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Profiling an invader - is the invasive cyanobacterium *Raphidiopsis raciborskii* on the path to ecological dominance in Australia in the context of environmental change?

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Statement of Originality

This is to certify that, to the best of my knowledge, the content of this thesis is my own work and has not been submitted for any degree at this, or any other institution.

I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

Thomas Jephcott
June 2019

Abstract

Biological invasions are commonly reported ecological phenomena and are universally accepted as symptoms of the Anthropocene. Microbial invaders are particularly difficult to study, but potentially represent the most serious group of invaders, due to their extremely rapid responses to changing conditions. In this group, phytoplankton are perhaps the most significant; they are responsible for most of the primary production in aquatic ecosystems and are therefore the cornerstone of aquatic food webs. However, they can also produce toxins that pose threats to water quality, particularly when they form extensive blooms. One species exhibiting invasive behaviour is *Raphidiopsis raciborskii*. Known for its production of potentially lethal toxins, and flexible physiology, it favours warming temperatures and stratification regimes. In Australia, these conditions are becoming more prevalent, and with the species already having a foothold in many Australian ecosystems, it may continue to spread and impact ecosystem servies and stability. In this thesis, we use a combination of historical, theoretical, laboratory, and monitoring data studies to examine the factors behind the success of *R. raciborskii* in the context of Australian ecosystems. This research provides novel contributions to the field by demonstrating that 'invasiveness' is a symptom linked to environmental change, that *R. raciborskii* exhibits metabolic plasticity under various conditions, that *R. raciborskii* may be implicated in changing the bacterial community structures of freshwater systems, and that the historic presence of *R. raciborskii* in Australian environments reinforces its theoretically and experimentally purported environmental niche. This knowledge facilitates discussion of Australian water security and ecological health, and the critical importance of phytoplankton communities, in the face of an increasing population and cascading environmental change.

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Thesis Structure

This thesis is formatted as a collection of pieces, including an introduction, a series of chapters, all of which are currently in preparation for submission as a published manuscript, and a conclusion. Where a chapter is referenced in another chapter, the chapter number will be noted as such: (Chapter #). The structure is as follows:

Thesis Introduction

Chapter 1 – a literature review addressing projected changes in Australian aquatic systems, biological invasion theory and its application to Australian systems, the activity of cyanobacteria in Australia, and key systems within Australia that may be susceptible to a combination of these threats.

Chapter 2 – a quantitative review examining drivers of four invasive species of phytoaplnkton, one of which is *Raphidiopsis raciborskii*, and comparing these drivers with the physical traits of these species and global aquatic system changes.

Chapter 3 – a laboratory study based on batches of *Raphidiopsis raciborskii* grown as monocultures, and treated with a combination of different temperatures and CO_2 concentrations, under both N-rich and N-poor scenarios.

Chapter 4 – a laboratory study based on laboratory ponds generated from an urban pond system in Sydney, where phytoplankton communities are monitored for changes in response to warming and the introduction of *R. raciborskii*.

Chapter 5 – a modelling study based on historical monitoring data from water bodies in the Australian states of Queensland and South Australia, with a focus directed at the incidence of *R. raciborskii* and the factors most strongly driving its distribution.

Thesis Conclusion

Table of Contents

Thesis Introduction

In order to locate a wild hive of honey bees, a process of triangulation is used, where the seeker of the hive will walk in a wide radius and watch for foraging bees. When a bee is spotted, the seeker waits until the bee finishes foraging, and notes which direction the bee flies in; presumably the direction of the hive. Repetition of this process, measuring distances walked, and drawing of the theoretical flight paths of the foraging bees, will allow the seeker to accurately pinpoint the location of the hive. However, the process tells the seeker nothing about how difficult the hive will be to access once found.

Harmful phytoplankton is defined in contemporary research as varieties of phytoplankton, most typically cyanobacteria, that produce compounds exhibiting deleterious effects on other organisms, and that are capable of forming masses of sufficient density to result in concentrations of deleterious compounds that have a direct impact on the safety, stability, or serviceability of the systems in which they act. Studying harmful phytoplankton involves the synthesis of several fields and perspectives; it lies at the intersection of microbial biology and ecology, ecosystem and food web dynamics, water quality and security, resource policy and management, and anthropogenic ecosystem change, including climate change and more localised change entities such as agricultural activity and urban development. It is truly a multi-disciplinary field with avenues of influence extending from social impact to theoretical biology; this makes it a complex and contentious subject of research. The increasing incidence of harmful cyanobacterial blooms worldwide has been most clearly linked to a combination of anthropogenic climate change combined with human influence on nutrient fluxes in freshwater bodies. These combine to make conditions ideal for exponential proliferation of cyanobacteria in water bodies, resulting in extremely high cell densities, with follow-on effects observed on ecosystem health and general functionality; for example bloom activity has been linked to large incidences of fish deaths worldwide due to their tendency to create anoxic conditions in the water column as heterotrophic bacteria respire and feed upon the masses of phytoplankton biomass readily available.

Distinct varieties of cyanobacteria are shown to occupy niche space according to the characteristics of the environment they are in, and the physiological characteristics of each species. Thus, dominant species of blooming cyanobacteria may be viewed as representative of the ecosystems they act in. Recently, researchers in the field have identified several

distinct changes in dominant phytoplankton species worldwide, with several species behaving invasively, meaning they are growing further afield in regions they have not been observed previously. These incidences of invasion seem to part of a growing trend in increasing activity of invasive species worldwide, as further indicators of unprecedented environmental change dramatically altering ecological dynamics and facilitating the creation of niche space. One variety of cyanobacteria, *Raphidiopsis raciborskii*, has received much attention over the past decade due to its migration from tropical areas into temperate regions, which have previously been considered as unsuitable for its growth. As the species produces potent hepatotoxic and neurotoxic compounds, its spread has caused considerable concern. Given the progressing shifts in climatic conditions globally, the spread of the species will theoretically be promoted over the mid- to long-term as environmental changes continue to occur as a resul of our activities. *Raphidiopsis raciborskii* appears to be migrating from several points where its populations have historically flourished; one of these points is Queensland, in the North-Eastern region of Australia. Due to Australia's erratic hydrological regimes and history of harmful cyanobacterial blooms, it is key that researchers identify potential threats to water quality in order to better manage our resources and environment.

This thesis attempts to address the question 'Is the invasive cyanobacterium *Raphidiopsis raciborskii* on the path to ecological dominance in Australia in the context of environmental change?'. This is a broad and far-reaching topic, with aspects of invasion biology, cyanobacterial dynamics, ecosystem community structure, climate change, and Australian hydrology, all making up a critical part of the whole. As such, the approach taken in this thesis is in some ways like the hive locating process described above. Below, chapters are presented that approach the overarching topic from different directions, in order to align findings with those obtained in other chapters. Alignments achieved can then be followed to a logical statement, which will go towards outlining part of the answer to the thesis question, or a novel issue in the field that commands further investigation.

Firstly, a literature review has been conducted. The aim here is to summarise contemporary knowledge on the key discipline areas that form part of the thesis question, namely how aquatic systems are changing in response to shifts in climate and our management regimes, how invasive species are at the forefront of environmental change and are a symptom of ecological disruption, and how cyanobacteria are a key threat to water security and ecosystem productivity and stability, all within the context of the Australian biome. Finally, key systems

within Australia are highlighted as being vulnerable to the pressures described above. The central questions being addressed here are:

1) *How are Australian aquatic systems changing?*
2) *Why are Australian aquatic systems vulnerable to change?*

Secondly, a quantitative review is used to synthesise information gathered on invasive phytoplankton globally and link their success to specific measures associated with environmental change. One of these invaders is *Raphidiopsis raciborskii*, the organism of focus within this thesis. This chapter seeks to illustrate that although the focus of the thesis is on one species of phytoplankton, there are other species exhibiting similar expansion traits worldwide, and that there are consistent characteristics that are shared between them. The findings of this chapter will be used to support information presented in Chapter 1, and to further familiarise the reader with invasive phytoplankton and their driving factors. The central questions being addressed here are:

1) *Are specific environmental parameters more important in influencing invasive phytoplankton than others?*
2) *Do these parameters link with physiological traits possessed by invasive phytoplankton?*

Thirdly, *R. raciborskii* will be grown in monocultural batches, and subjected to different temperature, CO_2, and N regimes. This chapter will provide detailed information on the physiological responses of *R. raciborskii* to a combination of changing conditions, with the intent being to outline a flexible physiology that may explain why it is a successful invader. The central questions being addressed here are:

1) *How does R. raciborskii respond to shifts in temperature and CO_2?*
2) *How successful is R. raciborskii at maintaining its population densities under nitrogen poor scenarios compared to nitrogen rich scenarios?*

Fourthly, *R. raciborskii* will be introduced to freshwater laboratory ponds, which will also be heated at different temperatures. Here an environment will be provided based on an urban system that *R. raciborskii* has never been reported in, and a hypothetical invasion simulated. This chapter attempts to illustrate how a microbial community might be affected by a foreign cyanobacterial invader. It is a laboratory-based approach as is Chapter 3, however the results

garnered here will be applicable to freshwater communities. The central questions being addressed here are:

1) *How do freshwater microbial communities respond to warming conditions?*
2) *Do freshwater microbial communities exhibit changes in structure because of the presence of an alien invasive cyanobacterium?*

Fifthly, historical monitoring data that is comprised of physiochemical water measurements and phytoplankton cell counts will be analysed with respect to the drivers of *R. raciborskii*. Two large regions of data will be compared, the sub-tropical North-Eastern Australian state of Queensland, and the temperate Southern Australian state of South Australia. Results obtained in previous chapters will be compared to results obtained here to assess how laboratory studies portray *R. raciborskii*, against how the organism has behaved over a large temporal and spatial scale. The central questions being addressed here are:

1) *How do the key drivers of R. raciborskii in two regions with different climates?*
2) *Does R. raciborskii exhibit any relationships with other varieties of harmful algae over long temporal and spatial scales?*

Although the resounding theme of these chapters is *R. raciborskii* and its presence in Australia as a potentially harmful, invasive, bloom-forming cyanobacteria, the results of these chapters will also provide other insights into freshwater ecosystems, and valuable information on various approaches one can take in order to answer a biological question. Furthermore, each chapter is not without its limitations, and these are discussed in light of the results obtained. The thesis is concluded with a discussion, which will provide answers to the central questions presented above based on results garnered, synthesise their answers, and present areas for future investigation.

This thesis is about synergy, that is, how various influential factors meet to strengthen an overall result. Here, the meeting factors are invasive species, environmental change, cyanobacterial dominance, and the result being promoted is a species of cyanobacteria, *R. raciborskii*, that poses a hypothetical threat to water security in Australia. Although Australia is the region of focus here, the approaches and reasoning may be applied elsewhere in the world, as invasive, species, environmental change, and cyanobacterial dominance are global issues.

1. Harmful cyanobacteria, invasive species, and climate change in Australia – a deadly recipe?

1.1 Abstract

We live in a world of drastic environmental change. Although much effort is placed into understanding aspects of environmental change, the interaction between these aspects can often be disastrous for ecosystems. Two key relationships beginning to emerge from recent research are the emergence of invasive species and serious increases in the incidence of harmful phytoplankton blooms, because of shifting environmental conditions. Both dynamics can be identified in Australia, which is a region of environmental extremes. Australia has a dynamic and rich history of cyanobacterial activity; from Francis' first report of a toxic bloom in 1878 to the present, the presence of cyanobacteria in Australian waterways has inspired numerous studies, management strategies, and caused significant water quality and health issues. Due to our colonial history, and the evolution of highly specialised ecosystems because of our relative isolation, Australia is also inhabited by many foreign invasive species, which have had noteworthy detrimental effects on our environmental stability and diversity. These facets of Australia's environmental profile are further complicated by anthropogenic climate change, which is predicted to place strain on the stability of our natural resources, and the capability of our biota to adapt to conditions which are shifting at an increasingly rapid pace. Considering these issues, we compile a review of the literature to answer two questions: is climate change responsible for an increase in harmful phytoplankton blooms in Australia and are novel changes in cyanobacterial dynamics analogous to biological invasion? Our review seeks to not only answer these questions, but to investigate to what extent they are related. By compiling information about the history of cyanobacterial activity in Australia, and examining key systems that have been affected by cyanobacterial blooms, we find that changing system dynamics can be most readily linked to changing climatic dynamics, including storm events, rainfall fluctuations, and human management. Furthermore, the spread of novel cyanobacterial species may be symptoms of environmental change, rather than representative of novel range migrations. It is our hope that undertaking this work will promote further investigation into the relationships between factors of environmental change being documented today.

1.2 Introduction

Cyanobacteria are a principal water quality problem in Australia (Thompson et al. 2003, Bowling et al. 2013a, Williamson et al. 2018). They are diverse and widely spread, with blooms being reported in every mainland state (May 1980), and issues associated with freshwater blooms costing the Australian government $180 – 240 million each year (Atech 2000). If marine bloom effects are included in this assessment, the cost would be significantly higher (Davis et al. 2006). By their very nature, Australian terrestrial systems are well suited to algal proliferation. Low flows and elevated temperatures promote stratification, and large stores of sediment provide a phosphorus bank for phytoplankton to draw on, as well as a resting area for dormant cell stages (Carey et al. 2012). Toxins produced by cyanobacteria can pose a serious health hazard to water users, livestock, and aquatic organisms (Ingleton et al. 2008). The water quality issues that cyanobacteria present are compounded by the fact that, globally, cyanobacterial populations are likely to increase significantly with the onset of climate change (Elliott 2012). In contrast to European and American systems, Australia's freshwater, marine, and estuarine systems are lower in nutrients, which is partly due to less atmospheric N deposition, and partly because Australian catchments are, comparably, less disturbed (Rukminasari and Redden 2012). Thus, it is a reasonable expectation that Australia's cyanobacterial dynamics will be markedly different from those observed in other regions. In Australia, the main toxin producing cyanobacteria have been identified as *Dolichospermum circinalis*, *Microcystis aeruginosa*, and *Raphidiopsis raciborskii* (Shaw et al 1999, Bowling et al. 2013a, Main et al. 2018). Additionally, the species *Nodularia spumigena* has caused significant water quality issues but is generally confined to more saline environments such as estuaries (Boon et al. 2016).

Invasion biology is a field still coming to terms with its own definition, as there is a significant difference between artificial and natural range expansions, and empirically determining what classifies a species as invasive remains difficult (Catford et al. 2018). For the purposes of the work here, we are using the term invasive to denote a species that is exhibiting an unprecedented geographical expansion with associated ecological detriments, since without these detriments biological invasions would most likely go unnoticed. In Australia, such classic examples as the rabbit (Hall et al. 1964), mouse (Mutze 1989), European carp (Shearer and Mulley 1978), and contemporary examples such as the cane toad (Beckmann and Shine 2009), and lantana (Debuse and Lewis 2014) come to mind. The success of such species is determined by several factors, which can vary according to which

framework is applied, however they are, generally, 1) the characteristics of the invader species; 2) the characteristics of the novel location; and 3) the characteristics of the introduction, which commonly includes the population density of the initial invasion, also referred to as the propagule pressure (Blackburn et al. 2011). Identifying species that have a high potential of becoming future invaders is of immense practical significance, however this is challenging, as most biological invasions, excepting diseases of humans, are based on post hoc observations. However, characterising invaders as such can be simplified to a distinction between whether there was a compromising vulnerability present in the system in question, or whether the invader in question possessed extraordinary attributes (Mack et al, 2000).

The cyanobacterium *Raphidiopsis raciborskii* has drawn much attention and research over the past decade due to its rapid spread from a narrow tropical range to an established global distribution (Antunes et al. 2015). The species possesses several physiological traits that have been put forward as explanatory for its success, such as an affinity to inorganic phosphates, a strong growth response to warmer temperatures, diazotrophy, and cyst formation. Furthermore, the species has been the subject of several studies illustrating the avoidance of the species as food by grazer zooplankton and, when consumed as food, negative effects on growth and survival (Hong et al. 2015, Gebrehiwot et al. 2019). The combination of these traits is purported to give *R. raciborskii* a phenotypic flexibility that allows it to cope and flourish under shifting conditions. However, in addition to the citation of its physiological prowess, explanations for its sudden range expansion have also sited climate change and system disturbance as additional significant driving factors (Sinha et al. 2012). Furthermore, the application of current frameworks to characterise microscopic invaders is challenging; for example, how would one establish the propagule pressure of a starting population of *Raphidiopsis raciborskii*? Is *Raphidiopsis raciborskii* an alien invader, or is it ubiquitous and only now responding to recent shifts in environmental circumstances? This question is important, as it redirects focus back to humanity and how our activities are changing the environment around us, instead of the remarkable traits of certain species that allow them to flourish in a wide variety of conditions (Wood et al. 2014). These limitations being considered, a different approach is needed to consider the mechanisms behind the spread of invaders such as these. Current accounts of the activity of *R. raciborskii* agree that its dominance appears linked to anthropogenic environmental change. Thus, a potentially interesting question to ask is: will system disturbance linked to climate change increase the

vulnerability of systems to invasion by alien species, or is this a niche *R. raciborskii* is particularly suited to occupy?

