongoing decomposition of organic matter that deposits there (Raymond *et al.*, 2013). Thus, for *Nitzschia* sp., a high internal pH and large surface area to volume ratio may supply DIC effectively without much CCM activity when it inhabits deeper waters. Closer to the surface, higher light intensities provide energy to operate the CCM, thus balancing the reduction in free CO_2 with improved DIC affinity.

As atmospheric CO_2 concentrations rise, the biophysical consequence of a high SA:Vol ratio may result in a faster down-regulation of the CCM in low C_i environments, as these species will benefit more rapidly from the diffusion of CO_2 into the cell, or rather, the reduction in rate of diffusive CO_2 escape due to the smaller concentration gradient. However, the limited number of species used to collect this data means further work would enhance our understanding.

Species dominance is controlled by many factors. It is evident, from the results presented here, that DIC uptake and storage by the freshwater phytoplankton of Lake Wivenhoe is a key point of difference between species, which, in specific circumstances, may contribute to species dominance. While all species were shown to possess active CCMs, the kinetics of uptake varied broadly, which adds to the understanding of bloom formation by *Cylindrospermopsis raciborskii* during stratified summer months. Further, the SA:Vol ratio of a species was shown to correlate with internal bicarbonate concentration, which may have important consequences for these species as atmospheric CO_2 levels continues to rise. However, further work on more species is necessary to determine whether this trend applies to all pelagic species, and whether benthic species operate differently.

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Figures and tables - Paper 2

Table 1: Michaelis-Menten half saturation constants (μM) for inorganic carbon, and maximum photosynthetic rate (nmol O_2 · (10^7 cells) · min^{-1}) for the six species of microalgae examined. Values in brackets are SE (n ≥ 3).

	$K_{0.5(DIC)}$	$K_{0.5(CO_2)}$	$K_{0.5(HCO_3^-)}$	P_{max}
C. raciborskii	19.0[a] (3.1)	1.3[a] (0.2)	17.73[a] (2.9)	5.5[a] (0.7)
Cyclotella sp.	11.0[a] (1.7)	0.7[a] (0.1)	10.28[a] (1.6)	38.5[a] (12.6)
Nitzschia sp.	18.4[a] (1.7)	1.2[a] (0.1)	17.14[a] (1.5)	24.0[a] (3.1)
Stichococcus sp.	79.9[b] (3.7)	5.3[b] (0.2)	74.3[b] (3.4)	36.7[a] (4.9)
Staurastrum sp.	80.8[b,c] (8.3)	5.3[b,c] (0.5)	75.28[b,c] (7.7)	850.2[b] (140.2)
Monoraphidium sp.	53.1[c] (11.3)	3.5[c] (0.8)	49.44[c] (10.6)	24.3[a] (5.8)

Table 2: Values for internal pH, total internal DIC (µM), internal CO_2 (µM), and CO_2 concentration factor (ratio int:ext) for the five species of microalgae examined. Values in brackets are SE (n=3). External DIC concentration 180 µM, pH 7.35.

	Internal pH	*Internal DIC*	*DIC ratio (int:ext)*	*Internal CO_2*	*CO_2 ratio (int:ext)*
C. raciborskii	$7.32^{a,b}$ (0.03)	2257^{a} (246)	12.0^{a} (1.3)	223^{a} (34)	14.2^{a} (2.2)
Cyclotella sp.	7.19^{a} (0.04)	729.6^{b} (157)	3.8^{b} (0.8)	97^{b} (29)	5.4^{b} (1.7)
Nitzschia sp.	7.53^{c} (0.05)	632^{b} (15)	3.4^{b} (0.1)	40^{b} (5)	2.6^{b} (0.3)
Stichococcus sp.	7.35^{b} (0.01)	$1080^{b,c}$ (389)	$5.6^{b,c}$ (2.0)	99^{b} (36)	$8.2^{a,b}$ (3.0)
Staurastrum sp.	7.71^{d} (0.04)	$1896^{a,c}$ (41)	$9.8^{a,c}$ (0.2)	80^{b} (8)	4.9^{b} (0.5)

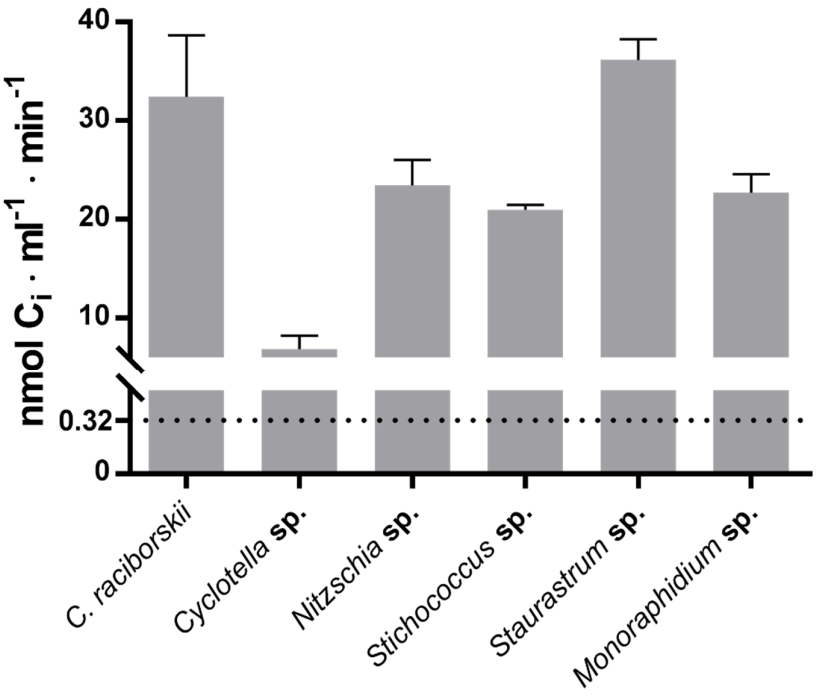

Fig. 1: Rates of C drawdown (nmol $CO_2 \cdot mL^{-1} \cdot min^{-1}$) at 180 µM DIC for each species, estimated from oxygen evolution data. The dotted line represents the rate of CO_2 supply from the uncatalysed dehydration of HCO_3^- at a DIC concentration of 180 µM (pH 7.35, 25 ºC). Cell densities were 10^7 cells · mL^{-1} for *C. raciborskii*, 10^6 cells · mL^{-1} for *Cyclotella* sp., 10^7 cells · mL^{-1} for *Nitzschia* sp., 8 x 10^6 cells · mL^{-1} for *Stichococcus* sp., 5 x 10^5 cells · mL^{-1} for *Staurastrum* sp., and 10^7 cells · mL^{-1} for *Monoraphidium* sp.. Error bars are SE, $n \geq 3$.

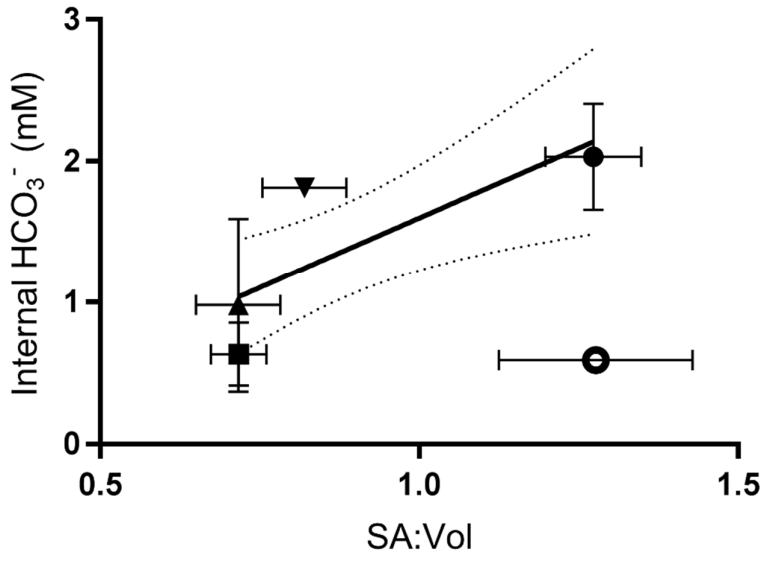

Fig. 2: The internal HCO_3^- concentration (mM) vs surface area to volume ratio for each species. Symbols are; ●, *C. raciborskii*; ■, *Cyclotella* sp.; ⭘, *Nitzschia* sp.; ▲, *Stichococcus* sp.; ▼, *Staurastrum* sp.. *Nitzschia* sp. was excluded for curve fitting (see text for justification). Linear regression, $R^2 = 0.48$, $F_{1,10} = 9.37$, P = 0.012. Dotted lines are the 95% confidence limits.

