From Ecosystem to Cell: Assessment of a brackish lake system and key environmental drivers of algal autecology

Thesis submitted for the degree of Doctor of Philosophy

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Abstract

Microalgae are vital components of most aquatic ecosystems. Algae are responsible for approximately 50% of global primary production, and as such, account for significant drawdown of anthropogenic CO_2 from the atmosphere. In addition, phytoplankton primary production is the main source of energy fuelling most aquatic food webs. In recent decades aquatic systems worldwide have suffered from recurring algal blooms, with an apparent increase in harmful algal blooms (HABs). Increased eutrophication in coastal and inland waters, enhanced stratification and warming oceans all contribute to the rise of algal blooms in aquatic ecosystems. In this thesis I aimed to explore how changes in environmental factors impact/influence microalgal growth, physiology, cellular composition and behaviour. Accordingly, Chapter 2 reports on investigations of the seasonal changes in the physico-chemical conditions of a brackish lake system in SE Australia, the Gippsland Lakes, and discusses how these changes influence the natural phytoplankton assemblage. We also describe key indicators that led to a bloom of the non-toxic cyanobacterium *Synechococcus* sp. throughout the lakes. Chapter 3 describes how key environmental drivers namely light, salinity and temperature affected the growth and physiology of four microalgae recently isolated from the Gippsland Lakes. The final two chapters examine how these four microalgae responded to environmental change through chemical and behavioural acclimation strategies. In Chapter 4, I report on studies in which I utilized Attenuated Total Reflectance - Fourier Transform Infrared (ATR-FTIR) spectroscopy to look at changes in internal cellular composition under environmental change. Lastly, the work described in Chapter 5 investigated vertical migration as a behavioural strategy to deal with separation of key growth resources (light and nutrients) for a HAB species. The results of this thesis demonstrated that light, salinity and temperature are key environmental drivers that influence the growth, physiology, and cellular composition of microalgae. We observed changes in natural phytoplankton biomass and species diversity, largely related to changes in temperature in the Gippsland Lakes. Microalgae were able to redirect their energy into different macromolecules in order to acclimate to variations in environmental conditions. Furthermore, we provided the first report of vertical migration patterns of the toxic, motile, haptophyte *Prymnesium parvum*; a HAB species with increased occurrences worldwide. This work demonstrates a greater understanding of how phytoplankton respond to changes in their environment, providing important quantitative ecological data to incorporate into predictive models used to assess how phytoplankton may respond to future environmental changes.

General 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 1 original paper currently under review in a peer reviewed journal and 3 unpublished publications. The core theme of the thesis is the impact of climate change on phytoplankton. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the School of Biological Sciences under the supervision of Professor John Beardall and co-supervision of Associate Professor Perran Cook.

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 chapter number 2-5 my contribution to the work involved the following:

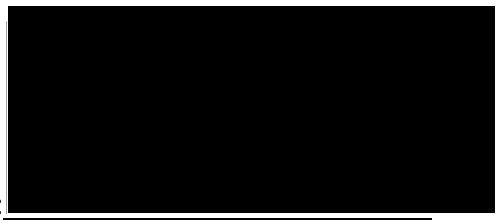
Thesis Chapter	Publication Title	Publication Status	Candidate's Contribution	Co-author names	Co-author Monash Student?
2	Seasonal and spatial changes of the physico-chemical and phytoplankton assemblages in a brackish lake system in SE Australia	In prep	Design, data collection, data analysis, and writing of manuscript 85%	1. John Beardall, 10% 2. Perran Cook, 5%	No
3	An autecological study of microalgae isolated from a brackish lake system: effects of light, salinity and temperature on growth and photophysiology	Under Review	Design, data collection, data analysis, and writing of manuscript 85%	1. John Beardall, 10% 2. Perran Cook, 5%	No
4	Characterizing compositional changes in response to key environmental drivers in four microalgae isolated from a brackish lake system	In prep	Design, data collection, data analysis, and writing of manuscript 85%	1. John Beardall, 5% 2. Perran Cook, 5% 3. Phillip Heraud, 5%	No
5	Assessment of vertical migration patterns of the toxic phytoflagellate *Prymnesium parvum* (Haptophyta) in a stratified water column	Under Review	Design, data collection, data analysis, and writing of manuscript 85%	1. John Beardall, 10% 2. Perran Cook, 5%	No

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

Student signature:  **Date:** June 25, 2018

Melissa L. Wartman

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:** June 25, 2018

Prof John Beardall

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When I started my PhD journey, I said to John that while I am happy to do lab work, I would also love to be able to get out into the field. Luckily John knew just the right person (Perran Cook) to help me achieve this and brought on as my co-supervisor. Perran, your expertise on the Gippsland Lakes was invaluable throughout the project. Your insights and feedback on my work not only improved my research output, but helped me develop as a scientific writer and critical thinker. Again, you made my PhD experience one of ease and delight and I thank you greatly for that.

As I was heading down a research tangent in one of my chapters, I was fortunate enough to end up at the Centre of Biospectroscopy. There I had the privilege of collaborating and working with Phil Heraud, Finlay Shanks, and Bayden Woods. Thank you for all your support with learning the instrumentation, software and which wavelengths correspond to which macromolecule. You have opened my eyes up to the endless possibilities FTIR spectroscopy.

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The Monash University I started out at is one that is significantly different to the one I finish at thanks to all the never ending construction. Fortunately the important things have remained unchanged, and I am forever grateful to my PhD friends for their support and guidance over the years. Thanks for all the talks, and laughs, Lotte van Boheemen, Qamariya Nasrullah, Kelsey Roberts, Julie Groce, Emily De Stigter, Louise Nøergaard, Stephen Gipson, Helena Chan, Rowan Mott, Franciscus Scheelings, Ash Herrod, Will Sowersby, Nataly Hidalgo-Aranzamendi, Stefanie Rog, Nancy Van Nieuwenhove, Shane Baylis, Candice Bywater, Cathy Cavallo, Sonia Sánchez, Amanda Pettersen, and the many others I met during my time at Monash.

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Contents

Chapter 1

INTRODUCTION

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MICROALGAE & CLIMATE CHANGE

Microalgae are ubiquitous in almost all aquatic environments. Through the process of photosynthesis, they are responsible for 50% of global primary production (Field et al., 1998). As such, they are vital in the drawdown of atmospheric carbon dioxide (CO_2), assimilating about half the anthropogenic CO_2 (Falkowski et al., 2000, 1998) through processes such as the biological CO_2 pump (Bowler et al., 2010), assimilation and regeneration (Eppley and Peterson, 1979). The phylogenetic and morphological diversity of microalgae is vast (Naselli-Flores et al., 2007), with each species playing an important role at the base of most aquatic food webs by fixing carbon into organic matter, that is then used to fuel higher trophic levels. Furthermore, microalgae have long been used as indicators of water quality and ecosystem health, providing researchers with unique information regarding ecosystem conditions (McCormick and Cairns, 1994).

Although abiotic and biotic interactions are complex, the rate of which atmospheric CO_2 has been increasing since the beginning of the industrial revolution will likely pose problems for microalgae by changing environmental conditions (Beardall and Stojkovic, 2006). The foremost direct consequences of increased atmospheric CO_2 are increasing ocean temperatures and acidity. Additionally, increased temperatures have the potential to cause rising sea level, increased water column stratification, and altered patterns of ocean circulation, precipitation, and freshwater input, leading to changes in salinity, nutrient distribution and light penetration (Gruber, 2011). These environmental changes will affect phytoplankton distribution, ecosystem assemblages and individual cell physiology in complex manners. The ongoing role of climate change in altering ocean properties mentioned above is well documented through long term time-series observations and climate modelling (Doney, 2010). The driving force for this research comes from the need to develop our comprehensive understanding of how climate change is affecting marine primary producers like microalgae which is still not well understood. The rate of marine primary production is largely controlled by light, temperature, and nutrient availability all of which will be altered with climate change (Falkowski et al., 1998; Valiela, 2015).

Light is the ultimate source of energy for phytoplankton, but the availability and access to this vital resource for phytoplankton in the aquatic environment is very dynamic

(Huisman et al., 2004; Kirk, 1995). Light attenuation through water is impacted by the physical properties of water, suspended particles, and dissolved organic matter which act to reflect, refract, absorb, and scatter the incident radiation (Dennison et al., 1993). Temperature is another important physical factor influencing both chemical and transport pathways important for growth, as well as regulation of enzyme catalysis, in microalgae (Raven and Geider, 1988). The impacts of global warming will continue to contribute not only to the direct warming of surface waters, but also intensify vertical stratification in both freshwater and marine systems, and alter climate and ocean circulation patterns (Behrenfeld et al., 2006; Doney, 2006; Levitus et al., 2000). The salinity of freshwater, estuarine and brackish systems will likewise face fluctuations under climate change. Altered rainfall patterns, enhanced evaporation rates, rising sea levels, and ever-increasing demands on freshwater for drinking and irrigation purposes, will likely increase levels of salinity (Harley et al., 2006; Paerl and Paul, 2012). These changes in salinity can affect phytoplankton by inducing/causing osmotic stress, ion (salt) stress and changes in cellular ionic ratios (Kirst, 1989).

HARMFUL ALGAL BLOOMS

While phytoplankton are vital components of aquatic environments, Harmful Algal Blooms (HABs) occur when populations of algae grow in excessive amounts, producing toxins, depleting oxygen in the water column, contaminating drinking water, and producing negative effects on humans and other organisms inhabiting the environment (Sellner et al., 2003). The environmental damage and ensuing financial costs of HABs is large (Hoagland et al., 2002). The occurrence and intensity of HABs has been on the rise over recent decades (Hallegraeff, 1993), with numerous waterbodies suffering reoccurring HABs (Bricker et al., 2008; Cook and Holland, 2012; Finni et al., 2001). Unfortunately, environmental conditions that cause HABs, such as eutrophication, upwelling, stratification, warmer temperatures, will likely enhance as a result of climate change (Beardall and Raven, 2004; Paerl and Paul, 2012). Studies have already begun to observe shifts in species composition of typical blooms, linked to changes in environmental conditions (do Rosário Gomes et al., 2014; Kremp et al., 2008) with some regular blooms being dominated by more harmful species (Kosten et al., 2012; O'Neil et al., 2012). Shifts in community composition can impact the ecosystem by changing the food

quality available to the upper trophic levels and also affect the pathways of nutrient cycling. Furthermore, researchers have also found that enhanced CO_2 concentrations and nutrient limitation synergistically increase toxicity in some toxin producing phytoplankton (Fu et al., 2010; Tatters et al., 2012).

THE GIPPSLAND LAKES

The main study area for this thesis was the Gippsland Lakes, a group of temperate brackish lakes located in southeast Victoria, Australia. The three major coastal lakes include Lake Wellington, Lake Victoria and Lake King, covering a total area of 354 km^2. Lake Wellington is quite shallow with an average depth of 4 metres, but is the largest of the three lakes (Webster et al., 2001). Lake King is the deepest lake, with a maximum depth of 10 metres. Like many temperate lakes, Lake Victoria and Lake King experience stratification, typically during spring and summer periods (Boehrer and Schultze, 2008; Wüest and Lorke, 2003). The Gippsland Lakes are a complex system, influenced by both water exchange from a narrow channel to the ocean, as well as from surrounding tributaries. The Entrance Channel at Lakes Entrance is a permanent man-made connection to Bass Strait in the south-east corner of Lake King (Wheeler et al., 2010). This permanent connection created an environment that is substantially altered from its natural state (Boon et al., 2015; Collett, 1987), shifting the system from a series of freshwater lakes to its current state as a brackish water body.

Freshwater entering from several rivers influences both salinity and nutrient concentrations in the lakes. There are seven major rivers that feed into the Gippsland Lakes with a total catchment area of approximately 20,600 km^2 (Webster et al., 2001). River and nutrient loads and nutrient cycling in the Gippsland Lakes have been studied extensively (Cook et al., 2010; Cook and Holland, 2012; Grayson et al., 2001; Longmore, 1994; Webster et al., 2001). These monitoring studies have found that nutrient loading into the lakes is primarily controlled by riverine input (Cook and Holland, 2012), with highest nutrient input occurring when riverine input is highest (typically winter and spring). Currently, the Gippsland Lakes are considered eutrophic, meaning that the nutrients loads and levels of organic matter entering the system are well above the natural assimilation capacity of the system (Webster et al., 2001). Eutrophication of this system can be attributed to land use changes in

the upper catchment, associated with urban, agricultural and industrial sources (Grayson et al., 2001; Holland et al., 2013).

Increased nutrient concentrations in aquatic systems can increase the probability of algal blooms (Anderson et al., 2002; Heisler et al., 2008). The first phytoplankton bloom in the Gippsland Lakes was recorded in 1965 (Solly, 1966). Since then there have been numerous recorded blooms of cyanobacterial and non-cyanobacterial species, with the non-cyanobacterial blooms usually comprising of diatoms or dinoflagellates (Day et al., 2011). Investigators have reported increased frequency of algal blooms over the years with the reoccurrence of the toxic cyanobacteria *Nodularia spumigena* and non-toxic *Synechococcus* sp. (Beardall, 2008; Holland et al., 2013). Several of these blooms have been estimated to have caused economic losses of millions of dollars, due to the restriction, when blooms are occurring, on the use of the lakes for both recreational and commercial purposes (Connolly et al., 2009; Gordon, 1988; Stephens et al., 2004). The preservation of the health of these lakes is critical as they provide a vital ecosystem for native flora and fauna, as well as an economic source to the region, bringing in an estimated $272 million annually (Department of Natural Resources and Environment, 2002).

CARBON ALLOCATION & MACROMOLECULES

Having evolved to live in dynamic environments, microalgae have strategies to enable acclimation to variable environmental conditions. One way is through regulation of metabolic processes (Palmucci et al., 2011a; Toseland et al., 2013). Through photosynthesis and nutrient acquisition from the surrounding environment, phytoplankton are able to form macromolecules such as proteins, lipids, carbohydrates and phosphorus containing molecules, including nucleic acids and protein synthesis. Proteins and carbohydrates are synthesized for a wide range of uses including osmoregulation, energy storage, buoyancy regulation in cyanobacteria, cell wall formation and maintenance, oxidative energy producing processes, metabolic processes mediated by enzymes and membrane structure (Dickson and Kirst, 1987; Finkel et al., 2016; Romans et al., 1994), while the main functional properties of lipids include structural components of the cell wall, membranes and energy storage (Guschina and Harwood, 2009).

Naturally, microalgae endeavour to maintain internal compositional balance, also known as homeostasis, in response to pressure exerted by the environment. However, when the threshold of an environmental factor exceeds the physiological limits of a cell, modification of internal macromolecular composition or allocation of resources into different molecular pools is possible, to help minimize the reduction in growth rate and reproduction potential (Giordano, 2013). The redistribution of internal carbon into different macromolecules is possible as the allocation of carbon into the various macromolecules is not energetically equivalent (Halsey and Jones, 2015), with substantial intraspecific (Dean et al., 2012) and interspecific (Dean et al., 2007) heterogeneity in allocation patterns. It is well known that environmental factors, particularly light, temperature, nutrient status and salinity, have the ability to affect not only growth and photosynthesis of microalgae, but also influence cellular metabolism thereby altering cellular macromolecular composition (Finkel et al., 2009; Giordano et al., 2001; Jebsen et al., 2012; Palmucci et al., 2011b; Sackett et al., 2013).

FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR)

There are a variety of methods available for the detection and measurement of macromolecular composition including Gas Chromatography (GC) and High Performance Liquid Chromatography (HPLC) mass spectrometry, UV-visible spectroscopy chromatography and infrared spectroscopy. This thesis employed Attenuated Total Reflectance-Fourier Transform InfraRed (ATR-FTIR) spectroscopy to investigate changes in the macromolecular composition of phytoplankton induced by alterations in environmental conditions. FTIR spectroscopy uses mid-infrared light, to characterise macromolecular composition of biological cells and tissues compounds using characteristic absorbance bands in the infrared spectrum (Giordano et al., 2001). Infrared spectroscopy offers numerous advantages over other available alternative methods including minimal sample preparation and sample volume needed, lower cost, and rapid acquisition of data.

Combining FTIR spectroscopy with chemometrics, researchers are able to obtain a snapshot of the compositional profile in response to environmental growth conditions (Heraud et al., 2006). The range of applications for FTIR spectroscopy as a tool in algal research continues to grow. Initially FTIR spectroscopy was utilized to measure changes in

macromolecular composition of cells under various nutrient treatments (Beardall et al., 2001; Giordano et al., 2001; Heraud et al., 2007, 2005; Hirschmugl and Gough, 2012; Sackett et al., 2014), as well as taxonomic classification of both microalgae and bacteria (Kansiz et al., 1999; Mariey et al., 2001). FTIR has now applicable for assessment of metal toxicity in microalgal cells (Dao et al., 2017), carbon productivity (Sackett et al., 2015) and most recently, the detection and prediction of cells exposed to oil and dispersant (Kamalanathan et al., 2018), highlighting the importance of this technique in monitoring the impacts of environmental change on microalgae composition. Infrared spectroscopy can even be applied to individual cells in natural populations when using micro-FTIR (Dean et al., 2012, 2007; Sackett et al., 2014), allowing researchers to investigate carbon allocation strategies in natural mixed-populations, an area of much-needed attention.

VERTICAL MIGRATION & WATER COLUMN STRATIFICATION

In addition to metabolic responses to environmental changes, phytoplankton are also capable of employing behavioural changes, with the focus of this thesis being on vertical migration. Most phytoplankton are non-motile, passively moving through the water column relying on turbulence to keep them re-suspended in the photic zone (Ross, 2006). However some phytoplankton species are capable of directed motility (Sohn et al., 2013). Several strategies for buoyancy regulation include flagella (Fenchel, 2001; Kamykowski, 1995), and specifically for cyanobacteria: gas vesicles (Kromkamp et al., 1986; Oliver and Walsby, 1984; Reynolds et al., 1987) in combination with density changes through carbohydrate ballasting (Cullen, 1985; Kromkamp and Mur, 1984; Villareal and Carpenter, 2003). Behavioural-focused research on numerous species of motile phytoplankton has established that vertical migration (VM) is an important aspect of their ecology (Cullen, 1985; Kromkamp and Walsby, 1990). VM is a behavioural mechanism which allows these motile species adaptive advantages, including avoiding passive sinking out of the water column, access to critical resources which may be spatially separated, migration to a depth of optimal resources for growth, predator avoidance, avoidance of high irradiance in the surface waters and escape from energetic turbulence (Bollens et al., 2011; Doblin et al., 2006; Durham et al., 2013; Tilney et al., 2015; Yoshiyama et al., 2009).

In estuarine and brackish systems, strong gradients of salinity (halocline) and temperature (thermocline) during summer can cause stratification of the water column, which can result in separation of essential resources (light and nutrients) that phytoplankton need for growth. While light decreases vertically from the surface (Kirk, 1995), nutrients are generally supplied from deeper water or bottom sediments (Scicluna et al., 2015), therefore forming opposing vertical gradients of light and nutrients. Motile species can overcome these opposing gradients by vertically moving through the water column, accessing light for photosynthesis in the surface during daylight, and storing nutrients from the bottom waters at night, a behaviour known as diel vertical migration (DVM) (Anderson and Stolzenbach, 1985; Cullen and Horrigan, 1981; Cullen and MacIntyre, 1998). Increasing our understanding of the ecological importance and impacts of vertical migration on phytoplankton population dynamics is important, as water column stratification is expected to intensify in the future. In coastal regions, increased temperature and precipitation will most likely intensify thermal stratification and further strengthen the salinity gradient and alter nutrient availability, which will likely select for motile phytoplankton species, a high percent of which are toxic (Smayda, 1997).

RESEARCH AIMS

Algae have long been used as indicators of water quality and ecosystem health. Since they are ubiquitous in almost all aquatic ecosystems, improving our understanding of their growth, physiology, and occurrences will help us in predicting how they may respond to future environmental change. The principal aim of this thesis was to assess how key environmental drivers from the Gippsland Lakes, mainly light, salinity, temperature, and nutrients, affect the growth, physiology, cellular composition, and motility of microalgae isolated from the lakes. My thesis is organized into the following chapters:

Chapter 2	Determines key environmental drivers of phytoplankton assemblages of the Gippsland Lakes. This chapter provides background on the lakes in terms of physico-chemical parameters over an annual cycle as well as understanding of the annual succession of species composition in the natural environment.
Chapter 3	Assesses the growth and photophysiology of four microalgal species isolated from the Gippsland Lakes, under different treatments of light, salinity and temperature.

Chapter 4	Evaluates biochemical changes that occurred in four microalgal species under the light, salinity and temperature treatments to assess chemical acclimation under different environmental conditions, using ATR-FTIR spectroscopy.
Chapter 5	Investigates *Prymnesium parvums* use of vertical migration as a behavioural acclimation strategy under environmental stresses including uneven resource distribution and salinity stratification in laboratory water columns.
Conclusion	Provides a general synthesis of all the key results from the previous chapters, and summarizes the main findings of this thesis. Future direction and weakness in research is also discussed in this chapter.

This thesis provides further understanding towards our comprehension of how microalgae deal with perturbation in environmental growth conditions. The experimental and observational data collected for this thesis, will be valuable for enhancing current models developed for the Gippsland Lakes for predicting physico-chemical changes and HABs. The results of this thesis will also be shared with the Gippsland Lakes environmental managers, to enable a greater understanding of the autecology of several species from the lakes.

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Chapter 2

SEASONAL AND SPATIAL CHANGES OF THE PHYSICO-CHEMICAL AND PHYTOPLANKTON ASSEMBLAGES IN A BRACKISH LAKE SYSTEM IN SE AUSTRALIA

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ABSTRACT

The Gippsland Lakes are a group of interconnected brackish lakes in SE Victoria, which suffer from eutrophication leading to recurring algal blooms. The seasonal and spatial variations of key physico-chemical variables and phytoplankton community composition was studied in the Gippsland Lakes over a 15 month period from 2017-2018. We investigated the occurrences of phytoplankton in relation to measured environmental parameters to assess which factors play the largest role in controlling growth and occurrence of microalgae in the lakes. The Gippsland Lakes are located in a temperate environment, reflecting the 21.8^{o}C temperature change between the maximum summer temperature (29^{o}C) and minimum winter temperature (7.5^{o}C). Salinity ranged from 32 psu to 2 psu throughout the lakes, with Lake Wellington being significantly fresher (2 to 16 psu) than the Lake King and Lake Victoria sampling sites (16 to 32 psu) during the study period. Summer 2017 chlorophyll *a* was relatively low, and did not significantly differ from winter 2017. However, December 2017 (early austral summer), an increase in chlorophyll *a* was observed, with a bloom of the non-toxic cyanobacterium *Synechococcus* sp. occurring throughout most of the 2018 summer. The dataset we present also details annual changes of inorganic carbon parameters in the Gippsland Lakes. We found that the photosynthetic biomass plays a large role in determining whether the lakes act as a source or sink of atmospheric CO_2. Our findings emphasize that the occurrence of phytoplankton is strongly influenced by temperature, and to a lesser extent salinity and nutrients. Furthermore, this study improves our understanding of the dynamics of the Gippsland Lakes which can aid in management decisions. Our results provide additional observations on the physico-chemical conditions that influence the planktonic communities in the Gippsland Lakes.

ABBREVIATIONS

Chl *a*, Chlorophyll *a*

CO_2, Carbon dioxide

CO_3^{2-}, Carbonate ion

DIC, Dissolved inorganic carbon

FRP, Filterable reactive phosphorus

F_v/F_m, Maximum quantum yield of Photosystem II

NO_x, Nitrogen oxides

OJIP, rapid fluorescence transient

pCO_2, Partial pressure of carbon dioxide

PSII, Photosystem II

TA, Total alkalinity

Ω_{Ca}, Calcite saturation state

Ω_{Ar}, Aragonite saturation state

INTRODUCTION

The Gippsland Lakes can be included in the growing list of coastal aquatic systems which suffer from recurring algal blooms (Smith, 2003). Eutrophication of estuarine and coastal aquatic ecosystems is ever-increasing, due to human alterations of aquatic landscapes through increases in population density, industrial activities and land use changes along the edges of these waterbodies (Jonge et al., 2002; Nixon, 1995). Compounding with eutrophication are the direct effects of climate change, resulting in higher water temperature, enhanced stratification, and changes in salinity and nutrient regimes. Eutrophication and the effects of climate change are leading to both algal and cyanobacteria blooms becoming predictable features in marine and freshwater environments (O'Neil et al., 2012; Paerl and Paul, 2012).

Phytoplankton and their community composition play an important role in ecosystem function, as they are essential for ecological and biogeochemical processes (Arrigo, 2005). In aquatic ecosystems, variability in environmental conditions such as temperature, salinity regimes, nutrient loads, light availability, competition, grazers and general hydrological conditions, can affect the abundance, composition, and distribution of phytoplankton (Boyd et al., 2010; Margalef, 1978). Furthermore, the growth and succession of phytoplankton species through time can alter environmental conditions, making it suitable for growth of other species (Cook et al., 2010). Therefore, there is a growing need to understand how these communities responded to environmental shifts, and to identify key indicators triggering the onset of algal blooms (Cook and Holland, 2012; Kruk et al., 2011). This information is vital for the management of these aquatic ecosystem.

A group of brackish lakes in south-eastern Victoria, Australia, the Gippsland Lakes, have suffered from reoccurring algal blooms over the past century with toxic cyanobacteria increasingly dominating these blooms (Cook and Holland, 2012). The resultant cyanobacterial blooms cause closure of the lakes for both commercial and recreational fishing as well as limiting use for recreational purposes. The closure of the lakes results in millions of dollars in lost revenue to the region due to decreased tourism (Connolly et al., 2009). It is likely that algal blooms will continue to affect the Gippsland Lakes as increased nutrient loadings from the rivers in the catchments have led the lakes to become eutrophic (Cook et al., 2010;

Stephens et al., 2004). Like many other coastal aquatic ecosystems, the Gippsland Lakes are important breeding grounds and nursery areas for a variety of coastal species: birds, fish and crustaceans, and numerous surrounding wetlands are protected Ramsar sites (Boon et al. 2015).

Our understanding of the effects of eutrophication in estuarine and coastal marine systems, hydrologically complex systems, is constantly advancing. To continue progress our understanding, the present work addresses the spatial and temporal extent of natural phytoplankton assemblages and discusses how a variety of physical and chemical environmental variables affect phytoplankton biomass and species diversity in a eutrophic brackish lake system in SE Australia. Here, we undertook a 15-month monitoring program of the Gippsland Lakes, we described the environmental conditions that led to the development and supported a cyanobacterial bloom of *Synechococcus* sp. over the 2018 summer period throughout the lakes. Furthermore, we looked at variations of inorganic carbon parameters throughout the Gippsland Lakes, and linked these to changes in phytoplankton biomass.