The present review will use the activity of *Raphidiopsis raciorskii* in Australian systems as a case study with the view to providing a preliminary attempt at answering the above question. We hope that not only will this analysis reveal patterns in pressures that environmental systems are subjected to and invaders that may reduce the ecological services these systems provide, we hope to also identify specific systems that could be at risk of invasion in the near future. The second is that although there is much information available regarding cyanobacterial bloom dynamics in Australia, it is impossible for us to cover all water bodies, and indeed all bloom events, as most nuisance blooms pass undocumented (Bowling 1994). Despite this, we feel that the review presented here provides a novel, valid, and much-needed approach to understanding and working with the intricacies of aquatic ecology, climate change, and water management in Australia.

1.3 Change in Australian Systems

Ecologists are faced with significant challenges when making decisions based on future projections. There are several methodologies that are used when trying to do this. Firstly, extrapolation of past environmental trends can give a depiction of future trends, however it is only potentially reliable over short time periods and generally does not enable non-linear patterns in parameters. Furthermore, predictions of climate over short time periods are difficult to interpret with respect to general changes, due to the presence of natural climate variability (Hobday and Lough 2011). Climate models can generate reasonably confident estimates of shifts on a continental scale, however because Australian exhibits such high regional climatic variability, there is a necessity to predict at a finer resolution, ideally at the system scale (Gillanders et al. 2011). One of the key projected changes that threaten Australian hydrological systems is significant alterations in the frequency and intensity of storms and primary rainfall events, with events expected to become more severe but less frequent. This places increasing pressure on ecosystems that depend on regularly occurring flood events, as wetlands, which represent a significant biodiversity component, are forced to go without replenishment for lengthier periods of time (Pittock and Finlayson 2011).

Australia's freshwater systems range from ephemeral billabongs and inland lakes, seasonal creeks and rivers, to permanent tropical rivers (Lough and Hobday 2011) and are heavily burdened by a variety of water quality and system degradation problems (Gippel 2001). Of these problems, cyanobacterial blooms have become an increasingly major component, and a symptom of poor river health (Edgar et al 2001). The increase in blooms have arisen primarily as a result of streamflow regulation, with the construction of many thousands of weirs, over 400 large dams, and the development of over 50 intra- and inter-basin water transfer schemes since 1857 (Arthington and Pusey 2003). Weirs in Australia often suffer from extensive cyanobacterial blooms due to their lack of flow, which usually acts as a mechanism of nutrient dispersal following rainfall events (Ganf and Rea 2007). To compound the issue of over-managed systems, Australia is a dry continent and an estimated 70% of river channels are intermittent (Datry et al. 2014). Furthermore, the large catchments and stream systems of Australia exhibit extremely high variability in runoff and generally low discharge levels and sediment delivery efficiencies, leading to large amounts of sediment accumulation (Rutherford and Gippel 2001). These sediment stores act as nutrient repositories that can release vast concentrations of inorganic nitrates and phosphates in the event of heavy storm, which can then lead to high density algal blooms. For example, Warragamba Dam, one of the largest domestic water storage areas in the world, supplying Sydney with up to 80% of its domestic water (Kristiana et al. 2011), suffered extensive and toxic blooms after 9 years of severe drought from 1998 – 2007 reduced water levels to a point that 1400 hectares of sediment were exposed. Heavy rainfall in June and July of 2007 caused these sediments to be mobilised into the lake, with the resultant blooms causing significant water quality issues to the Sydney region (Kristiana et al. 2011). The increased incidence of cyanobacterial blooms in Australia represents a considerable social and economic impact, with the disruption of businesses (Davis and Koop 2006), ecosystem services (Osborne et al. 2007), potential risks to human health (Svircev et al. 2017) and water quality (Barrington et al. 2014).

Australia's inland arid and semi-arid water systems are plagued with elevated salt levels, and shallow saline wetlands or salt lakes are numerous and widespread. These systems arise primarily as a result of the high evaporation rates and atmospheric accession of salt, or the presence of remnant marine deposits, which then enter the water table (Davis et al 2003). Thus, larger freshwater systems that are not intermittent or prone to such extreme salinities are heavily relied upon for drinking and irrigation purposes, and this reliance is increasing

due to population growth. For example, water withdrawal from the Murray-Darling basin increased substantially through the 20th century up until the 1990s, whereupon the Australian government put in place a restriction on extraction licenses to curb problems that had arisen in the system, such as salination, declines in biodiversity, and cyanobacterial blooms (Pittock and Finlayson 2011). Such extraction regimes, coupled with high temperatures and irradiances, promotes water body stratification, or the distinct separation of the upper and lower water layers. With the additional pressure of severe droughts that are increasing in frequency and intensity, the outlook for many Australian freshwater systems is bleak.

Estuaries are ecologically sensitive and vulnerable systems due to their transitional nature, being dependent on a consistent freshwater and saltwater mixing regime. Thanks to this dynamic, they are also highly complex and comprise some of the most productive ecosystems on the planet, acting as habitats for a large array of organisms (Beck at al. 2001). Australia has a diverse variety of estuaries which are influenced by coastal wave action, tidal movement, and river flow, as well as climatic and hydrologic factors including quality and quantity of riverine and groundwater flow, air-water fluxes of CO_2, air and water temperatures, atmospheric activity, and fluctuations in sea level (Koehn et al. 2011). These systems will be threatened by anthropogenically driven changes such as increases in atmospheric CO_2, warming of air and water temperatures, fluctuations in the stability of rainfall events and freshwater flows, and increased incidence of storm surges and intense wave conditions (Gillanders et al. 2011). Each of these shifting components represents a significant ecological stressor. Increases in CO_2 will acidify the brackish waters of estuaries; ocean pH in South West Australia is predicted to decline by as much as 0.08 units by 2030, and by as much as 0.15 to 0.33 units by 2090, which can be computed to an increase in acidity of 40 – 110% (Hope et al. 2015). Increases in air temperatures will lead to greater levels of evapotranspiration; this is critical for estuarine systems because evaporation of water will raise salinity levels significantly. Extending on this is the disruption of regular rainfall patterns, with most of Australia set to receive less rainfall, but heavier downpours when it does occur (Cook et al. 2016). This too will mean swings in salinity of greater magnitude than has previously been seen. Higher volumes of run-off occurring more irregularly will also result in larger swings in nutrient concentrations (Larned et al. 2004, Gu et al. 2019). This will particularly be the case in systems that lie adjacent to farming land (Shrestha et al. 2019).

The issues associated with anthropogenically driven change in Australian systems as outlined above are most easily quantified by the ecosystem services that will be threatened by these changes, and the monetary value these services represent. Ecosystem services can be split into two broad categories; functional, and recreational. The most obvious functional service is typically the reliance on large freshwater systems for drinking water, as is the case with Warragamba Dam in Sydney, however other functional services, such as fishing activities and the drawing of irrigation water, are of immense societal value (Koehn et al, 2011, Doody et al. 2015). Dams are critically important for continued access to freshwater for society, however they are also a primary driver of degradation in freshwater systems (Pittock and Hartmann 2011). Dams are typically designed without thought given towards natural behaviours of native organisms, for example fish migrate upstream to spawning grounds in order to reproduce but they are prevented from doing so by dams (Garcia de Leaniz 2009). Not only are natural life cycle stages impinged on, but dams also restrict the ability of aquatic organisms to migrate in response to changing conditions (Olden and Naiman 2009). Furthermore, dams promote the development of thermally and chemically stratified reservoirs that can significantly alter downstream water conditions, for example the storage of cold water from high altitude run-off by dams in the Murray-Darling Basin can lower the water temperature by as much as 12 °C, significantly impacting the breeding capabilities of native fish (Sherman et al. 2007). Why can ecological stability or system health be correlated to a systems ability to provide a service? In the case of freshwater systems, we are heavily dependent on other organisms, such as grazer zooplankton, to consume and redistribute energy from primary producers to higher level consumers; herbivores, or planktonic grazers, represents 60 – 70% of the consumption of phytoplankton primary production (Turner 2006). Many of these organisms are sensitive to shifts in the chemical makeup of systems, and the ecological collapse of these systems would promote excessive growth of phytoplankton, a significant component of which would be harmful cyanobacteria. Consequently, it has been suggested that algal blooms may occur as a result of shifting grazing regimes (Irigoien et al 2005). Whether by causing instability in systems through artificial fluxes in nutrients or climatic variables, or overdrawing water, the promotion of harmful cyanobacteria inevitably follows. From this effect all recreational services, which include businesses dependent on healthy freshwater systems for tourism-related activities, will be lost. It has been estimated that algal blooms in Australian freshwaters cost the community between 180 and 240 million dollars every year. If marine estuaries are included in this appraisal, the cost is much higher, and needs serious assessment (Davis and Koop 2006).

1.4 Invasive species in Australia

Australia has been identified as being especially vulnerable to biological invasions due to its relative geographic isolation, which allowed the evolution of highly complex and interdependent ecosystems with unique biota, but with very few predators (Phillips et al. 2010). The subsequent traffic brought about by European colonisation and the industrial revolution caused the introduction of many species to Australia, where they had few or no natural predators, and thereby flourished to the detriment of native species (Phillips et al. 2010). Of these invaders, the most notable example is typically the rabbit, a generalist consumer that has caused severe damage to crops and other native plants through over-grazing, thereby simultaneously depriving native species of resources. The activities of rabbits have also been implicated in soil erosion, as their grazing habits cause soil to be perpetually exposed to the harsh Australian elements (Cooke et al. 2013). The fox is another example; it has caused significant losses in native wildlife through predation (McLeod and Saunders 2011), and the cane toad, introduced to combat the crop-destroying cane beetle, is now out of control through the North-Eastern region of Australia, and spreading South and West across the continent (Videl-Garcia and Keogh 2017). Finally, lantana, a woody weed originating from South America, is now widespread through Australian forest and pasture systems, where it tends to cause a smothering effect via allelopathy and has been linked to declines in native plant diversity (Gentle and Duggin 1997). Australia is now amongst the most invaded land mass on the planet, by number of identified unique invaders, with an established increase in invaders predicted to continue as we enter an unprecedented era of globalisation (Rejmanek 2014). This is concerning not only from an environmental perspective, but a financial one too; it has been estimated that the cost of alien weeds to the agricultural sector alone is in excess of $4 billion annually (Sinden et al. 2004).

Why is the effect of invasive species in Australia so pronounced? It has been mentioned above that Australia regionally is subject to many limiting factors for the growth of biota; infrequent rainfall combined with old and heavily eroded soils means the continent is considerably limited in inorganic phosphorus (Lambers et al. 2010). Because of this, our plant and animal life are well adapted to coping with nutritional stress; eucalypts display various adaptations for water conservation such as pendulous leaf arrangements, sunken stomata and waxy cuticles, all which limit water loss via evapotranspiration (King 1997).

These adaptations come at a cost, however, which is namely relatively slow metabolisms and growth rates (Clarke et al. 2005). The agricultural development of Australia by European settlers involved heavy use of inorganic fertilisers and the drawing of water from freshwater rivers, which were necessary to ensure the production of vast amounts of food. This food, in the form of crops or stored grain, represented an unprecedented concentration of nutrients in the Australian environment. Due to the evolutionary history of the Australian biota, native organisms were poorly placed to take advantage of this boom in resources, however opportunistic alien invaders were perfectly placed, and used this agricultural development as a springboard into explosive population growth (Bradshaw 2012).

This dynamic, whereby the establishment and dominance of alien invasive species is facilitated by drastic shifts in environmental conditions due to human activity, has been replicated experimentally, with similar results being observed. In low-fertility sandstone soil systems from the Hawkesbury region in Sydney, Australia, added nutrients resulted in significantly increased activity of exotic invasive species, whereas native species did not respond and thereby gained no advantage from the increased availability of resources (Leishman and Thomson 2005). This example is rather specific to the Australian background of a transition from low to high nutrient statuses, however this could be looked at as one form of system disruption. One potential line of reasoning to consider is that global climate change is causing similar disruptive effects worldwide, which explains why the incidence of invasive species is increasing on a global scale (Simberloff et al. 2013). It thus stands to reason that the most vulnerable regions will be those that have the most introverted evolutionary history, as this represents a native population of biota that are adapted to living under a relatively narrow set of conditions; Australia is a prime example of this.

An identified feature of invasive species is that the longer the species manages to sustain an initially introduced population, the more likely the species will become invasive and continue to spread (Phillips et al. 2010). This can be seen as a certain ‘lag time’, whereby populations require a number of generations to pass before they are sufficiently acclimatised to the novel conditions of the invaded system and can begin expanding their range. Why this change occurs at all, and what separates species who merely maintain a population versus species that become an ecological nuisance, remains empirically unknown, though in plant species invasion success has been linked to high seed survivability in soil (Phillips and Murray 2012). Physiology is a commonly examined element of invasive species, namely flexibility; can a species remain viable through adverse conditions to take advantage of favourable

conditions that will inevitably follow? This line of argument pertains to the ecological niche, in that a species able to flourish only under relatively narrow conditions is less likely to continue to flourish through changing conditions (Higgins and Richardson 2014). To be more specific, the niche, from the perspective of an invasive species, can be described as fundamental, which refers to the absolute physiological preferences of the species, or realised, which refers to the niche the species is utilising due to limiting environmental factors that make the fundamental niche unobtainable. Invasive species that expand and succeed only according to the fundamental niche is considered as displaying niche conservatism, whereas species that demonstrate different responses to conditions in different environments are said to be displaying niche shifts (Hill et al. 2012). Thus, identifying whether invasive species are exhibiting niche conservatism or niche shifts can be an effective way to isolate the mechanisms behind the success of the species, though a main limitation here is that the fundamental niche can never be fully defined (Kearney et al. 2008). Here, the physiological characteristics of invasive species become important in discerning whether their behaviour is niche conservative or not.

1.5 Cyanobacteria in Australia

In Australia, the key species of harmful freshwater and estuarine bloom-forming cyanobacteria are the colonial unicellular *Microcystis aeruginosa* (Atkins et al. 2001), and the filamentous *Dolichospermum circinalis* (Mitrovic et al. 2011), *Nodularia spumigena* (Holland et al. 2012), *Raphidiopsis raciborskii* (Burford et al. 2016), and to a lesser extent *Chryosporum ovalisporum* (Cirés and Ballot 2016). Bloom formation is most often a mixed population of two or more of these species, and traits can vary quite significantly between strains of each species, making general bloom characterisation difficult (Al-Tebrineh et al. 2012). For example, each species consists of strains that either do or do not produce deleterious toxins (Willis et al. 2016). Blooms occur on a continental scale and are increasing in frequency and severity (Bowling et al. 2013a). They have been correlated with a wide array of environmental parameters, most commonly a combination of elevated temperatures over the warm season from October to February and eutrophication caused by large Summer rainfall events. This is especially the case in North Australia, which displays a semi-tropical to tropical climate, characterised by pervasive warmth and regular storms. Drought conditions exacerbate this trend, with many systems suffering from low flows that lead to

severe stratification, and also reduce the amount of water covering sediment deposits, making them easier to shift in the event of storms. Sediment agitation results in high concentrations of inorganic nutrients being mobilised and transported down-stream, which can lead to the formation of major blooms. Blooms have also been linked to management of freshwater systems, including dam construction and over-allocation of water, which leads to stiller and more stratified waters (Olden and Naiman 2009).