Supplementary data – Paper 2

Table S1: Proportions of oil mixture components used as the boundary between cell suspension and killing solution. No mixture was found to be effective for *Monoraphidium* sp. which was unable to pass through any achievable stable layer of oil.

	Dinonyl Pthalate (DP)	*Wacker Silicone fluid AR 20*	*Wacker Silicone fluid AR 200*
C. raciborskii	-	70	30
Cyclotella sp.	-	70	30
Nitzschia sp.	45	-	55
Stichococcus sp.	40	-	60
Staurastrum sp.	50	-	50

Chapter 4: Paper 3

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Elevated CO_2 has differential effects on five species of microalgae from a sub-tropical freshwater lake: possible implications for phytoplankton species composition

Impacts of high CO_2 on freshwater algae

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Summary

Rising atmospheric CO_2 partial pressures are predicted to have a significant impact on global phytoplankton populations. Of particular interest in freshwater systems are those species that produce toxins or impact water quality, though evidence for how these species, and many others, will respond is limited. This study investigated the effects of elevated CO_2 (1000 ppm) relative to current atmospheric CO_2 partial pressures (400 ppm), on growth, cell size, carbon acquisition and photophysiology of five freshwater phytoplankton species (a Cyanophyte: *Cylindrospermopsis raciborskii*, two chlorophytes: *Monoraphidium* sp., *Stichococcus* sp. and *Staurastrum* sp., and a diatom: *Cyclotella* sp.), from Lake Wivenhoe, Australia. Effects of elevated CO_2 on growth rate varied between species; notably growth rate was considerably higher for *Staurastrum* sp. and significantly lower for *Stichococcus* sp. and *C. raciborskii*. Cellular biovolume was significantly lower with elevated CO_2, for *Monoraphidium* sp. and *Stichococcus* sp., while surface area to volume ratio was significantly higher for all species except *Cyclotella* sp.. Photosynthetic affinity for CO_2 was lower with elevated CO_2 for all species, as indicated by higher Michaelis-Menten half saturation constants for CO_2-dependent O_2 evolution. The surface water CO_2 concentrations varied across the seasons in Lake Wivenhoe between 2008 and 2010, ranging from greater 200 µM in winter, to zero in summer. The timing of maximum cell concentrations of those genera studied in monoculture occurred in the lake in order of CO_2 affinity when free CO_2 concentrations dropped below air equilibrium. The results presented here suggest that as atmospheric levels of CO_2 rise, *C. raciborskii* may become less of a problem to water quality, while some species of chlorophytes may become more dominant during the periods when toxic algal blooms previously occurred, though not all chlorophytes will thrive. This has

implications for stakeholders of many freshwater systems as less expenditure on bloom prevention and minimisation may be necessary.

Introduction

Changes in species composition of phytoplankton populations are primarily driven by environmental factors such as inorganic carbon availability, nutrient concentrations, pH, temperature and light intensity, as well as the capacity of the different species of phytoplankton to acclimate to changes in these parameters (Huisman, 1999; Recknagel, Orr & Cao, 2014; Low-Décarie, Bell & Fussmann, 2015). Future predictions for atmospheric CO_2 partial pressures are for an increase from 400 ppm to 1000 ppm by the year 2100 (Meehl *et al.*, 2007). In any environment where the biota are dependent on (and potentially limited by) CO_2 for growth, it is feasible that this change will have dramatic impacts on those organisms and any species dependent upon them (Beardall & Raven, 2004). Of immediate interest are the toxic and economically costly species present in the waterways upon which humans are dependent for drinking water.

Lake Wivenhoe is a sub-tropical freshwater lake in south eastern Queensland, Australia and is a source of drinking water to up to three million people, as well as being an important site for recreation by the local population. Like most freshwater bodies, many species of phytoplankton inhabit Lake Wivenhoe, most of which currently pose little risk to the public. However, the cyanobacterium *Cylindrospermopsis raciborskii* (Wołoszyńska) Seenaya & Subba Raju, a widespread and increasingly invasive species (Neilan *et al.*, 2003) shown to produce cylindrospermopsins (CYNs), frequently forms toxic surface blooms during summer months (McGregor & Fabbro, 2000; Briand *et al.*, 2004). Such blooms are a health hazard to recreational users of the lake, and also add high costs to water treatment operators by increasing the necessary chlorination and consequential removal of trichloromethane (a by-product of chlorination) (Cheng *et al.*, 2009; Westrick *et al.*, 2010). Understanding the impacts of future CO_2 rises on *C. raciborskii* as well as on other species from the reservoir,

may aid mitigation strategies designed to minimise health risks to the public, or identify new species or groups of concern. Currently little information exists describing how freshwater species will respond to elevated CO_2 so it is impossible to know whether new strains may dominate the waterways and fill ecological niches currently held by different species.

Most phytoplankton use a CO_2-concentrating mechanism (CCM) to improve the effectiveness of the slow and kinetically poorly performing CO_2 fixing enzyme ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) (Giordano, Beardall & Raven, 2005; Raven, Beardall & Giordano, 2014). For most species, Rubisco is unsaturated at current air equilibrium levels of CO_2, thus a CCM is required to scavenge dissolved inorganic carbon (DIC), in the form of bicarbonate (HCO_3^-) and/or CO_2, from the surrounding medium in order to maximise rates of C fixation. CCM activity (and thus cellular affinity for inorganic carbon in photosynthesis) is regulated, *inter alia*, by the concentration of inorganic carbon (C_i) dissolved in the medium (Beardall & Giordano, 2002). Energetically, CCM operation is costly, and energy for its operation as ATP, is provided by cyclic electron flow around photosystem one (PSI) (Spalding, Critchley & Orgren, 1984; Ogawa, Miyano & Inoue, 1985; Palmqvist *et al.*, 1990). Hence, with increased CO_2 and a down-regulation of the energetically costly CCM, productivity should increase (Schippers, Lurling & Scheffer, 2004; Riebesell *et al.*, 2007) as energy can be used for other, growth, processes. The impacts of elevated CO_2 are well documented for marine species (Clark & Flynn, 2000; Burkhardt *et al.*, 2001; Czerny, Ramos & Riebesell, 2009). However, in freshwater systems evidence is less forthcoming. A number of studies have shown that, while cellular responses to elevated CO_2 levels can be varied, consistencies between different taxa have been observed, where cyanobacteria show a decline in competitive ability, compared to green algae, that improves their performance, while

diatoms show minimal differences in terms of competition (Low-Decarie, Fussmann & Bell, 2011; Low-Décarie, Bell & Fussmann, 2015; Ji *et al.*, 2017).

Further, regulation of CCM activity is controlled, not only by carbon availability and light intensity, but by other environmental factors (Beardall, 1991; Beardall, Johnston & Raven, 1998; Beardall & Giordano, 2002). Thus it is difficult to make useful predictions about how CO_2 will affect complex freshwater systems, and it is of importance to compare experimental observations with available environmental data.

Presented here, the effect of elevated atmospheric CO_2 was investigated by characterising a number of relevant species' carbon concentration mechanisms, growth and photosynthesis responses to an increase in CO_2 in the atmosphere. These results were compared to observations of the same genera in Lake Wivenhoe over the summer 2009/2010.

Materials and Methods

Strains and culturing. Originating from Lake Wivenhoe, south eastern Queensland (27° 16' S, 152° 32' E), unialgal cultures of a cyanobacterium; *C. raciborskii* (Class: Cyanophyceae), a diatom; *Cyclotella* sp. (Class: Coscinodiscophyceae), and 3 green algae; *Monoraphidium* sp. (Class: Chlorophyceae), *Staurastrum* sp. (Class: Zygnemophyceae, order: Desmidiales), and *Stichococcus* sp. (Class: Chlorophyceae) were used in this study. Jaworski's medium (Thompson, Rhodes & Pettman, 1988) supplemented with 300 µM Na_2SiO_3 (JM+Si), with an initial pH of 7.35 was used to culture all species. Other culturing conditions included a constant temperature of 25 ^oC, with a photon flux density (PFD) of 60 µmol photons m^{-2} s^{-1}(PAR 400-700 nm; measured with a LiCor Li188B and a cosine corrected quantum sensor), and a light: dark cycle of 12h: 12h, except for *C. raciborskii*, which was grown under a PFD of 20 µmol photons m^{-2} s^{-1} [preliminary experiments showed this was a low-light preferring strain (Willis *et al.*, 2016)]. Using an air pump, cultures were bubbled at approximately 4 L/hr with either filtered air (~400 ppm CO_2) or filtered air supplemented with pure CO_2, to a CO_2 partial pressure of 1000 ppm (v/v) using a gas mixer (Thermoline Scientific Equipment Pty Ltd, Melbourne Australia). High CO_2 air was varied minimally via the use of a large mixing vessel from which the air was pumped to the flasks via a humidifier.