MATERIALS AND METHODS

Site Description

The Gippsland Lakes are an open coastal lagoon system, permanently linked to the ocean (Bass Strait), through a narrow channel at Lakes Entrance (Fig. 1). Located in southeast Victoria, Australia, the three major lakes include Lake Wellington, Lake Victoria and Lake King. Lakes Victoria and King are both deeper (9 and 10 m respectively) than Lake Wellington (6 m). In the Gippsland Lakes, freshwater inputs from the surrounding tributaries, along with exchanges in oceanic waters from Bass Strait influence the salinity (Fryer and Easton, 1980). Due to limited exchange with the other lakes and distance from the entrance to the ocean (Bass Strait), Lake Wellington is usually much fresher, with a salinity around 0.5-16 psu (Webster et al., 2001). Changes in salinity are due to shifts in the balance between riverine input, evaporation and water exchange with the other lakes through McLennan's Strait. Lakes Victoria and King have much higher salinities ranging due their proximity to Bass Strait entrance. Salinity can range between 4 to 26 psu in the surface and 7 to 36 psu in bottom waters (Webster et al., 2001).

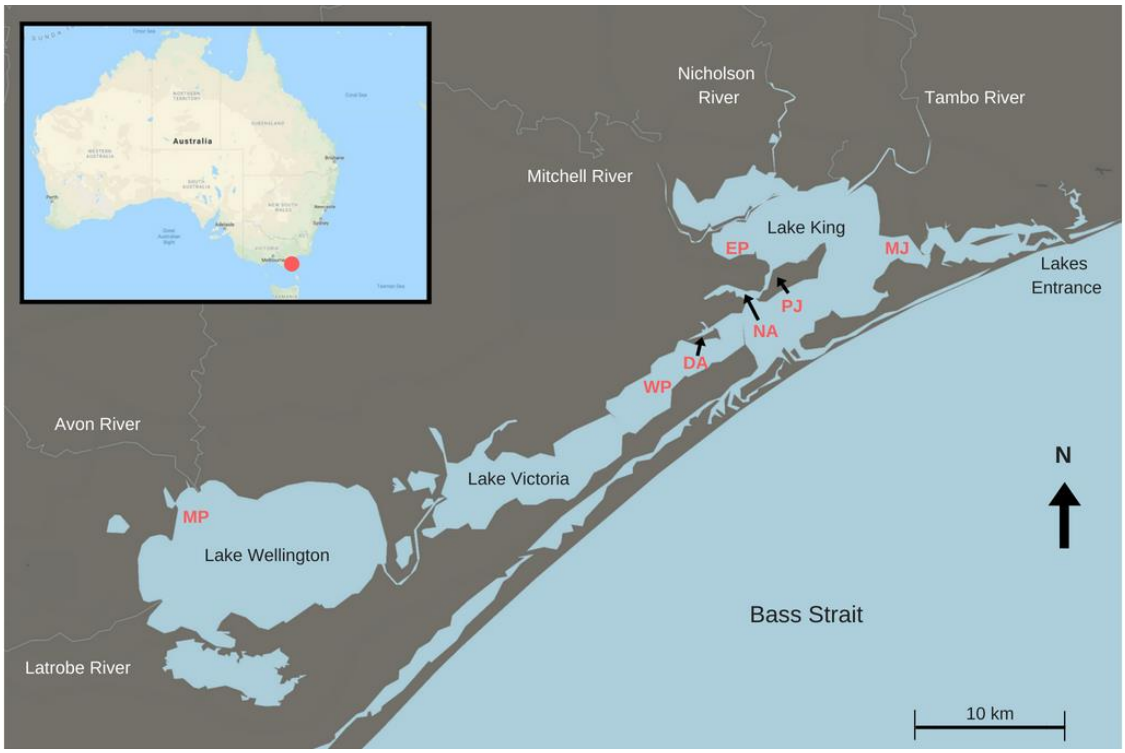

Figure 1. The Gippsland Lakes showing sampling locations around the lakes and the main rivers. Abbreviations: MJ, Metung Jetty; EP, Eagle Point; PJ, Progress Jetty; NA, Newlands Arm; DA, Duck Arm; WP, Wattle Point; MP, Marlay Point.

Sampling

Seven sites were chosen around the Gippsland Lakes (Table 1). Bi-weekly to monthly sampling was undertaken from January 2017 until April 2018. Rainfall data was sourced from the BOM webpage (http://www.bom.gov.au/climate/data/) for Bairnsdale Airport (ID: 085279). Samples were collected from the surface (0.5 m) at each of the sampling sites. Temperature was measured using a thermometer (Brannan; Cumbria, England), while salinity was taken using a portable refractometer (RHS-10ATC). Percent dissolved oxygen was measured using a Hydrolab Quanta multi-meter.

Table 1. Sampling locations of 7 sites around the Gippsland Lakes.

Sampling Location	Location ID	Latitude	Longitude
Metung (Lake King Jetty)	MJ	-37.892336	147.852461
Eagle Point Jetty	EP	-37.891695	147.684512
Paynesville (Progress Jetty)	PJ	-37.919196	147.719369
Newlands Arm	NA	-37.916148	147.695378
Duck Arm	DA	-37.951065	147.663846
Wattle Point Jetty	WP	-37.970687	147.634680
Marlay Point Jetty	MP	-38.059896	147.250479

Chlorophyll a

Extractible chlorophyll *a* (chl *a*) was used as a proxy for biomass. Triplicate volumes of lake water (between 20 mL and 400 mL) were filtered onto glass-fibre filters (Whatman GF/C) using a syringe filter. The filters were folded in half and wrapped in aluminium foil, then placed on ice while in the field. Upon return to the laboratory, samples were frozen at -20^{o}C until analysis. Chlorophyll *a* was extracted using 2 mL of dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany) with samples placed in a 70^{o}C water bath for 5 minutes. The sample was centrifuged for 10 min and the supernatant was then analysed spectrophotometrically (Cary-50 UV-Vis Spectrophotometer; Agilent Technologies, Santa Clara, CA, USA), using the equations of Seely et al. (1972).

Photophysiology

Several non-destructive PSII fluorescence measurements were measured on lake water samples from each sampling site. An AquaPen-C (AP-C 100; Photon Systems

Instruments, Czech Republic) was used to measure maximum quantum yield (F_v/F_m) analysis. Triplicates of 2 mL of lake water were dark acclimated for 10 minutes prior to analysis.

Dissolved Inorganic Carbon Parameters and pH

A sample (30 mL) of lake water was collected, then preserved with saturated mercuric chloride to kill any biological activity. On 4 occasions samples were filtered through a 0.45 µm syringe filter (PALL Life Sciences Acrodisc® Supor® Membrane Port, Washington, NY, USA) to remove biological organisms. A linear regression of the poisoning and filtering methods found there to be a strong fit between the both methods (R^2 = 0.9643). Samples were stored at 20°C in the dark until analysis, typically less than 48 hours. Samples for DIC were analysed following the protocol of Smith-Harding et al. (2017). Briefly, air was stripped of CO_2 by passing through an enclosed flask of soda lime (Chem-supply, Gillman, SA) prior to being bubbled into an enclosed glass chamber containing 20 mL of 0.1 M hydrochloric acid (HCl; Merck, Darmstadt, Germany). Two millilitres of sample was injected to the enclosed chamber through a one way sampling valve. Prior to reaching the gas analyser the air in the head space was passed through a desiccant chamber of magnesium perchlorate (Alfa Aesar, Haverhill, MA, USA) and cotton wool. The amount of CO_2 released from the reaction was analysed continuously by an Infrared Gas Analysis systems (LI-840A CO_2/H_2O Gas Analyzer; Licor, Lincoln, NE, USA). Sodium Bicarbonate ($NaHCO_3$) stock standards (3 mM, 1.5 mM, 0.75 mM and 0.375 mM $NaHCO_3$) were used to create a standard curve. The data were plotted in Microsoft Excel in order to calculate the area under the curve of the CO_2 vs time plot. This was then compared to the $NaHCO_3$ standard curve to calculate the amount of DIC in the sample. A calibrated sensION+ PH31 pH meter (Hach, Loveland, CO, USA) was used to measure pH of the samples. Total alkalinity (TA), calcite saturation (Ω_{cal}), and aragonite saturation (Ω_{arg}) state at *in-situ* temperature and pressure, were calculated from the DIC and pH measurements using the CO2SYS program (Lewis et al., 1998). The equilibrium constants (K1 and K2) of Mehrbach et al. (1973) as refit by Dickson and Millero (1987) were used in this calculation.

Nutrient Analysis

Water for nutrient analysis was collected in the field and directly filtered through a 0.45 µm syringe filter (PALL Life Sciences Acrodisc® Supor® Membrane Port, Washington, NY, USA), then stored on ice until return to the laboratory where samples were frozen at -20°C

until analysis. Nutrient samples were analyzed for ammonia (NH_3), nitrous oxides (NO_x), and filterable reactive phosphorus (FRP), using standard colorimetric methods (Hansen and Grasshoff, 1983), by a NATA accredited unit in the Water Studies Centre (Monash University).

Cell Identification

Vertical-tow net and bottle samples were collected for species analysis. Cells were identified by Jonathan A. Smith (First Alert Algae Monitoring, FAAM; SEAPro – South East Algae Project) as part of his routine monitoring program in the Gippsland Lakes. Cell count estimates were done using both improved Neubauer haemocytometer and Sedgewick Rafter chamber. Cell abundance was reported as present, common, very common and dominant. To quantify these estimates, we assigned numbers to each measure (present = 1, common = 2, very common = 4, dominant = 8) to estimate relative abundance.

RESULTS

Environmental Parameters

Surface-water salinity varied over the sampling period at all sampling locations (Fig. 2A). Marlay Point salinity was significantly lower than that of the other stations, with a minimum salinity of 2 psu and a maximum of 16 psu. The remaining sampling locations were also observed to have fluctuations in salinity, ranging from 18 psu to 32 psu across the monitoring period. River discharge into the lakes was relatively low throughout most of the sampling period, except for late winter (August 2017) and into early summer (December 2017) where maximum discharge (15 GL day^{-1}) was measured (Fig. 2A). The Mitchell River in the east had the highest discharge rates, while 2 western rivers (Latrobe and Thomson) also had high discharge rates during the same period. The temperate waters of the Gippsland Lakes were observed to show the characteristic seasonal variation in temperature (~ 21^{o}C) at all sampling locations (Fig. 2B). Maximum surface temperatures (29^{o}C) occurred during the austral summer periods, and the coldest temperatures (7.5^{o}C) were found during the austral winter. The 2017 austral summer had slightly warmer summer temperatures than the 2018 austral summer. Rainfall was below average throughout 2017 and into 2018 (Fig. 2B). Late spring 2017 and into early summer had the highest amount of rain but, compared to mean rainfall since 1942, it was still below average (data not shown).

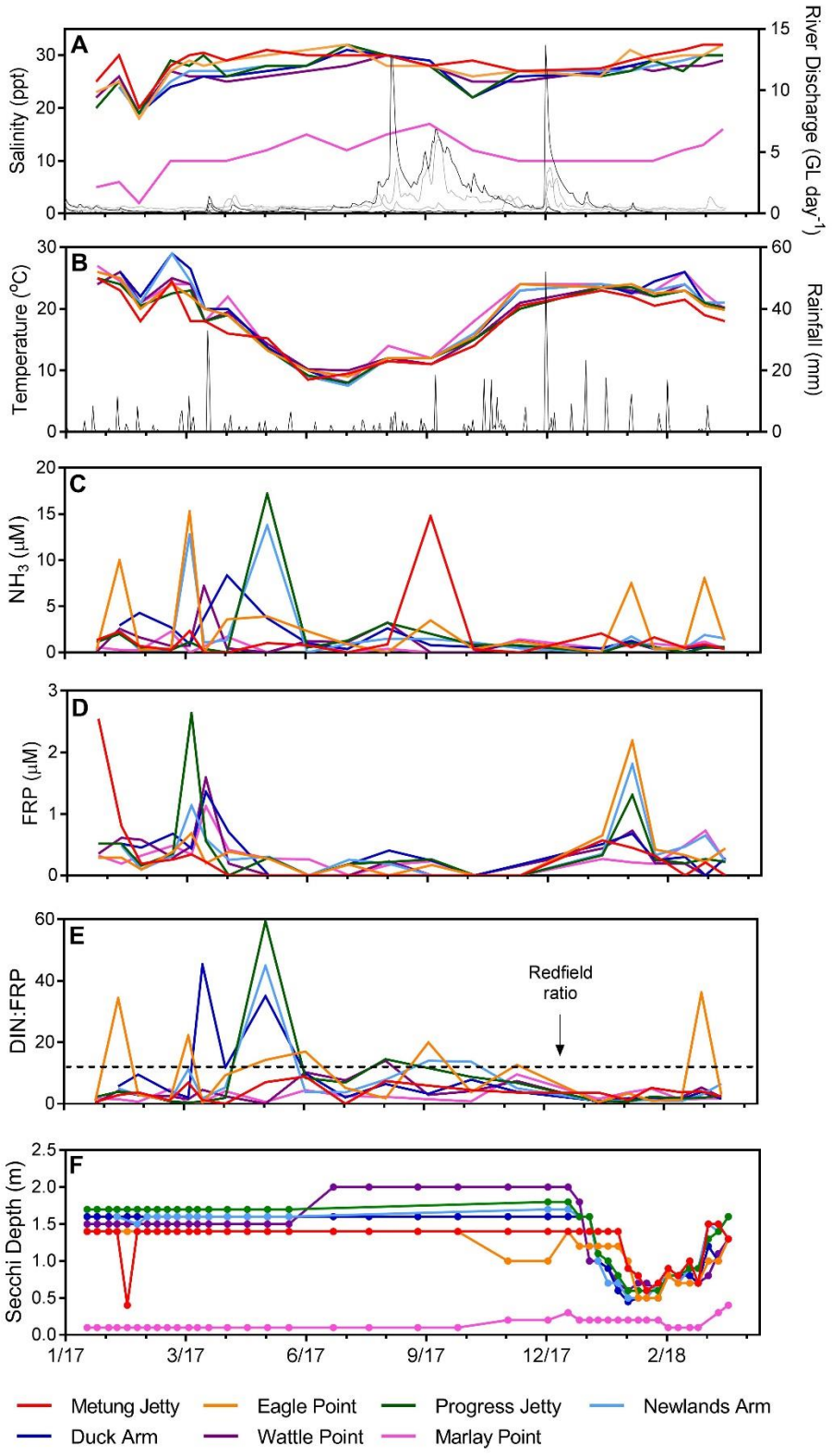

Figure 2. Physico-chemical conditions in the Gippsland Lakes during the study period. (A) Salinity and river discharge, western rivers (grey), eastern rivers (black). (B) Surface water temperature and rainfall at Bairnsdale Airport. (C) Ammonia (NH_3) concentration. (D) Filterable reactive phosphorus (FRP) concentration. (E) The ratio of DIN:FRP (DIN = Dissolved inorganic nitrogen) in the surface water. Dashed line represents the Redfield ratio. (F) Secchi depth.

Seasonal variations in inorganic nutrients (FRP, and NH_3) can be observed in Figure 2. Measurements of NO_x were carried out, but NO_x remained undetectable throughout the entire sampling period. Concentration of NH_3 varied across both site locations and sampling period. Concentrations ranged between <0.1 to 17 µM (Fig. 2C). Concentrations of FRP ranged between <0.1 to 2.5 µM, with the levels of FRP were undetectable throughout parts of the sampling period, mainly during the 2017 austral winter (Fig. 2D). The DIN:FRP ratios throughout the sampling period were generally below the Redfield ratio and values were on average 7 (Fig. 2E). Secchi depth remained relatively stable throughout the summer of 2017 until the summer of 2018 (Fig. 2F). In the summer of 2018 the Secchi depth rapidly shallowed, corresponding with the *Synechococcus* sp. bloom occurring during that time period. Coming into spring 2018, as the bloom was finishing, the Secchi depth was returning to its regular values. The Secchi depth at most sampling site was relatively similar, except for Marlay Point in the Gippsland Lakes. In this case, due to Lake Wellington's shallow depth throughout the majority of the lake, the water column is well mixed on most days with lots of sediment and sand in the water column.

Surface DIC samples were collected throughout the entire sampling period and TA was calculated based on DIC and pH measurements (Fig. 3). Their concentrations ranged between 720 and 2127 µmol DIC Kg^{-1} and between 743 and 2325 µmol TA Kg^{-1}, respectively (Fig. Xa). Lowest DIC and TA concentrations were found during the 2017 and 2018 summer period. Marlay Point was significantly lower ($p < 0.001$) in terms of both DIC and TA, than the other sampling locations. Calculated surface pCO_2 varied across the sampling period, with relatively high values between January and November 2017, and low pCO_2 values occurring during the 2018 austral summer. Both calcite (Ω_{cal}) and aragonite (Ω_{arg}) saturation states were generally saturated in the surface waters over the course of the sampling period for all sampling locations in Lakes Victoria and Lake King. However, Marlay Point in Lake Wellington, was under-saturated in respect to both Ω_{cal} and Ω_{arg} over most of the sampling period.

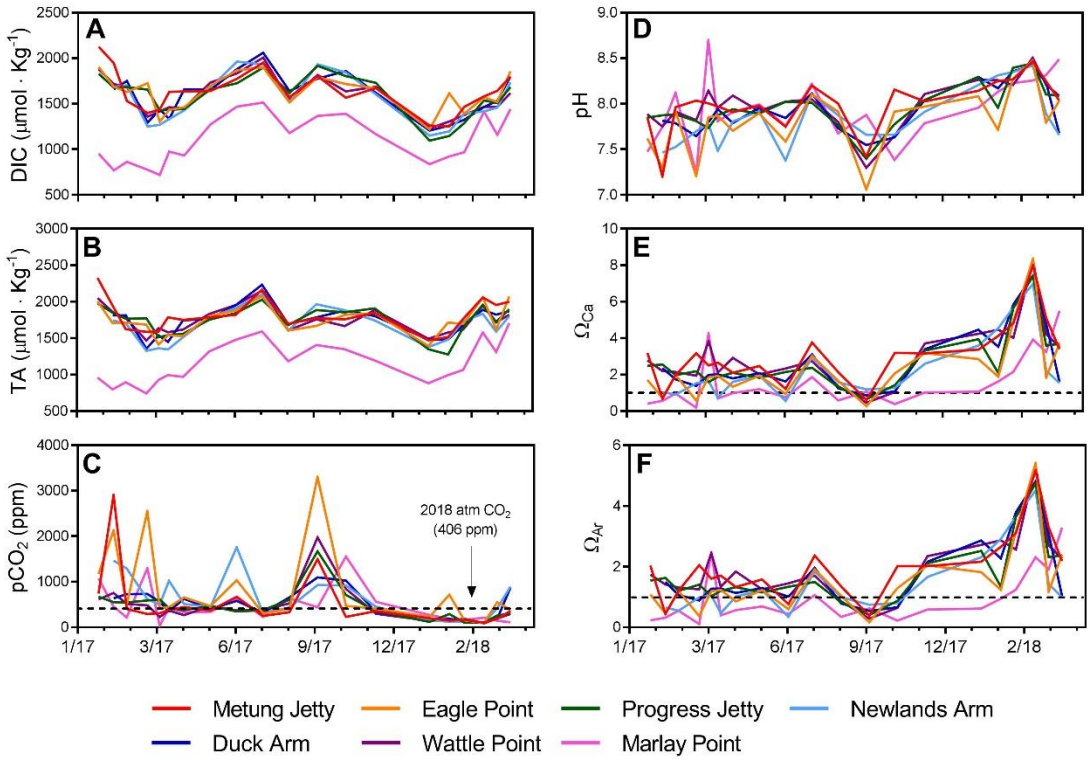

Figure 3. Inorganic carbon parameters at seven sampling locations (identified by colour) over a 15 month period in the Gippsland Lakes. (A) Dissolved inorganic carbon (DIC); (B) Total alkalinity (TA); (C) partial pressure CO_2 (pCO_2), dashed line represents 2018 atmospheric CO_2; (D) pH; (E) Calcite saturation state (Ω_{Ca}), dashed line represents equilibrium saturation (F) Aragonite saturation state (Ω_{Ar}), dashed line represents equilibrium saturation.

Biomass and species diversity

The austral summer of 2017 had relatively low levels of phytoplankton biomass, reflected in the relatively low chlorophyll *a* values (Fig. 4). The majority of sampling sites had chl *a* levels ranging from 2 to 5 $\mu g \cdot L^{-1}$ between January-March 2017. However, biomass was highest (16 $\mu g \cdot L^{-1}$) at Marlay Point, during this time period. Phytoplankton biomass remained low over the winter and into early spring. By early summer, the Gippsland Lakes were undergoing a pronounced algal bloom throughout most of the lakes. The high phytoplankton biomass was reflected in the high chl *a* concentration of 45 $\mu g \cdot L^{-1}$ at Marlay Point, and between 30 to 40 $\mu g \cdot L^{-1}$ at the other sampling sites. Peak maximum biomass occurred in January 2018 for Progress Jetty, Newlands Arm, Duck Arm, and Marlay Point, while Metung Jetty, Eagle Point, and Wattle Point had maximum biomass in February 2018. By mid-autumn (April 2018), chlorophyll *a* levels had almost returned to early spring levels.

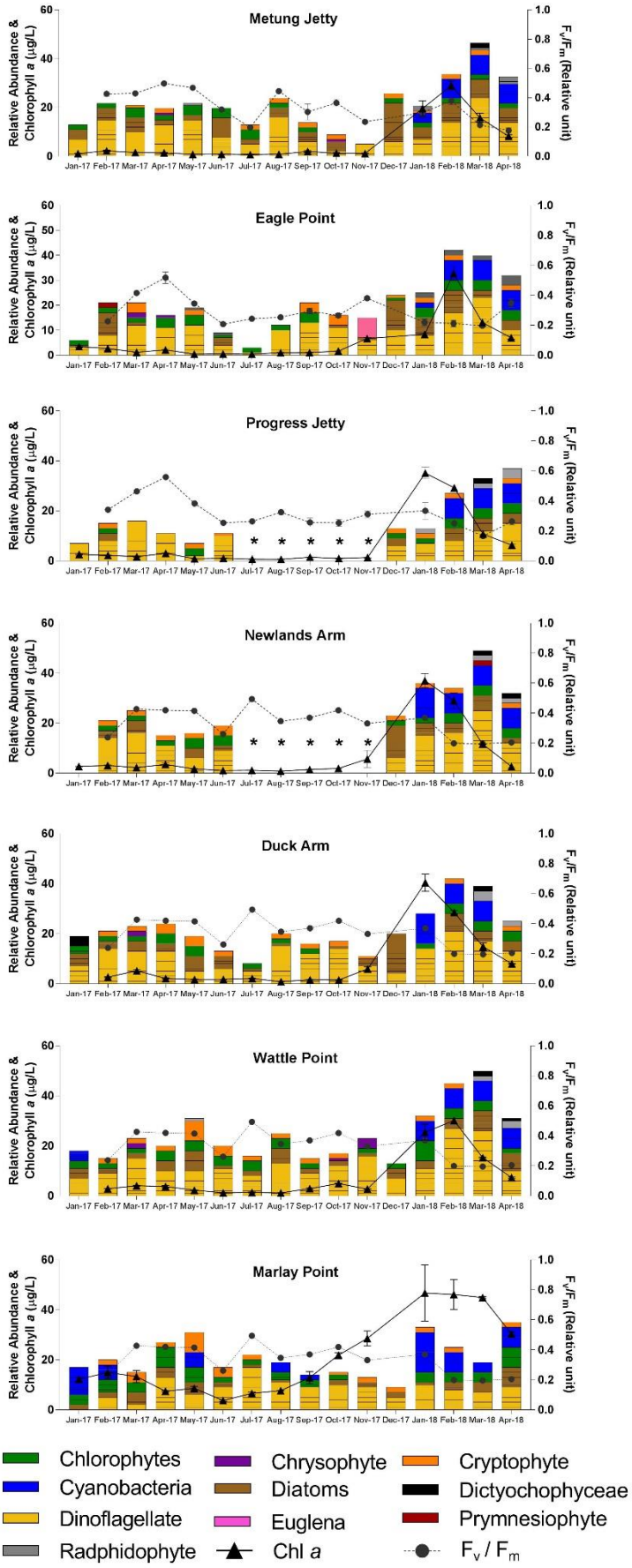

Figure 4. Biomass (chlorophyll *a*), species diversity, and maximum quantum yield (F_v/F_m) for the 7 sampling locations throughout Gippsland Lakes. Different algal taxonomic groupings are indicated by colour, with each box representing a different group identified in the sample, and the height of the box indicating the relative abundance. Chl *a* and F_v/F_m were measured in triplicate, with error bars representing standard error mean. Data not available represented by (*).

The mixed phytoplankton population throughout the Gippsland Lakes was largely dominated in the surface waters by species of dinoflagellates, and to a lesser extent, several species of diatoms (Fig. 4). Dinoflagellate diversity was high at most sites across the sampling period, with a maximum of 12 dinoflagellate species identified in March 2018. Several of the identified dinoflagellates include, *Dinophysis* sp., *Gymnodinium* spp., *Ceratium* sp., *Gyrodinium* sp., *Prorocentrum* sp., *Karlodinium* sp., *Alexandrium*, *Scrippsiella trochoidea* and *Heterocapsa triquetra*. The diversity of both individual species and divisions present in the surface waters were generally higher in the warmer summer months in both 2017 and 2018. In the summer of 2018, a cyanobacterial bloom, largely dominated by *Synechococcus* sp. but with *Synechocystis* sp. also present, dominated the water column at all sampling locations (Table 2). This cyanobacterial bloom was not strictly just cyanobacterial, as during this bloom there was also a high abundance of small flagellated chlorophytes, as well as other phytoplankton groups, present in the water column.

Table 2. Comparison of environmental conditions at the onset of *Synechococcus* blooms in the Gippsland Lakes.

	2007/2008	2017/2018
Duration	>1 year	~4 months
Nutrients	High	Low
Temperature	>20^{o}C	>25^{o}C
Salinity	~17 psu	>27 psu

Marlay point had more occurrences of cyanobacteria (Fig. 4), comprised of *Nodularia spumigena*, *Synechococcus* sp., *Anabaenopsis elenkinnii*, *Synechocystis* sp., *Oscillatoria* sp. and *Dolichospermum spiroides,* throughout the sampling period. However the cyanobacterial community at Marlay Point was largely dominated by *Nodularia* and *Synechococcus*, and sometimes *Synechocystis*. Dinoflagellates did not make up as large a portion of the phytoplankton assemblage at Marlay point, compared to the other sampling sites.