Australia is typically a P-limited environment, and logic would lead to the conclusion that because of this, bloom formation in Australia should most often be limited by low amounts of available phosphorus. However, phosphorus storage and release from sediments has been labelled as a secondary critical factor in the ongoing formation of harmful cyanoabacterial blooms (Carey et al. 2012). The binding of P to iron(III) oxyhydroxides is a mechanism whereby available P in lakes is limited, however anoxic or hypoxic conditions reduce these iron species to Fe(II), which is soluble, and the dissolution of the iron-phosphorus bonds cause the release of significant amounts of available P (Scicluna et al. 2015). Anoxic conditions are either caused by extensive blooms, the microbial breakdown of which involves sufficient respiration to take up most of the dissolved oxygen within the system, or mass entry of organic matter, which is similarly digested and causes oxygen uptake (Whitworth et al. 2012). This dynamic has had implications for bloom development in Australia, as growth of *Microcystis* is commonly N limited instead of P limited. McDougal and Ho (1991) reported an average extracellular N:P ratio of 4.9:1 in a study of the eutrophication of North Lake, and an estimated average cellular N:P ratio of 21:1 in samples of *M. aeruginosa*, with evidence that N was the primary factor limiting growth due to the high availability of phosphorus, which was observed at > 0.1 mg L^{-1}. The report stated that the large amounts of P available was probably due to the pH increase caused by high uptake rates of CO_2 from photosynthesis of algae, which caused liberation of phosphorus from sediments. pH values in the lake fluctuated between 7.5 - 10.9 and were strongly correlated with chlorophyll-a concentrations. This pathway of P-mobilisation has been reported in other studies (Carey et al. 2012). Nutrient enrichment in connection with *Microcystis* activity has also been reported in Lake Elphinstone by White et al (2003), with maximum total N concentrations of 3000 µg L^{-1}, and total maximum P concentrations above 200 µg L^{-1} recorded during 2002. Cyanobacteria were sampled in the lake in March, May, and July in 2002, and correlations between cyanobacterial characteristics and physical and chemical conditions were examined. During March the cyanobacterial community was dominated by *M. botrys*, however in May

this dominance had shifted towards *M. panniformis*. A corresponding change in toxicity was observed, with microcystin concentrations rising to exceptionally high concentrations (2500 μg L^{-1}). Other studies report on the influence of N:P ratios in determing successful varieties of cyanobacteria. *Dolichospermum* has been found to dominate under TN:TP (total N:total P) ratios of ~ 20, and DIN:PO_4 ratios of ~ 40, in floodplain lakes (Liu et al. 2019), complimenting the result found by Harris (1986) that suggested that diazotrophic cyanobacteria did not commonly dominate above a TN:TP ratio of 25 – 30, indicating that non-diazotrophic cyanobacteria would not be N-limited and thus diztotrophy would convey no ecological advantage (Smith 1983). Comparing these results to the tendencies of *Raphidiopsis*, it has been shown to succeed under a range of phosphorus concentrations; manipulation of phosphorus to achieve low and high TN:TP ratios has been shown to result in *Raphidiopsis* dominance in either case (Chislock et al. 2014). Applying variable phosphorus loads to cultured *Raphidiopsis raciborskii* has also shown that the morphology and growth rate of the species can change on a timescale of minutes to hours in response to nutrient availabilities (Amaral et al. 2014). It is worth considering that these studies were laboratory based; our field data demonstrates a clear negative trend between *Raphidiopsis* and phosphorus concentrations (Chapter 5), however other studies have reported positive correlations with turbidity and phosphorus, and, unintuitively, negative correlations with temperature (Kokocinski and Soininen 2012). These results suggest that locally-based dynamics play a role in the response of the species to driving factors, and that more reseach is needed to link the responses of the species with regional-based changes.

Cyanobacteria are well suited to proliferation in a rapidly changing environment due to several key physiological traits. Arguably the most well-known, and most socially impactful, but paradoxically least understood trait, is their production of harmful secondary metabolites, which are referred to as toxins due to their deleterious effects on other organisms (Downing et a. 2015). In Australia, issues with the presence of toxic cyanobacteria in drinking water revolve heavily around *M. aeruginosa*, *M. flos-aquae* and *M. panniformis*, the microcystins they produce, and their tendency to proliferate in supply reservoirs in temperate areas (Falconer 2001). The ability of the toxin to accumulate in high concentrations can be partly attributed to the self-shading effect of dense mats of *Microcystis*, which drastically increase water turbidity and decrease light penetration, thereby reducing toxin breakdown (White et al 2003). Toxin production is also observed in *Dolichospermum*, which causes intermittent blooms in water supply reservoirs, and whilst not as prevalent as *M. aeruginosa*, present a

serious health risk in the form of saxitoxins (Falconer 2001). Although worldwide *Dolichospermum* is known to produce the neurotoxins anatoxin-a and anatoxin-a(s), these are not present in Australia, and Australian isolates of *Dolichospermum* exclusively produce saxitoxin. The reason for this geographic variation is not known (Beltran and Neilan, 2000).

Cylindrospermpsis raciborskii is also a recognised toxin producer. The toxicity of *Raphidiopsis* is most commonly associated with the alkaloid toxin cylindrospermopsin, although the compound is also produced by *Chryosporum ovalisporum*, *Dolichospermum bergii*, *Umezakia natans*, *Raphidiopsis curvata*, *Chryosporum flos-aquae*, *Lyngbya wollei*, and *Dolichospermum lapponica* (Everson et al 2009). Production of cylindrospermopsin in *R. raciborskii* is also highly strain specific and appears to be heavily influenced by environmental conditions (Kokocinski et al. 2013, Rigamonti et al. 2018, Nor et al. 2019, Fu et al. 2019) (but see Pierangelini et al. 2015 and Willis et al. 2015). Concentrations of cylindrospermopsin in Queensland tropical reservoir systems are typically in the range of 1 - 10 µg/L, with 1 µg/L being associated with 20,000 cells/mL (McGregor and Fabbro 2000), however measurements as high as 100 µg/L have been observed in similar systems, significantly lower than is typically observed from *Microcystis*. *Raphidiopsis* has been labelled the cause of one of the most impactful documented human cyanobacterial poisoning incidents, with 149 people (mostly children) falling ill three days after copper sulphate treatments were applied to control a bloom in the local water supply on Palm Island in 1979 (McGregor and Fabbro 2000). *Raphidiopsis* has also been labelled as the cause of 'Barcoo fever' and 'Barcoo spew' that was a common malady in remote regions of central and northern Australia (Hayman 1992). *R. raciborskii* flourishes under warmer conditions; because of this, it is often found in stratified systems (Burford et al. 2016). These systems typically exhibit high irradiances because they are often low-flowing and nutrient poor, and *R. raciborskii* has been shown to exhibit increased growth rates at higher light concentrations (Nor et al. 2019). Thus, toxin production of *R. raciborskii* is often associated with shallow lakes and ponds in tropical and sub-tropical zones. Finally, *Nodularia spumigena* produces the hepatotoxic cyclic pentapeptide nodularin, which if ingested by animals can cause degradation of liver structure through inhibition of protein phosphatases. In severe cases this can lead to liver failure and death (Hobson et al 1999). The structure of nodularin and its protein phosphatase 1- and 2A-inhibitory action are similar to that of microcystin-LR (Fitzgerald et al 1999). Toxins have historically been the focus of much attention due to their adverse health effects on livestock and humans, however research has also examined their

ecological significance; defence against parasites (Rohrlack et al. 2013), deterrence against grazers (Rangel et al. 2016), and reduction of vitality in competitor phytoplankton (Leao et al. 2009).

Conditions can change rapidly in aquatic systems. A switch to unfavourable conditions can decimate previously flourishing populations due to the need in many organisms for consistent and ongoing nutritional intake (Miner et al. 2005). Cyanobacteria are not limited in this way because they are able to form resting cysts, also known as akinetes, in response to poor conditions not suited to growth. Akinetes are characterised by thick walls which allow them to survive turbulent conditions that may lead to cell desiccation, as well as cold conditions that would ordinarily kill vegetative cells (Kim et al. 2005). Akinetes are able to remain viable in a state of dormancy for decades and are thought to subsist within sediment deposits until conditions return to a more suitable state, at which time they will germinate into vegetative cyanobacterial cells (Ramm et al. 2017). However, it has been posited that akinetes submerged too deep within sediment deposits, particularly in deeper systems, will not be exposed to enough light to trigger germination; thus, only those akinetes that are exposed to sufficient light levels can be considered part of the akinete 'pool' that will contribute to a resurgence of Nostocales populations (Ramm et al. 2017). This suggests significant potential for more aggressive development of cyanobacterial populations in Australian systems due to the already high levels of evapotranspiration and irradiance that lead to shallow, stratified systems with low flow and high light penetration. Akinete production in filamentous cyanobacteria has been linked to physiological stress, such as cold temperatures, poor nutrition, or excessive grazing (Myers et al 2010). Although *Microcystis* do not differentiate akinetes, their cells do 'overwinter' in sediments in a vegetative state. This strategy may play an important role in bloom development; resting stage *Microcystis* from sediments have been associated with significant population development in the pelagic zone of water bodies (Stahl-Delbanco et al. 2003, Park et al. 2018). Cells in this state are more vulnerable to adverse conditions than akinetes are, however; warming has been shown to increase mortality in overwintering *Microcystis* cells. These declines in vegetative cell innocula have been shown to significantly reduce subsequent *Microcystis* populations (Verspagen et al. 2005), potentially exposing niche space for other varieties of cyanobacteria, such as *R. raciborskii* under warming scenarios.

Nitrogen fixation is arguably the most significant physiological feature of cyanobacteria in the context of environmental change. Also referred to as diazotrophy, nitrogen fixation is a

feature exclusive to the Nostocales, and involves the differentiation of thick walled heterocysts that can convert atmospheric N2 into NH_4^+. This gives diazotrophic cyanobacteria a significant advantage in N poor scenarios; decreasing nitrogen loading in lakes to mitigate cyanobacterial growth has been shown to have little effect on diazotrophic cyanobacteria (Higgins et al. 2018). The advantage nitrogen fixation conveys can be quite variable from species to species: *Dolichospermum* sp. typically dominate other non-diazotrophic cyanobacteria such as *Microcystis* under low N (Chia et al. 2018), whilst *Lyngbya* and *Cylindrospermopsis* typically dominate under low N and low P (Hannington et al. 2016, Fu et al. 2019). It is an energetically expensive process due to the need to break the triple bond present in the N_2 species, however has been shown to allow Nostocales cyanobacteria to maintain close to optimum growth levels whilst subsisting solely on fixed sources of N (Willis et al. 2016, Chapter 3). This would represent field scenarios such as oligotrophy brought on by extended periods of stratification, or by severe drought conditions, which would mean very little run-off making its way into systems, and thus a lack of nutritional replenishment. Both these situations are expected to increase in frequency and severity within Australian systems, suggesting that nitrogen fixing cyanobacteria may increase in abundance in Australian waters. Furthermore, many bloom management strategies have adopted a denitrification approach, where inorganic N inputs into a system are restricted to prevent excessive phytoplankton growth, however this would not limit diazotrophic cyanobacteria. Nitrogen fixation is an enzymatic process driven by nitrogenase and has been shown to increase in rate as temperatures increase (Chapter 3), thus warming, and nutrient-poor waters, are particularly suited to diazotrophic cyanobacteria.

Finally, cyanobacteria have several adaptations that allow them to cope with low concentrations of available P, which represents the most common limiting factor in freshwater systems, particularly those in Australia. Although eutrophication is commonly associated with combined loads of N and P (Smith and Schindler 2009), contemporary literature is increasingly highlighting the importance of P enrichment in encouraging biomass development (Schindler et al. 20008, Przytulska et al. 2017). Cyanobacterial adaptations to low P are the production of phosphatases, which are enzymes that allow cyanobacteria to hydrolyse phosphate from organic material which can then be utilised, and the ability to store excess P for later use (Reynolds 2006). Prolonged stratification and separation of the hypolimnion and epilimnion can lead to anoxic and nutrient rich conditions in the hypolimnion and nutrient poor conditions in the epilimnion (Carey et al. 2012).

Cyanobacteria, due to their luxury P storage and buoyancy regulation, can thrive in these conditions, whilst other varieties of phytoplankton that do not store excess P (Salmaso et al. 2018). Maintaining a standing population size under low phosphorus concentrations allow cyanobacteria to take advantage of future nutritional influxes derived from rainfall or storm events, which will also increase the likelihood of fringe phytoplankton varieties being outcompeted (Paerl and Otten 2016). More intense storm events will result in increases in mixing of system layers, and greater terrestrial runoff entering the system, both of which will result in higher overall available nutrient levels that will likely be utilised by dominant cyanobacteria present prior to the influx.

1.6 Focus Systems in Australia

1.6.1 The Myall Lakes

The Myall lakes consist of three shallow coastal lakes in East Australia: Myall Lake, Boolambayte Lake, and Bombah Broadwater. The lakes as a system have been the focus of a cyanobacterial study since 1999, when flood events caused a drop in salinity levels that allowed the development of a severe bloom of *Dolichospermum* in Bombah Broadwater, causing significant detriment to fishing and recreational activities in the area (Wilson 2008a). Additionally, the catchment of the upper Myall River has seen increases in agricultural activity, resulting in small increases in nutrient loads making their way into the system, which also contributed to the bloom event (Wilson 2008b). The lakes have historically been considered oligotrophic and pristine, with strong populations of macrophytes, and thus at low risk of cyanobacterial blooms (Dasey et al. 2005). The 1999 *Dolichospermum* bloom challenged this notion however, with concerns raised that the increased nutrient loads from the developed catchment areas would result in excessive phytoplankton growth and resultant shading and decline of macrophyte communities. Sediment data has revealed, however, that macrophyte communities have increased in abundance post European settlement, likely due to agricultural nutrient enrichment of waters, and a high enough salinity to prevent excessive dominance by *Dolichospermum* (Leyden et al. 2012). Between wet and dry seasons, which cause salinity to fluctuate, dominant phytoplankton taxa changes between *Dolichospermum* and the salt-resistant *Chroococcus* (Ryan et al. 2008). Interestingly, though the lakes have been classified as brackish, there has never been any history of activity by *Nodularia*. Finally,

the lakes are an excellent example of the presence of a thriving charophyte community in freshwater systems, as the charophytes act as a significant P-sink, whereby the nutrient status of the lake is kept relatively oligotrophic. Notably, charophytes are usually able to bind P through the formation of calcium carbonate, which causes the precipitation of $CaPO_3$, which is biologically unavailable. However, elevated levels of Mg^+ in freshwater from drinking water inhibits the formation of calcium carbonate in charophytes, and so in the case of Myall Lakes, charophytes act as a P-sink only through the binding of P in organic matter, which then forms part of the sediment body (Siong and Asaeda 2009). This bound P could potentially be released through mobilisation and digestion catalysed by storm events.

1.6.2 The Lower Lakes

Lakes Alexandrina and Albert have an extensive history of *N. spumigena* cultivation. Francis' 1878 report of a bloom of *Nodularia spumigena* in lake Alexandrina, South Australia, was the first scientific documentation of the serious water quality issues that cyanobacteria can cause, with several hundred cattle, sheep, and horses dying as a result of acute poisoning from drinking contaminated water (Francis 1878). He attributed water levels that were unusually low and warm, with surface and depth temperatures of 76º and 73º F respectively, as factors that were responsible for the bloom. During the serious bloom events in the Murray Darling system in 1989 - 1990, Lake Alexandrina was again blooming with *Nodularia*, alongside Lake Albert (Falconer 1991). At the time of Francis (1878), it is quite likely that the lake were estuarine due to seawater intrusion. A series of barrages now prevent seawater from entering the system, however, and the systems are mostly fresh, being used for potable supplies to towns and communities in the area (Heresztyn and Nicholson 1998). This anthropogenic intervention and consequent change in water quality has appeared to have little effect on occurrence of *N. spumigena* blooms, suggesting the development of an ecotype in the system. Investigations specifically applied to cyanobacterial dynamics in Lake Alexandrina are limited, although blooms of *N. spumigena* are pervasive in the system. The report by Francis (1878) attributes water level and temperature as being the dominant factors in bloom formation in the lake, however the study was of a more speculative and observational nature and yields no concrete data to indicate the possibility of other factors being involved. In more contemporary studies, the proliferation of *Nodularia spumigena*, and indeed cyanobacteria on a general level, is attributed to water quality decline through nutrient

enrichment as a result of anthropogenic activity (Codd et al 1994). The South Australian EPA (Environmental Protection Authority) has actively monitored water quality parameters in Lake Alexandrina since early 2008, including pH, turbidity, salinity, alkalinity, total nitrogen, total phosphorus, and chlorophyll-*a* (SAEPA 2012). In a more recent study, Li et al. (2018) found that Lake Alexandrina was eutrophic both through and after the drought period of 2008 – 2010. Sediment sampling in the Lower Lakes has revealed a historic increase in both organic particle and fine particle abundance, which has been linked to a shift from macrophyte to phytoplankton dominance (Skinner et al. 2014). This increase in organic matter within the sediment body suggests an increased potential for hypoxia and P liberation from storm-drive sediment mobilisation. The potential for *R. raciborskii* to invade these brackish systems appears to be low, as the genus does not have a high salt tolerance (Engstrom-Ost et al. 2015).