Prior to any experimental analysis, cultures were acclimated to the respective atmospheric CO_2 conditions over a period of at least 30 days. From an acclimated culture growing in exponential phase, flasks (250 ml) containing 100 ml fresh medium were inoculated for each experiment. At mid-late exponential growth phase, at least three independent cultures for each species underwent physiological analysis.

Culture enumeration. An improved Neubauer haemocytometer (Boeco, Germany) was used to determine cell concentration. For *C. raciborskii*, cell and trichome lengths were measured

using a Zeiss Axioskop optical microscope (Zeiss, Göttingen, Germany). The average trichome length was divided by the average cell length to give the average number of cells per trichome. A final cell concentration was determined by multiplying this value by the number of trichomes per millilitre (via haemocytometer).

To determine specific cell division rate inoculated cultures were sampled daily and cell concentrations determined. The specific cell division rate was determined as $\mu_{max} = [\ln(C_f)-\ln(C_0)]/(T_f-T_0)$, where $\ln(C_f)$ and $\ln(C_0)$ are the natural log of the cell concentration at the end, and beginning of exponential growth phase respectively, and T_f-T_0 is the difference (in days) between sampling points.

P vs I. The relationship between net oxygen production and light intensity was determined by performing photosynthesis vs. irradiance (P vs I) curves. At constant temperature (25 ^oC), pH (20 mM HEPES buffer, 7.35) and saturating DIC supply (2 mM), a temperature-regulated Clark-type oxygen electrode (Hansatech, Norfolk UK) was used to measure oxygen production by the algal cells in response to increasing intensities of light provided by a halogen lamp (OSL1-EC Fibre Illuminator, Thorlabs, USA). Cells were dark acclimated for at least ten minutes, which allowed measurement of the steady state dark respiration rate (R_d) before they were then exposed to light intensities from 10 to 2400 µmol photons m^{-2} s^{-1} until rates of O_2 evolution were stable. The equation $P = (I - I_c) / [\ a\ (I - I_c)^2 + b\ (I - I_c) + c]$ as described by Eilers & Peeters (1988), was used to model the photosynthetic response (oxygen evolution) to increasing light intensity, where P is the photosynthetic rate (in nmol O_2 (10^7 cells)$^{-1}$ min^{-1}), I is the incident light intensity (in µmol photons m^{-2} s^{-1}) and I_c is the compensation light intensity (in µmol photons m^{-2} s^{-1}). The least sum of square differences method was used to fit the curve to the data points by solving for a, b and c using the software Graphpad Prism7. I_c was derived by interpolating between the oxygen evolution rate

in the absence of light (dark respiration rate, I = 0 µmol photons m^{-2} s^{-1}) and the lowest tested light intensity (I = 10 µmol photons m^{-2} s^{-1}) to identify the theoretical light intensity at which the oxygen evolving process (photosynthesis) balances the oxygen consuming process (respiration). After fitting the curve, the maximum photosynthetic rate (P_{max}) and light harvesting efficiency (α) were calculated using the equations: $P_{max} = b + 2\sqrt{ac}$ and $\alpha = 1 / c$. Rates of photoinhibition (β) (the decline in oxygen production rate at high light intensities) were calculated as the slope of the fitted curve, at light intensities from 1200 to 2400 µmol photons m^{-2} s^{-1}.

P vs. DIC. A temperature controlled Clark-type oxygen electrode (Hansatech, Norfolk UK) was used to determine rates of oxygen production in response to increasing doses of DIC (0 - 1360 µM) at constant temperature (25 ºC), buffered pH (20mM HEPES, pH: 7.35) and saturating light intensity (from prior P vs. I experiments). Photosynthesis vs. DIC curves were fitted to a Michaelis-Menten model (Graphpad Prism 7), from which half -saturation constants ($K_{0.5(DIC)}$) were determined. CO_2 and HCO_3^- affinities were calculated from the equivalent DIC values using the references below.

For all calculations, aqueous CO_2 and HCO_3^- concentrations were determined from total DIC concentration using the dissociation constants of Millero *et al.* (2006) and Dickson & Riley (1979), solubility constants of Weiss (1974) in combination with pH, and temperature.

Chlorophyll Fluorescence. The parameters $rETR_{max}$ and non-photochemical quenching (NPQ) for each strain were measured with a Phyto-PAM phytoplankton analyser (Heinz Walz GmbH, Effeltrich, Germany), using the method described by Pierangelini *et al.* (2014b). Samples of culture in growth medium were taken simultaneously with samples used for oxygen evolution experiments, and rapid light curves carried out using the Phyto-WIN software with increasing levels of actinic light for 30 s at each intensity. Relative electron transport (rETR) was

determined as rETR = $\Delta F/ F_m'$ x PAR x 0.5 where $\Delta F=F_m'-F_0$, and F_m' is the maximum fluorescence in the presence of actinic light. The light saturated rates of relative electron transport ($rETR_{max}$), were then calculated using the equations of Eilers & Peeters (1988). NPQ was calculated using the Stern–Volmer equation; NPQ = $(F_m/F_m') - 1$ under an actinic light intensity of 610 µmol photons m^{-2} s^{-1}.

Biovolume. Cellular biovolume was determined microscopically, using geometric formulae described in Hillebrand *et al.* (1999) and Vadrucci, Mazziotti & Fiocca (2013). The formula for a prolate spheroid was applied to *Stichococcus* sp., the formula for a cylinder was applied to both *Cyclotella* sp. and *C. raciborskii*, a monoraphidioid formula was applied to *Monoraphidium* sp., while the formula for volume of two truncated cones, with four attached cylinders was used for *Staurastrum* sp.. Where possible, the dimensions of individual cells were measured, and population means taken. For all species except *C. raciborskii*, volumes were taken for each cell and the resulting values averaged. In the case of *C. raciborskii*, due to its size and structure, cell widths and lengths were taken (n > 50), and averages of these dimensions used to determine cellular biovolume.

Lake Wivenhoe timescale data. Nutrient and biovolume data from Lake Wivenhoe were collected for a period of three years beginning 6th January 2009. Due to flooding of the area in late 2010, much data, including pH and alkalinity is missing during this period, thus only data from summers where complete data sets are available are presented here. Two sampling sites were used in the study; Site A (location: 27°23'17.00"S 152°36'45.65"E), Site B (location 27°19'57.50"S 152°33'29.51"E). Temperature was averaged from the surface to a depth of 5 m. Nutrient data (including alkalinity) consisted of 4×5 m depth integrated hosepipe subsamples, providing nutrient levels representative of the average of the top five

metres of water. Average pH of the surface water was calculated by averaging the free hydrogen concentration as determined each metre for five metres, before calculating pH.

Statistical analysis. Multiple T-tests were used to determine whether differences between species means were significant. Statistical analysis was performed using Graphpad Prism 7 software. The accepted level of significance was $p \leq 0.05$. All data are reported as means with standard error, from a sample size of at least three replicates.

Results

The effects of elevated CO_2 on the five species differed. Specific cell division rates, measured during the exponential phase of growth (Table 1), were significantly lower, by 18% ($p < 0.01$) for *Stichococcus* sp., by 21% ($p < 0.05$) for *C. raciborskii* and significantly higher, by 66% ($p < 0.01$) for *Staurastrum* sp. under elevated CO_2 partial pressures compared to values for cells grown at present day (400 ppm) CO_2. There was no significant difference observed in specific cell division rate with CO_2 partial pressure for the other strains. Cellular biovolume was significantly lower at high CO_2 partial pressure for both *Monoraphidium* sp. and *Stichococcus* sp., by 36% ($p < 0.01$) and 55% ($p < 0.01$) respectively, but did not change significantly for other species. Changes in the surface area to volume ratio were significant for all species except *Cyclotella* sp.. The high CO_2 treatment resulted in higher SA:Vol ratios by 14 % for both *Monoraphidium* sp. ($p < 0.05$) and *Stichococcus* sp. ($p < 0.01$), ranging to 24 % for *Staurastrum* sp. ($p < 0.01$).