Maximum quantum yield (F_v/F_m) gives an indication of phytoplankton physiological health. F_v/F_m was quite varied over the sampling period (Fig. 4), with no seasonal trends observed. F_v/F_m varies across algal groups (Flameling and Kromkamp, 1998), which likely contributed to the variation observed throughout the sampling period.

DISCUSSION

The purpose of this study was to examine seasonal patterns in phytoplankton in the Gippsland Lakes to develop a more comprehensive understanding of biomass development and the likely environmental drivers behind summer algal blooms.

Summer *Nodularia* bloom

The Gippsland Lakes have suffered from reoccurring cyanobacterial blooms (Cook and Holland, 2012; Holland et al., 2013), with the toxic *Nodularia spumigena* being the dominant species. *Nodularia* was present at low abundance at certain time points throughout the 2017-2018 sampling period, however there was no observations of a major bloom. Previous work in the Gippsland Lakes identified key physico-chemical indicators for a *Nodularia* bloom to be low salinity (< 20 psu), warm air temperatures (> 20^{o}C) and a low N:P ratio (< 5) caused by internal nutrient cycling (Cook et al., 2010; Cook and Holland, 2012). In this study, conditions during the summer of 2018 were ideal in terms of nutrients and temperature, however salinity throughout all the basins was quite high (> 27 psu) and remained so throughout the summer. High salinity is well-known to severely limit the growth of *Nodularia* (Blackburn et al., 1996), especially in the Gippsland Lakes (Myers, 2008). This is the likely determining factor preventing *Nodularia* from blooming during this study period.

Summer *Synechococcus bloom*

Picocyanobacteria of the *Synechococcus* type are common features of oceanic phytoplankton assemblages (Murphy and Haugen, 1985; Partensky et al., 1999), and coastal and estuarine environments (Agawin et al., 1998; Cook and Holland, 2012; Phlips et al., 1999; Wang et al., 2011). In the Gippsland Lakes the first recorded bloom of *Synechococcus* occurred in late 2007 and lasted through to April 2008 (Beardall, 2008; Cook and Holland, 2012). Wildfires in the upper catchment burnt 32% of the land leading to a large input of nutrients during a 2007 flood into the lakes prior to the onset of the bloom (Cook and Holland, 2012). The longevity of the bloom was due to the fact that the small cell size of *Synechococcus* allowed efficient recycling of nutrients in the stratified water column (Finkel et al., 2009; Shuter, 1978). Conditions that were thought to lead to the 2007/2008 bloom were warm temperatures (> 20^{o}C), a salinity of 17 psu and high TN (> 70 µM) and TP (> 4 µM; Beardall, 2008; Cook and Holland, 2012).

In contrast to the 2007/2008 *Synechococcus* bloom (Table 2), the bloom in summer 2018 lasted only several months (January-early April). Conditions leading up to the bloom included high temperature (>25^{o}C), high salinity (> 27 psu) at most sampling locations and low nutrients (nitrogen limiting). Observations from both *Synechococcus* blooms in the Gippsland Lakes (Beardall, 2008; Cook and Holland, 2012), as well as other previous research, found temperature to be the main controlling factor over the occurrence and distribution of *Synechococcus* (Flombaum et al., 2013; Phlips et al., 1999; Wang et al., 2011). Optimal growth for Synechococcus has been reported at temperatures > 25^{o}C, with limited growth below 10^{o}C (Agawin et al., 1998; Kuosa, 1991). Furthermore, Flombaum et al. (2013) found that light was also a key driver behind *Synechococcus* abundance and distribution. As irradiance is highest during the summer period, it is likely a contributing factor for bloom stimulation. The reported *Synechococcus* bloom in this study was observed to reside at the surface, as measurements were taken in the upper 0.5 m of the water column. Previous research supports this notion has *Synechococcus* has been found to be quite tolerant of high light levels, likely due to the increase in synthesis of photoreactant D1 proteins (Kulkarni and Golden, 1994; Kuosa, 1991; Modigh et al., 1996).

Distribution models for *Synechococcus* found no clear relationship between nutrients and *Synechococcus* abundance, concluding that nutrients did not play a significant role in bloom formation of the species (Flombaum et al., 2013). Observations from the Gippsland Lakes are consistent with this interpretation as during the 2007/2008 bloom, the lakes were considered hypereutrophic (Cook and Holland, 2012), while in the summer of 2018 nutrients were low and nitrogen-limited. The picoplankton *Synechococcus* is extremely small in size (< 2 µm), and has a large surface to volume ratio, and cells therefore are capable of rapid nutrient uptake rates (Stockner, 1988). Unsurprisingly, nutrients may contribute to the persistence of *Synechococcus* blooms in the lakes. High levels of nutrients in the Gippsland Lakes allowed the Synechococcus bloom to persist for over a year, while the 2018 summer bloom with low levels of nutrients saw the termination of the bloom after several months.

Salinity has also been proposed as a contributing factor to phytoplankton growth and distribution (Kirst, 1989). However our results suggest that the ecological distinctiveness of certain areas of Gippsland Lakes in terms of salinity does not impact the growth of *Synechococcus*. During the 2018 summer bloom, *Synechococcus* was the dominant species at

all sampling locations, including Marlay Point. The salinity difference between Marlay Point (lowest salinity; 15 psu) and Eagle Point (highest salinity; 32psu) was 17 psu. Furthermore, at the onset of the 2007/2008 bloom salinity was approximately 17 psu (Cook and Holland, 2012). Previous studies have also found no connection between variations in salinity and *Synechococcus* abundance (Modigh et al., 1996). Whether different ecotypes present play a role in the species appearance to be quite tolerant of a range of salinities, salinity is likely not a controlling factor for the onset of *Synechococcus* blooms in the Gippsland Lakes.

Lastly, water exchange and water column stratification have been found to play important roles in the formation of cyanobacterial blooms (Paerl and Huisman, 2009). Phlips et al. (1999) found that limited restriction of water exchange between partially enclosed water bodies and the oceanic environment can contribute to the build-up of cyanobacteria (*Synechococcus*) due to the high residence times. Similar to water exchange observations made in Florida Bay (Phlips et al., 1999), the Gippsland Lakes have a relatively low flushing period. The average retention time of the lakes between 1975 and 1999 was 206 days (Webster et al., 2001). Seawater exchange into the Gippsland Lakes is a result of seasonal changes in sea level (30 cm), with minimal influence from tidal flushing due to the low tidal amplitude of the region and the narrow entrance channel to Bass Strait (Cook et al., 2010). Furthermore, Cook and Holland (2012) found that water column stratification was high at the onset of cyanobacterial blooms of *Nodularia* and *Synechococcus* in the Gippsland Lakes. There was observations of salinity stratification in the Gippsland Lakes, during the study period, at the onset of the *Synechococcus* bloom, and through the duration of the bloom (data not shown). Water column stratification has been shown to be beneficial to *Synechococcus* dominance of the water column, as its small size allows for a slow sinking rate compared to larger competitors who do not have vertical migration strategies to maintain the position in the surface (Bouman et al., 2011).

Inorganic carbon parameters

Upon examination of pCO_2 values over the sampling period, most of the sampling sites had surface waters supersaturated in CO_2 during the austral winter months. It is likely that during this period the Gippsland Lakes were a possible source of CO_2 to the atmosphere. The CO_2 emissions of estuarine environments into the atmosphere have been estimated to

be +0.27 ± 0.23 PgC yr^{-1} (Laruelle et al., 2010). Previous studies and modelling have found that the majority of inland waters are net sources of CO_2 to the atmosphere (Cole et al., 1994; McDonald et al., 2013). However, during the austral 2018 summer period in the Gippsland Lakes, CO_2 saturation dropped below atmospheric equilibrium (~ 406 µatm), possibly switching over to acting as a sink for atmospheric CO_2. The low pCO_2 values observed during the summer corresponded to a period of enhanced primary production as indicated by increased surface chl *a*. These findings are consistent with previous research describing the role that primary producers play in determining whether water bodies act as a source or sink of CO_2 (Balmer and Downing, 2011; Gu et al., 2011; Talling J.F., 1976; Tranvik et al., 2009). In addition, the elevated pH of surface waters throughout the Gippsland Lakes during the summer of 2018 also suggested high photosynthetic activity, as did the high-percentage oxygen saturation, which was supersaturated in the surface waters (data not shown). These findings agree with the general consensus that unproductive ecosystems tend to act as net CO_2 sources, while highly productive ecosystems act as CO_2 sinks (Duarte and Agusti, 1998).

CONCLUSION

This study described the phytoplankton population in the Gippsland Lakes, an important group of interconnected brackish lakes that continue to suffer from recurring cyanobacterial blooms. Over the course of the sampling period, we observed the biomass standing crop to generally be dominated by dinoflagellates. However during the summer of 2018 the lakes suffered from a non-toxic cyanobacteria bloom of *Synecochoccus* sp. From our observations temperature appeared to be the main controlling factor of *Synechococcus* blooms in the Gippsland Lakes. Our observations, along with previous research found no clear relationships between the onset of *Synechococcus* blooms and nutrients and salinity. The biological activity from the Synechococcus bloom, as indicative by the reduction in pH in the surface waters, played a role in the large variability observed in the inorganic carbon system of the lakes. The remarkably high abundance of *Synecochoccus* during the summer period in the Gippsland Lakes likely caused the lakes to temporary switch from being sources of atmospheric CO_2 to become sinks.

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Chapter 3

AN AUTECOLOGICAL STUDY OF MICROALGAE ISOLATED FROM A BRACKISH LAKE SYSTEM: EFFECTS OF LIGHT, SALINITY AND TEMPERATURE ON GROWTH AND PHOTOPHYSIOLOGY

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ABSTRACT

In coming decades, algal blooms globally are expected to increase in frequency and intensity, resultant from the numerous effects of climate change. Among the expected changes are increased temperature and in coastal regions, because of alterations in rainfall and run-off, salinity. Providing insight into the ecophysiology of a variety of algae will better help understand bloom triggers and progression. The role of light, salinity and temperature on growth and photosynthesis was studied for four recent isolates of phytoplankton, namely, *Chlorella* sp., *Kirchneriella* sp., *Prymnesium parvum*, and *Sarcinochrysis* sp. Batch cultures were grown under 8 light and salinity treatments, and 3 temperature treatments. The results of this study demonstrate that the four phytoplankton investigated have broad tolerances to a range of light, salinity and temperature, a reflection of the conditions experienced in their natural environment. Growth rate data showed a preference for low salinity in the two chlorophytes, though both were capable of tolerating salinities up to 25 psu. The *Sarcinochrysis* sp. isolate showed the highest rates of growth and photosynthesis of all the species under almost all treatments. Furthermore, results suggest that this brackish lake system provides ideal growth conditions for the toxic *P. parvum*, and raises concern for future blooms. Indeed, the presence of *Sarcinochrysis* and *Prymnesium* has not previously been reported for this lake system. The findings of this study highlight the physiological and growth characteristics of four different microalgae found in a brackish lake system, providing crucial insights into how they may respond to future climatic changes.

ABBREVIATIONS

Chl *a*, chlorophyll *a*

F_v/F_m, maximum fluorescent yield

HABs, Harmful Algal Blooms

I_C, light compensation point

I_K, light saturation point

NPQ, non-photochemical quenching

OD, optical density

P_{max}, maximal photosynthetic capacity

R_d, dark respiration

α, light harvesting efficiency

INTRODUCTION

Over the past several decades, researchers have observed an escalation in the number of algal blooms occurring globally, with blooms becoming more persistent and the involvement of toxic species increasing (Hallegraeff, 1993). While algae are naturally occurring organisms, present in almost all waterbodies and supporting ecosystems' food webs as a result of their primary production, unrestricted growth in the form of algal blooms can have large scale negative impacts on the environment, humans and the economy (Anderson et al., 2002; Hoagland et al., 2002). It is thought that these increased algal blooms can be attributed to eutrophication of the water bodies through agriculture, mining, land use changes and a number of other factors (Heisler et al., 2008). However, light, temperature and salinity are key drivers that impact the growth and persistence of phytoplankton species (Andersson et al., 1994; Falkowski and Owens, 1980; Lionard et al., 2005; Morton et al., 1992; Thomas et al., 2012).

Knowledge of the environmental limitations of phytoplankton to light, salinity and temperature is key for understanding species ecology in the natural environment. As sunlight is the main energy source for all phytoplankton species, the amount of light available to harvest is critical to satisfying energetic costs. Each species has specific light requirements and optima, and species-dependent photoacclimation strategies are used. For example, Richardson et al. (1983) identified broad differences in light requirements for growth at the level of different algal classes and Pierangelini et al. (2014) found strain specific photoacclimation strategies in the cyanobacterium *Cylindrospermopsis raciborskii*. Salinity has been found to regulate the distributional range of phytoplankton (McLachlan, 1961). Salinity can also affect growth and photosynthesis by osmotic and ionic stress (Murphy et al., 2003) as well as inhibiting photosystem II activity (Sudhir and Murthy, 2004). Coastal regions have highly variable salinity; therefore, species that inhabit this environment must be capable of tolerating a range of salinities. Furthermore, climate-change-driven alterations in rainfall and evaporation are likely to influence salinity regimes in estuaries and lake systems in the future. Reduction in growth rates in order to maintain osmotic adjustment, and changes in turgor pressure or volume changes are a few acclimation strategies that are engaged in order to survive under variations in salinity (Kirst, 1989). Changes in temperature can affect numerous chemical and transport pathways important for growth and photochemistry, as

well as enzyme catalysis (Raven and Geider, 1988). Predicted future increases in global average temperatures associated with climate change could result in an increased growth of algal blooms (Beardall and Raven, 2004), particularly cyanobacterial blooms (Jöhnk et al., 2008).

Throughout Australia, numerous inland waters and estuarine systems have suffered from reoccurring algal blooms (Beardall, 2008; Davis and Koop, 2006; Hallegraeff, 1992; Holland et al., 2013). One such system is the Gippsland Lakes in south-eastern Australia, a group of interconnected coastal brackish lakes with a range of salinities. Over the last several decades, nuisance algal blooms in the Gippsland Lakes alone have caused economic losses of millions of dollars each (Connolly et al., 2009; Stephens et al., 2004). Due to its very high environmental, social and economic value (Boon et al., 2015), this coastal lagoon system has been subjected to numerous studies monitoring the long-term health of the lakes. Eutrophication of global coastal waterways has been a major driver of nuisance algal blooms worldwide (Davis and Koop, 2006; Paerl, 1988). Numerous studies have investigated the limnology of the Gippsland Lakes (Cook et al., 2010; Fryer and Easton, 1980; Harris et al., 1998; Webster et al., 2001), with clear indications that nutrient loading into the lakes is primarily controlled by riverine input (Cook and Holland, 2012), with the highest nutrient input occurring when riverine input is highest (typically winter and spring).

Understanding environmental optima for algal species provides ecosystem managers with a predictive power, critical for monitoring these key indicators. With reoccurring blooms of *Nodularia spumigena*, and a major *Synechococcus* bloom in 2007/2008 in the Gippsland lakes, previous studies on these particular species have led researchers to identify key indicators of bloom conditions now being used by lakes managers as monitoring guidelines (Beardall, 2008; Cook and Holland, 2012; Holland et al., 2013; Myers et al., 2010). Previous work has uncovered key environmental indicators for the risk of a summer *Nodularia* bloom in the Gippsland Lakes to be, dissolved inorganic nitrogen concentrations less than 0.4 µmol/L and salinity below 20 psu (Cook and Holland, 2012). Myers et al. (2010) found that akinete germination for *Nodularia* in the Gippsland Lakes was strongly influenced by salinity and light, with optimal salinity for germination between 5 to 25 psu. High temperature and were thought to be the cause of the major *Synechococcus* bloom in the Gippsland Lakes (Beardall, 2008).

As part of the efforts to better understand the autecology of phytoplankton from the Gippsland Lakes, four recent isolates were investigated for this study, namely *Chlorella* sp. (ChlorGL11), *Kirchneriella* sp. (KirchGL02), *Prymnesium parvum* (PrymGL20), and *Sarcinochrysis* sp. (SarcinGL07). The two chlorophytes are typically present throughout the lakes, at low standing crop abundance during winter periods, but have been seen to increase in abundance during the summer (Jonathan Smith, unpublished data). While this study is the first report the occurrence of *Prymnesium parvum* in the Gippsland Lakes, this species has been of increasing interest due to reports of its ability to form toxic blooms in parts of the USA and worldwide (Edvardsen and Paasche, 1998; Roelke et al., 2007). It is known to produce a number of toxins (Shilo, 1981) and to cause large scale fish kills (Roelke et al., 2010). The fourth alga, *Sarcinochrysis* sp. is a rare chrysophyte that has also not been previously reported as occurring in the lakes. To date, there have been relatively few studies on *Sarcinochrysis* sp., with the limited available studies mainly focused on pigment composition (Withers et al., 1981). Therefore, to our knowledge, this study is the first to present growth and physiological data on a *Sarcinochrysis* sp.

This study investigated the ecophysiological response of four phytoplankton isolated from the Gippsland Lakes in relation to light, salinity and temperature. The information gathered gives an overall understanding of these phytoplankton, and the key roles played by main abiotic elements, which can influence the occurrence of the species throughout the estuarine system. The results obtained provide broad data on environmental tolerances and optima of these species, appraising what environmental conditions have the potential to trigger blooms of these algae. From the results, we make predictions as to which of these species if any, could pose a potential bloom threat in the lakes in future years.

MATERIALS AND METHODS

Site Description

The Gippsland Lakes are a group of coastal brackish lakes located in southeast Victoria, Australia (Fig. 1). Located in a temperate climate, the lakes' water temperatures can range between 10°C to 25°C (Harris et al., 1998). The three major lakes that comprise the Gippsland Lakes include Lake Wellington, Lake Victoria and Lake King. Lakes Victoria and King are both deeper (9 and 10 m respectively) than Lake Wellington (6 m). In the Gippsland Lakes, freshwater inputs from the surrounding tributaries, along with exchanges in oceanic waters from Bass Strait influence the salinity (Fryer and Easton, 1980). Due to limited exchange with the other lakes and distance from the entrance to the ocean (Bass Strait), Lake Wellington is usually much fresher, with a salinity around 0.5-10 psu (Webster et al., 2001). Changes in salinity are due to shifts in the balance between riverine input, evaporation and water exchange with the other lakes through McLennan's Strait. Lakes Victoria and King have much higher salinities ranging due their proximity to Bass Strait entrance. Salinity can range between 4 to 26 psu in the surface and 7 to 36 psu in bottom waters (Webster et al., 2001).

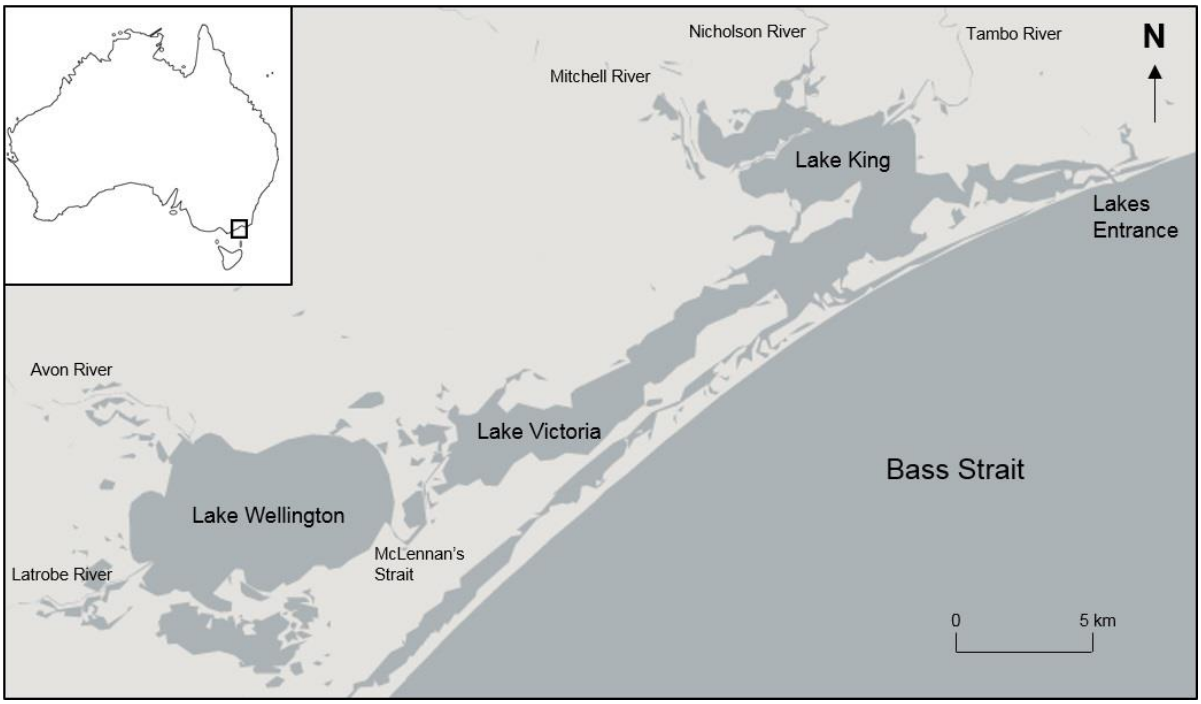

Figure 1. Map of the location of the Gippsland Lakes within Australia, and showing the three main lakes, Lake Wellington, Lake Victoria and Lake King. The Gippsland Lakes are fed by several tributaries including Tambo River, Nicholson River and Mitchell River from the eastern catchment, and Avon River and Latrobe River from the western catchment. The Gippsland Lakes are connected to Bass Strait by a narrow man-made entrance at Lakes Entrance.

Isolation of algal species

Water and sediment samples were collected from multiple locations in the Gippsland Lakes (37°53'S, 147°43'E), reflecting the different salinity environments found in the lakes. Samples were collected at three different sampling times (February and September 2015, and March 2016) in order to isolate a diverse range of species present in the lakes. Samples were collected in 500 mL plastic bottles and brought back to Monash University (Clayton, Victoria) and stored at 18°C under a continuous photon flux of 60 µmol photons · m^{-2} · s^{-1} until processing approximately 12 hours later.

Approximately 5 mL of water sample from each location was transferred to 250 mL flasks that contained 100 mL of sterilized f/2 medium (Guillard and Ryther, 1962). These cultures were grown for 7 days on a shaker, under 60 µmol photons · m^{-2} · s^{-1} of continuous light at 18°C. After 7 days of growth, several methodologies were employed to isolate a range of the species present. Serial dilution, streaking on agar, and micropipetting of individual cells were employed to isolate species. Once isolated, the cultures were sent to Microalgal Services (Ormond, Victoria) for identification. Single strains of each species were used for the subsequent experiments.

Culture conditions and experimental setup

Cell growth and photosynthetic performance were studied under various conditions of light, salinity, nutrient and temperatures (Table 1). To study the effects of light, we grew our four algal isolates under 8 different continuous light intensities viz. 20, 40, 60, 80, 100, 150, 200, and 400 µmol photons · m^{-2} · s^{-1}. For salinity variation experiments, the algae were grown at optimal light intensity under a range of 8 salinities which were 0, 5, 10, 15, 20, 25, 30, 35 psu, obtained by diluting full strength seawater with MilliQ water, and then amending with f/2 nutrients. Finally, for the temperature experiment the algae were cultivated, with optimal salinity and light, under three temperatures namely 15, 20 and 25°C. Cells were grown in f/2 medium. Once the cells were acclimated to the treatment (approximately 4-5 generations), experiments were initiated by inoculating culture tubes to 1 x 10^5 cells · ml^{-1}.

Table 1. Experiment regimes used to investigate the effects of light, salinity and temperature on four species of phytoplankton isolated from the Gippsland Lakes.

	Study 1: Light	**Study 2: Salinity**	**Study 3: Temperature**
Light (µmol photons $m^{-2} \cdot s^{1}$)	**20, 40, 60, 80, 100, 150, 200, 400**	ChlorGL11: 100 KirchGL02: 100 PrymGL20: 100 SarcinGL07: 150	ChlorGL11:100 KirchGL02:100 PrymGL20: 100 SarcinGL07: 150
Salinity (psu)	ChlorGL11: 20 KirchGL02: 20 SarcinGL07: 20 PrymGL20: 25	**0, 5, 10, 15, 20, 25, 30, 35**	ChlorGL11:20 KirchGL02:20 SarcinGL07: 20 PrymGL20: 25
Temperature (°C)	20	20	**15, 20, 25**

Cell growth experiments were carried out using a PSI Multi Cultivator 1000-OD (MC1000-OD, Photon Systems Instruments, Czech Republic). Cells were grown in eight 100 mL culture vessels, which were immersed in a temperature controlled water bath. Cultures were aerated with filtered ambient air from manifolds that attached to a main gas dispenser tube connected to a sterilized humidifier bottle. Each of the eight vessel slots contained its own LED panel with equally distributed cool white light that provided continuous illumination (24 hours). The PSI Multi-Cultivator was equipped with an optical density (OD) sensor that measured OD every 10 minutes in each culture vessel. Analyses of physiological parameters of phytoplankton were carried out on batch cultures under continuous illumination, when the cultures were in mid-exponential phase. Independent runs of cultures under each condition were carried out on three separate occasions.

Growth

Cell enumerations were counted daily. The number of cells per mL was counted microscopically on a Zeiss Axioskop optical microscope (Zeiss, Göttingen, Germany) using an improved Neubauer hemocytometer. Cell growth was also monitored by OD, that was measured every 10 minutes at 720 nm by the PSI Multi-Cultivator. For all treatments, there was a good correlation between OD (720 nm) and cell counts ($r^2 > 0.9669$). Maximum

specific growth rates (μmax, day^{-1}) were calculated from regression of the log OD values versus time, during the exponential phase of growth.

Pigment Analysis

Chlorophyll a and carotenoids were estimated by harvesting 15 mL of culture during exponential phase and concentrating the cells by centrifugation (4000 g, 10 min, Heraeus Multifuge 3SR+, Thermo Scientific). The supernatant was discarded and the sample was resuspended in 5 mL of 100% methanol (Merck, French Forest, NSW, Australia). The sample was placed in the dark at -20°C for 24 hours, before centrifuging again. The absorbance of the supernatant was measured in a Cary-50 UV-Vis Spectrophotometer at 665, 652, 632, 696, 470, and 750nm. Chlorophyll a was then calculated using the equations of Ritchie (2008). Carotenoid concentrations were calculated using the equation of Wellburn (1994).