1.6.3 The Gippsland Lakes

The Gippsland Lakes are the largest estuarine system in Australia (Zhu et al. 2017). Summer blooms of *Nodularia* occurred sporadically in the Gippsland Lakes region between 1988 and 2003, however no *Nodularia* blooms occurred from 2003 to the beginning of 2011. This lack of bloom formation is attributed to severe drought conditions over this period which resulted in high salinity and low nutrient input levels. In 2007 an extensive bloom of *Synechococcus* spp. occurred in the Gippsland Lakes system. The bloom occurred through the drought mentioned above and was caused by catchment wild fires which led to an unusually large load of N into the system (Cook & Holland 2012). Since the easing of the drought after 2011, the lakes have returned to a cyclic bloom structure, where floods through the Winter and Spring cause diatom and dinoflagellate blooms due to the nutrient influx and salinity reduction. The breakdown of these blooms created hypoxic conditions, which triggered the release of large amounts of phosphorus from the lake sediments. This phosphorus then facilitates extensive *Nodularia* blooms over the summer, which are considered as recurrent and nuisance (Zhu et al. 2012). Due to their recurrence, studies have illustrated that the diazotrophy of *Nodularia* is capable of providing nitrogen to the system that comprises roughly 75% of particulate N, suggesting that recurring diazotrophic blooms may have significant impacts on aquatic nitrogen budgets (Woodland and Cook). Although mostly dominated by *Nodularia,* the development of a bloom of *Synechoccus* sp. indicates the

possibility that, under the right circumstances, freshwater systems can take on marine cyanobacterial dynamics, since *Synechoccus* is extensively distributed in marine environments. This interesting change in dynamic occurred as a response to heavy rainfall in a drought period and is indicative of the dramatic effects climatic events can have when placed alongside one another. *Nodularia* growth in the Gippsland Lakes was shown to decrease with increasing levels of available N, as the additional N allowed other varieties of

Table 1.1: Systems of major cyanobacterial activity and ecological vulnerability in Australia (cont. on next page).

System	Location	Type	Average Depth (m)	Management	Dominant BGA	Causative factors	Effects	References
The Myall Lakes	NSW (east coast)	Fresh / brackish	2.85	None	*Dolichospermum* / *Microcystis*	Accumulation of nutrients due to the low flushing rate of the system	Restrictions to recreational use, possible fish, bird, and turtle kills, hindrance to oyster leases	Newcastle Herald 2008, Redden and Rukminasari 2008
The Lower Lakes	South-east SA	Brackish / Fresh	2.8	Water monitoring, construction of seawater barrages	*Nodularia spumigena*	Dynamics heavily seasonal, also influenced by events such as drought	Water supply switched to reservoirs, recreational use of the lakes restricted	Codd et al 1994, Heresztyn and Nicholson 1998, EPA SA 2014
The Gippsland Lakes	South-east VIC	Brackish	2.6 / 4.8 / 5.4	Construction of a permanent sea gate in 1889	*Nodularia spumigena*	Dominated by major episodes including rainfall, drought, and wildfires	Lost revenue from tourism and commercial fishing, potential bioaccumulation of nodularins in seafood from the region	Cook et al 2010, Cook and Holland 2012
The Murray-Darling Basin	SA / NSW / QLD	Fresh	2	Weir pools are present throughout the system. The catchment supplies a large agricultural area with irrigation water.	*Microcystis* / *Dolichospermum*	Management dominated system, excessive water withdrawal causes low flow conditions, weir pools provide ideal environments for blooms. The system is also heavily eutrophic from intensive agricultural activity within the basin	Water supply contamination, stock deaths	Bowling et al 2013

Peel-Harvey Estuary	West WA	Brackish	1	Dredging	*Nodularia spumigena*	Dominated by major episodes, most significantly heavy rainfall events	Loss of revenue from fisheries, loss of recreational utility	McComb and Humphries 1992
Swan-Canning Estuary	Western Australia	Brackish / fresh		Filling, draining, and modification of urban lakes in conjunction with development of Perth	*Microcystis aeruginosa*	Excessive use of fertilisers across the coastal plain causing large amounts of P to leach into the system though sandy soils	Disruption of recreational use of the system	Davis and Koop 2006, Kemp and John 2006;
Lake Torrens	Central SA	Brackish	0.5	None	*Microcystis aeruginosa*	Urban run-off	Business losses, recreational restrictions, lake closure due to health threats	Falconer 2001, news.com.au
Lake Cargelligo	Central NSW	Fresh	3	The lake is an artificial storage body off Lachlan River	*Dolichospermum circinalis*	Flooding causes additional water to enter the lake via overland flow, which significantly increases nutrient concentrations	Loss of water for irrigation, stock, domestic supply, and recreational purposes	Bowling 1994
Moreton Bay (and associated*)	QLD (east coast)	Marine	n/a	None	*Lyngbya majuscula*	Increased phosphorus, iron, and dissolved organics due to run-off from anthropogenically modified forests	Seagrass losses, altered marine plant commuity structure, lost revenue from commercial fishing, tourism, and beach clean-up efforts, health threats due to toxicity	Albert et al 2005

competing phytoplankton to grow (Holland et al. 2012). This suggests that *Nodularia*, like *Raphidiopsis*, performs well under nutrient limited conditions.

1.6.4 The Murray-Darling Basin

The extensive urban and agricultural management practices undertaken in the Murray-Darling basin have seriously compromised water quality, with significantly increased eutrophication, heavily regulated flow regimes, and larger sediment loads. Additionally, recent drought conditions have resulted in significantly poor overall river health in the system (Al-Tebrineh et al 2012). Although the diatom *Aulacoseira granulata* is typically the dominant phytoplankton variety in the upper half of the catchment under regular flow conditions, in recent years cyanobacteria have been reported as increasingly dominant due to the low flow conditions (Croome et al. 2011). *Dolichospermum* blooms occurred in weir pools of the Murray-Darling system in 1989/1990 and were the cause of significant complaints in neighbouring towns regarding the taste of tap water, which was sourced from the pools (Falconer 1991). The following year, a massive bloom predominately made up of *D. circinalis* occurred during November and December 1991, which at its peak covered much of the length of the Barwon-Darling River. The bloom was both neurotoxic and hepatotoxic, and was implicated in stock deaths at Louth, which resulted in a declaration of a state of emergency in NSW (Bowling and Baker 1996). During the summer of 1989/1990 major blooms of *Microcystis* occurred in headwater storage reservoirs of the Murray Darling system, which prompted radio broadcasts warning water users and the erection of warning signs (Falconer 1991). After a decade of drought from 2000 – 2010, flood events mobilised organic matter within the Southern Murray-Darling basin, causing hypoxia through the digestion of organic matter that lasted for 6 months (Whitworth et al. 2012). In 2009, the basin suffered from a severe bloom that covered over 1100 km of channel length, and was comprised mostly of *Microcystis* and *Dolichospermum*, however interestingly, *Raphidiopsis* was also present in the bloom community (Al-Tebrineh et al. 2012). The provision of EWPs in order to increase river flow and disrupt the formation of algal blooms has done little to alleviate the ongoing cyanobacterial dominance (Mitrovic et al 2006). Low flows have also impacted the zooplankton populations towards the mouth of the Murray river, with low community diversity, poorly represented copepods usually common to the area, and well represented rotifers, which are scavenging species most often found in water bodies with a

significant organic debris component (Geddes et al. 2016). This lack of zooplankton diversity is an additional factor promoting excessive cyanobacterial growth.

Recently, there has been a shift in the dominant blooming species in the Murray-Darling basin, from *Dolichospermum circinalis* to *Chryosporum ovalisporum*, with a significant bloom taking place in 2016 and covering ~ 2360 km of the system (Crawford et al. 2017). The reasons for the switch in dynamic are not yet known, but two limnological features, namely low salinity (40 – 60 µS cm^{-1}), and the presence of cold water moving through the lower layers of the system and causing the upwelling of nutrients, have been suggested as possibly causal (Bowling et al. 2018). This shift is highly unusual as *C. ovalisporum* blooms are generally infrequent on a global level (Cirés and Ballot 2016), and only ever making up a small proportion of blooms to date in the Murray system (Bowling et al. 2018).

1.6.5 The Peel-Harvey Estuary

The Peel-Harvey Estuary supports a growing local community with its provided services and has over the past several decades suffered extensive anthropogenic impact (Metcalf et al. 2014). *Nodularia spumigena* is reported to cause massive and prolific blooms in the system, with four dense blooms on record since monitoring of algae in the area began in 1976. The main cause of these blooms is high in phosphorus inputs into the estuary, caused by nutrient rich run-off from land utilised for agricultural purposes (Sellner 1997). The proliferation of cyanobacterial blooms in the area prompted the construction of a man-made channel in 1994 which allowed tidal flushing of the estuary with saline water. This has reduced the occurrence of blooms in the area, although the ecological dynamics of the estuary have deteriorated markedly since (Wildsmith et al. 2009). Fish species commonly associated with macrophyte communities were present in relatively large numbers in the 1980s, however recent surveys illustrate their decline in favour of marine opportunistic species in response to the eutrophication and increase in salinity in the estuary (Potter et al. 2016). The existence of a large sub-population of *N. spumigena* in the form of akinetes, which lie in the sediment and germinate into new filaments given the appropriate conditions, has also been cited as a significant factor in bloom development in the system (Huber 1984). Furthermore, the estuary is subjected to occasional dredging, which results in short-term increases in nitrate, phosphate, and ammonium concentrations, and flocculant addition also causes mobilisation of nutrients from the sediment (Chopppala et al. 2018). In order to combat eutrophication, a

weir was trialled to reduce surface water run-off entering the estuary, with results suggesting that it was effective in reducing concentrations of total nitrogen and phosphorus loads (Ocampo et al. 2015), however as noted in earlier sections, weirs are ideal environments for cyanobacterial growth due to their low flow and high nutrient input, and solutions of this nature may inadvertently facilitate development of dense populations of harmful algae.

1.6.6 Lake Cargelligo

In November 1990 a bloom of *Dolichospermum circinalis* occurred in Lake Cargelligo, central New South Wales, which functions as an off-river storage midway along the Lachlan River. Cell count peaks were recorded which exceeded 100,000 cells.mL^{-1}. The Lake water is sourced from a weir across the river, via an inflow channel, and from two smaller lakes (The Sheet of Water and Curlew Water). Additionally, during flood periods, water enters the lake via overland flow. Lake Cargelligo has a maximum storage of 36,000 ML, a maximum depth of 5 m, covers an area of 1500 ha, and used for irrigation supply to the lower Lachlan River valley, town water supply, local stock and domestic supply, and recreation. Samples taken from each area of the lake corresponding to these four uses illustrate explosive growth of *Dolichospermum* in mid-November, 1989, with cell counts rising from negligible amounts to over 100,000 cells mL^{-1} within the space of a week (Bowling 1994). Scant literature is available on the lake due to its relatively remote location, however we here identify it as a key system to protect against currently projected changes, as it provides much of the local area with amenities and revenue.

1.6.7 The Torrens River

The Torrens River is critically important to the economic and environmental profile of South Australia, providing diverse ecosystem services to the city of Adelaide, and acting as an important source of floodwater for the Adelaide Plains (Gale et al. 2006). It bisects Adelaide and pools in an artificial lake within the city, which suffers periodic algal blooms and hypoxic blackwater events due to nutrient rich urban run-off from storm events. Management strategies implemented to improve water quality, including the establishment of macrophyte beds and aeration, have been largely ineffectual (Wallace et al. 2016), although reintroduction of course particulate organic matter has seen some success at stream

rehabilitation (Aldridge et al. 2009). Tributaries to the river exhibit highly variable annual flow, with changes in regular rainfall regimes and the presence of agricultural dams for irrigation purposes causing significant losses of flow within the catchment (Aldridge 2005). Pressures on the catchment will increase in the near future due to heightened intensity of storm events and temperature, and with the river delivering water to a densely populated urban centre, protection of this system from harmful algae will need dedicated attention.

1.6.8 The Swan-Canning Estuary

The Swan-Canning Estuary and its catchment in South-Western Australia have undergone significant modification since European settlement, with land clearing, shoreline construction, surrounding agricultural development, and the installation of stormwater and wastewater drains that empty into the system (Hallett et al. 2015). The system has a history of *Microcystis* dominance in its waters. In 2000 a severe bloom of *Mirocystis aeruginosa* occurred in the estuary with cell counts reaching 130,000,000/mL in shallow bay areas, and integrated water cell counts of 15,000/mL, making it the largest bloom of toxic cyanobacteria ever recorded (Robson and Hamilton 2004). The Swan-Canning system meanders through the Swan Coastal Plain and Perth and is subject to a variable climate exhibiting cold wet winters and hot dry summers, with regular south westerly sea breezes. The cause of the blooms was attributed to two highly irregular rainfall events, which caused large fluctuations in nutrient inputs, and lowered the estuary salinity to below the critical level for *Microcystis* (5 – 10 ppt) (Atkins et al. 2000). The river is a shallow tributary of the Swan River, with an average maximum depth of 2.5 m, and exhibits highly regulated and seasonal flows, with high discharge during winter and often no discharge during summer. During seasonal flow fluctuations, weir boards are erected to ensure the upstream impoundment water remains free of intruding sea waters. Nutrient loads are mainly sourced from sediment release during no-flow periods. The system is subject to high insolation, high nutrient concentrations, and warm water temperatures (Thompson et al 2003). During the 2000 Swan River *Microcystis* bloom, various management strategies were utilised by the Waters and Rivers Commission of Western Australia (WRC) and the Swan River Trust (SRT) in an attempt to reduce cell concentration levels. These included physical removal of bloom material via pumping and land disposal, surface application of modified clays for removal of cells via flocculation and sedimentation, and mixing surface waters with extracted saline water in order to raise the

salinity levels above the *Microsystis* tolerance threshold. None of these treatments showed any signs of being successful during the bloom, the reasons for which have not been identified (Orr et al 2004).

1.6.9 Moreton Bay

This semi-enclosed shallow bay on the North-Eastern Australian coast suffers sewage-derived nutrient enrichment from wastewater discharge and runoff (Abal et al 2001), and within the last twenty years has experienced increasingly frequent blooms of the filamentous toxin producer *Lyngbya* (Ahern et al. 2007, Wulff et al. 2011). Additionally, the neighbouring catchment areas, including the large Burdekin (133,000 km^2) and Fitzroy (143,000 km^2) catchments, has been extensively modified since European settlement, and suffer frequent and dense blooms, most commonly of *Raphidiopsis* (Bormans et al. 2004). Grazing of cattle for beef production is the largest single land use, being considerably more extensive than cropping, mainly of sugarcane, and residential development, and resulting in large loads of nutrient-rich terrestrial run-off entering the catchment (Davis et al. 2017). Wide-spread soil erosion has occurred due to land clearing for grazing and erosion from cattle, exporting large amounts of eroded deposits into the coastal system, which further promote turbid conditions and elevated nutrient concentrations in the bay. These conditions have contributed to deterioration of seagrass ecosystems in the area (Saunders et al. 2017). Due to the sub-tropical climate of Northern Australia, this region is particularly susceptible to flash-flooding events; these result in large deposits of sediment being transported to coastal waters in a relatively short amount of time. Pollutants from these sediments, including heavy metals, have serious impacts on local businesses that thrive on the fishing industry in the region (Coates-Marnane et al. 2016). This region is arguably the centre of dominance for *Raphidiopsis* in Australia; thus its conditions can act as an archetype of what to examine when considering the invasion potential in other regions.