As proxies for carbon fixation and release, rates of oxygen production and consumption were measured and calculated both on a 'per cell' basis, and 'per cellular biovolume' to better compare changes between species (Table 2). Maximum rates of oxygenic photosynthesis (P_{max}) varied from 5.9 nmol O_2 min^{-1} $(10^7$ cells$)^{-1}$, for *C. raciborskii* at 400 ppm CO_2, to 1067 nmol O_2 min^{-1} $(10^7$ cells$)^{-1}$ for *Staurastrum* sp. at 1000 ppm CO_2. At 1000 ppm CO_2, P_{max} was significantly lower, by 43% for *C. raciborskii* ($p < 0.01$) and higher, by 45% ($p < 0.05$) and 129% ($p < 0.01$) for *Staurastrum* sp., and *Cyclotella* sp. respectively. When calculated on a 'per cubic millimetre' (P_{max} vol^{-1}) basis, high CO_2 resulted in a 33% ($p < 0.05$) lower P_{max} for *C. raciborskii*, and a 77% higher P_{max} for *Staurastrum* sp. ($p < 0.05$), to 242% ($p < 0.01$) for *Stichococcus* sp.. At elevated CO_2 partial pressures, dark respiration was fourfold higher for *C. raciborskii* from 0.2 to 0.9 nmol O_2 min^{-1} $(10^7$ cells$)^{-1}$, a significant increase ($p < 0.01$).

Cyclotella sp. also experienced a significant increase in R_d of 82% ($p < 0.05$). Per unit volume these changes become significant for *Monoraphidium* sp. and *Stichococcus* sp., with differences of 132 % and 194 % respectively ($p< 0.05$) between atmospheric and 1000 ppm CO_2 partial pressures. With elevated CO_2, respiration as a percentage of P_{max} was significantly higher [7.5 fold ($p < 0.01$)] for *C. raciborskii* but did not change for any other species.

Light harvesting efficiency (α) was significantly higher ($p < 0.05$) with 1000 ppm CO_2, for all strains except *Stichococcus* sp. (Table 3). Light harvesting efficiency of *C. raciborskii* was 199% higher than the atmospheric control, while *Monoraphidium* sp., *Cyclotella* sp., and *Staurastrum* sp. also presented with higher light harvesting efficiencies of 51%, 119% and 72% respectively. Beta (β), the rate of decrease in oxygen production with increasing light (photoinhibition) varied with CO_2 partial pressure for all species, being significantly higher for *Cyclotella* sp. ($p < 0.05$). The compensation light intensity, I_c varied with elevated CO_2, between 1 and 6 µmol photons m^{-2} s^{-1} for all species. The maximum change in I_c was less than 1 µmol photons m^{-2} s^{-1} and was not significant.

The PhytoPAM was used to measure chlorophyll fluorescence (Table 3) and determine relative electron transport rates, non-photochemical quenching, and quantum yield, to better provide an explanation as to where photosynthetically derived energy is being diverted. Elevated CO_2 saw significantly lower values for $rETR_{max}$ for both *C. raciborskii* ($p < 0.01$) and *Monoraphidium* sp. ($p < 0.01$), and higher $rETR_{max}$ for *Cyclotella* sp. ($p < 0.01$). The only significant effect of high CO_2 on NPQ was observed in *Cyclotella* sp., where it was 54% ($p < 0.01$) lower.

Cellular affinity for CO_2 was significantly lower (t-test, $p < 0.05$) with higher CO_2 partial pressures for all strains (Table 2). The largest change occurred in *Stichococcus* sp., where the

$K_{0.5(CO_2)}$ changed from 5 µM to 105 µM (a 20-fold increase), while CO_2 affinity for *C. raciborskii* changed the least of all species, from 1.3 µM to 3.3 µM.

To provide a perspective on the annual variation within the surface water of Lake Wivenhoe, surface water data from Lake Wivenhoe are presented in Fig. 1, including temperature, pH and bicarbonate concentration. The data were collected between January 2009 and April 2010. Minimum and maximum temperatures were 15.6 and 28.6 ºC respectively, with a mean of 22.8 ºC. The surface water pH had a mean of 7.9 and varied between 7 in May 2009, and 9.58 in December 2010. Bicarbonate concentration showed a minimum of 0.8 mM and a maximum of 1.5 mM with a mean of 1.17 mM.

To identify potential correlations between CO_2 availability and species composition, free CO_2 and air-equilibrium CO_2 concentrations are plotted, along with species biovolume in the populations, in Fig. 2. Non-normalised values for genus biovolume can be seen in Table 4. At sites A and B, free CO_2 had a minimum of 0 for both sites, a maximum of 0.14 and 0.2 mM respectively, with means of 0.02 and 0.03 mM respectively. Air equilibrium CO_2 concentration had a minimum, maximum and mean of 0.012 mM, 0.014 mM and 0.013 mM respectively at both sites. To confirm observations were not location-specific, the appropriate data from both sites A and B are presented. During summer months (vertical grey bars), for periods of weeks to months, free CO_2 levels can be seen dropping below air equilibrium, which implies Lake Wivenhoe becomes a CO_2 sink. At other times of the year, the free CO_2 in the surface water is far above the air equilibrium concentration, indicating the reservoir is a CO_2 source to the atmosphere. Peak biovolumes of the various genera occurred in order of their $K_{0.5(CO_2)}$values (as determined when grown at 400 ppm CO_2).

Discussion

The effect of elevated CO_2 varies across the species studied here, adding weight to the evidence that elevated atmospheric CO_2 will affect taxa differently, and thus adding to the challenge of predicting climate change outcomes. As a general indicator of the impact of elevated CO_2, growth rate changes provide an insight into potential shifts in the phytoplanktonic population that may occur in the future. Changes in the growth of species studied here are varied. A 66% increase in the growth rate of *Staurastrum* sp. with high CO_2, identifies it, and species with similar physiology, as potential beneficiaries of the changing atmospheric composition. Existing research into the effect of inorganic carbon availability on growth varies between species, though trends between classes of phytoplankton are beginning to emerge (Rost *et al.*, 2003; Riebesell, 2004). However, the complex interactions between the underlying drivers of this change are less clear. Work by Low-Decarie, Fussmann & Bell (2011) with freshwater phytoplankton species at high CO_2, showed a decrease in the competitiveness of cyanophytes, minimal change for diatoms, and an increase in competitiveness in chlorophytes. More recent work has also shown cyanophytes are likely be less competitive at high CO_2, while the inverse is true for chlorophytes (Ji *et al.*, 2017). These results are similar to what has been observed in this study, both in the monoculture experiments, as well as in the Lake Wivenhoe data. However, with *Stichococcus* sp. as an example, some species, though belonging to a more CO_2 tolerant class, may not thrive into the future. Despite its decrease in growth rate, as evidenced by its isolation from the reservoir, *Stichococcus* sp. does appear to persist through periods of elevated CO_2, despite its absence at detectable levels during the summer period shown in Fig. 2. Differential effects of CO_2 have also been observed in the cyanophytes *Microcystis aeruginosa* (Kützing) Lemmermann and *Nodularia spumigena* Mertens ex Bornet & Flahault (Qiu & Gao, 2002;

Czerny, Ramos & Riebesell, 2009), while Van de Waal *et al.* (2011) showed that with changes in carbon availability, differences between strains of the same species can predictably define dominance in mixed cultures.

Under elevated CO_2 treatment we observed smaller cell sizes, and a significant increase in cellular surface area to volume ratio, for almost all species. This is likely to have impacts on many factors affecting phytoplankton growth, if these changes occur in the environment as CO_2 levels rise. Nutrient acquisition by larger cells, via an increase in boundary layer diffusive restriction, requires an up-regulation of uptake mechanisms (Flynn, 1998; Flynn *et al.*, 1999). Sinking rate decreases with smaller cell size and higher SA:Vol ratio, following Stokes' law, which will affect cellular distribution in the water column as well as nutrient cycles in freshwater bodies (Waite *et al.*, 1997). Metabolic costs of intracellular transport decrease with decreases in cell size (West, Brown & Enquist, 1999; Banavar *et al.*, 2002). Predation of phytoplankton by zooplankton will also be affected by a reduction in cell size and morphology, which generally makes them more susceptible to predation by small grazers (Beardall *et al.*, 2009). Thus, these changes will be experienced throughout the food web (Finkel *et al.*, 2009). Finally, carbon uptake, storage and diffusive CO_2 loss, as well as photosynthesis (Finkel & Irwin, 2000) will be influenced by a reduction in cell size and increase in SA:Vol ratio. Stress, whether from pH or salinity, is likely to increase with SA:Vol ratio, as diffusion rates increase, and thus so does the cost of homeostasis.