Photosynthetic parameters

The protocol of Pierangelini et al. (2014) was followed, with modifications. A Clark-type oxygen electrode (Hansatech, Norfolk, UK) was used to measure oxygen evolution as a function of light (producing P vs. I curve), and dark respiration (R_d). Dark respiration was measured first, by dark acclimating the cells for 10 min. The R_d rate was then measured over the subsequent 5 min. Oxygen evolution was measured at 8 different light intensities over a range from 10 to 1200 μmol photons $\cdot m^{-2} \cdot s^{-1}$. The rate of oxygen evolution was measured for approximately 5 min at each light intensity. If O_2 concentration increased above 250 nmol ml^{-1}, the sample was bubbled with N_2 gas to bring the O_2 concentration down to 30% of air equilibrium. P vs. I curves were created using the equation $P = (I - I_c) / a (I - I_c)2 + b (I - I_c) + c$ (Eilers and Peeters, 1988). P_{max}, α and I_k were calculated using the equations $P_{max} = b + 2\sqrt{ac}$, $\alpha = 1/c$ and $I_k = c/b + 2\sqrt{ac}$ (Eilers and Peeters 1988).

Photosystem II (PSII) Parameters

The method for PSII fluorescence measurements and calculations were adapted from Pierangelini et al. (2014), using a Phyto-PAM Phytoplankton Analyzer (Heinz Walz, Effeltrich, Germany). Concentrating cells from each treatment was not required for the four species used in this study. After 15 min of dark adaptation, 3 mL of culture sample was placed in the

PhytoPAM cuvette equipped with a stirring bar. A measuring light beam of low intensity (2 µmol photons · m^{-2} · s^{-1}), too low to cause any photochemistry, was switched on and gave the minimal fluorescence value F_o. A saturation pulse of maximum intensity (2600 µmol photons · m^{-2} · s^{-1}) and 300 ms duration was applied to determine maximum fluorescence (F_m). From these values, maximum quantum yield (F_v/F_m) was determined, with $F_v = F_m - F_o$. Actinic light sufficient to saturate electron transport rate (480 µmol photons · m^{-2} · s^{-1}) was then provided and a saturating pulse was given every 20 seconds for 10 min. Once F_m' stabilized, the value was used to calculate NPQ using the Stern-Volmer equation $NPQ = (F_m - F_m') / F_m'$.

Statistical Analysis

The variation among means in relation to light, salinity and temperature and between species was tested using two-way ANOVA followed by Tukey's multiple comparison test. All analyses were carried out using GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA), with a significance level of $P < 0.05$.

RESULTS

Growth Rates

Irradiance had a significant effect on growth rates for all four species (Two-way ANOVA: $F_{7,48} = 36.81$, $P < 0.0001$). Generally, the four microalgae showed a broad tolerance, growing under various light levels (Fig. 2 A). Growth rate during exponential phase increased with increasing light intensity in each species, until maximum growth at light saturation was reached. Light saturation for growth occurred around 100 µmol photons · m^{-2} · s^{-1} for ChlorGL11, KirchGL02, and PrymGL20. However light saturation for growth occurred at a higher light intensity of 150 µmol photons · m^{-2} · s^{-1} for SarcinGL07. Overall SarcinGL07 had much higher rates of growth compared to the two chlorophytes and the haptophyte (Two-way ANOVA: $F_{3,48} = 382$, $P < 0.0001$). Maximum growth rate for ChlorGL11 was 0.36 day^{-1} and for KirchGL02 0.29 day^{-1} when grown at 150 µmol photons · m^{-2} · s^{-1} (Table 2). PrymGL20 had a maximum growth rate of 0.28 day^{-1} when grown at 100 µmol photons · m^{-2} · s^{-1}. Finally, SarcinGL07 achieved a maximum growth rate off 1.5 day^{-1} when grown at its optimum of 200 µmol photons · m^{-2} · s^{-1}.

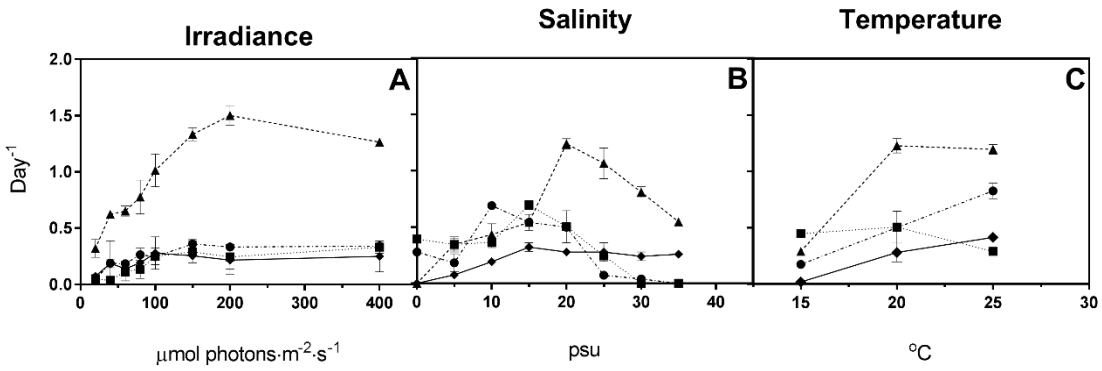

Figure 2. Exponential growth rates for ChlorGL11 (circle), KirchGL02 (square), PrymGL20 (diamond) and SarcinGL07 (triangle), under a range of (A) irradiance, (B) salinity and (C) temperature treatments. Vertical bars represent standard error mean of three replicates.

Table 2. Treatment conditions which were found to promote maximum growth rates for the four phytoplankton isolates grown in culture. The corresponding maximum growth rate (d^{-1}) is found in brackets.

	Irradiance (µmol photons $m^{-2} \cdot s^{-1}$)	**Salinity (psu)**	**Temperature (°C)**
ChlorGL11	150 (0.36)	10 (0.69)	25 (0.83)
KirchGL02	150 (0.29)	15 (0.70)	20 (0.50)
PrymGL20	100 (0.28)	15 (0.32)	25 (0.41)
SarcinGL07	200 (1.50)	20 (1.24)	20 (1.23)

All four-species had significant growth responses when grown under a range of salinities (Two-way ANOVA: $F_{7,64} = 37.9$, $P < 0.0001$). Two of the species investigated (SarcinGL07 and PrymGL20) exhibited no growth at 0 psu, while the other two species (ChlorGL11 and KirchGL02) showed no growth at 35 psu (Fig. 2 B). ChlorGL11 and KirchGL02 showed rapid growth rates when grown in salinities between 10-20 psu. PrymGL20 exhibited overall lower growth rates compared to the other three species, but grew best when grown in 15 to 35 psu. SarcinGL07 demonstrated increased growth rates with increasing salinity up 25 psu, but above that it had slightly decreased growth rates.

The growth rates of all four isolates were significantly impacted by temperature (Two-way ANOVA: $F_{2,24} = 68.99$, $P < 0.0001$). All species were able to grow over the temperature range investigated (Fig. 2 C). Three of the four species were found to exhibit higher growth in warmer temperatures, whereas KirchGL02, exhibited a lower growth rate at higher temperatures. At 25°C ChlorGL11 and PrymGL20 had a two-fold increase in growth rates compared to 15°C ($P < 0.0001$, $P = 0.0037$). KirchGL02 had similar rates of growth at 15°C and 20°C. At a higher temperature of 25°C, a decrease in growth rate was observed, however this was not statistically significant ($P = 0.3400$). Maximum growth occurred between 20°C and 25°C for SarcinGL07 and this isolate had significantly reduced growth at 15°C ($P < 0.0001$).

Pigments

Cellular pigment concentration was significantly affected by irradiance (Fig. 3; Chl *a*, Two-way ANOVA: $F_{7, 61} = 14.19$, $P < 0.0001$; Carotenoids, Two-way ANOVA: $F_{7, 64} = 22.37$, $P < 0.0001$), and salinity (Chl *a*, Two-way ANOVA: $F_{7, 64} = 30.42$, $P < 0.0001$; Carotenoids, Two-way ANOVA: $F_{7, 64} = 26.86$, $P < 0.0001$). Chl *a* and carotenoid concentrations per cell decreased with increasing light intensity for all four species. Growth under 20 µmol photons $\cdot m^{-2} \cdot s^{-1}$ led to increased chl *a* and carotenoids per cell, while the higher light treatments resulted in decreased amounts of both pigments per cell (Fig. 3 A and D).

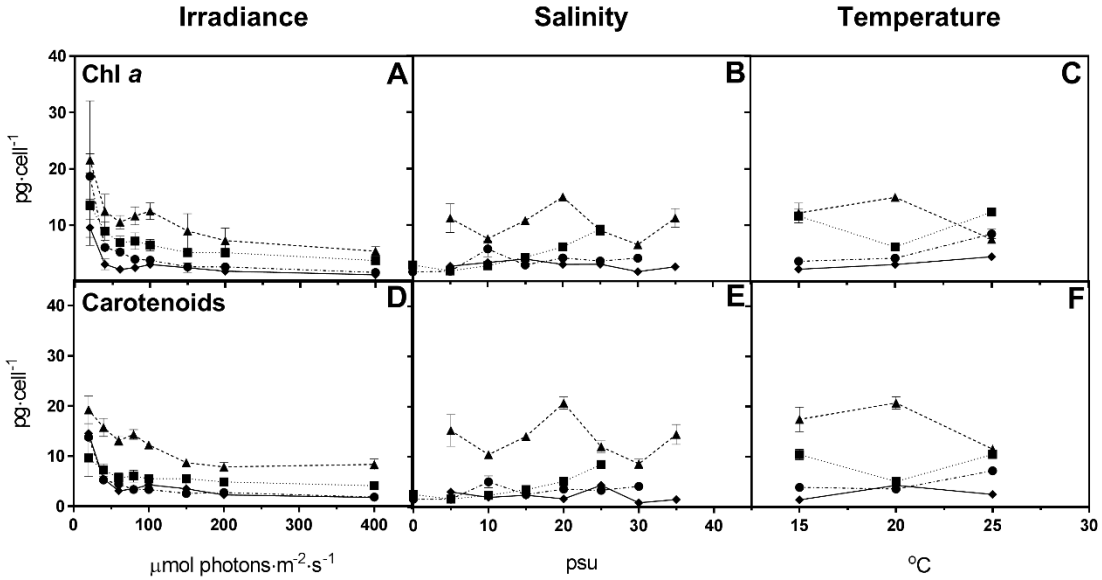

Figure 3. Cell concentration of two main pigment components of ChlorGL11 (circle), KirchGL02 (square), PrymGL20 (diamond) and SarcinGL07 (triangle). Chlorophyll *a* concentration per cell under (A) irradiance, (B) salinity and (C) temperature treatments. Carotenoid concentration per cell under (D) irradiance, (E) salinity and (F) temperature treatments. Vertical bars represent standard error mean of three replicates.

We observed an increase in chl *a* and carotenoid concentration per cell with salinity for KirchGL02 (Fig. 3 B and E; Two-way ANOVA: $F_{7, 64} = 30.42$, $P < 0.0001$). However, ChlorGL11 and PrymGL20 did not show significant variations in either chl *a* or carotenoid content when grown in different salinities ($P = 0.6766$). Chl *a* and carotenoids varied with salinity for SarcinGL07. A maximum peak for both pigments was observed at 20 psu for SarcinGL07.

While no significant differences in pigment concentration between temperature treatments was found using two-way ANOVA ($F_{2, 24} = 2.384$, $P = 0.1137$), planned multiple comparisons did reveal differences between treatments. Chlorophyll a and carotenoids were

the same for ChlorGL11 at 15°C and 20°C (Fig. 3 C and F; P > 0.9999). At 25°C chl *a* reached a significant maximum (P = 0.0045). KirchGL02 had maximum pigment levels for both pigment types at the temperature minimum and maximum treatments, with a significant decrease in pigments at 20°C (P = 0.0010). SarcinGL07 had elevated pigment levels at 15°C and 20°C, and decreased chl *a* and carotenoids at 25°C (P < 0.0060). PrymGL20 did not significantly vary pigments across the temperature treatments (P > 0.5000).

PSII Efficiency

The results of measurements of maximum photosynthetic efficiency and NPQ are shown in Figure 4. F_v/F_m remained relatively constant between light treatments for growth, however statistical significance was found between F_v/F_m values for each of the different species (Two-way ANOVA: $F_{3,63} = 33.82$, P < 0.0001). There were significant differences across salinity treatments for some of the species (Fig. 4 B; Two-way ANOVA: $F_{7,62} = 261.9$, P < 0.0001). ChlorGL11 had significant decreases in F_v/F_m at 25 and 30 psu (P = 0.0001) though KirchGL02 appeared to maintain its photosynthetic efficiency across the range of salinities. PrymGL20 had relatively constant F_v/F_m across salinities but had a significant drop in F_v/F_m at 5 psu (P = 0.0002). SarcinGL07 showed a slight increase in F_v/F_m with increasing salinity (P <0.0001). F_v/F_m was significantly affected by temperature treatments for two species (Fig. 4 C; Two-way ANOVA: $F_{3,24} = 23.89$, P < 0.0001). ChlorGL11 and PrymGL20 experienced statistically significant decreases in F_v/F_m at 15°C (P = 0.0117; P < 0.0001). In contrast, temperature had no effect on F_v/F_m of KirchGL02 (P = 0.9997) or SarcinGL07 (P > 0.9999).

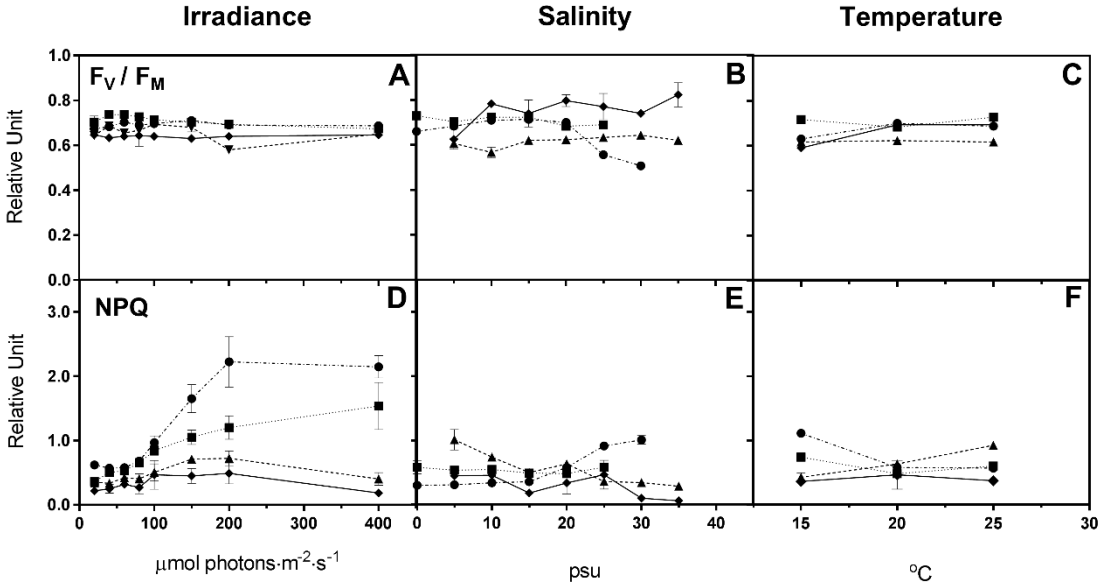

Figure 4. Photosynthetic efficiency (F_v/F_m) of ChlorGL11 (circle), KirchGL02 (square), PrymGL20 (diamond) and SarcinGL07 (triangle) under (A) irradiance, (B) salinity and (C) temperature treatments. Non-Photochemical Quenching (NPQ) of the four species of phytoplankton under a range of D) irradiance, E) salinity and F) temperature. Vertical bars represent standard error mean of three replicates.

Growth light intensity affected NPQ capacity (Two-way ANOVA: $F_{7, 63} = 20.08$, $P < 0.0001$); NPQ increased with increasing growth light levels for all species, however the greatest increase was seen in ChlorGL11 and KirchGL02 (Fig. 4 E). NPQ values differed significantly between species at light levels above 100 µmol photons $\cdot m^{-2} \cdot s^{-1}$ (Two-way ANOVA: $F_{7, 64} = 26.61$, $P < 0.0001$). PrymGL20 had the overall lowest NPQ values, while ChlorGL11 had the maximum value at high light treatments. Salinity affected NPQ differently for each species (Fig. 4 E; Two-way ANOVA: $F_{3, 63} = 26.61$, $P < 0.0001$). ChlorGL11, PrymGL20 and SarcinGL07 had increases in NPQ at suboptimal salinity for growth, while KirchGL02 did not show significant variation in NPQ across salinity treatments. Temperature did not have significant impacts on NPQ (Fig. 4 F; Two-way ANOVA: $F_{2, 24} = 2.83$, $P = 0.0788$). KirchGL02 and PrymGL20 did not significantly vary its NPQ between temperature treatments. SarcinGL07 increased NPQ with increasing temperature with maximal NPQ value at 25°C ($P = 0.0026$).

Photosynthesis and Dark Respiration

No significant differences between light treatments were found for maximum photosynthetic rate (P_{max}; Fig. 5 A; Two-way ANOVA: $F_{7, 63} = 1.195$, $P = 0.3186$). However,

there were significant differences in P_{max} between species (Two-way ANOVA: $F_{3, 63} = 98.79$, $P < 0.0001$). SarcinGL07 had the highest observed P_{max} across all light treatments, while ChlorGL11, and PrymGL20 had similar, low, P_{max} values. Dark respiration for all four species did not vary greatly with increased irradiance, however R_d varied between species, with PrymGL20 having the highest rate (Fig. 5 D; Two-way ANOVA: $F_{3, 61} = 76.77$, $P < 0.0001$). Alpha decreased with increasing light treatment for all species, except PrymGL20 which remained constant (Fig. 5 G; Two-way ANOVA: $F_{7, 62} = 21.28$, $P < 0.0001$). The opposite trend was observed for I_k (Fig. 5 I; Two-way ANOVA: F7, 64 = 18.99, $P < 0.0001$).

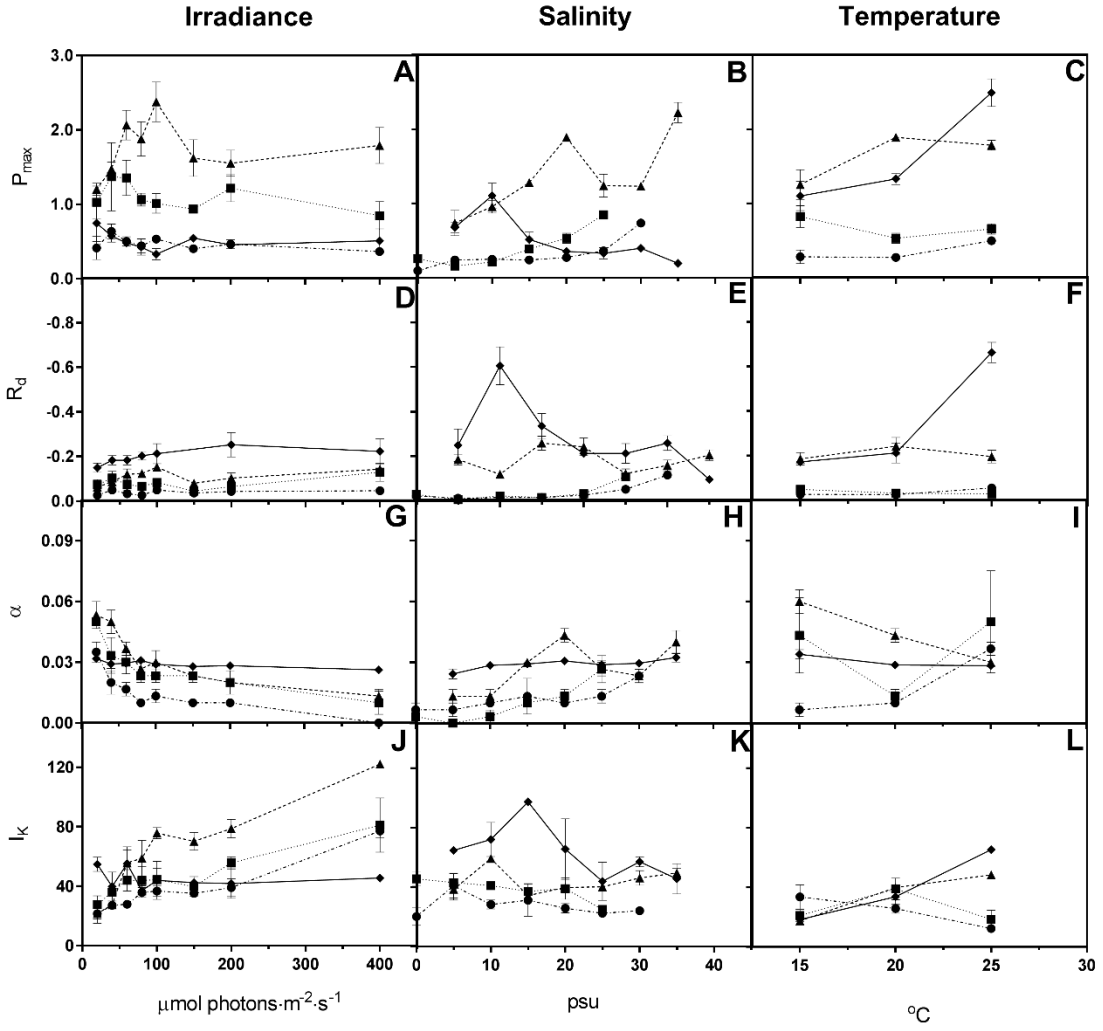

Figure 5. P_{max} and dark respiration in ChlorGL11 (circle), KirchGL02 (square), PrymGL20 (diamond) and SarcinGL07 (triangle), during exponential phase. (A-C) Maximum photosynthetic rate, P_{max} (nmol O_2 $(10^6 \cdot \text{cells})^{-1} \cdot \text{min}^{-1}$); (D-F) Dark respiration, R_d (nmol $O_2 \cdot (10^6$ cells$)^{-1} \cdot \text{min}^{-1}$); (G-I) Light harvesting efficiency, α (nmol $O_2 \cdot (\mu\text{mol photons} \cdot \text{m}^{-2})^{-1} \cdot (10^6 \text{ cells})^{-1}$); (J-L) Light saturation parameter, I_K (μmol photons·m^{-2}·s^{-1}); grown under a range of irradiance, salinity and temperatures. Vertical bars represent standard error mean of at least three replicates.

P_{max} was significantly affected by salinity (Two-way ANOVA: $F_{7, 64} = 45.5$, $P < 0.0001$). With increasing salinity, ChlorGL11, KirchGL02, and SarcinGL07 showed increased P_{max} (Fig. 5 B). PrymGL20 showed an opposite trend, with a maximum P_{max} between a salinity of 5-10 psu, followed by a decrease in P_{max} with increasing salinity. SarcinGL07 had significantly higher maximum photosynthetic rates at higher salinities, compared to the other species (Two-way ANOVA: $F_{7, 64} = 45.5$, $P < 0.0001$). Dark respiration followed the same significant trend observed for P_{max} for ChlorGL11 and KirchGL02 (Two-way ANOVA: $F_{7, 64} = 15.69$, $P < 0.0001$): increasing R_d with salinity (Fig. 5 E). SarcinGL07 R_d varied over the range of salinities with maxima observed at 15 and 20 psu. PrymGL20 had a maximal R_d value at 10 psu, which was 5-fold larger than that for the two chlorophytes and slightly higher than that of SarcinGL07 (Two-way ANOVA: F3, 64 = 123.5, $P < 0.0001$). Alpha values for all species were typically significant lower in the low saline treatments and highest in the brackish to saline treatments (Fig. 5 H; Two-way ANOVA: $F_{7, 64} = 22.33$, $P < 0.0001$). The I_k values significantly varied between salinity treatments and species, but no clear trend was apparent (Fig. 5 J; Two-way ANOVA: $F_{7, 64} = 3.734$, $P = 0.0019$; Two-way ANOVA: $F_{3, 64} = 45.5$, $P < 0.0001$).

P_{max} varied with temperature and species (Fig. 5 C; Two-way ANOVA: $F_{2, 24} = 18.39$, $P < 0.0001$; Two-way ANOVA: $F_{3, 24} = 95.08$, $P < 0.0001$). ChlorGL11 showed a gradual increase in P_{max} with temperature, while KirchGL02 had maximal P_{max} at 15^{o}C and decreased P_{max} at the other two temperature treatments. SarcinGL07 and PrymGL20 both had higher P_{max} than the two chlorophytes at all temperatures. Maximum P_{max} values for SarcinGL07 and PrymGL20 were attained at high temperatures. There was significant increase in R_d with increasing temperature for PrymGL20 (Fig 5 F; Two-way ANOVA: $F_{2, 24} = 28.78$, $P < 0.0001$). However R_d in ChlorGL11, KirchGL02, and SarcinGL07 did not vary significantly. SarcinGL07 and PrymGL20 had much higher rates of Rd compared to ChlorGL11 and KirchGL02 (Two-way ANOVA: $F_{3, 24} = 105.1$, $P < 0.0001$). Alpha (Fig 5 I) was not significantly influenced by temperature treatment (Two-way ANOVA: $F_{2, 24} = 2.264$, $P = 0.1256$), while I_k (Fig 5 L) was affected by temperature (Two-way ANOVA: $F_{2, 24} = 11.99$, $P = 0.0002$).

DISCUSSION

Effects of Irradiance

From our results, it was clear that irradiance affected both growth and photophysiology. When grown under light limitation, the rate of photon capture was too low to sustain the maximum growth rate. As a result growth rates of the four species studied increased with increasing light. This occurred until an increase in irradiance no longer resulted in an observed increase in growth, a common pattern observed in algae when grown under increasing light levels (Taylor et al., 2001). In all four cultures, photosynthetic efficiency, as evident by stable F_v/F_m values, was maintained over all the light treatments, indicating a fully functional reaction centre for photosystem II. The maintenance of photosynthetic ability across a range of light intensities suggests the use of an effective photoacclimation strategy as a way to acclimate to the different light conditions. To capture more light for photosynthesis, the cells increased chl *a* and carotenoid content, a well-documented adaptive response to light limitation (Beardall and Morris, 1976; MacIntyre et al., 2002; Richardson et al., 1983). The observed increase in chl *a* and carotenoid content under light limiting growth conditions in the species studied is supported by low α values. α is an indication of how efficient plants and algae are at harvesting light (Henley, 1993), and under low light there are limited photons available for capture, so the ones that are available need to be captured efficiently. Under the high light treatments there is a surplus of photons available to the cell, therefore the cell is not required to work as efficiently at capturing the photons, resulting in a decrease in α, which was observed in all four species.