1.7 Conclusion

The areas we have introduced above are broad and far-reaching. The intention of this overview is to demonstrate how these areas may intersect as a synergistic threat to water quality and security in Australia, in the form of a toxic invader, *Raphidiopsis*. We have

introduced areas of change expected to impact Australian aquatic ecosystems, including rising temperatures, increased storm intensities, population growth causing more severe water extraction regimes, and continued channel erosion and sediment mobilisation due to land clearing. These factors result in systems with lower flow, and longer periods of stratification, where a greater degree of separation is observed between the upper (epilimnion) and lower (hypolimnion) layers of freshwater streams, lakes and reservoirs. When taking Australia's already erratic rainfall regime into account, Australian freshwater systems are particularly vulnerable to these shifts. Dramatic changes in environmental conditions disrupt local ecological communities and can create niche space for foreign invasive organisms, which can reshape ecological landscapes and cause losses in diversity. Concurrently, overuse and inappropriate management of freshwater systems, and urbanisation and agricultural development, have resulted in exacerbated cyanobacterial blooms worldwide, and due to the ephemeral nature of Australian freshwater systems, cyanobacterial blooms have been a common historical nation-wide occurrence. The increasing pressure Australian aquatic systems are being subjected to will theoretically promote shifting phytoplankton communities, and here we identify *R. raciborskii* as a potential candidate to take advantage of these conditions. Supporting this notion is the findings of this review, which are that the spread of *Raphidiopsis* is difficult to characterise in the terms of biological invasion, that it possesses a flexible physiology allowing it to succeed under a range of conditions, and that systems exhibiting significant cyanobacterial activity are mostly event dominated, with additional evidence pointing to the influence of management approaches. These findings suggest *Raphidiopsis* and other novel biological agents can be viewed as symptoms of change rather than novel invading species. Thus, in the following sections of this thesis, we will pose the question: could *R. raciborskii* achieve ecological dominance in Australian waters in the future? To provide a position on this question, we will use a variety of quantitative and laboratory-based methods to examine the global ecological context of the organism, its responses to shifting climatic and nutritional conditions, its behaviour in and effect on freshwater community environments, and its historical presence in two contrasting areas of Australia. As a product of collecting, examining, presenting and summarising these approaches, we will garner other insights into cyanobacterial and freshwater dynamics that can be applied in the broad area of freshwater ecology, and we aim to identify these areas as promising avenues of further research in the field.

2. Learning from invasive phytoplankton – comments on invasion, physiology, and ecosystem change in the Anthropocene

2.1 Abstract

Phytoplankton posing potential threats to ecosystem services, such as those that exhibit invasive behaviour, or which produce deleterious toxins. Consequently, these phytoplankton are the subject of much attention, and can become valuable models for examining community dynamics and change in aquatic systems. Anthropogenic environmental change is immensely complex, and the success and characteristics of invasive species may be a useful proxy for the state of change on a vast scale. In the present study, we have pooled data from 68 field studies concerning the invasive phytoplankton species *Raphidiopsis raciborskii*, *Didymosphenia geminata*, *Gonyostomum semen*, and *Prymnesium parvum*, combined with results identifying environmental drivers that are significantly correlated with their abundance. We tested two hypotheses, namely, whether drivers were significantly different between invasive species, and which drivers were most significant across invasive species. We find that temperature and phosphorus are the two most significant variables associated with the spread of the species are temperature and phosphorus, and that the success of toxin producers *R. raciborskii* and *P. parvum* are more well understood than *D. geminata* and *G. semen*. Our data also suggests that differences in physiological strategies, namely toxin production and mixotrophy, are not significantly correlated with relative significance differences. These results suggest that key environmental drivers, namely temperature, community structure, and phosphorus levels, are heavily involved in determining the success of invasive phytoplankton species. However, driver significance varied significantly between species, suggesting that ecophysiology is also a significant determining factor. We link our findings with current knowledge regarding the influence of humanity on environmental conditions and suggest that invasive species are an accessible and interpretable tool for characterising shifts in environmental conditions. Documentation of novel behaviour in phytoplankton is increasing; as more data becomes available, our study provides a robust framework to examine these community shifts in the context of other shifts taking place globally.

2.2 Introduction

Phytoplankton are one of the cornerstones upon which aquatic ecosystems are built. They are diverse, ancient, and ubiquitous, and in recent years their importance as model organisms has been recognised and explored in the context of various ecological paradigms (Ostrovsky et al. 2013). Harmful algae, so called for their ability to produce toxins that have deleterious effects on other biota, have emerged as a dominant research priority, due to their often-serious effects on ecosystem services (Paerl et al. 2016). This realisation of the potential significance of proliferating toxic phytoplankton has recently extended to the consideration of biological invasions, as natural range expansions are today a commonly observed ecological phenomenon and are associated with human activity and declines in biodiversity (Butchart et al. 2010, Angeler and Johnson 2013).

In recent years, four species of phytoplankton have received significant attention for their perceived invasiveness and dramatic spread from their native ranges. These species are the cyanobacteria *Raphidiopsis raciborskii* (Sinha et al. 2012, Antunes et al. 2015**)**, the diatom *Didymosphenia geminata* (Kirkwood et al. 2007, Bothwell et al. 2014), the raphidophyte *Gonyostomum semen* (Hagman et al. 2015, Sassenhagen et al. 2015b), and the golden alga *Prymnesium parvum* (Hambright et al. 2010, Roelke et al. 2016). *Raphidiopsis raciborskii* is a filamentous diazotrophic cyanobacteria that has received the most attention out of the four species, due to a combination of its strong responses to warming temperatures, its range expansion and extremely flexible physiology, and its production of the harmful secondary metabolites cylindrospermopsin, and saxitoxin, which has resulted in severe wildlife losses (Svirčev et al. 2016), losses in drinking water security (Lei et al. 2014), and one very serious human poisoning event (Hawkins et al. 1985). Similarly, the golden alga *Prymnesium parvum*, a species historically associated with marine and brackish conditions, has also been implicated in an invasion through freshwater systems in North America, with resultant severe fish mortalities due to the alga's production of prymnesins, which are powerful fish-killing toxins, also known as ichtyotixins (Hambright et al. 2010). *P. parvum* has also incited significant interest due to its unusual tendency to form blooms and produce heavy concentrations of toxins under very nutrient poor conditions (Roelke et al. 2012), contrary to the traditional association between harmful algae and eutrophication. This link with oligotrophic conditions has also been found in the invasive diatom *D. geminata*, which forms very thick mats that cause a smothering effect in freshwater streams (Bothwell and Kilroy

2010). Its ability to anchor itself to stream rocks and to germinate prolific basal stalks facilitating high phosphatase affinity, combined with anthropogenic introduction to foreign environments, has allowed the species, once limited to cool oligotrophic mountain streams, to dominate in many freshwater systems worldwide (Bray et al. 2016). Finally, the mixotrophic raphidophyte *G. semen* has exhibited a dramatic expansion throughout Norway, Sweden, and Finland, with dominance resulting in water fouling from the ejection of slime threads from trichocysts, causing skin irritations and preventing use of systems by bathers, clogging water filters, and threatening potential negative impacts on freshwater communities (Hagman et al. 2015).

Factors driving the spread of invasive species are complex and difficult to elucidate, particularly in the context of global climate change (Hellmann et al. 2008). In order to work towards resolving this issue, syntheses of available data and literature, and simultaneous comparisons between invasion cases, are a transparent and accessible way forward. Here, we have quantified significance and insignificance in unmodified field studies reporting relationships between abundance and environmental parameters for the four most widely reported species of invasive phytoplankton, to characterise links between invader, physiology, and environment. Our approach allows a clear and concise examination of the role specific environmental variables are playing in driving the abundance of invasive species, as well as allowing a simultaneous comparison of the success and characteristics of individual species. In this study, we test two hypotheses regarding the proliferation of invasive phytoplankton: (a) Drivers of invasive phytoplankton do not differ significantly across species; and (b) Specific environmental drivers of invasive phytoplankton have equal importance when compared across species. We then use our results to discuss the respective roles played by ecophysiology and environmental change in promoting the spread of these species from their native ranges.

2.3 Method

2.3.1 Data collection

Table 2.1: Taxonomic and ecophysiological characteristics of four major invasive phytoplankton and number of studies from which data was extracted for this study (n).

Invader	Class	Other name	Strategies	n
Raphidiopsis raciborskii	Cyanobacteria	Blue-green alga	Diazotrophy Toxin production Resting cells High P affinity	20
Didymosphenia geminata	Bacillariophyceae	Diatom	Anchorage Stalk production High P affinity	24
Gonyostomum semen	Raphidophyte		Mixotrophy Slime-threads	14
Prymnesium parvum	Prymnesiophyceae	Golden alga	Toxin production Salt tolerance Mixotrophy	10

We collected over 200 studies on our chosen species, with data on 26 environmental parameters. We collected our studies by searching for the name of the invader genera in the Web of Science database and selected for field-based papers that had documented physio-chemical or community characteristics of water bodies affected by the proliferation of our chosen species. There were many studies that implemented experimental treatments in a field setting; these studies were discarded to avoid any bias towards treatment selection by authors in our results. After this selection process, we were left with a total of 64 studies (Supp. Tab. 2.1). Since these were field studies with no treatment structures, they generally corresponded to reports of range expansion or bloom activity of the focus species within certain systems.

2.3.2 Statistical approach

From these studies, we compiled significance results for physio-chemical and community measurements. We assessed significance towards cell counts or biomass of the target species,

and considered results gained from regression and ANOVA ($p < 0.05$), principal component analyses (eigenvector direction or dominant eigenvalues), and classification trees. Where enough data was accompanied by no statistical assessment of significance, the data was extracted via visual interpolation software and modelled using linear regression (Supp. Tab. 2.1). Through this methodology, we constructed a data set comprised of counts of the environmental variables that were significance/non-significant drivers for each invader. For studies where multiple ecosystems, such as various streams or ponds in a region, were assessed, and the variably was alternatively significant and insignificant depending on the system examined, we took a significant or insignificant result if there were a majority of systems that presented one or the other.

To assess parameter importance, we calculated a relative significance difference (RSD) for each parameter at the species level:

$$RSD_{i,p} = \frac{n_{p(significant)} - n_{p(insignificant)}}{n_{i,p}}$$

Where $RSD_{i,p}$ is the relative significant difference for a species i and an environmental parameter p, $n_{p(significant)}$ is the number of significant counts for a parameter p, $n_{p(insignificant)}$ is the number of insignificant counts for parameter p, and $n_{i,p}$ is the total number of counts for a particular parameter p and species i. Relative difference ratios are common in various forms in the literature, see for example Burns and Strauss (2012) and Gibb et al. (2013). This method has the advantage of removing any cases where the number of significant counts equalled the number of insignificant counts and avoiding over-inflation of data with results that equated to zero. It also allows negative results to be obtained for situations where the number of insignificant counts exceeded the number of significant counts. We determined whether there were significant differences between RSD profiles for each invader using a Fisher's exact test, and for significant differences between RSD profiles for each parameter we used a simple linear regression between an ordered vector of the total RSDs for each parameter and a dummy variable representing each parameter.

Table 2.2: Layout for the conversion of counts of significance and non-significance for each functional group, for a single parameter, to proportions of significance and non-significance for that parameter for each functional group. The structure is based on that outlined by Baker (2018).

Event	Group	
	Functional class 1	**Non-functional class 1**
Yes (significant)	n_{11}	n_{12}
No (non-significant)	n_{21}	n_{22}
Total	N_1 or N_p	N_2 or N_q

For an analysis of parameter importance based on physiological characteristics, including toxin production and mixotrophy, we computed fixed-effects log-odds ratios and variances according to the formulas outlined by Baker (2018) and followed the methodology outlined by Borenstein et al. (2009). Briefly, our counts of significance and non-significance were converted into 2 x 2 tables and totalled for each functional group, for each parameter, following the structure of Tab. 2.2. Then, the probabilities of observing significance for each functional group for parameter *i* are given by:

$$p = \frac{n_{11}}{N_1} \text{ and } q = \frac{n_{12}}{N_2}$$

Log-odds ratios θ were calculated for parameters i, as

$$\theta_i = \ln \frac{q(1-p)}{p(1-q)}$$

Where q is the proportion of significant results observed for the functional group members (n_{12}) over the total number of results for the functional group members (N_2), and p is the proportion of significant results observed for the remaining members outside the functional group (n_{1i}) over the total number of results for the members outside the functional group (N_1). Model variances σ_i^2 were calculated as:

$$\sigma_i^2 = \frac{1}{q(1-q)N_q} + \frac{1}{p(1-p)N_p}$$

The functional groups selected were toxin producers, which included *R. raciborskii* and *P. parvum*, and mixotrophs, which included *G. semen* and *P. parvum*, and the parameters investigated were conductivity, nitrogen, phosphorus, pH, temperature and turbidity. By using log-odds ratios, we did not intend to calculate an overall effect; rather we used this technique to isolate environmental parameters that had disproportionate contributions to significance across different groups. Because of limitations in the number of counts we obtained, the parameters phytoplankton, streamflow, stratification, invertebrates, light, water colour, zooplankton, calcium, chlorine, sulfate, sodium, dissolved organic carbon, magnesium, silica, salinity, stream geology, higher trophic levels, potassium, carbonate, and iron could not be examined using this method. All data visualisation and modelling were done using the statistical software R using the package "ggplot2" (http://ggplot2.org/).

2.4 Results

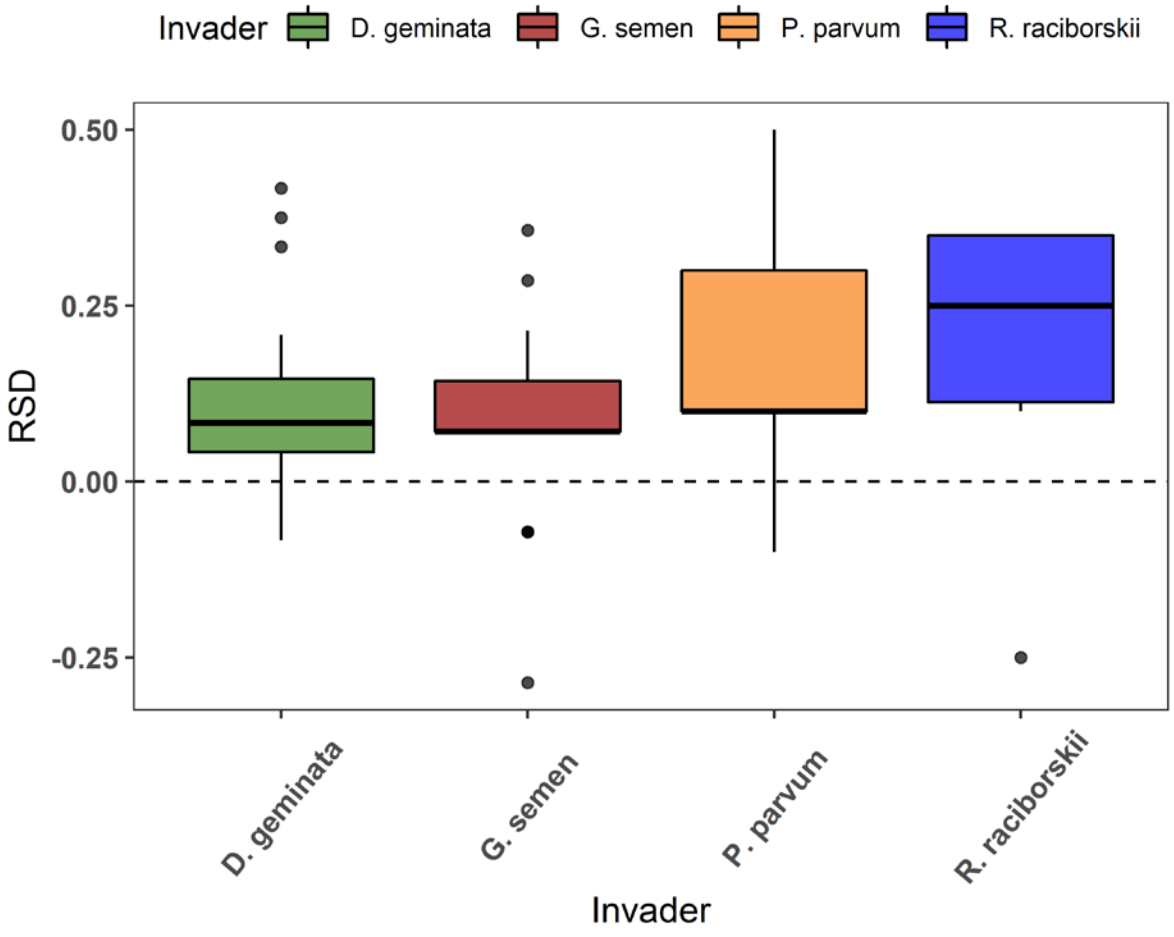

Figure 2.1: Boxplot of RSDs (relative significant differences) summarising all environmental parameters for each species. In order from left to right, Cr = *Raphidiopsis raciborskii*, Dg = *Didymosphenia geminata*, Gs = *Gonyostomum semen*, and Pp = *Prymnesium parvum*.