The effect of elevated CO_2, and the resultant decrease in pH of the aquatic environment should be considered when discussing its physiological effects. Extracellular pH, as it decreases with elevated CO_2 levels would have immediate impacts on CCM operation (Mangan *et al.*, 2016). However, due to cellular pH homeostasis, an increase in CO_2 concentration from 400 ppm to 1000 ppm will unlikely affect internal pH (Bown, 1985). The

mean pH of Lake Wivenhoe over the period presented here, was 7.9, higher than the internal pHs observed in the studied species. Thus, a reduction in external pH may reduce the metabolic cost of pH regulation, as the difference between external and internal pH decreases. However, this will depend on the external pH optima of the species and how this affects uptake of available nutrients and other homeostatic processes. Many non-photosynthetic processes in the cell, such as nitrogen assimilation, also affect cytoplasmic pH, thus internal pH regulation is largely independent of photosynthesis (Smith & Raven, 1979). From the Lake Wivenhoe observations, bicarbonate concentration never approaches zero (0.8-1.5 mM) throughout the year, while CO_2 concentrations vary extensively (0-200 µM) with thermal stratification, inflows, storms and wind. The dynamic pH range (7-9.6) of the reservoir suggests that the studied species should be capable of surviving through a high CO_2 induced pH drop, though this should be addressed in further research.

The lower NPQ, observed for most species with elevated CO_2, could be attributed to increased rates of C fixation providing an energy sink and reducing the need for NPQ. The down regulation of the CCM removes an energy sink, potentially increasing the need for NPQ under high light. It is possible that a decrease in chlorophyll and other light harvesting pigments in the cells, also resulted in a decrease in the excitation level of photosystem II, thus resulting in a decrease in energy supply and reduced requirement for NPQ. Chlorophyll content has been found to correlate well with cellular biovolume (Felip & Catalan, 2000). If the number of photosynthetic units per cell do not decrease with high CO_2, the observed decrease in NPQ could be due to a reduction in excitation pressure associated with lower concentrations of light harvesting pigment.

An increase in half saturation constants, relative to previous work by Lines and Beardall, (submitted, chapter 3 of this thesis) of cultures grown at atmospheric levels of CO_2, was

consistently observed, and significant for all species. The greatest absolute changes in CO_2 affinity were observed in the three green algae studied, suggesting that they possess a high level of regulatory control over their CCMs, and the reallocation of energy from carbon concentration to growth may be possible. *C. raciborskii*, with a high C_i affinity, increased its $K_{0.5(CO_2)}$ by 2-fold, much less than the other species, suggesting a high level of constitutive CCM activity in this strain. Though changes in C_i affinity support a shift in regulation of CCM machinery, any reduction in energy output is difficult to measure, though insights can be gained by observing rates of photosynthesis, respiration and growth.

The response of P_{max} to high CO_2 varied for the species in this study. For *C. raciborskii*, *Cyclotella* sp. and *Staurastrum* sp., growth rate loosely mirrors changes in both $rETR_{max}$ and P_{max}. Data from both *Monoraphidium* sp. and *Stichococcus* sp. go against this trend, though this may be due to complicating issues associated with cell size, and the elevated metabolic consequences of regulating internal pH. Studies of green algae, diatoms and cyanobacteria have shown both increases and decreases in P_{max} in response to elevated CO_2, depending on species (Burkhardt *et al.*, 2001; Yang & Gao, 2003; Collins & Bell, 2004; Pierangelini *et al.*, 2014a). Despite observed changes in P_{max}, no significant changes were observed in the compensation light intensity of any species, suggesting that, at low light levels, elevated CO_2 will not have a significant impact on rates of C fixation.

The diversity of observed responses to high CO_2 adds weight to the need to characterise individual species and even strains of phytoplankton, to better predict species succession into the future. For example, strains of the species *C. raciborskii* show differing responses to light and toxin production (Pierangelini *et al.*, 2014b; Burford *et al.*, 2016; Willis *et al.*, 2016).

The fluctuations of available CO_2 present in Lake Wivenhoe suggests that in real world conditions, the resident species may experience reduced selection pressure, and thus adapt to

future CO_2 levels at a slower rate than species in marine environments where CO_2 levels are more stable over longer time scales (bloom conditions excepted). In culture, the short period of acclimation to high CO_2 in this study would often be considered a limitation when attempting to extrapolate findings to the response of freshwater ecosystems 90 years into the future. However, available CO_2 in Lake Wivenhoe regularly reaches above 2000 ppm, thus acclimation of these species, for an extended period, would not have offered any benefit in terms of relating the results to environmental conditions. Adaptive evolution in response to elevated CO_2 appears to be limited (Collins & Bell, 2004; Low-Décarie *et al.*, 2013). Physiological changes such as smaller cell size, and higher rates of photosynthesis and respiration were observed in both the study by Collins & Bell (2004) and in some of the species studied here, though these are not necessarily linked to hereditable changes in the genomes of these species. This may suggest that freshwater species from similar environments may not show adaptive changes to CO_2, due to the dynamic habitat they were sourced from. The observations presented here, of higher SA:Vol ratio with elevated CO_2, were carried out in replete culture medium. Whether or not this trend may be observed in the natural environment, where many factors interact, is difficult to say. For species that prefer high CO_2 and DIC concentrations, rising atmospheric CO_2 partial pressures should pose no threat into the future. However, for species that inhabit the low CO_2 niche such as *C. raciborskii*, future levels of high CO_2 may be challenging if Lake Wivenhoe CO_2 concentrations also increase.

Based on the results presented here, it is likely elevated CO_2 partial pressures will impact these species into the future. The drivers behind these changes include shifts in photosynthetic capacity and respiration rates, as well as changes to cell surface area and volume. It is possible that, due to the generally high levels of CO_2 in Lake Wivenhoe,

adaptation by low CO_2 preferring species will be extremely slow, and species that prefer available CO_2 slightly higher than the current minimums will thrive as CO_2 partial pressures increase.

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Figures and tables – Paper 3

Table 1 Maximum exponential growth rates (n = 3), cellular biovolume (n ≥ 25), and surface area (n ≥ 25) for monocultures grown at 400 ppm and 1000 ppm CO_2 respectively. Data are means ± standard error (SE). Also shown are the differences (Δ, %) in each parameter between elevated and ambient CO_2 T-tests were performed to determine significance between treatment and control (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

	[CO_2]	Growth rate μ (d^{-1})			Cell Biovolume (μm^3)			SA: Vol (m^{-1})		
	ppm	Mean	SE	Δ%	Mean	SE	Δ%	Mean	SE	Δ%
C. raciborskii	400	0.26	0.01	-21	60.0	3.0	-8	1.27	0.02	18
	1000	0.20	0.01	*	55.0	2.3	n.s.	1.50	0.02	***
Monoraphidium sp.	400	0.97	0.03	10	106.9	9.2	-36	1.29	0.05	14
	1000	1.07	0.05	n.s.	68.8	4.1	***	1.46	0.04	*
Stichococcus sp.	400	1.24	0.03	-18	148.9	7.0	-55	0.72	0.01	14
	1000	1.02	0.02	**	67.6	1.8	***	0.82	0.01	***
Cyclotella sp.	400	0.62	0.01	3	621.3	31.7	2	0.72	0.01	-2
	1000	0.64	0.06	n.s.	635.5	25.1	n.s.	0.70	0.01	n.s.
Staurastrum sp.	400	0.36	0.02	66	2342.9	99.4	-18	0.70	0.01	24
	1000	0.59	0.03	***	1931.0	70.8	n.s.	0.87	0.01	***

Table 2 Michaelis-Menten half saturation constants ($\boldsymbol{K_{0.5\,(CO_2)}}$), for CO_2-dependent O_2 evolution (μM); maximum photosynthetic rate per cell, P_{max} [nmol O_2 min^{-1} $(10^7$ cells$)^{-1}$], and per unit biovolume; P_{max} vol^{-1} (nmol O_2 mm^{-3} min^{-1}); dark respiration rate per cell, R_d [nmol O_2 min^{-1} $(10^7$ cells$)^{-1}$], and per unit biovolume; R_d vol^{-1}(nmol O_2 mm^{-3} min^{-1}) and respiration as a percentage of photosynthesis R_d:P_{max} (%). Also shown are the standard error (SE, n = 3) and the differences (**Δ,** %) in each parameter between elevated and ambient CO_2. T-tests were performed to determine significance between treatment and control (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Table 2