Photoautotrophic organisms require light, however too much light can cause damage to both acceptor and donor components of PSII reaction centres (Vassiliev et al., 1994). Under high light treatments (150 to 400 µmol photons $m^{-2} \cdot s^{-1}$), observed increases in NPQ values and carotenoid:chl *a* ratios (data not shown), with increasing light, demonstrates the cells' capacity for dissipating excess energy which can cause physiological stress on the cell, or a reduction in growth rate due to photoinhibition. Managing excess light is achieved through implementing a photoprotective mechanism, NPQ, which dissipates excess energy as heat (Müller et al., 2001). This is possibly achieved through the xanthophyll cycle involving light-triggered concentration changes in certain carotenoids (Demmig-Adams and Adams,

1996). The two chlorophyte strains used in this study had significantly higher levels of NPQ when growth light was high, compared to PrymGL20 and SarcinGL07. Differences in NPQ capacity between species has been linked to differences in pigment composition and photosynthetic apparatus in algae (Casperlindley and Björkman, 1998).

Effects of Salinity

The experimental data demonstrated that most of the species examined were broadly tolerant of light and temperature, while salinity tolerance was more species specific. Salinity is an important abiotic factor which can limit algal distribution (Ahel et al., 1996; Maier Brown et al., 2006). Both SarcinGL07 and PrymGL20 grew better under higher salinities, while the two chlorophytes, KirchGL02 and ChlorGL11, grew well under lower salinities. In fact, all species were unable to acclimate and grow under the lower (SarcinGL07 and PrymGL20) and upper (ChlorGL11 and KirchGL02) salinity limits to which they were subjected.

The effects of salt stress were not only reflected in growth rate data, but also changes observed in PSII variable fluorescence; congruent to what has been previously reported in other algal species and higher plants (Sudhir and Murthy, 2004). A decrease in photosynthetic efficiency when cells experience salt stress was observed in this and other studies (see review by Kirst, 1989) and can be caused by disruption of energy transfer to one or both photosystems (Ferroni et al., 2007). Varying salinity did affect P_{max} and R_d in all species, though the response was species specific. Joset et al. (1996) reported increases in respiration rates in cyanobacteria that experienced salt stress. This was found for the species tested in this study, as respiration rates increased at suboptimal salinities. A possible explanation is the pumping of excess sodium against the concentration gradient which requires energy, part of it in the form of ATP (Joset et al., 1996; Parida and Das, 2005).

Effects of Temperature

Under the three temperature treatments tested, all four species were able to maintain growth, indicating a general tolerance and adaptability to changes in temperature between 15^{o}C to 25^{o}C. The range of temperatures used in this study is typically encountered in the Gippsland Lakes (Fryer and Easton, 1980). ChlorGL11, PrymGL20 and SarcinGL07, increased their growth rates with increasing temperature. Sorokin and Krauss (1962), Baker et al. (2007) and Mills (1972) have described comparable temperature ranges for each of

these species respectively. KirchGL02 maintained similar growth rates between 15-20°C, but at 25°C had decreased growth rates, which was also mirrored in its maximum photosynthetic capacity. Our results are the first report of decreased growth rates at higher temperature for *Kirchneriella* sp. Previous studies of *Kirchneriella* abundance and growth have only been reported in tropical lakes throughout Africa (Ferroni et al., 2007). This could be suggestive of the species' adaptability to different environmental conditions or simply represents the characteristics of the particular strain isolated from the Gippsland Lakes.

The increase of P_{max} observed in PrymGL20 and SarcinGL07 at 20°C and 25°C respectively, indicates the ability of these species to effectively photosynthesize in warmer waters, typically experienced during the summer in the Gippsland Lakes. The enhanced photosynthesis could contribute towards the observed increase in growth rate with temperature for these two species, though growth rate is not simply a direct reflection of photosynthesis (Evans, 2013). On the contrary, the increase in growth rate with temperature observed in ChlorGL11 was not reflected in its P_{max} and R_d. It is clear from this study that the strains of the four species we examined have the ability to grow and photosynthesize over the range of temperatures encountered in the Gippsland Lakes. From our results KirchGL02 has the competitive advantage over the other species when temperatures are cooler, but once the lakes warm up it could swiftly be out-competed by the other species' photosynthetic and growth enhancement in warmer conditions. The temperate environment in which the lakes are situated allows for a diverse range of species/strains to coexist throughout the year. However, with the threat of global warming in the coming decades, the Gippsland Lakes could see a decrease in the diversity of microalgae that are competitively advantageous in cooler conditions.

SarcinGL07 Summary

With very limited data on *Sarcinochrysis* sp., this study highlights several interesting characteristics of this uncommon species. SarcinGL07 grew rapidly and had a two- to three-fold higher growth rate under most conditions compared to the other species studied. It appears to be well adapted to high light levels (150-400 µmol photons $m^{-2} \cdot s^{-1}$), which can likely be contributed to the high carotenoid content of the cell. Several types of carotenoids are known to act as a protective mechanism under exposure to high light (Demmig-Adams

and Adams, 1996). From the observed growth rates under the different salinities, it is clear that SarcinGL07 is best suited to brackish and marine environments, as at lower salinities it had significantly reduced growth rates and was unable to grow in freshwater. Mills (1972) found similar results with *Sarcinochrysis edaphica* with most growth occurring between 22-29 psu. The preference of SarcinGL07 for warmer waters, is shown through a large increase in growth rate from 15^{o}C to 20^{o}C, similar to temperatures (18 to 25^{o}C) found by Mills (1972).

The rapid growth rates for SarcinGL07 observed in this study, are conditions typically experienced in the Gippsland Lakes. With no previous reports of this species occurrence in the Gippsland Lakes, and very rare reports of *Sarcinochrysis* species globally (Mills, 1972), it begs the question as to what constraints are preventing it from producing high levels of biomass throughout the Gippsland Lakes. From looking at its growth rate and photophysiology observed in this study, SarcinGL07 rapid growth and ability to maintain photosynthetic rates under a range of environmental conditions is suggestive that species would be a good competitor. Therefore we hypothesize that external pressures due to its preferential selection by grazers could be keeping SarcinGL07 biomass in check. Previous studies have found that zooplankton grazers can preferentially graze on select phytoplankton species, leading to a competitive advantage to those species that are avoided (Holland et al., 2012; Porter, 1973). High nutritional value of certain species over others (Ahlgren et al., 1990), poor digestion, cell size (Bartram, 1980), as well as unpredictable occurrences of species (Holland et al., 2012) are several factors thought to cause selective grazing.

Moreover, when grown under two different nitrogen sources, *Sarcinochrysis edaphica* has been shown to exhibit better growth when grown with nitrate, as used in this present study, while ammonium caused lower growth rates (Mills, 1972). The main form of nitrogen in the Gippsland Lakes can vary between ammonium and nitrate; the dominant form dependent on inflow from surrounding tributaries and anoxic bottoms waters (Holland et al., 2010). Future studies on macromolecular composition and nutrient impacts on growth is needed to elucidate the controlling factors on growth.

Bloom Formation Scenarios

Reports on *Chlorella* blooms are rare, and the ones that are reported have not been shown to have largescale negative impacts on the environment (Asha et al., 2015). Our

culture of ChlorGL11 had high growth and productivity under high light, saline conditions between 10 to 20 psu, and warmer temperatures. Ideal conditions in the Gippsland Lakes for high biomass to occur would therefore be during summer during a wetter year when salinity in the lakes remains low. As Lake Wellington is the freshest basin of the three main lakes, it would be the most likely location in this estuarine system that this species can thrive in. Chlorella sp. has been reported in Lake Wellington during spring and summer periods, however no significant level of biomass to classify as a bloom has been reported (Jonathan Smith, unpublished data).

Various species of *Kirchneriella* have generally been reported and isolated from freshwaters globally (da Silva et al., 2013; Ferroni et al., 2007; Soylu and Gönülol, 2010). To our knowledge, there are no reports of major blooms from this species. Our study is the first to study a brackish water isolate. From our results, it is clear that while this species can survive in saline conditions up to 25 psu, it is better adapted to less saline conditions. Similar to ChlorGL11, KirchGL02 would most likely thrive in Lake Wellington, or inlet basins throughout the lakes, which are fed by nearby rivers. The observed decrease in growth rate at higher temperatures was unexpected, as this species is typically recorded in tropical latitudes (Ferroni et al., 2007; Frampton et al., 2013). However, the strain we used may have adapted to the cooler water temperatures during the spring, typically when the Gippsland Lakes experience influx of freshwater (Cook et al., 2010).

Results from this and other studies (Baker et al., 2007; Larsen and Bryant, 1998) found that ideal bloom conditions for *P. parvum* would be those conditions found in an estuarine system, during summer with warm water temperatures. The strain isolated from the Gippsland Lakes was slower growing than the isolate of *P. parvum* from the Colorado River (Baker et al., 2007), which achieved maximum growth rates of 0.94 d^{-1}. Maximal growth rates of approximately 0.5 d^{-1} were achieved by our strain under high light, salinity ranging between 15-35 psu and warmer temperatures. Blooms of *Prymnesium* sp. have been reported in both brackish estuarine and coastal systems (Johansson and Granéli, 1999; Otterstrøm and Nielsen, 1939), as well as in freshwater lakes and rivers (Baker et al., 2007). Observations from this study and previous research clearly demonstrate that *P. parvum* is extremely euryhaline. The plasticity of PrymGL20 to grow under wide range of salinity, would allow this species to occur throughout most of the Gippsland Lakes. Particularly, Lake King

and Victoria would be ideal environments as salinities in these lakes range between 20 to 30 psu. With significantly reduced growth rates at low temperatures, PrymGL20 would be less likely to bloom during winter and spring, when water temperature in the lakes range between 10-15^{o}C.

CONCLUSIONS

The present study provides an understanding of how four different species of phytoplankton grow under a range of light, salinity and temperature conditions. While the algal isolates are specifically from the Gippsland Lakes, and each species here is represented by only one strain, the results can be related to other systems with similar environmental conditions. Despite the differences in growth and physiology observed between the species, similar photoacclimation strategies were utilized by all four species when grown under low and high light conditions. SarcinGL07 grew rapidly, faster than the other three species, under almost all of the treatments conditions. Despite its rapid growth, no blooms of SarcinGL07 have been reported in the lakes. Further studies on nutrient requirements and grazing could therefore be informative. This study demonstrated that salinity is an important parameter for controlling growth of all four species at their suboptimal levels. The two chlorophytes are competitively advantageous in less saline conditions, however potential blooms from either species is unlikely. The observed conditions that promoted high growth from the toxic PrymGL20 in this study, raises concerns of the potential bloom threat in the future, to the Gippsland Lakes.

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Chapter 4

CHARACTERIZING COMPOSITIONAL CHANGES IN RESPONSE TO KEY ENVIRONMENTAL DRIVERS IN FOUR MICROALGAE ISOLATED FROM A BRACKISH LAKE SYSTEM

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ABSTRACT

Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) spectroscopy was used to assess changes in macromolecular composition of microalgae, under changes in light, salinity and temperature. The aim of this work is to provide a better understanding of microalgae acclimation to key environmental drivers, mainly light, salinity and temperature. Four recent isolates of algal species, from a eutrophic, estuarine system in SE Australia, were grown in batch monocultures under 7 light, 7 salinity and 3 temperature treatments. Characterization of changes in macromolecular composition was completed by obtaining ATR-FTIR spectra of cells in exponential growth. Deviations from optimal growth conditions for light, temperature and salinity resulted in distinct spectral profiles for each species. These differences were seen in most spectral band regions, representing the major macromolecules. Principal Component Analysis (PCA) revealed specific clustering among the treatment levels, which varied between species. Generally, lipids increased under suboptimal growth conditions, while increased proteins under optimal growth conditions for *Kirchneriella* sp. and *Sarcinochrysis* sp. *Prymnesium parvum* displayed a high degree of cellular homeostasis, which we attributed to its mixotrophic behaviour. The results indicate species specific strategies for coping with changes in the environment. The observed reallocation of carbon into different macromolecules can not only impact the individual cell, but also the energy transferred to higher trophic levels.

ABBREVIATIONS

ATR-FTIR, Attenuated Total Reflectance-Fourier Transform Infrared

EMSC, Extended Multiplicative Signal Correction

FTIR, Fourier transform infrared

PCA, Principal component Analysis

SIMCA, soft independent modelling by class analogy

INTRODUCTION

The rapid change in the global environment owing to the impacts of anthropogenic climate change is unprecedented. Therefore, investigating of the effects of climate change on the aquatic environment will increase our understanding of how to better conserve these important ecosystems. The research presented in this article focusses on the impacts of different environmental conditions on the lowest trophic level, microalgae, isolated from a brackish lake system. Coastal waters, including estuaries and brackish lakes, already experience drastic fluctuations in water column light, stratification, temperature, salinity, and eutrophication (Cook et al., 2010; Day et al., 2012), however climate change is expected to exacerbate these changes further (Harley et al., 2006; Rabalais et al., 2009). Microalgae living in these ecosystems need the capability to adapt to these fluctuations, otherwise shifts in community structure may occur (Hays et al., 2005; Scheffer et al., 2001).

Microalgae play a vital role in aquatic ecosystems, as they form the base of most aquatic food webs. Through their photosynthetic ability, the energy that they produce is transferred further up the food chain. It is estimated that they are responsible for approximately 50% of global primary production (Field et al., 1998). The energy phytoplankton create through photosynthesis in combination with nutrients acquired from the surrounding environment play an important role in forming macromolecules, such as proteins, lipids, carbohydrates and phosphorylated molecules. Proteins and carbohydrates are synthesized for wide range of uses including osmoregulation, energy storage, buoyancy regulation, cell wall maintenance, and membrane structure (Dickson and Kirst, 1987; Finkel et al., 2016; Romans et al., 1994), while the main functional properties of lipids include structural components of the cell wall and energy storage (Guschina and Harwood, 2009). However, carbon allocation to specific macromolecules can be disrupted when the cell experiences perturbations in its external environment (Dickson and Kirst, 1987; Kamalanathan et al., 2015; Rhee, 1978).

Phytoplankton energy acquisition, primary production, can be affected by a variety of factors including: temperature, light, salinity, nutrient availability and even cell size (Beardall and Raven, 2004; Finkel et al., 2009; Malerba et al., 2017; Sackett et al., 2013). Naturally, microalgae endeavor to achieve constant internal compositional homeostasis, as under some

conditions it is competitively advantageous to maintain a constant internal organic composition (Montechiaro and Giordano, 2010). However, when perturbation in environmental conditions beyond a species optimal growth or physiological threshold occurs, the cells may reorganize macromolecular composition or allocate resources into different molecular pools, in order to minimize the impact on their growth and reproduction potential. The redistribution of internal carbon into different macromolecules is possible as the allocation of carbon into the various macromolecules is not energetically equivalent (Halsey and Jones, 2015). Microalgae have been observed to show high flexibility with carbon allocation patterns (Giordano et al., 2017) with the duration of changed growth conditions determining whether the cells regulate, acclimate or adapt to the altered condition (Giordano, 2013). Under exposure to stress conditions, phytoplankton have been observed to switch from making complex molecules like proteins, to simpler ones like carbohydrates or lipids (Palmucci et al., 2011), as proteins contain nitrogen which is difficult to obtain and get rid of, and it is easier for the cells to extract energy from lipids and proteins. The resulting changes in macromolecules can affect the amount or quality of energy phytoplankton produce, causing a cascading effect on the export production to higher trophic levels, as well as biogeochemical cycling (Finkel et al., 2016).

Over the last few decades, FTIR spectroscopy has become an emergent technique for studying macromolecular composition in microalgae (Beardall et al., 2001; Giordano et al., 2001; Heraud et al., 2007, 2005, Sackett et al., 2015, 2014). FTIR spectroscopy uses mid-infrared light, to characterize macromolecular composition of biological cells and tissues compounds using characteristic absorbance bands in the infrared spectrum. Combining FTIR spectroscopy with multivariate data analysis, researchers are able to obtain a snapshot of the metabolic profile in response to environmental growth conditions, and can be used to model data trends (Sackett et al., 2015). Attenuated Total Reflectance FTIR (ATR-FTIR; Blum & John, 2012), which couples an ATR accessory to a FTIR spectrometer has the potential for better sample-to-sample reproducibility and less user-to-user spectral variation than transmission measurements and has been employed in cyanobacterial and microalgal studies (Bounphanmy et al., 2010; Dao et al., 2017).

The use of FTIR spectroscopy as a tool in algal research continues to expand, being applied to a variety of scenarios. FTIR spectroscopy has been used in several studies to

measure changes in macromolecular composition of cells under different nutrient status (Beardall et al., 2001; Giordano et al., 2001; Heraud et al., 2008, 2005; Hirschmugl et al., 2006; Sackett et al., 2014). Dao et al. (2017) investigated the impacts of lead toxicity in two green species of microalgae and found the FTIR technique suitable for the detection of metal-contaminated cells. Additionally, FTIR spectroscopy has been used to classify both microalgae and bacteria (Kansiz et al., 1999; Mariey et al., 2001). Recently, Kamalanathan et al. (2018) were able to detect and predict cells that were exposed to oil and dispersant, highlighting the importance of this technique in monitoring the impact of future oil-spills. When combined with a microscope (FTIR microspectroscopy), the potential increases further, allowing researchers to look at changes in macromolecular structure of single cells in natural populations (Dean et al., 2012, 2007).

The aim of this study was to evaluate the impacts of changing environmental conditions on the macromolecular composition of four recent isolates of microalgae from a brackish lake system in southeast Australia. We utilized the rapid analysis of ATR-FTIR to obtain high resolution spectra which were processed using a multivariate analysis to detect the changes in macromolecular composition. Specifically, we investigated how changing light, salinity and temperature caused shifts in cellular macromolecular composition for each species. In particular we evaluated if all three treatments (light, salinity and temperature) would elicit similar changes in macromolecular composition in each of the species, as well as assessing whether if the changes observed in macromolecular composition were species specific.

MATERIALS AND METHODS

Species and growth conditions

Recent algal isolates from the Gippsland Lakes, namely *Chlorella* sp. (ChlorGL11), *Kirchneriella* sp. (KirchGL02), *Prymnesium parvum* (PrymGL20), and *Sarcinochrysis* sp. (SarcinGL07), were grown in batch cultures in a multi-cultivator (MC1000, Photon Systems Instruments, Czech Republic) as described previously by Wartman et al. (under review). Briefly, cells were grown under 7 irradiance, 7 salinity, and 3 temperature treatments. The algae were grown under continuous light and collected for analysis in mid-exponential phase. Triplicate cultures were used for each treatment.

Sample preparation

A volume of culture between 20 and 50 mL was collected and centrifuged (3000g, 10 minutes; Heraeus Multifuge 3SR+, Thermo Scientific). The pellet was washed three times with Milli-Q grade water. Once washed, the pellet, along with 15 µl of Milli-Q grade water, was frozen at -20^{o}C until analysis. Just prior to analysis, the pellet was defrosted and 3 µl of cells were pipetted onto a glass microscope slide (Menzel-Gläser, Lomb Scientific Pty Ltd, Taren Point, NSW). There were 5 to 10 pseudo replicates pipetted onto the slide, a procedure which was repeated for each of the three replicates. The slide was then placed in a dehydrator for 24 hours in the dark.

ATR-FTIR spectroscopy

Prior to measuring the absorbance spectra of each dried sample of cells on the slide, a blank background of the cleaned diamond window was taken. The spectrometer was controlled by OPUS (OPUS Spectroscopy Software, Bruker, Billerica, MA, USA). The slide was then placed cell-side down onto the sample stage of the FTIR spectrometer (Burke Equinox 55; Bruker Optics Inc., Etlingen, Germany) which was coupled to an attenuated total reflective (ATR) accessory (Golden Gate; Thermo Fisher Scientific, Inc, Waltham, MA, USA) and cooled using a liquid nitrogen cooled MCT detector. The absorbance spectra were collected at a spectral resolution of 8 cm^{-1} with 50 scans between 4000 cm^{-1} to 600 cm^{-1}. Between 5 and 10 spectra were taken for each replicate culture sample. An ATR correction was then completed on the absorbance spectra in OPUS. The extended ATR correction that

was applied was for a diamond ATR window using an internal reflection with a 45^{o} angle of incidence of IR beam and 1.5 mean refraction index of the sample (Smith, 2011). The ATR correction algorithm applied to the data corrects for band intensity distortion, peak shifts, and non-polarization effects.

Chemometrics

ATR corrected spectra were analysed using Unscrambler®X 10.3 software (Camo, Oslo, Norway). Spectral pre-processing, multivariate analysis and macromolecular estimation was followed using the methods described in Dao et al. (2017). Briefly, using the Savitsky-Golay algorithm, a second derivative (3 point polynomial) with 9 points of smoothing was calculated. Derivatizing the ATR corrected spectra not only smooths the data, but also increases the complexity of the derivative spectra which aids in qualitative analysis by revealing significant differences potentially not seen in the raw absorbance (Owen, 1995). Using the second derivative, Extended Multiplicative Signal Correction (EMSC) was applied to adjust for differences in sample thickness (Heraud et al., 2006). A Principal Component Analysis (PCA) was then performed in the biologically active regions (3050-2800 cm^{-1} and 1800-800 cm^{-1}) with mean centered data.

Scores plots were created to visualize clustering of data, while loadings plots were used to determine the spectral region that contributed most to the variance seen in the scores plot. Data points that are positively scored (right side of PCA scores plot), correspond to negative loadings (right side of bar graph. For ease of interpretation the negatively scored loadings values (bar graph) have been plotted on the right, to correspond to the positively scored values in the PCA plot, due to derivatization of the spectral data). The outcome of taking the second derivative of the spectral data results in a negative band with minimum at the same wavelength as the maximum on the absorbance spectra (Owen, 1995). Therefore, data points of the left side of the PCA scores plot saw increases in concentration of the macromolecules of the specific bands which had loadings on the left side of the bar graph, and vice versa for the right hand side of both the scores and loadings plot.

Integrated peak area calculations

Estimation of relative macromolecular concentrations was done by calculating peak area, according to the Beer- Lambert Law, where intensities of the bands, assigned to their functional group, are directly proportional to their concentration (Guillen and Cabo, 1997). Calculations of peak area was performed on 2nd derivative data. As the absorbance spectrum obeys Beer's law, there is a similar linear relationship between concentration and amplitude for 2nd order derivative (Owen, 1995). For relative lipid estimation we used the calculated peak area assigned to wave number 1750 cm^{-1}, for proteins we used the sum of amide I (1650 cm^{-1}) and amide II (1520 cm^{-1}) peak areas, and for carbohydrates we used the calculated peak area at 1155 cm^{-1}. The variation among peak area means in relation to light, salinity and temperature treatments was tested using two-way ANOVA followed by Tukey's multiple comparison test. All analyses were carried out using GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA), with a significance level of $P < 0.05$.

RESULTS

This study endeavored to detect shifts in macromolecular composition of four phytoplankton species in response to varying environmental conditions. Spectra were taken for each of the four algal species under the various treatments during mid-exponential phase. We used Principal Component Analyses (PCA), an unsupervised multivariate analysis method, to interpret the changes in chemical composition that occurred under light, salinity and temperature treatments.

Macromolecular 2nd Derivative Spectra

The averaged processed second derivative spectra of cells grown under optimal growth conditions, high light (200 µmol photons · m^2 · s^{-1}), high salinity (25 psu) and low temperature (15^{o}C) treatments for ChlorGL11 (Fig 1A), KirchGL02 (Fig. 1B), PrymGL20 (Fig. 1C) and SarcinGL07 (Fig. 1D), showed strong absorbance for functional groups of proteins (1650 - 1520 cm^{-1}), lipids (1750 cm^{-1}), phosphorylated molecules (1235 cm^{-1}), and carbohydrates (1200 - 900 cm^{-1}). Specific band assignments are shown in Figure 1 and can be found summarized in Table 1. There was clear variation in absorbance by most functional groups between treatments (light, temperature and salinity) for each species. This is suggestive of changes in macromolecular composition under the different growth regimes.

Table 1. Band assignment for major bands present in the spectra of the algae in this study.