We found significant differences in the RSD profiles between each invasive species (p = 0.03517, Fisher's Exact Test). RSDs were on average greater in *R. raciborskii* and *P. parvum* than in *D. geminata* and *G. semen*, although several outliers were present in the latter two species (Fig. 2.1).

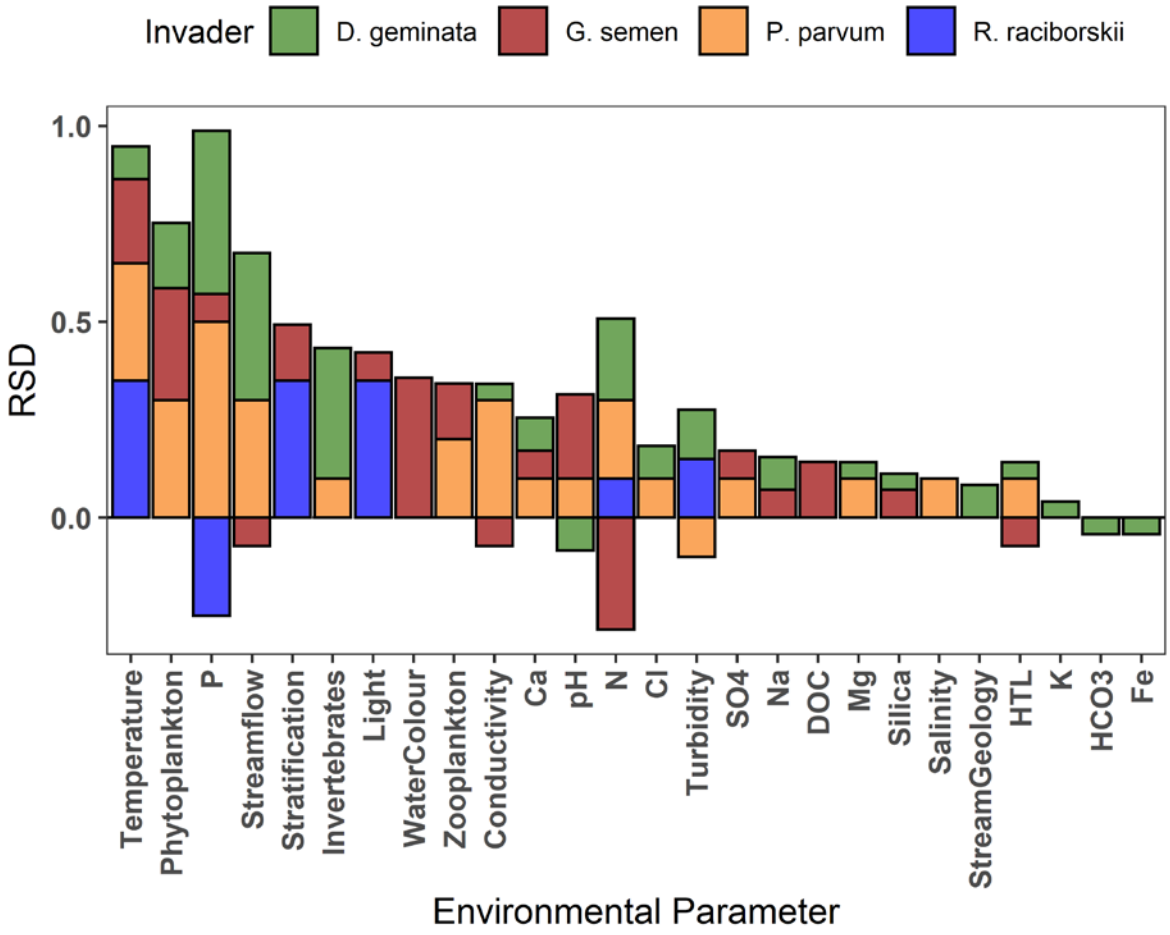

Figure 2.2: Cumulative RSDs (relative significant differences) for each parameter and each invader, sorted by highest to lowest total RSD for each parameter. Negative RSD values indicate that the parameter is not significant overall and positive means the parameter is significant overall. Cr = *Raphidiopsis raciborskii*, Dg = *Didymosphenia geminata*, Gs = *Gonyostomum semen*, Pp = *Prymnesium parvum*.

Linear regression found significant differences between the total RSDs for each parameter ($p < 0.01$), with temperature, phytoplankton, phosphorus, streamflow, and stratification being the most important parameters (Fig. 2.2).

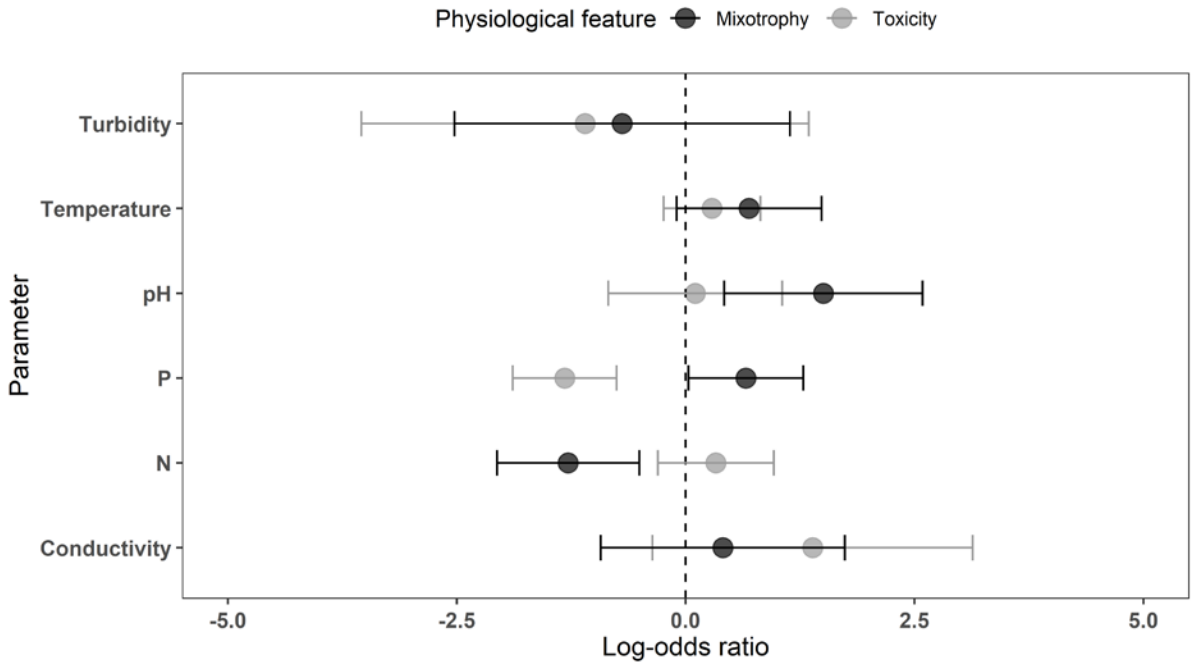

Figure 2.3: Log-odds ratios for selected parameters and functional combinations. Error bars represent computed variances. The log-odds ratio usually represents an arbitrary effect size of a study on the overall effect size representing the combined effect of all studies, with sizes ~ 0 representing little difference between the results for each group. Here they represent whether the likelihood of finding a significant result for a parameter driving members of a functional group is different between members of that functional group, and those outside that functional group.

Log-odds ratios revealed that across the two functional groups, most parameters containing enough data to be analysed contributed little to an overall significance difference (Fig. 2.3). There was roughly equal likelihood of turbidity being a significant driver of either mixotrophs or non-mixotrophs, whilst there was less likelihood of turbidity being significant for toxin producers versus non-toxin producers. Mixotrophs were less likely to be driven by nitrogen than non-mixotrophs, whilst toxin producers and non-toxin producers seemed equally driven by nitrogen. Whilst pH was more likely to drive mixotrophs, there was roughly equal likelihood that it would drive toxin producers compared to non-toxin producers. Conductivity was not more likely to drive mixotrophs, however it did seem marginally more likely to drive toxin producers compared to non-toxin producers.

Phosphorus represented the greatest divide between the two groups, with mixotrophs more likely to be driven by P, whereas toxin producers were less likely to be driven by P than non-toxin producers. Finally, temperature appeared to moderately drive mixotrophs more than non-mixotrophs, however there was no difference between toxin producers and non-toxin producers.

2.5 Discussion

From our results, both our hypotheses will be rejected, as drivers do differ significantly across species (Fig. 2.1) and individual drivers carry significantly different importance (Fig. 2.2). The significance results for each invader suggests that historically we have been more likely to identify significant parameters driving *R. raciborskii* and *P. parvum*, and less likely in *D. geminata* and *G. semen* (Fig. 2.1). Curiously, this result aligns with toxin production, as both *R. raciborskii* and *P. parvum* produce potentially harmful secondary metabolites that can affect ecosystem services (Antunes et al. 2015, Roelke et al. 2016). This may reflect the presence of more field studies that focus their attention on toxic phytoplankton activity than on more innocuous phytoplankton, as it is these bloom events that arguably result in the most readily identifiable impacts on both ecosystem health and human well-being. We can infer some significance from the relative importance of environmental parameters considering the physiological strategies employed by each invader. Total N in aquatic systems is highly insignificant for explaining the abundance of *G. semen*, as the raphidophyte is mixotrophic and can source N from organic matter in low N conditions, or actively take up available N in high N conditions. Following on from this, the significance of water colour, or turbidity for *G. semen* is intuitive, as the parameter is a proxy for the levels of organic matter within water bodies (Hagman et al. 2015). P again is highly insignificant in explaining *C. raciborski* abundance, since the cyanobacteria has a reportedly high storage capacity of and affinity to phosphates (Burford et al. 2016), however P is highly significant in explaining *D. geminata* abundance because the species has only been reported to be successful in systems with very low dissolved reactive phosphorus (DRP), due to its slow growth and massive stalk production stimulated by oligotrophic conditions (Bray et al. 2017). Finally, *P. parvum* is significantly driven by both temperature and P; like *G. semen* it is a mixotroph and can utilise alternate strategies in nutrient poor conditions (Lundgren et al. 2015). Furthermore, unlike *R. raciborskii*, there is much evidence to suggest the toxins produced by *P. parvum* have deleterious effects on potential grazers (Hambright et al. 2010), and that higher levels of toxin

production are consistently observed under oligotrophy (Hambright et al. 2014). This may also have been reflected in the higher likelihood of P driving toxin producers (Fig. 2.3). These links between our results and the physiology of our invasive phytoplankton are reassuring, as we would expect such associations.

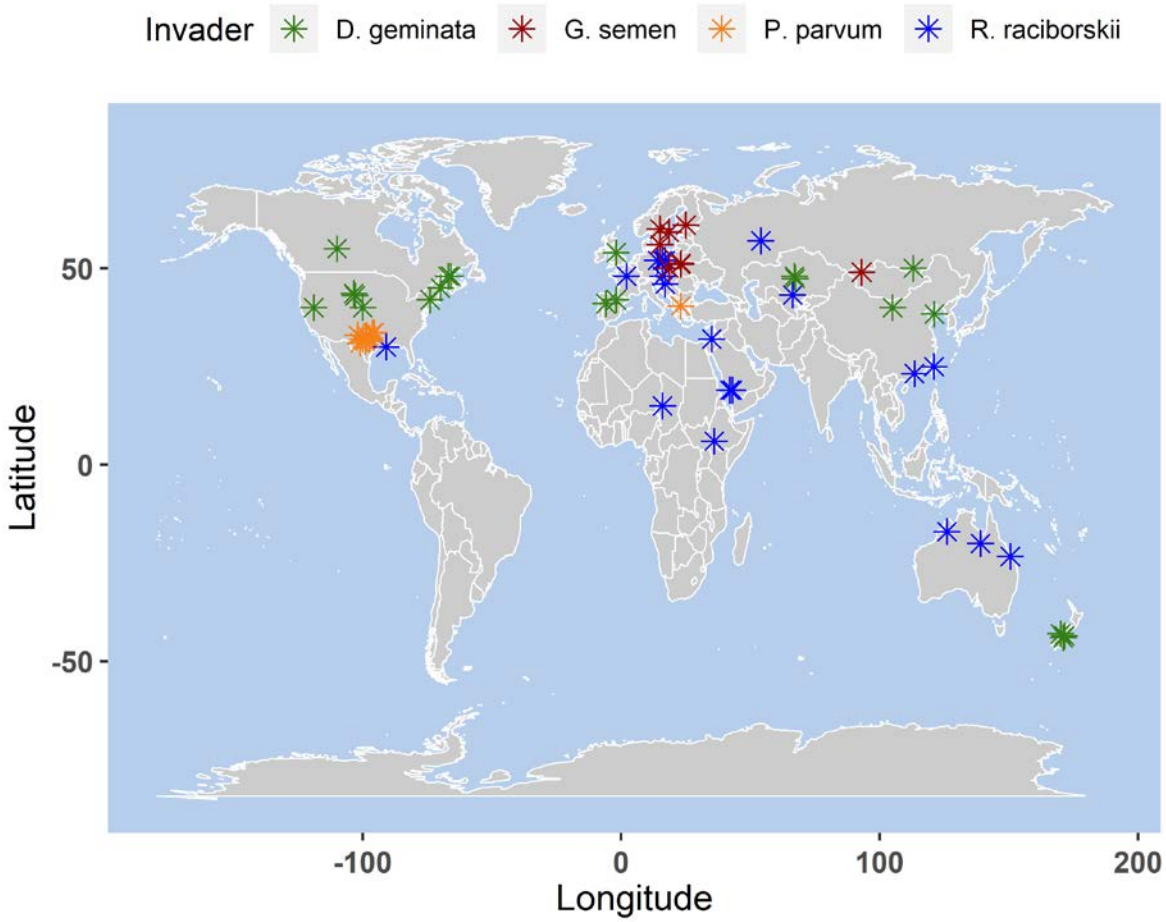

Figure 2.4: World map showing the locations of the systems from which data was extracted for this study, and the corresponding variety of phytoplankton that was the subject of each study.

The place of temperature as the most important variable, according to the RSDs (Fig. 2.2), suggests that warming or cooling is most responsible for the sudden increase in invasiveness in aquatic ecosystems, and based on contemporary literature, warming would seem the clear culprit here (Chapter 1). Whether this is a result of native species losing fitness due to temperature changes, invasive species gaining fitness, or a combination of both, is a deep question and cannot be answered with our data set. However, the importance of temperature in all invaders tested here suggests that thermal change is occurring on a global level and is

not confined to certain regions (O'Reilly et al. 2018). The result of phytoplankton as the next most important variable may suggest that community structure is important when considering vulnerability to invasion in aquatic ecosystems but is problematic considering our invaders have a strong capacity to influence the community structure around them (Trigal et al. 2011, Davis et al. 2015, Elwell et al. 2014, Figueredo et al. 2016). This leaves open the possibility of a significant variable caused by, but not driving, invasive species, and thus the phytoplankton will not be discussed further. Finally, P as the third most important variable is impactful; recent research illustrates that it is most often P, and not N as was typically thought in the past, that drives the activities of blooming cyanobacteria (Chapter 1). Therefore, from our data, the two strongest themes are increasing temperatures in water systems worldwide, and the ability of species to proliferate under P-poor conditions (except for *G. semen*). A question arises from this; are increasing temperatures driving more aggressive bloom cycles in lakes, and thereby leading to larger nutrient-depleted windows for such invaders to use as footholds in alien territory? Another possibility could be stratification, which is known to result in a greater distinction between the nutrient poor epilimnion and nutrient rich hypolimnion, and which is being increasingly altered on a global scale by temperature changes (Kraemer et al. 2015). Stratification is a factor that was found to be a significant driver of *R. raciborskii* and *G. semen*; *R. raciborskii* can dominate in the epilimnion due to diazotrophy and buoyancy regulation (Burford et al. 2016) while *G. semen* is mixotrophic and motile, allowing it to readily migrate to lower layers to search for food (Sassenhagen et al. 2015b). It is worthwhile to note that we obtained no data on whether stratification was a driver of *D. geminata* or *P. parvum*, so this phenomenon could potentially be highly significant.