	[CO_2] ppm	$K_{0.5}(co_2)$			P_{max}			P_{max} vol^{-1}			R_d			R_d vol^{-1}			Resp.: P_{max}		
		Mean	SE	Δ (%)	Mean	SE	Δ (%)	Mean	SE	Δ (%)	Mean	SE	Δ (%)	Mean	SE	Δ (%)	Mean	SE	Δ (%)
C. raciborskii	400	1.3†	0.2	164 ***	10.2	0.8	-43 **	17.1	1.6	-33 *	0.19	0.02	396 **	0.31	0.03	485 **	1.81	0.22	767 **
	1000	3.3	0.1		5.9	0.3		11.5	0.7		0.92	0.14		1.81	0.30		15.70	2.58	
Monoraphidium sp.	400	3.5†	0.8	442 ***	34.1	2.6	6 n.s	17.1	1.6	208 *	0.54	0.08	49 n.s.	0.50	0.09	132 *	1.57	0.27	41 n.s.
	1000	19.0	0.9		36.2	1.4		52.6	3.7		0.80	0.09		1.16	0.15		2.21	0.26	
Stichococcus sp.	400	5.3†	0.2	1896 ***	36.5	1.7	55 *	24.5	1.6	242 **	1.47	0.22	34 n.s.	0.99	0.15	194 *	4.03	0.63	-14 n.s.
	1000	105	13.3		56.6	6.0		83.8	9.1		1.96	0.37		2.91	0.55		3.47	0.75	
Cyclotella sp.	400	0.73†	0.1	892 ***	64.9	13.0	129 *	10.4	2.2	124 *	10.72	2.88	82 *	1.73	0.47	78 n.s.	16.52	5.55	-20 n.s.
	1000	7.2	0.2		148.7	16.2		23.4	2.7		19.55	0.76		3.08	0.17		13.15	1.53	
Staurastrum sp.	400	5.3†	0.5	777 ***	735.4	31.1	45 **	31.4	7.0	77 *	27.84	3.32	25 n.s.	1.19	0.30	52 n.s.	3.79	0.48	-14 n.s.
	1000	46.8	5.2		1069.9	46.0		55.4	3.1		34.90	3.85		1.81	0.21		3.26	0.39	

† Data from Lines and Beardall (submitted)

Table 3 Photosynthetic characteristics of each species. From P vs. I curves: α (light harvesting efficiency, initial slope of the P vs. I curve, (nmol O_2 min^{-1} (10^7 cells)$^{-1}$) (µmol photons m^{-2} s^{-1})$^{-1}$), β (rate of photoinhibition, final slope of the P vs. I curve, same units as α), and I_c (compensation light intensity, µmol photons m^{-2} s^{-1}). From rapid light curves of the PhytoPAM: $rETR_{max}$ and NPQ. Also shown are the standard error (SE, n = 3) and the differences (**Δ,** %) in each parameter between elevated and ambient CO_2. All species cultured at 400 ppm or 1000 ppm CO_2 at 25 °C, pH 7.35 in modified JM+Si media. T-tests were performed to determine significance between treatment and control (* $p < 0.05$, ** $p < 0.01$).

Table 3

	$[CO_2]$	α			β			I_c			$rETR_{max}$			NPQ		
	ppm	Mean	SE	Δ (%)	Mean	SE	Δ (%)	Mean	SE	Δ (%)	Mean	SE	Δ (%)	Mean	SE	Δ (%)
C. raciborskii	400	0.11	0.01	199	-4.9E-04	4.1E-05	-43	2.54	0.14	8	52.5	0.8	-10	0.23	0.02	-50
	1000	0.32	0.04	**	-2.8E-04	6.8E-05	n.s.	2.73	0.21	n.s.	47.2	0.2	**	0.11	0.06	n.s.
Monoraphidium sp.	400	0.29	0.01	51	-2.7E-03	5.7E-04	-4	1.60	0.33	-36	64.4	0.8	-12	0.57	0.03	-12
	1000	0.44	0.03	**	-2.6E-03	2.4E-04	n.s.	1.03	0.15	n.s.	56.6	0.3	**	0.50	0.02	n.s.
Stichococcus sp.	400	0.87	0.08	-31	-3.3E-03	1.0E-03	-39	1.99	0.20	53	51.3	0.9	-6	0.33	0.06	54
	1000	0.60	0.10	n.s.	-2.0E-03	6.5E-04	n.s.	3.03	0.37	n.s.	48.3	0.6	n.s.	0.50	0.13	n.s.
Cyclotella sp.	400	1.39	0.28	119	-2.6E-03	1.5E-03	216	6.00	1.38	-1	36.6	0.9	30	1.27	0.11	-54
	1000	3.04	0.35	*	-8.2E-03	4.7E-04	*	5.96	0.44	n.s.	47.5	1.6	**	0.59	0.03	**
Staurastrum sp.	400	4.79	0.07	72	-6.9E-02	1.7E-02	70	4.09	0.40	-19	71.2	13.3	15	0.88	0.36	-29
	1000	8.24	1.05	*	-1.2E-01	6.5E-03	n.s.	3.33	0.03	n.s.	81.6	6.8	n.s.	0.63	0.01	n.s.

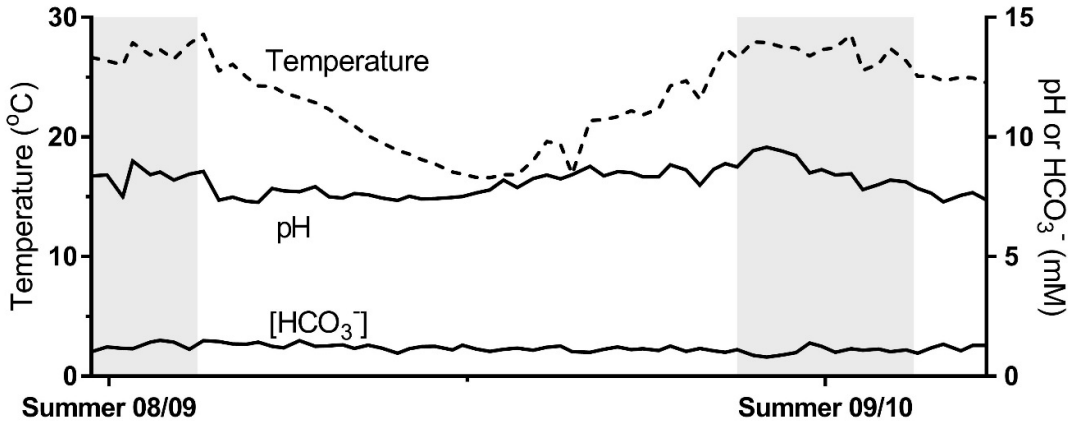

Fig. 1 Temperature (left Y-axis), pH (right Y-axis) and bicarbonate concentration (right Y-axis) of the surface water of Lake Wivenhoe at site A. Temperature and pH were measured continuously. Bicarbonate concentration was calculated via alkalinity (measured once daily). Shading indicates summer months.

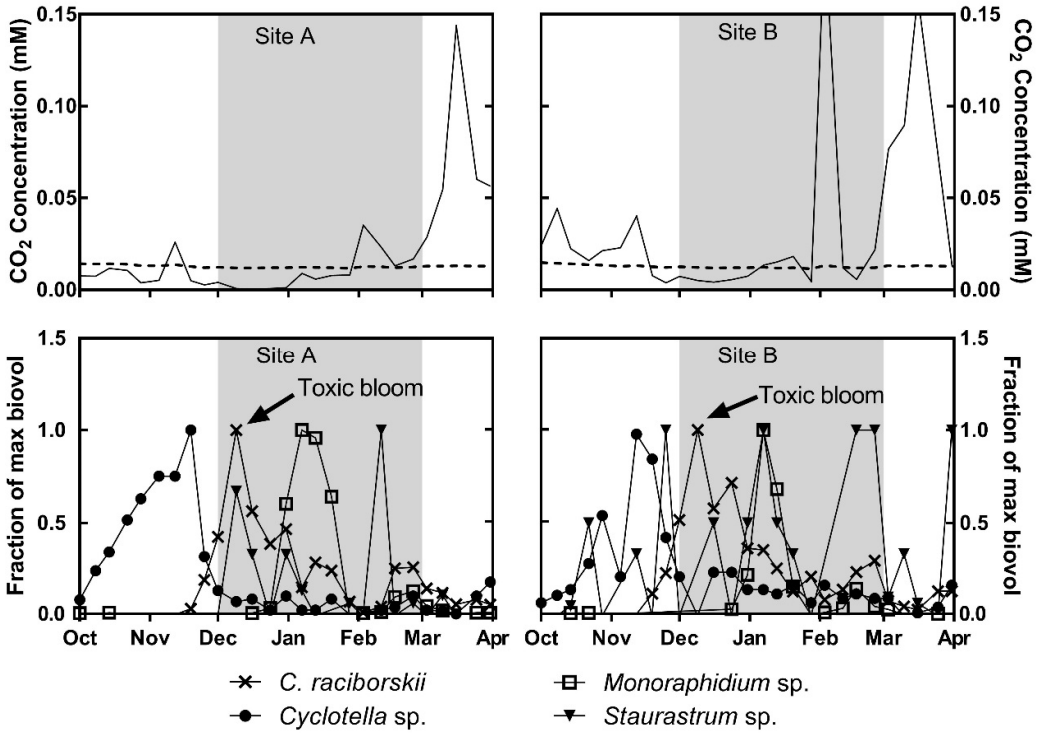

Fig. 2 CO_2 and biovolume data from Lake Wivenhoe between October 2009 and April 2010 at sites A (left panels) and B (right panes).Top panels: Free CO_2 (solid line) and air equilibrium CO_2 concentration (dashed line) at the Lake's surface (top 5m averaged). Bottom panels; Species biovolume as fraction of max cell density. *C. raciborskii*, *Monoraphidium* sp., *Stichococcus* sp., *Cyclotella* sp., and *Staurastrum* sp.. *Stichococcus* sp. was not identified in the Wivenhoe data set during this period, though it did occur at other times. Shading indicates summer period.