Wavenumber (cm^{-1})	Functional Group	Biological molecule
~ 3010	C=C–H	Unsaturated fatty acids
~ 2960	υ_{as} (CH_3)	Mainly methyl groups from lipids
~ 2920	υ_{as} (CH_2)	Saturated fatty acids
~ 2850	υ_{s} (CH_2)	Saturated lipids
~ 1750	υ_{s} (C = O)	Ester carbonyls from lipids and fatty acids
~ 1655	υ (C = O)	Amide I from proteins (α-helices)
~ 1620	υ (C = O)	Amide I from proteins (β-pleated sheets)
~ 1540	υ_{as} (C-H) & δ(N–H)	Amide II from proteins
~ 1515	υ_{as} (C-H) & δ(N–H)	Phenol Group
~ 1450	δ_s CH_3 and δ_s CH_2	Methyl/ Methylene (proteins)
~ 1380	δ_s (C-H) of CH_3 or CH_2	Methyl (protein)
~ 1235	υ_{as} P = O	Phosphorylated compounds
~ 1155	υ (C-O-C)	Polysaccharide (carbohydrates)
~ 1080	υ (C - O) or υ_{s} (P = O)	Polysaccharide (carbohydrates) Stretching phosphodiester
~ 1050	υ (C-O-C)	Polysaccharide (carbohydrates)
~ 1020	υ (C-O-C)	Polysaccharide (carbohydrates)

υ_{as}, asymmetric stretch; υ_{s}, symmetric stretch; δ_{as} asymmetric deformation (bend); δ_{s} symmetric deformation (bend).
Band Assignments based on (Bandekar, 1992; Giordano et al., 2001; Heraud et al., 2005; Vongsvivut et al., 2012; Wong et al., 1993; Zeroual et al., 1994)

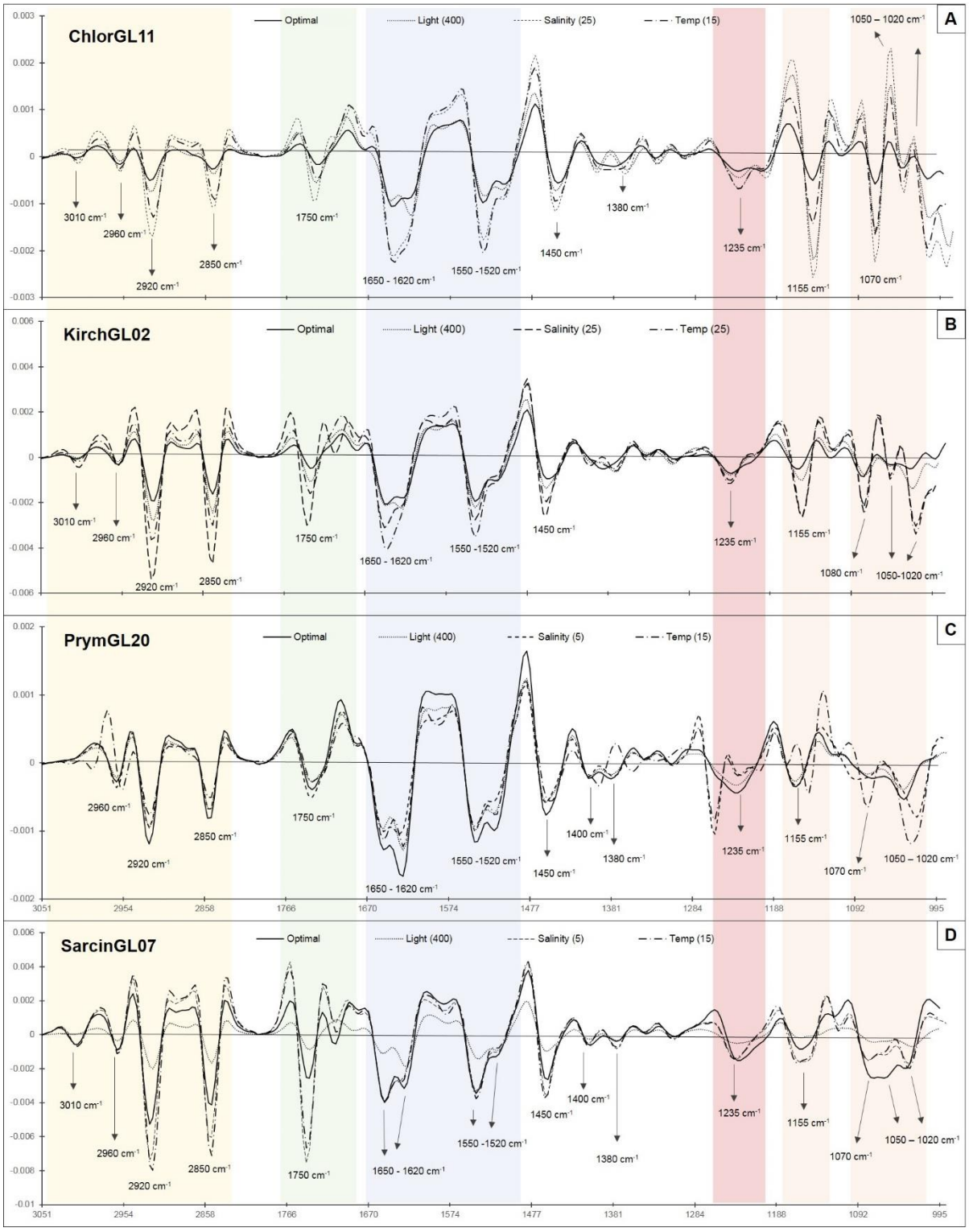

Figure 1. Averaged second derivative spectra of the biologically relevant bands (3055 cm^{-1} – 950 cm^{-1}) for ChlorGL11 (A), KirchGL02 (B), PrymGL20 (C) and SarcinGL07 (D) for optimal (solid line), light stressed (dotted line), salinity stressed (dashed line) and temperature stressed (dot and dash line) growth conditions.

Principal Component Analysis Figures

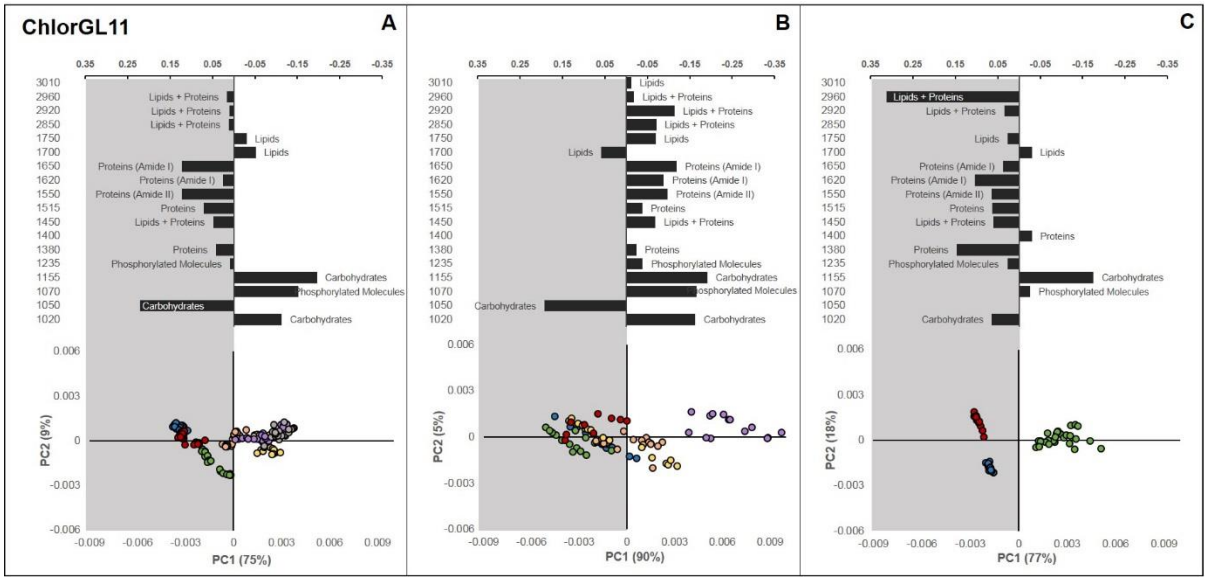

Figure 2. ChlorGL11 ATR-FTIR spectroscopy data showing Principal Component Analysis (PCA) and loadings plot for (A) light treatments 40 (blue), 60 (red), 80 (green), 100 (orange), 150 (yellow), 200 (purple) and 400 µmol photons • m^2 • s^{-1} (grey); (B) salinity treatments 0 (blue), 5 (red), 10 (green), 15 (orange), 20 (yellow), 25 (purple) and 30 psu (grey); (C) temperature treatments 15 (blue), 20 (red), and 25°C (green). For ease of interpretation the negatively scored loadings values (bar graph) have been plotted on the right, to correspond to the positively scored values in the PCA plot, due to derivatization of the spectra.

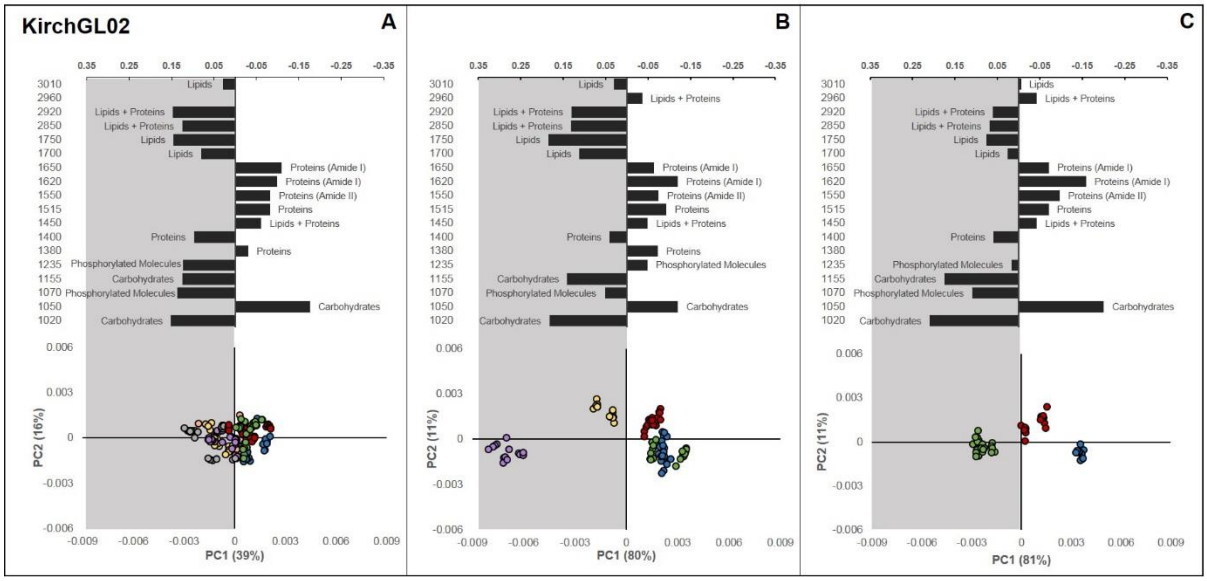

Figure 3. KirchGL02 ATR-FTIR spectroscopy data showing Principal Component Analysis (PCA) and loadings plot for (A) light treatments 40 (blue), 60 (red), 80 (green), 100 (orange), 150 (yellow), 200 (purple) and 400 µmol photons • m^2 • s^{-1} (grey); (B) salinity treatments 0 (blue), 5 (red), 10 (green), 15 (orange), 20 (yellow), and 25 psu (purple); (C) temperature treatments 15 (blue), 20 (red), and 25°C (green). For ease of interpretation the negatively scored loadings values (bar graph) have been plotted on the right, to correspond to the positively scored values in the PCA plot, due to derivatization of the spectra.

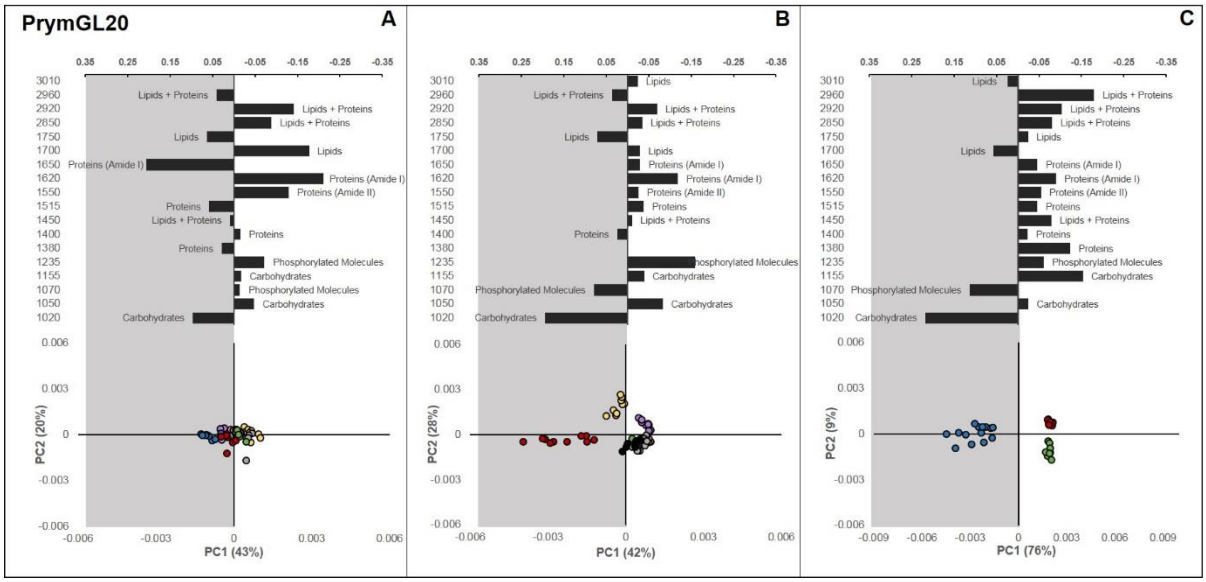

Figure 4. PrymGL20 ATR-FTIR spectroscopy data showing Principal Component Analysis (PCA) and loadings plot for (A) light treatments 40 (blue), 60 (red), 80 (green), 100 (orange), 150 (yellow), 200 (purple) and 400 µmol photons • m^2 • s^{-1} (grey); (B) salinity treatments 5 (red), 10 (green), 15 (orange), 20 (yellow), 25 (purple), 30 (grey) and 35 psu (black); (C) temperature treatments 15 (blue), 20 (red), and 25°C (green). For ease of interpretation the negatively scored loadings values (bar graph) have been plotted on the right, to correspond to the positively scored values in the PCA plot, due to derivatization of the spectra.

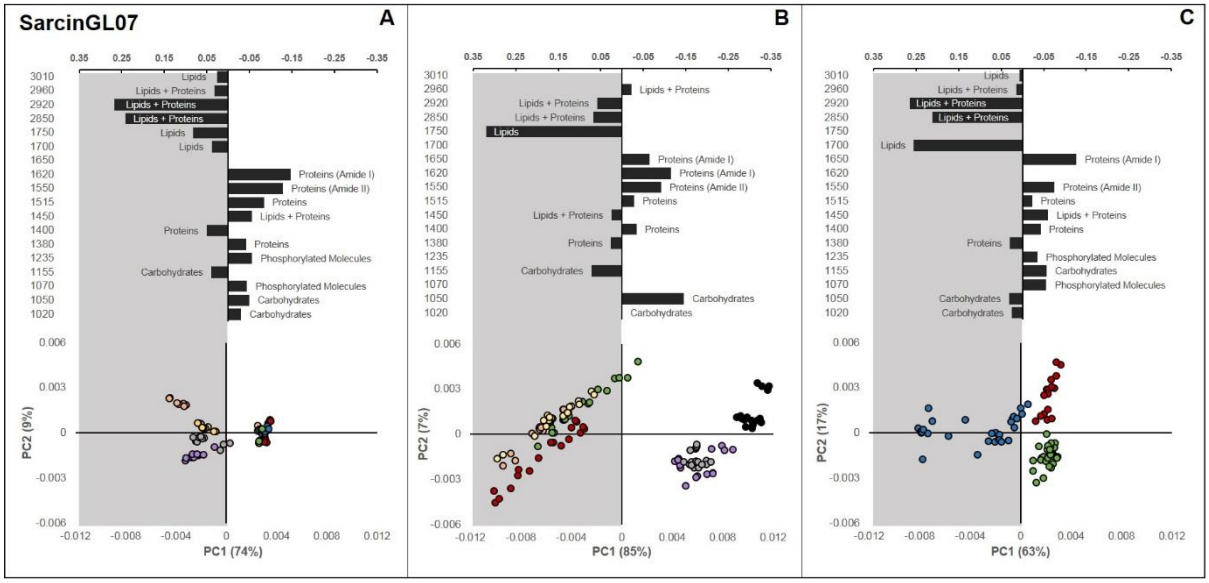

Figure 5. SarcinGL07 ATR-FTIR spectroscopy data showing Principal Component Analysis (PCA) and loadings plot for (A) light treatments 40 (blue), 60 (red), 80 (green), 100 (orange), 150 (yellow), 200 (purple) and 400 µmol photons • m^2 • s^{-1} (grey); (B) salinity treatments 5 (red), 10 (green), 15 (orange), 20 (yellow), 25 (purple), 30 (grey) and 35 psu (black); (C) temperature treatments 15 (blue), 20 (red), and 25°C (green). For ease of interpretation the negatively scored loadings values (bar graph) have been plotted on the right, to correspond to the positively scored values in the PCA plot, due to derivatization of the spectra.

Principal Component Analysis of Light Treatments

PCA results revealed clear separation in scores plots of spectral clusters for different light treatments in ChlorGL11 (Fig. 2A), KirchGL02 (Fig. 3A), and SarcinGL07 (Fig. 5A), signifying differences in macromolecular composition between growth treatments. Loadings plots for PC1 were analyzed, with negatively scored values corresponding to the strong positively loaded bands, and vice versa. Principal Component 1 (PC1) explained 75 % (ChlorGL11), 39 % (KirchGL02) and 74 % (SarcinGL07) of the variance between high- (400 to 150 µmol photons · m^{-2} · s^{-1}) and lowlight (100 to 40 µmol photons · m^{-2} · s^{-1}) growth treatments. The low light growth treatments for ChlorGL11, KirchGL02 and SarcinGL07 had the most pronounced response protein region (1650 - 1450 cm^{-1}), as well as the carbohydrates at 1050 cm^{-1}. SarcinGL07 also showed an increased carbohydrate signal at 1020 cm^{-1} and that of phosphorylated molecules (1235 cm^{-1} and 1080 cm^{-1}) under low light growth treatments. Exposure to high light growth conditions, saw an increase in lipids for ChlorGL11, KirchGL02, and SarcinGL07, as well as for carbohydrates and phosphorylated molecules for ChlorGL11 and KirchGL02. Contrary to the changes observed in the species described above, PrymGL20 PCA (Fig. 4A) had no differential clustering across light growth treatments.

Principal Component Analysis of Salinity Treatments

Separate clustering spectra corresponding to different salinity treatments was observed in the PCA scores plots for all four species. PCA scores for ChlorGL11 (Fig. 2B), KirchGL02 (Fig. 3B) and SarcinGL07 (Fig. 5B) revealed separation of clusters of spectra from high and low salinity growth treatments, primarily along PC1. The explained variance for PC1 was 90%, 79%, and 85% respectively. For ChlorGL11 the loadings plot for PC1 revealed a sharp maximal positive band loading assigned to 1050 cm^{-1} assigned to the C-O stretching of carbohydrates, as the main driver for the separation of negatively scored low salinity growth treatments. Low salinity growth conditions for KirchGL02 were positively scored, with the negatively loaded amide I and amide II proteins bands (1650-1450 cm^{-1}) being the major determinant of differential clustering, together with carbohydrates at 1050 cm^{-1}. The negatively scored, high salinity growth conditions for KirchGL02 cells corresponded to the positive PC1 loadings reflected in lipids (2920-1700 cm^{-1}) and carbohydrates at 1155 and

1020 cm^{-1}. Conversely, the clustering of positively scored spectra from high salinity growth treatments for SarcinGL07, were attributed to the negatively loaded proteins and carbohydrates (1050 cm^{-1}), while spectra from low salinities had higher loadings at 1750 cm^{-1} which has been assigned to lipids (v (C=O) of lipid esters). Despite the fact there was distinct clustering of spectra from different salinity treatments for PrymGL20 (Fig. 4B) along PC1 (42% of explained variance), there was no clear differences between spectra from the different growth treatments.

Principal Component Analysis of Temperature Treatments

PC1 accounted for 77%, 79%, 76%, and 63% of the explained variance observed in the scores plots for ChlorGL11 (Fig. 2C), KirchGL02 (Fig. 3C), PrymGL20 (Fig. 4C) and SarcinGL07 (Fig. 5C) respectively. Separation along PC1 for ChlorGL11 (Fig. 2C) occurred between the positively scored 25°C growth temperature and the negatively scored 20 and 15°C growth temperatures. The loadings plot for PC1 showed changes in proteins (methyl stretching at 2960 cm^{-1}, symmetric methylene and methyl bending modes at 1380 cm^{-1}, amide I and II modes) explained clustering patterns in scores plots at 15°C and 20°C, while the carbohydrate band at 1155cm^{-1} was the largest contributor to the spectral clustering observed at 25°C. Analogous to ChlorGL11, KirchGL02 (Fig. 3C) had clear separation of spectra from different temperature treatments along PC1, with 15 and 20°C treatments positively scored, and 25°C negatively scored. The loadings plot for PC1 suggested that the spectral clustering observed with KirchGL02 at 15 and 20°C were attributed to protein changes (amide I and II; 1650-1450 cm^{-1}) and carbohydrates at 1050 cm^{-1}. Spectral clustering for the 25°C treatment were explained by changes in carbohydrate bands (1155 cm^{-1} and 1020 cm^{-1}), phosphorylated molecules (1070 cm^{-1}), as well as for lipid band (2920 cm^{-1}; 1750 cm^{-1}).We observed two cluster groupings for temperature treatment for both PrymGL20 (Fig. 4C) and SarcinGL07 (Fig. 5C). Cells grown at 15°C were negatively scored, while cells grown at 20 and 25°C were positively scored. For PrymGL20, the positive loadings suggested that the major source of variance at the 15°C growth temperature came from the carbohydrate signal at 1020 cm^{-1} as well as phosphorylated molecules (1070 cm^{-1}). However for SarcinGL07, the 15°C growth temperature was correlated largely with lipids at 1700, 2850 and 2920 cm^{-1}.

Conversely 20 and 25°C loadings were associated with proteins at 1650, 1550 and 1515 cm^{-1}, phosphorylated molecules at 1235 and 1080 cm^{-1}, as well as carbohydrates at 1155cm^{-1}.

Relative changes in macromolecular composition

The Beer-Lambert Law states that infrared absorbance is directly proportional to analyte concentration. Here we used the integrated area of bands in mean second derivative spectra as a measure of the relative absorbance and hence, relative concentration, of the assigned macromolecular components (Fig. 6, Dao et al., 2017). For ChlorGL11, KirchGL02 and PrymGL20, increasing growth light had no significant changes in lipids. However SarcinGL07 had significant increase in lipids when grown under high light treatments (150 – 400 µmol photons · m^{-2} · s^{-1}; Two-way ANOVA: $F_{6, 89} = 7.852$, $P < 0.0001$). The mean integrated peak area for protein bands under the light treatment showed no significant changes for ChlorGL11 and KirckGL02. Conversely for PrymGL20 and SarcinGL07 there was a significant increase in relative protein concentration with increasing light flux (Two-way ANOVA: $F_{6, 79} = 9.442$, PrymGL20 $P < 0.0001$; SarcinGL07 $P = 0.0001$). The opposite was observed for carbohydrates under higher flux light treatments. PrymGL20 and SarcinGL07 showed no difference in carbohydrates that were statically significant, whereas an observed increase in carbohydrates with increasing growth light occurred for ChlorGL11 and KirchGL02 (Two-way ANOVA: $F_{6, 79} = 9.442$, ChlorGL11 $P < 0.0001$; KirchGL02 $P = 0.0001$).

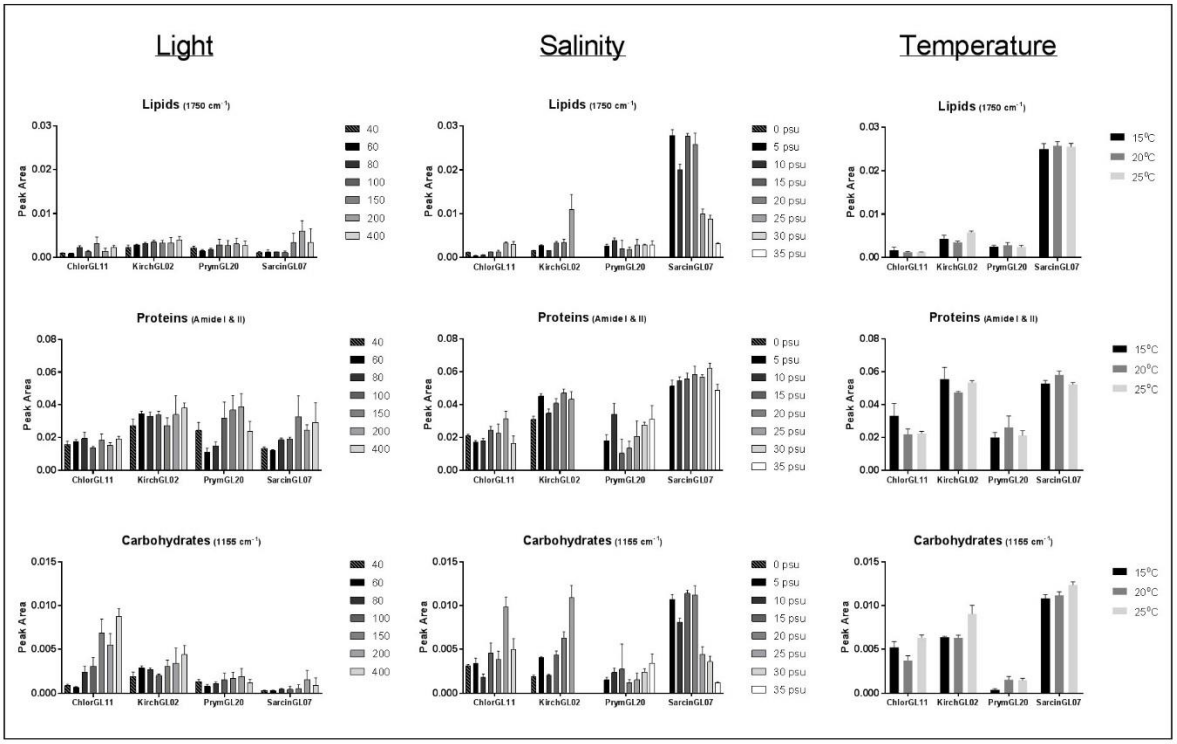

Figure 6. Integrated peak area of second derivative spectra for ChlorGL11, KirchGL02, PrymGL20 and SarcinGL07, under different light, salinity and temperature growth conditions. Integrated peak area for was selected at 1750 cm^{-1} for lipids, the sum of Amide I (1655 cm^{-1}) and Amide II (1540 cm^{-1}) for proteins and 1155 cm^{-1} for carbohydrates. Vertical bars represent standard error mean of at least three replicates.

Under salinity treatments, mean integrated peak area showed no significant differences in lipids for both ChlorGL11 and PrymGL20. KirchGL02 had a significant increase in lipids at 25 psu (Two-way ANOVA: $F_{7, 106} = 77.04$, $P < 0.0001$), whereas SarcinGL07 showed a decrease of lipids in high saline growth conditions (Two-way ANOVA: $F_{7, 106} = 77.04$, $P < 0.0001$). ChlorGL11 displayed a general increase in proteins with increasing salinity with a maximum at 25 psu, and a subsequent drop at 30 psu (Two-way ANOVA: $F_{7, 100} = 45.06$, $P < 0.007$). Both KirchGL02 and PrymGL20 had increased protein levels in higher saline growth conditions, but also had high protein levels at 5 psu (Two-way ANOVA: $F_{7, 100} = 45.06$, KirchGL02 $P < 0.001$; PrymGL20 $P < 0.05$). SarcinGL07 showed a decrease in protein levels at the minimum and maximum salinities (Two-way ANOVA: $F_{7, 100} = 45.06$, $P < 0.002$). For both chlorophytes, carbohydrate levels increased significantly under increasing salinity (Two-way ANOVA: $F_{7, 107} = 85.06$, ChlorGL11 $P < 0.001$; KirchGL02 $P < 0.0001$). Conversely, there was a decrease in carbohydrate levels with increasing salinity for SarcinGL07 (Two-way ANOVA: $F_{7, 107} = 85.06$, $P < 0.001$). PrymGL20 showed significant changes in carbohydrate levels with

changes in salinity (Two-way ANOVA: $F_{7, 107} = 85.06$, $P < 0.05$), with carbohydrates increasing with salinity, followed by a decline at 20 psu, with a subsequent rise again.