Several studies have developed theoretical frameworks to classify the actions of invasive species, with specific regard given to the effects of climate change. Rahel and Olden (2008) concluded that climate change would have five main effects on aquatic ecosystems: altered thermal regimes, reduced ice cover, altered streamflow regimes, increased salinity, and increased water development. Similarly, Hellmann et al. (2008) found that five consequences derived from climate change would be altered pathways for invaders, altered environmental constraints on invasion, altered distributions of existing invasive species, altered impacts of existing invasive species, and altered management strategy effectiveness for invasive species. Invasive species may simply be those species that are suited to taking advantage of a multitude of changing conditions and opportunities that are being presented to them, at the

expense of the opportunities available to native species (Butchart et al. 2010, Mollot et al. 2017). Curiously, a resounding theme in these four species is an ability or preference to thrive under low-P and/or low-N conditions. This is particularly concerning, as it suggests that systems in good nutritional balance or health, and which may be relatively free from other more common toxic cyanobacteria, may also be vulnerable to these species due to other extraneous factors, such as temperature and salinity, catchment processes such as fires and storms, and continuous human intervention in systems, such as channel alteration, land clearing, and agricultural development (Bowling et al. 2013a, Smith et al. 2012).

Perhaps the success of our invaders is simply a litmus test for phenotypic plasticity; in a rapidly changing environment, organisms with the most flexible phenotype will have maximal fitness (Piccini et al. 2011). However, data suggests that under nutrient limitation native species perform just as well, if not better than invaders (Davidson et al. 2011), leading to the conclusion that nutrient deprivation alone cannot explain the success of our invaders. This rests nicely with the evident conclusion that changes in ecosystems as a result of anthropogenic activity are multifaceted and cannot be expressed as a single driving factor (Fig. 2.2). Despite this, it has been noted that in the specific case of *P. parvum,* without the allelopathic benefits of its toxin production, which is brought on by stress, its slow growth and cell size would make the species a poor competitor (Roelke et al. 2016). Even under favourable conditions, the maximum growth rate of *P. parvum* has been logged at ~ 0.1 d^{-1} (Grover et al. 2010). The concept of synergy may be applied in this situation, which refers to significant effect derived from relatively minor contributions by a variety of factors. This concept has recently been brought forward to explain recent honey bee population losses (Raimets et al. 2018) and may be applied here to describe the synergistic interactions between rapid system change, human interventions such as those mentioned above, and key physiological traits. It is also worthwhile examining the term ‘invasion’ here, used to denote range expansion by species into areas that they previously did not occupy. Invasion is closely associated with vectors of transport, natural and unnatural, such as dispersal by animals, or by mechanical vehicles (Davidson et al. 2015). By extension, it also implies a certain inherent vulnerability in the system in question, as theoretically the system has existed with unutilised niche space which will be occupied by incumbent invaders. However, if synergistic influences are also present, such as human pollution, then invasion is a symptom of change; the provision of niche space that was not there previously (Chapter 1). Contemporary literature focusing on invasive phytoplankton operates almost exclusively on incidence of

blooms by species not previously observed as blooming, as opposed to identification of species not previously present in the system (Naselli-Flores and Padisak 2016). Moreover, phytoplankton communities are highly complex and not typically examined, and minor components of communities can easily be overlooked in favour of dominant blooming components. Whilst this is clearly not the case with respect to *Didymosphenia geminata* in New Zealand, which represents a genuine vector-based invasion (Bray et al. 2017), other species may be in fact minor cosmopolitan species that are able to transition to major species due to human induced environmental change. Emergence, rather than invasion, may represent a more accurate conceptual underpinning here, which casts focus on this change instead of system vulnerability, which is not necessarily a function of the Anthropocene (Harrison et al. 2017).

Matching parameter-invader specific responses to geographical distribution can hint at environmental changes currently underway in these regions, which may be helpful where the reasons for changes in phytoplankton composition have not yet been recognised. Although most available data on the driving factors of *Prymnesium parvum* is concentrated around Southern United States of America (Fig. 2.4), blooms of *P. parvum* have been reported in regions of Europe and Asia, with mass fish kills and threats to sensitive and diverse ecosystems mentioned as the key factors of concern (Wagstaff et al. 2018). For example, Hickling Broad, in England, a shallow, brackish lake, demonstrated a dramatic shift in phytoplankton composition through the 1970's, changing from a relatively pristine charophyte dominated system, to a cyanobacteria-dominated system with turbid water, and annual damaging *P. parvum* blooms. The reasons proposed for this change were that increases in roosting birds increased phosphorus loads into the system through defecation, and that the system chlorinity had risen by over 200% due to saline groundwater being pumped into the system to increase the arability of the surrounding catchment (Irvine et al. 1993). Based on our data, we could surmise that the establishment of *P. parvum* blooms was due to the rising salinity, which would have also reduced the fitness of the native community, allowing niche space for other competitors. Furthermore, the increased loads of P are unlikely to have driven *P. parvum* based on studies associating the species with oligotrophic conditions; rather, the declines in macrophytes were more likely correlated with the smothering varieties of cyanobacteria promoted by excessive P. The recent proliferation of *G. semen* through Scandinavian regions (Fig. 2.4) has been attributed to progressive water brownification in the region, which has provided the alga with a rich food source

(Sassenhagen et al. 2015a). Previous accounts, however, attempted to focus on eutrophication as explanatory, due to the classic association with eutrophication and phytoplankton blooms. Uncertainty surrounding simultaneous changes in Finnish waters, including increased fish farming causing elevated nutrient loads, and increased peat extraction resulting in increased levels of organic matter in water streams, is another example of the complexity surrounding harmful phytoplankton and their physiologies (Lepistö at al. 1994). Based on our data, we can conclude that peat extraction may have acted as the primary variable in driving growth here, as *G. semen* is mixotrophic and eutrophication may not be so relevant to this species. Whilst the likelihood of mixotrophs being driven by P is greater than non-mixotrophs (Fig. 2.3), this result is being driven by the data acquired on *D. geminata* (Fig. 2.2) and illustrates that caution must be applied when combining species based on physiology or functional traits.

Our method of examining the significance of parameters in driving phytoplankton invasion, while novel, is not without its limitations. First and foremost is the sampling bias that the authors of the papers we selected may suffer from. Sampling efforts in aquatic systems are often driven by events that directly lead to tangible or serious consequences, such as loss of livestock or ecosystem services usually delivered by these aquatic systems. As such, although we are capturing a wide variety of data documenting the drivers of each invasive species, our data is likely skewed towards those systems with which humans have regular contact with, and which may be undergoing some sort of change. Another limitation is the relationship between phytoplankton activity and antecedent environmental conditions. Field sampling efforts can only ever take immediate data, and although correlation between lagged variables and phytoplankton biovolumes is practised for the construction of forecasting models, it is rarely used as a technique for the analysis of ecological dynamics. This can be extended to the effects of weather events on phytoplankton; severe storms can mobilise large amounts of nutrients through sediment agitation and run-off and can significantly alter the composition of phytoplankton communities. Here, we generally have no indication of antecedent conditions prior to sampling. Finally, there is a paucity of studies undertaking dedicated field sampling for the purposes of monitoring the spread of these species and the driver parameters measured are often limited to those that are easy and cheap to measure. This is reflected in the parameters we collected data for, but which were unable to be included in the log-odds ratio analysis due to lack of data. Being able to examine these parameters against the physiological characteristics of the species would give us greater insight into how physiological

characteristics influence niche establishment. To truly understand the invasiveness of species, laboratory studies are essential for the assessment of specific responses, and it is this data that needs to be compared with field studies, in order to establish environment-specific responses. This is particularly relevant where phytoplankton are concerned; many biological factors, such as parasitism, grazing, co-evolution, and allelopathy, have yet to be explored in the context of invasion (Jephcott et al. 2017).

2.6 Conclusion

By examining four distinct varieties of invasive phytoplankton, and identifying common drivers of their proliferation, we can connect these findings with key areas of change within our ecosystems, and strengthen awareness surrounding our impact on the proliferation of these invasive species. We can also use these findings to reflect on localised aspects of environmental change. The identification of temperature as the most influential driver of these species points at the serious effects climate change is potentially having on species distribution and abundance. Furthermore, the role of phosphorus as a significant driver can be linked to our influence on nutrient fluxes within freshwater systems, as well as physiological characteristics of phytoplankton that allow them to take advantage of these fluxes. The approach taken in this study could be extrapolated to other varieties of invasive species, to gain insights into changes at higher trophic levels, and how these changes correlate up and down the food chain. Furthermore, this approach benefits from the availability of more data as more field studies are conducted to examine shifting community dynamics. Such insights can act as powerful motivators for dedicated action at local, regional, and global levels. It is becoming more apparent as time goes on that action of this nature is crucial for the preservation of ecological stability.

3. Interactive effects of climate change factors and nitrogen availability on the freshwater cyanobacterium *Raphidiopsis raciborskii*

3.1 Abstract

The concurrent increases in atmospheric CO_2 levels and temperature are expected to affect the occurrence, magnitude and toxicity of harmful cyanobacterial blooms. The tropical and potentially toxic cyanobacterium *Raphidiopsis raciborskii* is an emerging species in many temperate freshwater systems worldwide, a process that is presumed to be favored by climate change. Yet, responses of this species to elevated CO_2 levels and warming remain poorly understood. Here, we expose three Australian cylindrospermopsin-producing strains of *R. raciborskii* to temperatures of 18, 24, and 30°C and atmospheric CO_2 levels of ~ 80, 300, and 1000 ppm, in both N-replete and N-depleted conditions. Our results demonstrate a consistent increase in growth with warming for both N treatments in all tested strains, while the response to elevated CO_2 levels depended on N availability and increased under N replete but decrease under N depleted conditions. Optima in cylindrospermopsin quota were observed under the ambient temperature and CO_2 conditions, and tended to be highest under N-deplete conditions. N_2 fixation did not occur under N replete conditions, as well as in the lowest temperature treatment. With warming, N_2 fixation showed a consistent increase in all strains, followed by an increase in net N production rates and growth rate. Together, our results show a close association between growth, N assimilation and cylindrospermopsin content under N deplete but not under N replete conditions. We demonstrate plausible links between environmental conditions and toxin synthesis in *R. raciborskii*, and the ability of the species to achieve growth rates comparable to optimal conditions whilst relying solely on diazotrophy. We further demonstrate that the internal stoichiometry is influenced by both physiological and environmental factors, as found elsewhere in the literature. However, our findings exhibit moderately suppressive effects of both low and high CO_2 concentrations on growth and nitrogen fixation of *Rahpidiopsis*, raising key questions about its ecological performance given our climate change forecasts. Our data supports current hypotheses that the spread of *R. raciborskii* is driven by a flexible physiology, which is particularly favored by warming and stratification of freshwater bodies.

3.2 Introduction

The global climate is changing rapidly, with ongoing increases in atmospheric CO_2 levels accompanied by warming. This climate change is expected to affect the structure and functioning of aquatic ecosystems worldwide (Bonilla et al. 2012, Lürling et al. 2013). Phytoplankton represent a key component of aquatic ecosystems, and thus their responses to

climate change may cascade throughout the food web. For instance, elevated CO2 levels is causing ocean acidification with a wide range of impacts on marine primary production and consequent food web interactions (Alvarez-Fernandez et al. 2018). Similarly, warming was shown to result in reduced primary production (Velthuis et al. 2017). Furthermore, both elevated CO2 levels and warming are predicted to favor the occurrence of harmful cyanobacterial blooms in freshwater ecosystems (Paerl and Huisman 2009; Visser et al. 2016). Harmful cyanobacteria possess key physiological traits that allow them to take advantage of these altered climatic conditions, such as adjusting buoyancy, storing phosphorus, fixing atmospheric nitrogen (diazotrophy), and differentiating resting cysts, or akinetes, in response to unfavourable conditions (Carey et al. 2012). Under bloom conditions, CO_2 is often limiting, and thus a combination of elevated CO_2 and temperature may facilitate the intensification of harmful cyanobacterial blooms (Verspagen et al. 2014; Wells et al. 2015) and affect their toxicity (Van de Waal et al. 2009; Gehringer and Wannicke 2014; Liu et al. 2016). It has also been found that elevated CO_2 concentrations may bestow a further advantage on diazotrophic filamentous cyanobacteria by enhancing nitrogenase activity in heterocysts, fueling significant increases in growth (Hutchins et al. 2013, Eichner et al. 2014). Conversely, however, Law et al. (2012) reported no effects of elevated CO_2 on N-fixation in unicellular marine phytoplankton.

The cyanobacterium *Raphidiopsis raciborskii* is a filamentous diazotrophic species that is mostly recognised for its production of the hepatotoxic alkaloid cylindrospermopsin and the potent neurotoxin saxitoxin (Mowe et al. 2015a). The species is also regarded as invasive and responding strongly to global temperature increases, with reports documenting its range extension in Central and North America, Western Europe, and Australia (Sinha et al. 2012). This expansion is accompanied by follow-on consequences for water quality and ecosystem services due to the species' toxin production (Sukenik et al 2015), particularly in areas severely affected by eutrophication, such as China and South America (Soares et al. 2013a). Despite much literature reporting *R. raciborskii* as invasive, some evidence suggests that it may alternatively be emergent. For instance, a sudden surge in the activity of *R. raciborskii* in New Zealand waters was linked to changing conditions rather than biological invasions, as phylogenetic analyses showed that *R. raciborskii* has always been present (Wood et al. 2014).

R. raciborskii's physiology allows it to persist under a wide range of conditions (Antunes et al. 2015). Curiously, *R. raciborskii* only ever differentiates a maximum of two heterocysts, which are always positioned at the ends of the filament. The species compensates for this

limitation in the number of heterocysts by possessing an extremely efficient NH_4^+ transport mechanism, which allows further energy to be shunted into photosynthesis rather than heterocyst production (Plominsky et al. 2014). Other studies have suggested that the species couples this with a very high affinity for ammonium, phosphates, and warm temperatures, a resistance to grazing, and a long water residence time, which likely contribute to its success (Burford et al. 2006; Hong et al. 2013; Antunes et al. 2015; Burford et al. 2016). Toxins produced by harmful cyanobacteria present a variety of problems, namely a severe health risk to wild and domesticated animals and a drastic loss of ecosystem services in affected areas (Paerl and Otten 2013). Additionally, cylindrospermopsin has been implicated in potentially negative impacts on cropping plants (Freitas et al. 2015) and accumulation in the tissues of other animals such as snails and fish (Kinnear 2010). The exact ecological function of toxin synthesis in cyanobacteria remains unknown. Investigations conducted on the potential role played by cylindrospermopsin in the success of *R. raciborskii*, have suggested that the compound helps inhibit competing phytoplankton (Rzymski et al. 2014) and may provide resistance to zooplankton grazing (Rangel et al. 2016).

The recent rapid expansion of *R. raciborskii* suggests that it may be responding positively to changing conditions in aquatic ecosystems which have been taking place in recent decades. Whether this is analogous to anthropogenic environmental change remains to be seen. While various studies have investigated the responses of *R. raciborskii* to temperature, very few studies have addressed the impacts of N-availability, and no studies to date have addressed the combined effects of temperature and N-availability, and CO2 and N-availability. Here, we exposed three *R. raciborskii* strains to three temperature and three CO2 treatments, both under N-replete and N-depleted conditions. As such, we tested how *R. raciborskii* will respond to climate change and how this response may be impacted by need to fix N2. We predict that *C. raciborksii* will respond positively to elevated CO2 levels and to warming, as has been demonstrated in the literature for other cyanobacteria species. We also expect that this positive response will be particularly facilitated under N limiting conditions, where both warming and higher CO2 levels may compensate for high metabolic costs associated to N2 fixation.