Table 4 Maximum and mean biovolumes ($mm^3 L^{-1}$) for each species presented in Fig. 2 during the period present (October 1st 2009 to April 1st 2010). Minimum value for each species was 0 $mm^3 L^{-1}$.

	Site A		Site B	
	Max	Mean	Max	Mean
C. raciborskii	3.52	0.64	3.23	0.68
Monoraphidium sp.	0.29	0.04	0.39	0.04
Cyclotella sp.	1.09	0.23	0.69	0.15
Staurastrum sp.	0.12	0.01	0.08	0.02

Chapter 5

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Conclusions

This thesis investigated the physiology of previously unstudied strains of phytoplankton (with the exception of *C. raciborskii* which has not been studied using the same methods) that were isolated from Lake Wivenhoe, and considered the effects of elevated CO_2 partial pressures on their physiological characteristics. A focus of this thesis was the concept of competitive dominance, and how these differing physiological characteristics may contribute to the dominance of one species over another, depending on the environmental conditions.

Two main areas of physiology were considered in this research; carbon acquisition and photo-physiological aspects of photosynthesis. As CO_2 levels in the environment rise, the immediate impacts will depend upon a species' response to said changes in CO_2 concentration. Photosynthesis provides energy for growth of these species, while the amount of energy devoted to carbon acquisition may change with elevated CO_2 levels (Raven *et al.*, 2012).

Using recently isolated strains of phytoplankton from three classes, the photo-physiology of six species was investigated to determine photosynthetic characteristics of existing inhabitants of Lake Wivenhoe.

The carbon acquisition characteristics of these species were determined to establish the activity of the carbon-dioxide concentrating mechanisms (CCMs). The internal pH and carbon pools were determined using radiometric techniques to allow determination of concentration factors for CO_2 by the CCMs in the different species under optimal growth conditions.

By culturing these strains of algae at atmospheric and elevated levels of CO_2, an understanding of how photosynthesis and carbon acquisition might be affected into the future was gained. Environmental and biological observations from Lake Wivenhoe were used to

further clarify aspects of the phytoplankton populations' response to changes in available carbon.

Summary of key findings

Overall this thesis has explored the physiology of a number of freshwater phytoplankton from Lake Wivenhoe. Paper 1 focussed on the growth and photo-physiology of the species. Paper 2 focussed on the inorganic carbon uptake of the algae, as well as how their morphology may explain trends observed in their internal carbon pools. Finally, the effect of elevated CO_2 on algal growth and physiology was investigated in Paper 3.

Paper 1

Consistent with the ecological niches to which the studied species belonged, their photosynthetic characteristics were complementary. Perhaps the best example of this was observed in the light harvesting efficiencies and rates of photoinhibition of the species. Relatively high light harvesting efficiencies were observed in the large green alga; *Staurastrum* sp., and the diatom *Cyclotella* sp., which inhabit well mixed surface waters, where light intensity is dynamic, and often low. A high light harvesting efficiency is beneficial in these conditions, as it is allows high rates of photosynthesis when light becomes available. Photoinhibition, a result of exposure to damaging intensities of light is less of an issue for these species. *C. raciborskii*, conversely, has the lowest light harvesting efficiency, consistent with its niche at surface waters during summer. In these conditions, photoinhibition is a significant problem, against which a low light harvesting efficiency is a possible defence.

Like *C. raciborskii*, *Nitzschia* sp., a diatom, also has a relatively low light harvesting efficiency. However, *Nitzschia* sp., different in habitat from all other species studied here, is generally expected to be found at the bottom of the water column or on sediment surfaces. In contrast to *C. raciborskii*, *Nitzschia* sp. may be able to compensate for its low light harvesting efficiency by using its motility to adjust its light exposure, moving towards the sun during light limitation, and away when intensities are more than saturating. In doing so, less energy expenditure is necessary for varying the composition of the photosynthetic apparatus components, though more energy does need to be spent on motility.

The way in which these species responded to light will improve the predictability the population dynamics of Lake Wivenhoe, and will lead to a better understanding of the drivers of population change, into the future.

Paper 2

The carbon acquisition and storage characteristics of the studied species were investigated to improve the understanding of their CO_2-concentrating mechanisms (CCM). By comparing the $K_{0.5(CO_2)}$ to the air equilibrium concentrations of CO_2, the activity of the CCM could be estimated. All species were saturated with CO_2 at concentrations below ambient levels, indicating their capacity to actively accumulate DIC internally for carbon fixation. The species with the lowest $K_{0.5(CO_2)}$ and hence the most active CCM, was *Cyclotella* sp. with a saturation concentration of 0.7 µM CO_2. With a $K_{0.5(CO_2)}$ of 5.3 µM, *Stichococcus* sp. would still be saturated at common air-equilibrium CO_2 concentrations of 12 µM.

We also investigated the internal pool of dissolved inorganic carbon (DIC) concentration and determined the intracellular concentrations of the different DIC species. Internal pHs ranged from 7.2 to 7.7 which critically define the equilibrium of DIC species. A low internal pH

pushes the equilibrium to favour CO_2, the most membrane permeable of the DIC species. A high internal pH favours bicarbonate, which must be converted by carbonic anhydrase to make CO_2 available for Rubisco. Internal DIC concentrations ranged from 0.6 - 2.2 mM resulting in internal CO_2 concentrations ranging from 47 - 223 µM.

Nitzschia sp. possessed the lowest observed internal pH and CO_2 concentration, further consistent with its preference for a benthic habitat. In fact, the internal CO_2 concentration of all the green algae and diatoms investigated here were similar. In contrast though, *C. raciborskii* had the highest CO_2 and DIC affinity, internal bicarbonate concentration and internal CO_2 concentration. *C. raciborskii*'s accumulation of bicarbonate internally, allows the saturation of Rubisco with CO_2 via controlled release of CO_2 with carbonic anhydrases, while minimising diffusive losses of CO_2. Previous research has shown that the CO_2 affinity of cyanobacterial Rubiscos is far lower than those found in diatoms and green algae generally, requiring many fold the CO_2 concentration to saturate carboxylation (Badger *et al.*, 1998). Thus, with a kinetically inefficient Rubisco, *C. raciborskii* concentrates bicarbonate to high levels internally, as it does not diffuse through the cell membrane. This in turn enables localised saturation of Rubisco with CO_2 in the carboxysomes. Based on published literature, the other species studied are predicted to have relatively efficient Rubisco enzymes (Badger *et al.*, 1998, Rost *et al.*, 2006, Young *et al.*, 2016), thus it is not necessary to concentrate DIC to such high levels in order to still saturate Rubisco.

Having collated these results, it was observed that bicarbonate concentration may correlate with the surface area:volume ratio of the cells. This correlation was found to be statistically significant. We suggest that, as the SA:Vol ratio of a cell increases, the rate of diffusive loss of CO_2 must increase proportionately, which would be of more energetic cost to the cell than

the simultaneous small increase in diffusion rate of CO_2 into the cell. As a biochemical strategy to minimise diffusive losses of CO_2 from the cell, we propose that bicarbonate might be the favoured form of DIC in cells with a relatively high SA:Vol ratio.

A better understanding of the carbon uptake and utilisation of the studied species may help to explain current ecological phenomena, such as toxic blooms, and aid predictions of the effect of a changing atmosphere. The suggested correlation between internal bicarbonate concentration and cellular surface area to volume ratio, with further work, might lead to improvements in growth models and thus, enhance predictions of the impact of a changing atmosphere.