There was no significant changes in lipids, proteins and carbohydrates under temperature treatments for PrymGL20 and SarcinGL07. Likewise, mean integrated peak area showed no significant changes for ChlorGL11 and KirckGL02 in relative lipid and protein levels. By contrast, ChlorGL11 showed a relative decrease in carbohydrate levels at 20^{o}C (Two-way ANOVA: $F_{2, 37} = 14.39$, $P = 0.0031$) whereas KirchGL02 showed an increase at 25^{o}C (Two-way ANOVA: $F_{2, 37} = 14.39$, $P = 0.001$).

DISCUSSION

Microalgae play a critical role as the lowest trophic level in most aquatic food webs (Carpenter et al., 1985), and have long been studied as important indicators of water quality and ecosystem health (Domenighini and Giordano, 2009; McCormick and Cairns, 1994). The necessity to understand the response of microalgae to changes in their growth environment is unequivocal, as climate change is expected to bring about direct and indirect changes in light, temperature and salinity (Harley et al., 2006). Changes to these key drivers will likely have large scale impacts on algal growth and physiology, consequently altering their functional role in food webs and the carbon cycle (Beardall and Stojkovic, 2006; Wartman et al., n.d.; Wilken et al., 2013). Investigating how these changes influence macromolecular composition, growth and physiology of microalgae is key to elucidating the response to future change, in order to enhance our understanding of the conservation of aquatic environments and aid in resilience and sustainable management practices.

Treatment clustering reflects optimal growth conditions

Optimal growth for both chlorophytes (ChlorGL11 and KirchGL02) was found to be in lower salinity waters, while SarcinGL07 and PrymGL20 showed a preference for higher salinity treatments (Chapter 3, Wartman et al., unpublished data). The clustering of treatment levels for light, salinity and temperature, seen in the PCA scores plot for ChlorGL11, KirchGL02 and SarcinGL07, were largely related to the finding of optimal and suboptimal growth conditions for each species. Our previous laboratory work on growth and photophysiology of these microalgae isolates, tested under a range of environmental treatments, observed optimal growth that was comparable to the conditions they typically experience in the Gippsland Lakes (Chapter 3, Wartman et al., unpublished data). Earlier research has investigated the use of FTIR spectra to develop growth prediction models, for microalgae growing under variations in light and nutrients (Jebsen et al., 2012). These researchers found that the predicted growth models created using the spectral data were highly correlated with the actual measured growth, regardless of environmental factors tested and species of algae used. The findings of Jebsen et al. (2012) along with our results

highlight the ability of FTIR spectroscopy to be used as a tool to monitor growth under different environmental conditions.

Characteristic stress response in suboptimal growth conditions

Stress can be defined as a reduction in maximum growth rate caused by changes in environmental condition experienced by microalgae (Giordano, 2013). The PCA clustering of high and low light, salinity and temperature treatments, in ChlorGL11, KirchGL02 and SarcinGL07, demonstrates that these isolates have an environmental optimum for the balance of internal macromolecular composition. However when a perturbation in the environment beyond their threshold occurs, adjustment in the macromolecular pools is possible to minimize the reduction of growth. We observed a similar stress response in the PCA loadings plots of SarcinGL07 and KirchGL02, in which the two species clearly switched from allocation of carbon into proteins, to producing more carbohydrates and lipids for carbon storage, when exposed to environmental conditions beyond their optimum. This is a typical stress response observed in a diverse range of microalgae when exposed to stressful growth conditions (Dao et al., 2017; Giordano et al., 2001; Minhas et al., 2016; Stehfest et al., 2005). The redirected allocation of carbon into carbohydrates and lipid pools is advantageous because of their capability for energy storage. The energy stored in carbohydrates and lipids can be used more effectively than proteins to lessen the effect of change, due to smaller amount of energy required to breakdown (Fanesi et al., 2014; Palmucci et al., 2011). It has been shown that some species are capable of sustaining long-term adaptation to environmental change through phenotypic variation alone (Green et al., 2008). A study on Southern Ocean diatoms suggested that diatom species with higher levels of phenotypic plasticity may have greater capacity to adapt to environmental change associated with glacier melt (Sackett et al., 2013).

Species specific macromolecular responses to changes in environmental conditions

The understanding of resource allocation patterns in response to changes in environmental conditions in relation to taxonomy, was an objective of this research. While different species can allocate carbon to the same pools when exposed to perturbations, the extent to which they do can vary among species (Palmucci and Giordano, 2012). There was a

clear difference between the extent to which KirchGL02 and SarcinGL07 allocated resources into lipids, with SarcinGL07 typically allocating a larger percentage. While there can be some genotypic constraint in algal cell composition (Palmucci and Giordano, 2012), cells are also able to adjust their phenotypic plasticity in response to perturbation in their environment (Sackett et al., 2013). While different species may be adjusting their cellular pools in similar manners, the extent and the specific molecules of the macromolecule may differ, leading to a species-specific response. From our results, it is clear that the four species investigated not only had clear differences in macromolecular composition between treatment levels, but this also varied between species. This is partly related to different phyla have fundamental differences in cellular architecture, which has been shown to dictate cell composition (Finkel et al., 2016).

Temperature is linked to major changes in macromolecular composition

From the PCA analysis, temperature appeared to have the greatest impact on macromolecular structure. There was distinct differential clustering in the PCA analysis of the three growth temperatures for each species. This suggests that under variations in growth and temperature, species are adjusting to these changes by altering patterns of carbon allocation into different macromolecules. There is a large body of research that has shown temperature can affect cell composition, by altering enzyme-mediated biochemical reactions (Raven and Geider, 1988; Raven and Johnston, 1991; Renaud et al., 2002). In the four species studied, under temperature changes, alterations in lipids and carbohydrates were the greatest source of variance between treatments. Nitrogen metabolism has been shown to be strongly limited by temperature in diatoms and dinoflagellates (Berges et al., 2002; Lomas and Glibert, 1999). A decrease in nitrogen metabolism can significantly impact protein production.

However, inspection of integrated peak areas revealed that the most significant changes in macromolecular composition for all four species studied was associated with changes in salinity. This is a consequence of integrated peak area only taking into account individual band assignment. Owing to the diverse and complex allocation patterns of microalgal macromolecular composition, the utilization of the entire spectra has been found to be a more accurate approach (Heraud et al., 2006; Jebsen et al., 2012; Kansiz et al., 1999).

Maintaining cellular homeostasis in response to changes in light attributed to mixotrophy

Microalgae cells have an intrinsic tendency to preserve cellular homeostasis to aid in minimizing the cost of responses and reversal of acclimation to perturbations (Giordano, 2013; Montechiaro and Giordano, 2010). In this study, the three autotrophic species (ChlorGL11, KirckGL02 and SarcinGL07) rearranged their carbon allocation to different macromolecular pools in response to variation in growth light conditions. However, there was no observed differential clustering in the PCA analysis for PrymGL20 and integrated peak area showed no significant changes in lipids and carbohydrates under light limitation. This could be characteristic of *P. parvum*'s mixotrophic ability. Heterotrophic and mixotrophic species are known to maintain cellular homeostasis (Persson et al., 2010). The mixotrophic *P. parvum* has been described by Bold and Wynne (1985) as photosynthetic with possible heterotrophic ability in the form of phagotrophy. Previous research observed changes in cellular composition in marine planktonic diatoms when light decreased, concluding that decreased energy availability acts as a constraint on the maintenance of cellular composition (Post et al., 1985). While this may hold true for autotrophic species, *P. parvum* (PrymGL20), may have switched to heterotrophic behavior induced by light-limited conditions, to compensate for the decrease in photosynthesis, by consuming bacteria present in the water column (Jones, 2000, 1994; Rottberger et al., 2013).

Additionally, growth rates have been recognized as an important factor for carbon allocation and links to homeostatic behavior (Sterner and Hessen, 1994). A recent study on *Tetraselmis suecica* found that cells with lower growth rates had a higher tendency for cellular homeostasis when exposed to external environmental perturbation (Fanesi et al., 2014). The *P. parvum* isolate in this study, PrymGL20, had a significantly slower rate of growth than of the three other microalgae, as well as other isolates from Europe and the United States (Wartman et al., unpublished; Baker *et al.*, 2007). The link between growth rates and homeostatic behavior is a consequence of species with slower growth, having a shorter fraction of generation time being affected by the perturbation, compared to those species with faster growth. In this study, growth rate may not be the main cause of the cellular homeostasis of *P. parvum*, as the isolate was acclimated to the environmental perturbation for 4 to 5 generations (Wartman et al., unpublished).

CONCLUSIONS

Previous research, together with our results has highlighted the control that environmental factors, particularly light, temperature, and salinity, exert not only on growth and photosynthesis of microalgae, but also on cellular metabolism and thus dynamic cell composition. This study identified species-specific variations in cellular levels of the main macromolecules in response to light, salinity and temperature regimes. The results highlight the practicality and usefulness that ATR-FTIR provides as a diagnostic tool for microalgae research. Multivariate analysis on the biologically relevant regions of the spectra allowed visualization of related treatment clusters of optimal and suboptimal growth conditions, and further identified the macromolecular shifts that triggered the variation under those environmental conditions. The limitation of this research comes from investigating these environmental changes independently. Future investigation into multi-stressors on macromolecular composition is recommended to develop a comprehensive understanding of how natural microalga assemblages may respond to climate change, as shifts in the assemblage composition will likely alter flow of energy to higher trophic levels.

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Chapter 5

ASSESSMENT OF VERTICAL MIGRATION PATTERNS OF THE TOXIC PHYTOFLAGELLATE *PRYMNESIUM PARVUM* (HAPTOPHYTA) IN A STRATIFIED WATER COLUMN

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ABSTRACT

With global water column stratification expected to increase as a consequence of climate change, the separation of resources in the water column will likely increase. This could favour the selection of motile microalgae in aquatic environments. Occurrences of harmful algal blooms (HABs) of the motile and toxin-producing alga *Prymnesium parvum* have been increasing worldwide, yet there are no studies that have documented the vertical migration patterns of this species. Therefore, we studied the vertical movement of *P. parvum,* isolated from a brackish lake system in SE Australia, in laboratory plankton columns under a sinusoidal light/dark cycle, with salinity and nutrient-stratified water columns. *P. parvum* did not undertake any cyclic diurnal vertical migration during the experiments, even when surface waters were nutrient depleted. However, we would classify *P. parvum* as a 'layer former' as the population aggregated at mid-depth in the water column and remained there regardless of time of day. The depth of aggregation was found to correspond to optimal light for growth. Nutrient depletion in the surface waters caused an increase in descent rate to values similar to that of the theoretical sinking rate of *P. parvum* based on Stokes' Law; however the cause behind this increase was not determined. The results of this study provides the first ecologically-relevant data on the swimming patterns of this toxic bloom forming flagellate. This newly reported behavioural response can provide valuable ecological information to environmental managers.

ABBREVIATIONS

NH_3, Ammonia

DVM, Diel vertical migration

HABs, Harmful Algae Bloom

FRP, Filterable reactive phosphorus

F_t, Instantaneous chlorophyll fluorescence

NPQ, Non-photochemical quenching

NO_X, Oxidized nitrogen

PSII, Photosystem II

VM, Vertical migration

INTRODUCTION

Water quality issues are receiving growing attention due to the apparent rise in harmful algal blooms (HABs) over the recent decades (Hallegraeff, 2010, 1993). While phytoplankton play a critical role at the base of most aquatic food webs, as the dominant primary producer (Field et al., 1998), HABs can have devastating impacts on the environment (Sellner et al., 2003), resulting in damage and degradation of the environment with an associated large financial costs (Hoagland et al., 2002). Research predicts that HABs will continue to wreak havoc in the aquatic environment worldwide, as climate change could be a catalyst favouring their growth, occurrence and persistence (Moore et al., 2008). Unfortunately, environmental conditions that cause HABs (e.g., eutrophication, upwelling, stratification) are complex and often can be species specific, causing challenges for management and prediction of HABs.

Adding to the complexity are motile phytoplankton, many of them HAB-forming, which possess the ability to regulate their depth in the water column. Understanding the distribution and movement of phytoplankton in the water column is challenging, due to the complex, multi-scale interactions between biotic and abiotic processes. Predicted increases in water column stratification owing to climate change impacts (Hallegraeff, 2010), will likely lead to greater separation of vital resources, i.e. nutrients and light (Yoshiyama et al., 2009), resulting in unfavourable growth conditions, leading to physiological stress and reduced growth, affecting species fitness and succession.

Phytoplankton have, however, evolved numerous adaptive strategies to help regulate environmental stressors. By exhibiting plasticity in several functional traits, including depth regulation strategies (Cullen and MacIntyre, 1998), rearrangement of internal cellular composition (Palmucci et al., 2011) and photoacclimation (Beardall and Morris, 1976; Wartman et al., unpublished), phytoplankton can better acclimate to perturbations from ambient conditions. The ability to migrate vertically in the water column has important adaptive advantages, including the avoidance of passive sinking out of the water column, access to critical resources which may be spatially separated, predator avoidance, avoidance of high irradiance in the surface waters and escape from energetic turbulence (Bollens et al., 2011; Doblin et al., 2006; Durham et al., 2013; Tilney et al., 2015; Yoshiyama et al., 2009).

The motility of phytoplankton may confer selective advantage within the ecosystem, affecting phytoplankton growth, survival and coexistence strategies (Fogg, 1991; Margalef, 1978).

A species of particular concern, and the focus of this paper, is *Prymnesium parvum*, a mixotrophic, motile, haptophyte microalga capable of producing a number of toxins (Shilo, 1981). This species has a very wide-spread geographic distribution, and is capable of tolerating a range of environmental conditions (Baker et al., 2007; Brooks et al., 2011). When nutrients become limiting, *P. parvum* has the ability to switch to heterotrophic behaviour and ingest other planktonic microorganisms through phagotrophy (Brutemark and Granéli, 2011; Skovgaard and Hansen, 2003; Tillmann, 2003). Furthermore, numerous studies have reported increases in toxicity of the species under nutrient deficiency (Johansson and Granéli, 1999). It also possesses the capability to affect co-existing phytoplankton by allelopathy through cell lysis from its toxin production, and deter grazers (Granéli et al., 2012; Granéli and Salomon, 2010). *P. parvum* has been the perpetrator in several mass fish mortalities throughout parts of Europe and the Southern USA (Roelke et al., 2010). While *P. parvum* autecology has been the focus of considerable research over recent decades (reviewed by Granéli et al., 2012), the potential importance of depth regulation in *P. parvum* has not been addressed.

To our knowledge, evidence of vertical migration (VM) behavioural responses from *P. parvum* to strong vertical gradients in the water column does not exist. Therefore, the present study was undertaken to gain greater insight into how vertical gradients, particularly with respect to growth-limiting light, salinity and nutrient resources, influence the vertical movements of *P. parvum*. Understanding and quantifying this process could help generate ecologically relevant knowledge and aid in elucidating strategies responsible for the development or persistence of *P. parvum* blooms. Therefore, we studied VM patterns of the toxic *P. parvum* in vertically stratified laboratory water columns under different nutrient and salinity conditions. We aimed to determine (1) under what environmental conditions *P. parvum* regulates it position in the water column, (2) if *P. parvum* undergoes diel vertical migration (DVM) and (3) understand the implications of any active movement strategies on niche diversification and the adaptive advantages to this species' survival.

MATERIALS AND METHODS

Isolation and Cultivation of Cells

The *Prymnesium parvum* culture (PrymGL20) was recently isolated from the Gippsland Lakes, Victoria, Australia during late 2016 as described in Wartman et al. (unpublished). The isolate was identified by Microalgal Services (Ormond, Victoria). Cells were grown in f/2 medium adjusted to optimal salinity (25 psu), under a 14:10 sinusoidal light:dark cycle.

Tower Design

To examine the vertical migration patterns of *Prymnesium parvum*, we built six plankton towers. Towers were made from clear acrylic tubes which were 2 m high by 10 cm in diameter, and held approximately 14 L of culture (Domtech Ptv. Ltd., Victoria, Australia). Sampling ports (2 mm width) were drilled every 5 cm, and a sterile hypodermic needle (19 gauge; TERUMO, Shibuya, Tokyo, Japan) inserted and then sealed with silicone (Selleys 3 in 1 Silicone Sealant). The columns were kept in an 18^{o}C controlled temperature room. Individual tower covers were made from blackout material (Elija Montgomery, Melbourne, Australia) which ensured a unidirectional light source. The towers were illuminated from above by white LED lights (B-100, Valoya, Finland), that were attached to a digital controller (constructed in house by the Science Workshop, Monash University, Australia) and a dimming module (AquaController-Apex Jr., VDM-Module, Neptune Systems, CA). The lighting regime was set to a 14:10 sinusoidal light:dark cycle, with sunrise occurring at 07:00 h, and sunset at 21:00 h. Dusk and dawn had a light intensity of 100 µmol • photon • m^{-2} s^{-1} which then ramped up to 1200 µmol • photon • m^{-2} s^{-1} for a mid-day maximum. Irradiance was measured through the water column with a waterproof spherical PAR sensor (Aquation, NSW, Australia).

Tower Sterilization

Columns were scrubbed with a solution of hypochlorite, then rinsed three times with tap water, then washed once with 0.2 M HCl, and rinsed a final three times with deionised water. Once sterilized, the towers were sealed until the experiment. Seawater was amended with f/2 media and filtered sterilized with a 0.2 µm filter (Merek Millipore, Australia) into an

autoclaved sterile 10 L polycarbonate Nalgene carboys (Thermo-Scientific). The columns were filled using sterilized silicone tubing (Masterflex) attached to the 10 L carboy and an inflow valve on the top of the sealed tower, using a peristaltic pump (MasterFlex® L/S Economy Drive; Gelsenkirchen, Germany) to minimize bacterial contamination. The silicone and hypodermic needles used for sampling were replaced for every experiment as to not allow contamination to be carried over.

Experimental Setup

Three tower experiments were completed, each with three replicates. The water column in the tower was set up for each experiment according to Table 1. Once the water columns were set up, mid-exponential phase *P.parvum* culture was equally divided between the three replicate towers and was inoculated into the surface of each tower. Cells were allowed to acclimate to the towers for a 24-hour period, prior to commencement of sampling. For the nutrient deplete experiments, cells were transferred to depleted nutrient media for 48 hours prior to being put in column.

Table 1. Experimental setup for the three tower experiments completed. Each experiment was run in triplicate.

Experiment	Salinity	Nutrients	Light
1			
Surface	25	replete	Sinusoidal
Bottom	25	replete	14:10 LD
2			
Surface	27	deplete	Sinusoidal
Bottom	32	replete	14:10 LD
3			
Surface	25	replete	Sinusoidal
Bottom	35	replete	14:10 LD

Sampling

Sampling occurred three times over a 24-hour period for the experiment duration of 7 days. Samples were collected 1 hour prior illumination (dark acclimated cells), at mid-day (maximum light intensity) and 1 hour prior to dark (minimum light intensity). Samples were collected from each depth, therefore 40 samples per tower were collected at each time point. Samples were drawn out of the tower using a sterile syringe (TERUMO, Shibuya, Tokyo,

Japan) inserted into a one-way luer port (Value Plastics, Nordson Medical, Loveland, CO, USA) attached to the hypodermic needle head that was secured to the side of tower. The column was defined into 3 sections: surface (0 to 67 cm), middle (67cm to 124 cm) and bottom (124 to 200 cm) for calculations of the various parameters.

Biomass

To track the locations of the cells in the water column, for the first experiment we collected samples for cell counts at each sampling port and also used an Aquapen-P (Photon Systems Instruments, Czech Republic) to measure instantaneous chlorophyll fluorescence (F_t) adjacent to the port. We found good correlation between F_t and cell counts (R^2=0.9533, data not shown), so for the remainder of the experiments we only used the Aquapen-P to monitor the biomass, as this was much more time efficient for sampling.

Physiology

At each sampling point we used the Aquapen-C to perform several non-destructive PSII fluorescence measurements including F_v/F_m. The samples were dark acclimated at 18^{o}C for 5 minutes, prior to measurements. Samples were taken from the surface (5 cm below water level), middle (100 cm) and bottom of the tower (190 cm).

Physico-chemical parameters

Once the water column was filled with medium, a salinity profile was obtained by taking measurements from each sampling port and using a portable refractometer (RHS-10ATC). Samples (3 mL) were taken from various depths and pH determined using a sensION+ PH31 pH meter (Hach, Loveland, CO, USA). We took pH measurements both at the beginning and at the end of the experiment. Nutrient samples were also taken at the beginning and end of the experiment. For this, a 20 mL sample was taken from selected depths and directly filtered through a 0.45 µm syringe filter (PALL Life Sciences Acrodisc® Supor® Membrane Port, Washington, NY, USA), then frozen at -20^{o}C until analysis. Nutrient samples were analyzed for ammonia (NH_3), oxidized nitrogen (NO_x), and filterable reactive phosphorus (FRP), using standard colorimetric methods (Grasshoff 1983), by a NATA accredited unit in the Water Studies Centre (Monash University).

Calculations

Vertical Migration Patterns

To calculate whole population DVM, we used the method from Tilney et al. (2015), adapted from Pennak (1943), with minor modifications. Using this method allowed for observations over time of whole population changes in depth in the water column, allowing investigation of the entire population vertical migration patterns. In brief, F_t measurements from each depth were standardized to a percentage of total water column F_t, which was then used to calculate a cumulative percent distribution throughout the whole column. Pairwise linear regressions were used to interpolate the depth (in cm) of the upper (75%; Q1), median (50%; Q2) and lower (25%; Q3) quartiles of the population. The interquartile ranges were then plotted to interpret vertical movement patterns of the entire population.

Stokes' Law

The theoretical sinking rate was calculated using the Stokes' Law equation ($\mu = 2gr^2 (\rho' - \rho)/9\eta$). Where g is the gravitational acceleration, r is the cell radius, ρ' is the cell density, ρ is the density of the water and η is the viscosity of the water.

Accumulation Rates

The accumulation rates were calculated using the specific growth rate equation. $\mu_c = [\ln (F_{t\,t1}) - \ln (F_{t\,t0})]/(t_1 - t_0)$. We defined it as accumulation rate, as we are aware that cells are both growing and also changing in cell number per unit volume by passively sinking out and actively moving through the water column. Measured water column F_t values were calibrated to the corresponding cell number from a calibration curve previously calculated between F_t and cell number (data not shown).

Rate of Descent

The rate of descent was calculated from a linear regression of the interquartile range F_t values from the initial 100 hours of the experiment, using the calculated slope as the rate of descent.

Statistical Analysis

Differences between treatments were assessed using one-way ANOVA (Prism 7, Graphpad Software; La Jolla, CA, USA). When significant differences were detected in the ANOVA test, Tukey's HSD post hoc test was used for multiple comparisons of means. All statistical analyses were done with a $p < 0.05$ level of significance.

RESULTS

Measurements of salinity, pH and nutrients were completed through the water column, prior to commencing the experiment (Fig. 1A-C) as well as after the experiment was finished (Fig. 1D-F). In a well-mixed water column (Fig. 1A), we observed an increase in pH over time throughout the whole water column (Fig. 1D), with a larger increase in the upper portion of the water column. For nutrients represented in Fig 1 as FRP (though NO_X was measured and was found to follow the same trend, data not shown). Nutrient levels were depleted throughout the whole water column from the initial levels (34 µM FRP), but the greatest depletion was found to be in the middle of the water column. In the stratified water column (Fig. 1B), there was a large increase (0.78 units) in pH in the upper portion of the water column at the end of the experiment (Fig. 1E). While nutrients did slightly decrease in the upper portion, the largest decrease was seen at the bottom, where nutrients were initially replete. Finally, in a nutrient replete, but salinity stratified, water column (Fig. 1C), the largest increase in pH of approximately 1 pH unit was observed in the upper portion of the water column (Fig. 1F). This corresponded with a large reduction in nutrients in the upper portion, and a relatively slight decrease in bottom nutrients.

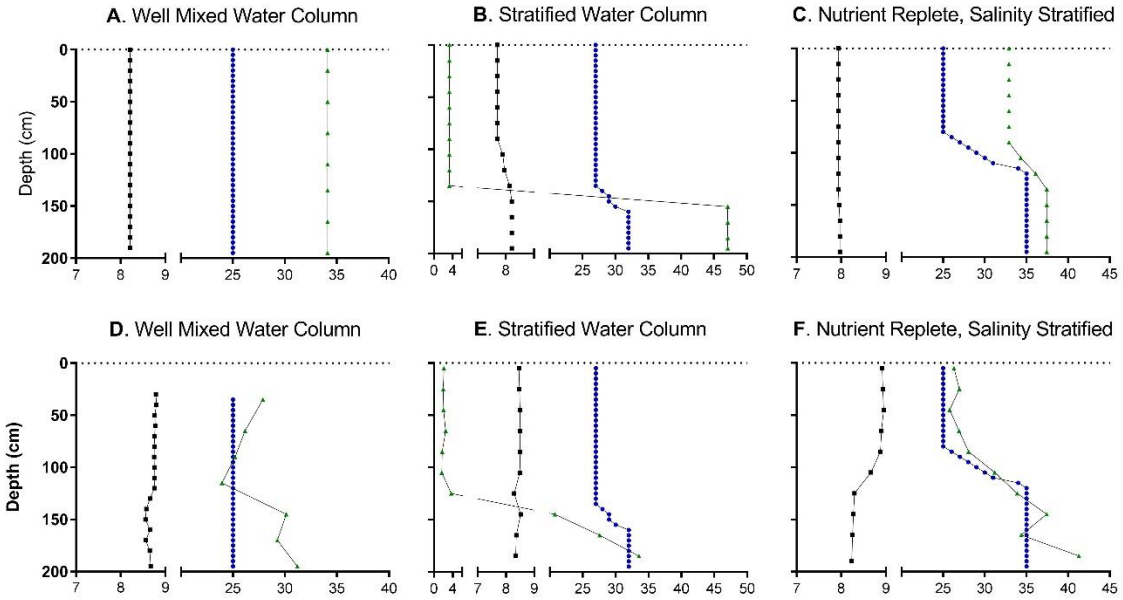

Figure 1. Laboratory water column setup at the start of the three experiments (A, B and C). Measurements were made of pH (black square), salinity (blue circle) and nutrients (phosphorus plotted, green triangles). A. the water column was well mixed with a salinity of 25 psu, and nutrients replete throughout. B. Stratified water column with a salinity of 27 psu in the upper 2/3 of the water column and nutrients depleted, and a salinity of 32 psu in the bottom with nutrients replete. C. Salinity stratified water column with a salinity of 25 psu in upper portion and 35 psu at the bottom, nutrients were replete throughout. Changes in pH and nutrients were measured at the end of each experiment (D, E and F).

Prymnesium parvum cells in the well-mixed, and both nutrient deplete and nutrient replete stratified water columns, did not exhibit any diel vertical migration behaviour (Fig 2). Instead *P. parvum* cells, in all experimental water columns, accumulated at a mid-depth level of approximately one metre, and remained there regardless of time of day. The dispersion between the interquartile ranges of the *P. parvum* population remained relatively constant throughout the three experiments. The theoretical sinking rate according to Stokes' Law was calculated and plotted to demonstrate the rate of which the cells would sink out of a low energy water column if they were not actively maintaining their position in the water column.

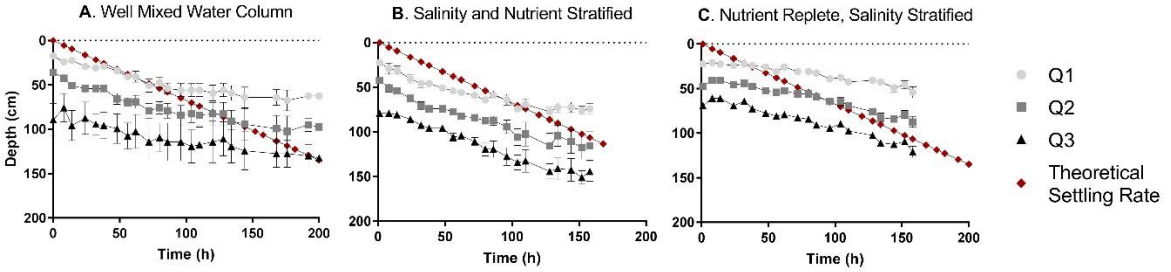

Figure 2. Vertical migration patterns of *Prymnesium parvum*, under three laboratory water column conditions including: A) well mixed water column, B) salinity and nutrient stratified and C) salinity stratified and nutrient replete. The upper (Q1), median (Q2), lower (Q3) of cell distributions, and theoretical settling rate were used to interpret whole population vertical movement patterns. Experiments were run in triplicate with error bars representing standard error of the mean.

Accumulation rates were calculated for the surface, middle and bottom portion of the water column (Fig. 3). In a well-mixed water column (Fig. 3A), the middle and bottom portion of the water column had a significantly higher accumulation rate than the surface ($p < 0.03$). While the middle waters showed the highest accumulation rate, it was not statistically significantly greater than that of the bottom portion of the tower ($p = 0.081$). In a stratified water column with nutrient depletion in the surface (Fig. 3B), cells at the surface had a negative accumulation rate, significantly lower ($p < 0.01$) than in the middle and bottom of the towers. The bottom waters appeared to have the highest accumulation rate of approximately 0.2 per day, but this was not statistically significant from the middle water accumulation rate ($p = 0.2074$). With a strong halocline (Fig. 3C), accumulation rates of *P. parvum* cells showed a similar pattern of accumulation to that of the well-mixed water column (Fig. 3A). Likewise, the surface water had a significantly lower accumulation rate of cells than the middle portion of the water column ($p = 0.0031$), however there was no statistical difference found between the surface and bottom accumulation rates ($p = 0.0627$).

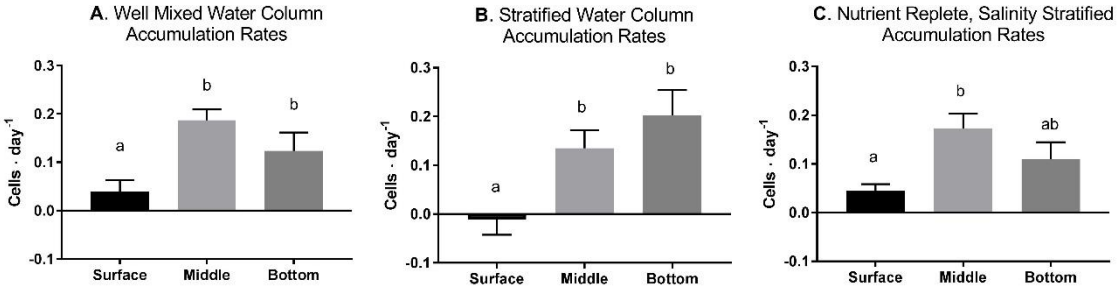

Figure 3. Accumulation rates of *Prymnesium parvum* in the surface, middle and bottom of three laboratory water columns including: A) well mixed water column, B) salinity and nutrient stratified and C) salinity stratified and nutrient replete. Experiments were run in triplicate with error bars representing standard error of the mean.

The rate of descent of cells for the first 100 hours of the experiments was observed to be slowest through a strong halocline water column ($p < 0.01$), while a salinity stratified water column with nutrient depletion in the surface resulted in a significantly faster rate of *P. parvum* descent ($p < 0.05$; Fig 4). The rate of descent through a nutrient depleted, stratified water column was most similar to the rate of descent of the theoretical sinking rate.

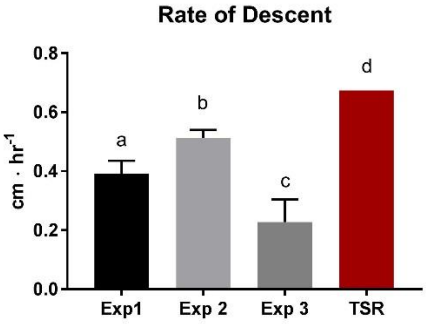

Figure 4. Rate of descent for *Prymnesium parvum* in the laboratory water columns experiments including: Exp 1) well mixed water column, Exp 2) salinity and nutrient stratified, Exp 3) salinity stratified and nutrient replete and TSR (Theoretical Sinking Rate according to Stokes' Law). Experiments were run in triplicate with error bars representing standard error of the mean.

In-situ surface irradiance did not differ between columns with only f/2 medium and columns containing medium with *P. parvum* at depths less than 0.25 m (Fig. 5). However, water column irradiance was decreased by approximately 50% relative to water columns with just f/2 medium at depths below 0.25 m. Surface irradiance was around 600 µmol photons $m^{2} \cdot s^{1}$, while light decreased exponentially through the water column, with the bottom receiving approximately 5 µmol photons $m^{2} \cdot s^{1}$.

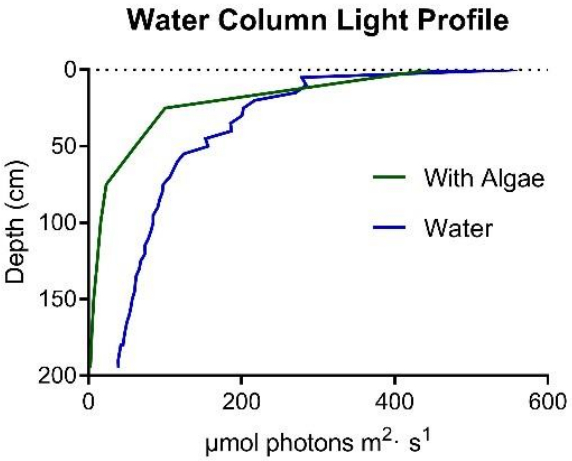

Figure 5. Water column light profile through f/2 medium (blue) and f/2 medium with *Prymnesium parvum* (green) at the end of experiment 1.

DISCUSSION

Observations of *P. parvum* distributions in the three types of water column showed that maximum cell abundances were found at a depth of approximately one metre, typically where light was around 100 µmol photons $m^{2}\cdot s^{1}$ or less. Previous studies on this strain have found optimal light for maximum growth to be 100 µmol photons $m^{2}\cdot s^{1}$ (Wartman et al., unpublished). While this strain of *P. parvum* showed some ability to photoacclimate to high light through increasing non-photochemical quenching (NPQ) with increasing light intensity, growth rate and photosynthetic rates were reduced when exposed to higher irradiance (Wartman et al., unpublished). The accumulation rates observed in this study also support the notion of decreased growth at higher light intensities, as seen in Fig 3, the surface was observed to have the lowest accumulation rate. Previous research has shown that while motile flagellates may possess the ability to photoacclimate to higher light intensities, there is a stronger tendency to adjust their position in the water column, avoiding the surface where the high light can be damaging (Hader and Worrest, 1991; Hall and Paerl, 2011; Heaney and Talling, 1980). Furthermore, toxins and allelochemicals produced by *P. parvum* cells were found to be sensitive to high irradiance. High light contributed to the transformation/degradation of *P. parvum* allelopathic compounds within hours of exposure, while toxicity of the cells was found to be higher in strains under low light intensities between 30 - 100 µmol photons $m^{2}\cdot s^{1}$ (Granéli and Salomon, 2010). It is possible that *P. parvum* cells may be descending to a depth where light is optimal for growth and production of allelopathic compounds and toxins, which would then confer a competitive advantage over their competitors and grazers.

The depth-regulating behaviour exhibited by our strain of *Prymnesium parvum* could lead to it being categorized as a layer-former, as it segregates vertically into a distinct stratum, generally governed by local conditions of optimal growth in a low energy, stratified water column. As discussed previously, *P. parvum* was observed to aggregate at a level of optimal light, and maintained this position in the water column. Other phytoplankton known to utilize this strategy includes *Gyrodinium aureolum* (=*Gymnodinium mikimotoi*) (Holligan, 1978), *Gymnodinium splendens* (Cullen and Horrigan, 1981), and *Ceratium tripos* (Eppley et al., 1984; Falkowski et al., 1980). Adaptations beneficial to species like *P. parvum* that utilize the layer formation strategy include depth regulation and mechanisms to deter predators

(Cullen and MacIntyre, 1998). *Prymnesium* spp. have effective physiological control on depth regulation by possessing two smooth flagella for motility as well as a third appendage, a haptonema, that can be used for attachment to a substrate (Manton and Leedale, 1963). In addition, *P. parvum* produces allelochemicals to repel grazers and other competitors which may inhabit the same niche (Granéli and Salomon, 2010).

Diel vertical migration has been commonly cited as advantageous to motile phytoplankton when vital resources are separated in the water column (Doblin et al., 2006; MacIntyre et al., 1997; Salonen et al., 1984). Researchers have found different DVM strategies between species (Jephson and Carlsson, 2009; Tilney et al., 2015), highlighting that DVM behaviour conforms to each species' physiology. However, we did not observe any DVM by *P. parvum*, even when resources were separated in the water column. The lack of DVM behaviour exhibited by *P. parvum* could likely be due to its mixotrophic ability. *P. parvum* is photoautotrophic, but is also capable of heterotrophic behaviour through ingestion of organic particles (i.e. bacteria, and other microorganisms) in the form of phagotrophy (Carvalho and Granéli, 2010; Stoecker et al., 2017). When nutrients become depleted in the surface waters, instead of migrating down to nutrient rich bottom waters, *P. parvum* possess the ability switch over to heterotrophy, ingesting bacteria and other microorganisms present in the water column. During the experiment, bacteria were attempted to be kept as low as possible, there was an accumulation of bacteria in the columns, which could act as a possible source of organic matter for *P. parvum*. Previous studies have found that *Prymnesium* sp. maintains heterotrophic behaviour even when nutrients are replete (Carvalho and Granéli, 2010), although this behaviour is typically increased when nutrients are deficient (Legrand, 2001). While it may be energetically expensive to maintain both heterotrophic and autotrophic machinery for *P. parvum*, as well as an energetic cost associated to switching between the two behaviours, algae capable of mixotrophy can gain a competitive advantage over other species who do not possess this ability (Stoecker et al., 2006).

We observed that *P. parvum* exhibited a rate of descent similar to its theoretical sinking rate when nutrients were deficient in the upper water column. It is generally accepted that the physiological health of phytoplankton has influence over its sinking rates (Bienfang, 1981; Smayda, 1970), although it is still debated whether nutrient depletion increases or decreases sinking rates (Bienfang et al., 1982; Kromkamp and Walsby, 1992;

Pantorno et al., 2013; Titman and Kilham, 1976). Furthermore, the nutrient status of cells can influence their capacity for photochemical work (Beardall et al., 2001). Eppley et al., (1967) hypothesized that higher sinking rates would occur with declining physiological activity. We also observed a decrease in F_v/F_m values in this study (data not shown) when the cells were nutrient starved, signifying a decline in physiological activity in *P. parvum*. On the contrary, *P. parvum* may have redirected its energy into producing toxins, as typically seen during stress conditions (Johansson and Granéli, 1999), instead of into energy required for flagella operation and associated cell components. However theoretical estimates of the metabolic cost of motility suggest a maximum energy cost of about 1% for a cell of 100 µm diameter, and much less for a cell of 10-20 µm diameter, like *P. parvum* (Crawford, 1992). Conversely, it could be the direct lack of nitrogen needed not only for making the flagella but also for generating energy needed for operating the flagella (Raven and Richardson, 1984). It appears that nutrient deficiency in *P. parvum* impacts its motility, causing a faster rate of descent through the water column. However further investigation into the impact of nutrient limitation on swimming speeds of *Prymnesium* sp. is warranted.

In aquatic systems, algae are grazed by zooplankton, which naturally aids in controlling the development of substantial biomass. However, phytoplankton have been found to use vertical migration as a mechanism for predator avoidance (Bollens et al., 2012, 2011). Just as numerous species of zooplankton have developed migration strategies to avoid predation (Bollens and Frost, 1989; Hays, 2003), phytoplankton also use a similar strategy to avoid being grazed. As *P. parvum* cells displayed little to no migration movement through the water column once it reached optimal depth, this may not be a strategy used by this species. Given allelopathic abilities to deter and kill grazers, it might be argued that it may be more strategic and easier to kill your predator than too actively swim through the water column to avoid them. A review of the literature demonstrates that cultures of *P. parvum*, and even just the culture filtrate, can immobilize and/or kill grazers (Fistarol et al., 2003; Tillmann, 2003). Tillmann (2003) even observed that grazers killed by toxins produced by *P. parvum* were then eaten by the algal cells. Clearly the allelopathic behaviour of *Prymnesium parvum* may provide a better survival strategy as opposed to using its motility to avoid being grazed. Further experiments using predators and *P. parvum* in laboratory water column studies would provide more insights into this.

CONCLUSION

We have here presented the first assessment of vertical distribution patterns in laboratory water columns of *Prymnesium parvum* isolated from a brackish lake system. *P. parvum* can be classified as a layer former, as we observed it to descend to a distinct depth, and maintained its position there. This layer formation strategy is ideal for *P. parvum* as they possess adaptive strategies like flagella for motility to remain at optimal depth, and allelopathic chemicals to deter competitors and grazers from infringing on its niche. *P. parvum* displayed no DVM behaviour, even when faced with nutrient deficient conditions, as it was likely to be able to compensate for lack of available nutrients through ingestion of bacteria present in the water column. *P. parvum* exploits a vertical migration strategy in a way that is enhanced by its mixotrophic behaviour, and production of chemical deterrents, making it a worthy competitor in most aquatic systems, suggesting a high likelihood for continued blooms in the future. Further experiments in laboratory water columns looking at grazing and competition experiments would be beneficial for further understanding patterns of vertical migration for *P. parvum*.

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Chapter 6

CONCLUSIONS

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The overall objectives of this research were to investigate how key environmental drivers, mainly light, salinity and temperature, affect microalgal growth, physiology, cellular composition and behavioural mechanisms. Microalgae are universally present in almost all aquatic environments, playing pivotal roles at the base of most aquatic food webs (Field et al., 1998), and aiding in the drawdown of anthropogenic CO_2 (Falkowski et al., 1998). Thus, enhancing our knowledge of their growth, physiology, and occurrences is critical to establishing how these species may respond to environmental change. Any impacts of environmental change on microalgae may have cascading effects on the entire ecosystem. Initially, Chapter 2 investigated the spatial and seasonal changes of physico-chemical parameters and phytoplankton assemblages in the Gippsland Lakes over a 15 month period. Chapter 3 then investigated the impacts of key environmental drivers of the lakes on the growth and physiology of four phytoplankton species isolated from the lakes. Chapter 4 then examined chemical acclimation strategies of the four microalgae, utilizing ATR-FTIR to observe the reallocation of internal carbon pools in response to key environmental factors. Finally, Chapter 5 addresses behavioural acclimation strategies in response to the separation of essential nutrients (light and nutrients) caused by stratification of the water column in the toxic, motile, haptophyte *Prymnesium parvum*.

In the work reported in Chapter 2, I investigated the seasonal and spatial variations of key physico-chemical variables and phytoplankton community composition over a 15-month period in the Gippsland Lakes, a group of interconnected brackish lakes in SE Victoria, which suffer from eutrophication leading to recurring algal blooms. Phytoplankton biomass, reflected in chlorophyll *a* measurements was low during the summer of 2017 and remained low over the winter period. This is likely a reflection of the low nutrient concentrations in the lakes at the time, which were found to be N-limited. The biomass of the standing crop was largely dominated by dinoflagellates over this time. However, from December 2017 (early austral summer), a bloom of the non-toxic cyanobacterium *Synechococcus* sp. occurred and persisted throughout most of the 2018 summer, despite nutrient concentrations remaining low. It is likely that the small size of *Synechococcus* sp. (< 2µm) contributed to efficient nutrient acquisition from the environment, not possible by larger species (Finkel et al., 2009; Shuter, 1978). Furthermore, from my observations, temperature appeared to be the main controlling factor of *Synechococcus* blooms in the

Gippsland Lakes, with blooms of *Synechococcus* occurring during summer periods when temperature are warm (Beardall, 2008; Flombaum et al., 2013). Additionally, this research, along with previous investigations, found no clear relationships between the onset of *Synechococcus* blooms and nutrients and salinity (Flombaum et al., 2013). Lastly, I found that the biological activity from the *Synechococcus* bloom, in combination with the freshwater input, played a role in the large variability observed in the inorganic carbon system of the lakes. The photosynthetic biomass in the Gippsland Lakes may cause the lakes to become a temporary sink for atmospheric CO_2 when that biomass is high (Balmer and Downing, 2011; Gu et al., 2011).

After assessing the environmental drivers controlling the natural phytoplankton assemblage in the Gippsland Lakes, I investigated the role of light, salinity and temperature on growth and photosynthesis in four recent phytoplankton isolates, namely, *Chlorella* sp., *Kirchneriella* sp., *Prymnesium parvum* and *Sarcinochrysis* sp. The results of this study demonstrated that the four phytoplankton species investigated have broad tolerances to a range of light, salinity and temperature, reflecting the fluctuating conditions experienced in their natural environment. Growth rate data showed a preference for low salinity in the two chlorophytes, though both were capable of tolerating salinities up to 25 psu, while *Sarcinochrysis* sp. and *P. parvum* had a preference for higher salinity. Despite the differences in growth and physiology observed between the species, similar photoacclimation strategies, producing more chl *a* under low light and increasing NPQ under high light, were utilized by all four species when grown under different light conditions. The *Sarcinochrysis* sp. isolate (SarcinGL07) showed the highest rates of growth and photosynthesis of all the species under almost all treatments. Despite its rapid growth, no blooms of SarcinGL07 have been reported in the lakes. Further studies on nutrient requirements and grazing could therefore be informative. Furthermore, results suggest that this brackish lake system provides ideal growth conditions for the toxic *P. parvum*, and raises concern for future blooms. Indeed, the presence of *Sarcinochrysis* and *Prymnesium* has not previously been reported for this lake system. The findings of this study highlight the physiological and growth characteristics of four different microalgae found in a brackish lake system, providing crucial insights into how they may respond to future climatic changes. This study demonstrated that salinity is an important parameter for controlling growth of all four species at their suboptimal levels. The

two chlorophytes are competitively advantageous in low salinity conditions, but due to the generally brackish salinity throughout the Gippsland Lakes, potential blooms from either species is unlikely. The salinity and temperature treatments in this study that promoted high growth from the toxic and euryhaline PrymGL20 are similar to the summer month conditions typically found in the Gippsland Lakes, raising concerns of the potential bloom threat in the future.

In order to detect the impacts of climate change there is a need for increased and more sophisticated monitoring. Utilizing ATR-FTIR spectroscopy allows researchers to rapidly gain valuable information on cellular composition with minimal sample volume and preparation. Using this technique allowed us to investigate changes in macromolecular composition of the four phytoplankton species from the Gippsland Lakes studied in Chapter 3 in response to environmental factors. The results of this chapter highlighted the control that environmental factors, particularly light, temperature, and salinity, exert not only on growth and photosynthesis of microalgae (Chapter 3), but also on cellular metabolism. Macromolecular composition showed clustering under treatment levels for light, salinity and temperature, seen in the PCA scores plot for each species. Alteration of macromolecular composition was found to be largely related to optimal and suboptimal growth conditions for each species. Two species, *Kirchneriella* sp. and *Sarcinochrysis* sp. demonstrated the characteristic stress response in suboptimal growth conditions (Dao et al., 2017; Giordano et al., 2001; Minhas et al., 2016; Stehfest et al., 2005), whereby the allocation of carbon into proteins was switched towards production of more carbohydrates and lipids (for their larger carbon storage capacity) (Fanesi et al., 2014; Palmucci et al., 2011). In contrast, I found that *Prymneisum parvum* maintained cellular homeostasis in response to changes in light, which I attributed to its mixotrophic ability. In summary, this study identified species-specific variations in cellular levels of the main macromolecules in response to light, salinity and temperature regimes. Furthermore, the results highlight the practicality and usefulness that ATR-FTIR provides as a diagnostic tool for microalgae research.

Lastly, Chapter 5 presents the first report on the vertical distribution patterns of *Prymnesium parvum*. In recent decades, there have been increased reports of damaging blooms of *P. parvum* in coastal and inland waters (Brooks et al., 2011). In addition, *P. parvum* is thought to be expanding its territory worldwide (Roelke et al., 2007). Understanding how *P.*

parvum utilizes its motility to move through, and affect its distribution in, the water column can help in elucidating mechanisms behind the success of this species. *P. parvum* did not undertake diel vertical migration, a behaviour commonly observed in some species of dinoflagellates and phytoflagellates (Cullen and MacIntyre, 1998). Instead, *P. parvum* accumulated at mid-depth in the water column, which was found to be a depth of optimal growth light (Chapter 3). Even nutrient depletion in the surface waters did not trigger *P. parvum* to undergo DVM in order to acquire nutrients from the bottom waters. It is hypothesized that the likely cause of this is *P. parvum*'s mixotrophic ability, in that it is capable of switching to heterotrophy and ingesting bacteria in the water column (Carvalho and Granéli, 2010) to compensate for the lack of nutrients available. Under nutrient limitation I observed an increase in rate of descent through the water column. Several hypothesis behind this apparent increase are proposed, however future work to confirm the main cause is needed. I classified *P. parvum* as a layer-former, as it remained in a distinct strata governed by optimal light for both growth (Chapter 3) and toxin production (Granéli and Salomon, 2010). Furthermore, this species possesses several attributes, key for being a successful layer-former, including flagella to maintain its position (Manton and Leedale, 1963) and the ability to produce allelopathic chemicals to supress competition and repel and kill predators (Tillmann, 2003). As a layer-former, it is unlikely that *P. parvum* acts as a transport vector, actively moving material and energy through the water column, unlike other vertically migrating species, which feed or photosynthesize at one depth and then move to another depth to respire, excrete, or be preyed upon (Salonen et al., 1984). I conclude that *P. parvum* utilizes its motility in a way that exploits its adaptive strategies to their full potential, providing advantages in the competition of growth and resource acquisition.

In conclusion, this research highlights various strategies utilized by four phytoplankton species in response to key environmental factors. Specifically, it was found that both behavioural and cellular strategies are used to regulate growth and physiology under different levels of light, salinity, temperature, nutrients and stratification. Together, the results of my thesis provide further insights into our understanding of phytoplankton ecology and contributes to the ongoing research into how phytoplankton will respond to future environmental changes brought on by climate change. The knowledge and data from

this thesis will be passed onto the Gippsland Lakes environmental managers to use for informing on policy design and creating alerts for conditions ideal for blooms of the species studied, as well as, to researchers creating a 3-D coupled hydrodynamic biogeochemistry water quality model.

LIMITATIONS & FUTURE WORK

The measurements in this thesis used reliable and well-known techniques to investigate photo-physiology and macromolecular composition, including PAM fluorescence, oxygen electrodes and ATR-FTIR spectroscopy. There were however some limitations to the experimental design. Only one strain of each species was studied in Chapter 3 and 4, and recent work has shown that many species of phytoplankton are found as multiple strains, each capable of exploiting slightly different physiological niches (Islam and Beardall, 2017; Olofsson et al., 2016; Pierangelini et al., 2014), therefore future studies assessing more strains would be beneficial to evaluate how the species as a whole may respond to future changes. Furthermore, Chapter 3 only looked at 4 species isolated from the Gippsland Lakes, not including *Nodularia*, *Synechococcus* and a diatom. *Nodularia* from the Gippsland Lakes had been previously studied (Myers, 2008), and delays in isolating a diatom and *Synechococcus* prevented these two species from being included in experiments. Lastly, while this study investigated important factors affecting primary production that are influenced by climate change (light, salinity and temperature), these properties were studied independently. Climate change alters oceanic conditions in a complex manner, simultaneously altering multiple properties (Boyd et al., 2015), therefore, it is recommended that future work incorporates multi-stressors into experimental design.

My original aim of this PhD was to assess the vertical migration patterns of *Nodularia spumigena*. Unfortunately, due to issues isolating this species and growing it in culture, I was unable to achieve this goal. Future work on vertical migration patterns of *Nodularia* would help in elucidating the role, if any, that vertical migration plays in nutrient transport from the bottom waters to the surface in the Gippsland Lakes. Toxic *Nodularia* blooms are a worldwide issue, as the toxin nodularin can interfere with human activities, contaminate drinking water and kill pets and livestock (Voß et al., 2013). Therefore understanding the adaptive significance that vertical migration plays in bloom formation and persistence would be beneficial to the global community. Furthermore, assessment of the growth, physiology and cellular composition under variations in key environmental parameters would be valuable for *Synechococcus* sp. isolated from the Gippsland Lakes. As blooms of this non-toxic cyanobacterium are increasing in occurrence the lakes, having a better understanding of its autecology would be beneficial for environmental managers of the lakes (Beardall, 2008). I

was able to successfully use FTIR spectroscopy on laboratory monocultures, therefore future research utilizing FTIR spectroscopy to assess natural populations would be valuable.

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