Paper 3

In an attempt to improve understanding about the impact of climate change on freshwater systems, we chose to test the effect of elevated CO_2 on the physiology of these freshwater species of phytoplankton, as well as to examine how these strains behave in Lake Wivenhoe in relation to ambient but variable CO_2 levels. Each strain was cultured by bubbling media with either air, or air supplemented with CO_2 to 1000 ppm. By electing not to buffer the cultures, changes in pH were allowed to occur and potentially affect the cell's growth and physiology which allowed more realistic replication of future freshwater systems to be measured. At 1000 ppm CO_2, growth rates, photosynthetic characteristics and DIC affinities were measured and compared to the air-bubbled controls.

With high levels of CO_2, growth rates varied between species. The growth rate was significantly lower for C. *raciborskii* and *Stichococcus* sp., while *Staurastrum* sp. had a significantly higher growth rate under the same (1000ppm CO_2) conditions. Cellular

biovolume decreased significantly for both *Monoraphidium* sp. and *Stichococcus* sp., and the surface area:volume ratios were significantly higher, at high CO_2 for all species except *Cyclotella* sp.. These morphological shifts could potentially affect cellular function, sinking rates, and predation, and, should the same changes occur in the natural environment, may lead to changes in the ecosystem.

Photosynthetic affinity of the cells for CO_2 was significantly lower for each species when cultured in the CO_2-supplemented conditions. This increase in $K_{0.5(CO_2)}$ (Michaelis-Menten half saturation constant) was expected, as the CCM components are down-regulated as external CO_2 levels increase. It also implies that some shift in energy collection or utilisation was occurring. Consistent with this finding, photosynthetic rates of the high CO_2 cultures, were significantly higher for most species, except in *Monoraphidium* sp. where no difference was observed, and in *C. raciborskii*, where a significantly lower rate was observed, compared to the control.

With observations of the surface water characteristics in Lake Wivenhoe between December 2008 and April 2010, we showed that surface temperatures reach almost 30 ^oC during summer and 16 ^oC during winter. Surface pH was at a low of 7 in autumn, increasing to its peak of 9.6 in early summer. Bicarbonate concentration had an average concentration of 1.2 mM throughout the time period, and did not fluctuate by more than 0.4 mM.

From data for the period between October 2009 and April 2010 we presented cell concentrations at two sampling sites, normalised to their maximum concentration, and compared them to the CO_2 concentration of the lake. Blooms of *C. raciborskii* can be observed only when the CO_2 concentration falls below the theoretical air-equilibrium level of CO_2, indicating that either *C. raciborskii* requires these conditions to bloom, or that during a

bloom CO_2 is depleted from the surface water faster than the air can resupply it. It was interesting to note that as the CO_2 in Lake Wivenhoe decreased below, and then increased above, air equilibrium levels, the peak biovolumes of detectable species occurred in order of their $K_{0.5(CO_2)}$ (when grown at atmospheric levels of CO_2).

This work suggests that C. *raciborskii* will diminish in its capacity to form blooms, as atmospheric CO_2 levels continue to rise into the future. Other species are likely to dominate this niche if *C. raciborskii* does not adapt effectively.

Strengths and limitations

This thesis employed well-established and reliable methods to measure the physiology of freshwater microalgae. The oxygen electrode based work (irradiance and carbon curves) provided valuable and reliable information on some of the most important processes of the cell, namely photosynthesis and respiration, carbon and light dependence. P vs. DIC curves, like P vs. I curves, provide a variety of characteristics of the cells, reliably and efficiently. However, when running experiments over eight hours, there was risk that diurnal variation in cell behaviour would reduce consistency.

The silicon oil centrifugation method for determining internal carbon pools and pH, though messy, time consuming and technical, provides rare information at a time when high throughput physical analyses are very common in this field, and fundamental physiology is often neglected. One of the limitations of this method is related to the cells for which it will work. Despite many days of optimisation and testing, *Monoraphidium* sp. would not work with any combination of available oils (to vary its viscosity). This is most likely due to the cell's morphology and density, which either prevents the cells from passing through the oil layer, or when using less viscous oil, the entire cell layer (including the media) pushes below the oil layer and mixes with the killing solution. .

By using a number of species from different classes of algae, we attempted to provide a broader perspective on potential changes in the phytoplankton population structure than would otherwise be gained studying only one or two species. However, like much research, the species selected here were limited to those that would grow successfully in a culture medium. Effectively this is a selection pressure that may bias the research towards strains

with a particular physiology that enables them to grow successfully in the lab. It is thus difficult to know how this affects the applicability of the research to real world conditions.

Though we use a number of species for this work, their identity was only narrowed to genus level. While we make these species available from the Monash culture collection, the absence of a precise species name is not ideal. Intraspecific variation between strains can be large, and the focus of this research was to identify potential trends between classes (with the exception of *C. raciborskii*, which is already known to be an invasive, toxic, nuisance algae), thus we believe this research is of significant value.

Following from this, the media used in the experiments (providing replete nutrients and trace metals), while allowing the algae to grow reliably, does not closely mimic the natural environment. It could be justifiably argued that the physiology of any of these species may change under changed nutrient conditions. Due to time and financial constraints, the ideal solution to this limitation (using filtered lake water to culture the algae) unfeasible. The focus of this work was not on nutrient limitation, however, and at least by removing this variable we were able to compare results.

The need to culture *C. raciborskii* under a lower light intensity to the other species was a significant limitation. Ideally a higher light tolerant strain would be used instead, however no such strain was available to us at the time of this research.

The simplicity of the experimental design resulted in the need for simple analytical and statistical methods which enhanced the transparency and make this work more understandable. Another strength of this research was its exploratory nature, which reduces the biases of the researchers, while sometimes leading to the development of novel concepts,

such as those relating internal bicarbonate concentration and surface area to volume ratio in Paper 2.

Significance of the findings and future research

The findings presented in each chapter of this thesis significantly add to the limited research available on the physiology of freshwater phytoplankton. It was shown that the photosynthetic characteristics of *C. raciborskii* support its bloom forming capacity in highly stratified surface waters, and why other species are less competitive in these scenarios, but more competitive in well mixed environments.

Future research would benefit from investigating the impact of nutrient limitation on the photosynthetic characteristics of these species in order to enhance our understanding of how these species respond to common environmental conditions. Similarly, testing the growth of each species at a variety of light intensities and mixing regimes would confirm our hypothesis about the favoured niches of these phytoplankton.

Each species studied showed strong CCM activity and the capacity to utilise bicarbonate as a carbon source. This is important in the context of climate change where elevated atmospheric levels of CO_2 are expected to have an impact on photosynthetic organisms. This study also suggested a correlation between surface area to volume ratio of the cell, and internal bicarbonate concentration. At high CO_2 partial pressures, growth rates generally differed from the atmospheric controls, however no common trend was apparent. Generally, higher photosynthetic rates, lower CCM activity and a higher surface area:volume ratio were common amongst the species studied.

Investigation of the internal carbon pools after culturing at high CO_2 would add to this work. This data, in combination with any observed changes in SA:Vol, would also contribute to the understanding of the relationship between internal DIC pools and the morphology of the cell. Following from this work, a meta-analysis could be carried out by collating already published literature on these cellular characteristics, and adding them to the curve in an attempt to model the theory.

In an attempt to measure changes in competitive behaviour and thus population structure of the phytoplankton in Lake Wivenhoe, microcosm experiments testing the effect on community composition of an elevated CO_2 atmosphere would be valuable. This would be of particular interest during summer when surface CO_2 concentrations become very low, prior to and during *C. raciborskii* blooms. In monoculture, measuring the activity of CCMs under a range of environmental conditions would also be informative.

Concluding remarks

Exploratory research, investigating the potential impact of climate change, and adding to the understanding of microalgal physiology, is of vital importance. The biodiversity of microalgae is enormous, and the response of individual species to a changing climate and atmosphere is not easily predicted. This research adds to this knowledge base by characterising the physiology of photosynthesis and carbon uptake of a variety of species of algae. Further, by culturing these species at high (1000 ppm) and atmospheric (400 ppm) concentrations of CO_2, we were able to observe and characterise some of the changes that may occur into the future as CO_2 levels rise. Of critical importance, our findings suggest that the growth rate of *C. raciborskii* may significantly decrease as CO_2 levels increase, which, for stakeholders of Lake Wivenhoe and many other freshwater systems where toxic *C. raciborskii* blooms, would be a welcome change, and may reduce operation costs for water operators.

Chapter 6 - References

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