3.3 Method

3.3.1 Strain and culture conditions

Table 3.1: Strains of *Raphidiopsis raciborskii* used in the study, originating from Queensland (QLD), Australia.

Strain	Species	Location of isolation	Toxic	Trichome morphology
CS-505	*C.raciborskii* Woloszynska	Solomon Dam, Palm Island, QLD	Yes	Straight
CS-506	*C.raciborskii* Woloszynska	Solomon Dam, Palm Island, QLD	Yes	Coiled
CS-511	*C.raciborskii* Woloszynska	McKinlay, QLD	Yes	Straight

Experiments were conducted with three strains of *R. raciborskii* Woloszynska: CS-505 and CS-506, which were both isolated from Palm Island, Queensland, and CS-511, which was isolated from McKinlay, Queensland (Tab. 3.1). These three strains were chosen because they all produce cylindrospermopsins, are Australian, have been isolated from geographically similar locations, and display differences in morphology. Furthermore, the use of three strains facilitates an intuitive experimental structure based on triplicates. All cultures were obtained from the CSIRO Australian National Algae Culture Collection in Tasmania, Australia. Cultures were maintained on WC medium. We followed the original medium recipe (Kilham et al. 1998) except for leaving out available N for the N-depleted set of experiments, and doubling $NaHCO_3$ to mitigate alkalinity shifts caused by inorganic nutrient acquisition (Wolf-Gladrow et al. 2007).

3.3.2 Experimental structure

For the temperature experiments, three water baths were set to 18, 24, and 30°C, and cultures were introduced in stoppered 250 mL Erlenmeyer flasks. *Raphidiopsis* is rarely found growing actively below 18 ºC, and has a reported optimal growth temperature of ~ 30 ºC (Soares et al. 2013b, Burford et al. 2016). Furthermore, Recknagel et al. (2014) found a critical transissionary temperature for bloom formation in the species was 25.5 ºC; our temperatures were selected to cover this range reported in the literature. Bath water was agitated using aquarium pumps, and culture material was aerated using humidified compressed air sterilized by air filters. Compressed air or gas mixtures were delivered into the culture material of each individual flask via glass tubing that was driven through the cellulose stopper. Cultures were kept on a photoperiod of 14:10 h (light:dark) cycle, and light intensities were measured with an LI-250A light meter daily and kept at ~ 45 µmol photons m^{-2} sec^{-1}. Prior to the experiment, cultures were acclimated to the distinct CO_2 and temperature conditions for several weeks to ensure ~ 7 generations had been produced under experimental conditions. Triplicate cultures were started at a biovolume of 2 x 10^7 fl mL^{-1}

and harvested in exponential phase. For the CO_2 experiments, water bath temperature was set to 24°C, and distinct CO_2 concentrations were achieved by combinations of compressed air and pure CO_2 to achieve a concentration of ~ 1000 ppm, and compressed air and air devoid of CO_2 to achieve a concentration of ~ 80 ppm. The air mixtures were delivered to the cultures via glass tubing as described above, with mass flow controllers used to maintain constant ratios of the different air sources (delta smart II, Brooks Instrument). Unmodified compressed air served as the mid-level CO_2 treatment of ~ 300 ppm. These levels were chosen to represent concentrations well below pre-historic times of ~ 800,000 years ago (80 ppm) (Sigman et al. 2010), a present-day equivalent (300 ppm), and a future concentration that could feasibly be achieved by 2100 under certain emissions scenarios (1000 ppm) (Jones et al. 2013). Admittedly the present-day atmospheric CO_2 concentration is closer to 350 ppm than 300 (Anderson et al. 2016), however we deemed 300 ppm to be typically representative of modern concentrations prior to the dramatic increases observed since 1950 (Jones et al. 2013). CO_2 concentrations in the experiments were calculated based on pH (pH/cond 340i, WTW) and alkalinity (TIM 840 Titration Manager, Radiometer Analytical), using the CO2sys program developed for MS Excel, with freshwater settings used, and inputs of pH, alkalinity, temperature, pressure and salinity (Pierrot et al. 2006).

3.3.3 Sampling approach and analysis

During the acclimation and experimental periods, samples were taken from each replicate every two to three days. Samples were taken between 1 and 3 hours after the beginning of the photoperiod to account for the diurnal rhythm of the cultures. Culture biovolumes were measured using an automated cell counter (Casy 1 TTC, Schärfe System) with a 200-µm capillary, which had been calibrated using different concentrations of microbead solutions. Cell or heterocyst counts were not undertaken due to time constraints. Once the cultures reached the exponential phase, culture material was filtered over glass microfibre filters (Whatman GF/F, Maidstone, UK) and stored for later analysis. Filters were taken for intracellular C, N, P, δ13C, and cylindrospermopsin content.

For C and N, holes were punched in the filter which amounted to ~ 22.7% of the total filter; the remaining filter material was process for P. For intracellular C and N analysis, filters were oven dried at 60 °C and stored in a desiccator until analysis (Flash 2000 organic elemental analyzer, Interscience BV, Breda, the Netherlands). Intracellular P content was determined by first incinerating the samples for 30 minutes at 500 °C, followed by a 2% persulphate

digestion step in the autoclave for 30 minutes at 121 °C. Subsequently, the digested samples were analysed (QuAAtro segmented flow analyser, Seal Analytical Incorporated, Beun de Ronde, Abcoude, the Netherlands).

For intracellular cylindrospermopsins, filters were stored at -20 °C before analysis. Filters were lyophilised and then vortexed in a 2 mL Eppendorf tube with 500 µl of 0.1% formic acid for 15 seconds. Samples were then put in a water bath at 95 °C for 10 minutes, before being placed in the vortex again for 15 seconds. Supernatant from the samples was then transferred to an Eppendorf with a built-in cellulose acetate filter. This filter Eppendorf was then centrifuged, and the filtrate was transferred to a fresh 2 mL Eppendorf. This process was repeated three times. The pooled filtrate was then dried in a Speedvac (Thermo Scientific Savant SPD121P, USA). The dry samples were resuspended by vortexing with 50 µl of milli-Q. Filtrate was stored at -20 °C for soluble N and P, and extracellular cylindrospermopsin analyses via LC-MS.

Nitrogenase activity was measured by acetylene reduction assay (Kemp at al. 1997) with 99.6% acetylene gas. 75 mL sampling bottles sealed with red rubber septa were filled with 37.5 mL of culture material and injected with ~5.7 mL of acetylene, shaken thoroughly, and incubated for 10 hours, from 9 am until 7 pm. Results were measured using FID-GC.

3.3.4 Data analysis

Data were analysed by fitting linear mixed models to each measured response variable, with strain used as random effect, temperature and CO_2 set as numerical fixed effects, and growth medium set as an interactive factor. To deal with non-linearity in the data, a quadratic term was added and retained if significant. Linear mixed models were also run between certain variables, with strain retained as a random effect, and medium as an interactive factor. Models were constructed and assessed based on marginal and conditional r^2 parameters using the packages “lme4” and “piecewiseSEM” for R. For visualization of directional trends, principal component analyses were run on data from the temperature and CO_2 experiments, and results were presented in biplots. Data were analysed untransformed. Pairwise comparisons were assessed using Tukey’s Honest Significant Difference (HSD) test ($p < 0.05$) using the “lsmeans” and “multcompView” packages in R. All analysis and graphical production were done using the statistical software R (https://www.r-project.org/) and the imaging software GIMP (https://www.gimp.org/).

3.4 Results

Table 3.2: Least-squares means for factorial linear mixed models run on all treatments and variables, with standard errors in brackets. Cells highlighted in tan denote results where the interaction between the primary treatment (temperature or CO_2) and medium were not significant, and thus means are reported both for the main effects (primary treatment and the mediums). Results with similar superscript letters did not differ significantly across treatments (P > 0.05), based on Tukey's HSD test. ND = nitrogen deficient medium, NR = nitrogen replete medium, cyn = cylindrospermopsin, NF = nitrogen fixation.

	Temperature					
Variable	**18**		**24**		**30**	
	ND	**NR**	**ND**	**NR**	**ND**	**NR**
Growth (d^{-1})	0.129^{a} *(0.0261)*		0.233^{b}		0.381^{c}	
	0.176^{a} *(0.0255)*	0.320^{b}	0.176^{a}	0.320^{b}	0.176^{a}	0.320^{b}
NF (nmol N $mm^{3\,-1}$ h^{-1})	n/a	n/a	7.614^{b} *(1.802)*	0.483^{a}	17.733^{c}	1.0766^{a}
C:N (molar)	7.350^{a} *(0.326)*	8.984^{b}	7.564^{a}	6.894^{a}	7.013^{a}	6.767^{a}
P (µmol $mm^{3\,-1}$)	0.0165^{a} *(0.00483)*	0.0330^{b}	0.0264^{ab}	0.0148^{a}	0.0260^{ab}	0.0130^{a}
δ13C:12C	27.255^{c} *(0.406)*	30.242^{b}	26.120^{cd}	31.939^{a}	25.003^{d}	32.360^{a}
Cyn (µg $mm^{3\,-1}$)	0.0166^{a} *(0.00606)*	0.0293^{ab}	0.0561^{c}	0.0477^{bc}	0.0254^{a}	0.0201^{a}
	CO2					
	80		**300**		**1000**	
	ND	**NR**	**ND**	**NR**	**ND**	**NR**
Growth d^{-1}	0.143^{a} *(0.0311)*	0.311^{b}	0.156^{a}	0.310^{b}	0.138^{a}	0.361^{b}
NF (nmol N $mm^{3\,-1}$ h^{-1})	4.510^{bc} *(1.251)*	0.806^{ab}	7.614^{c}	0.483^{a}	1.488^{ab}	0.194^{a}
CN (molar)	7.941^{ab} *(0.404)*		7.229^{a}		8.099^{b}	
	8.215^{b} *(0.382)*	7.298^{a}	8.215^{b}	7.298^{a}	8.215^{b}	7.298^{a}
P (µmol $mm^{3\,-1}$)	0.0544^{c} *(0.00691)*	0.0203^{a}	0.0264^{ab}	0.0148^{a}	0.0473^{bc}	0.0486^{bc}
δ13C:12C	25.537^{e} *(0.371)*	28.211^{c}	26.120^{de}	31.940^{b}	27.023^{d}	33.418^{a}
Cyn (µg $mm^{3\,-1}$)	0.0374^{a} *(0.00491)*		0.0519^{b}		0.0291^{a}	
	0.0448^{b} *(0.00442)*	0.0342^{a}	0.0448^{b}	0.0342^{a}	0.0448^{b}	0.0342^{a}

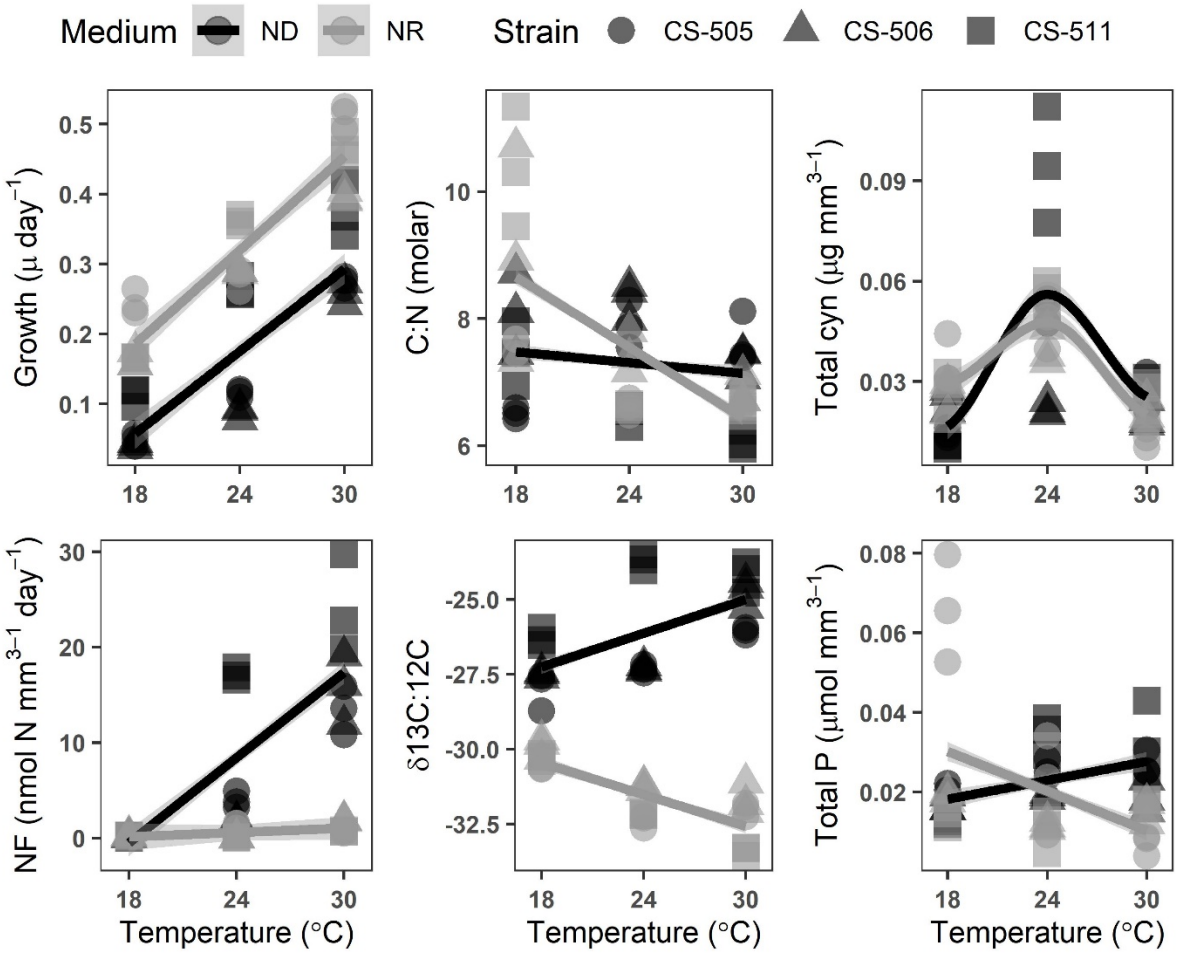

Figure 3.1: Modelled relationships between responses of the cultures to temperature and N-availability. From left to right, top to bottom, with units, marginal and conditional r^2 in brackets: top-left: Growth (μ d^{-1}) (0.82, 0.91), top-middle: cellular C:N (molar) (0.33, 0.39), top-right: total intra- and extracellular cylindrospermopsin (μg mm^{3-1}) (0.48, 0.58), bottom-left: N-fixation (nmol mm^{3-1} h^{-1}) (0.73, 0.81), bottom-middle: cellular δ13C (0.89, 0.91), and bottom-right: cellular P (μg mm^{3-1}) (0.21, 0.34). ND = nitrogen deficient medium, NR = nitrogen rich medium, cyn = cylindrospermopsin, NF = nitrogen fixation.

Except for toxin quotas, all data collected from the temperature experiments demonstrated strong linear trends (Fig. 3.1). Growth was highly correlated to temperature levels ($p < 0.001$) and was still increasing at 30 °C for all strains (Fig. 3.1 top-left). Dissolved inorganic nitrogen (DIN) availability was also found to be significant ($p < 0.001$), however the interactive effects between growth and medium were insignificant ($p = 0.24$). N2-fixation was significantly driven by temperature, DIN availability and their interactive effect ($p < 0.001$, $p = 0.0036$, and $p > 0.001$ respectively). Temperature and the interactive term between temperature and medium had significant effects on C_{13} fractionation ($p = 0$ and $p = 0$, respectively). Temperature had no significant effects on cellular C:N ($p = 0.43$), however

DIN availability, and the interaction between DIN availability and temperature, were found to be significant (p = 0.002 and p = 0.003, respectively). For intracellular P, no significance was found for either temperature or medium (Fig. 1f). Total intracellular cylindrospermopsin was significantly affected by both temperature and the quadratic temperature term (p = 0 and p = 0, respectively), and DIN availability (p = 0.027), however the interactive terms were all insignificant. Similarly, temperature and the quadratic temperature term had highly significant effects on total extracellular cylindrospermopsins (p > 0.001 in both cases), with all other terms found as insignificant.

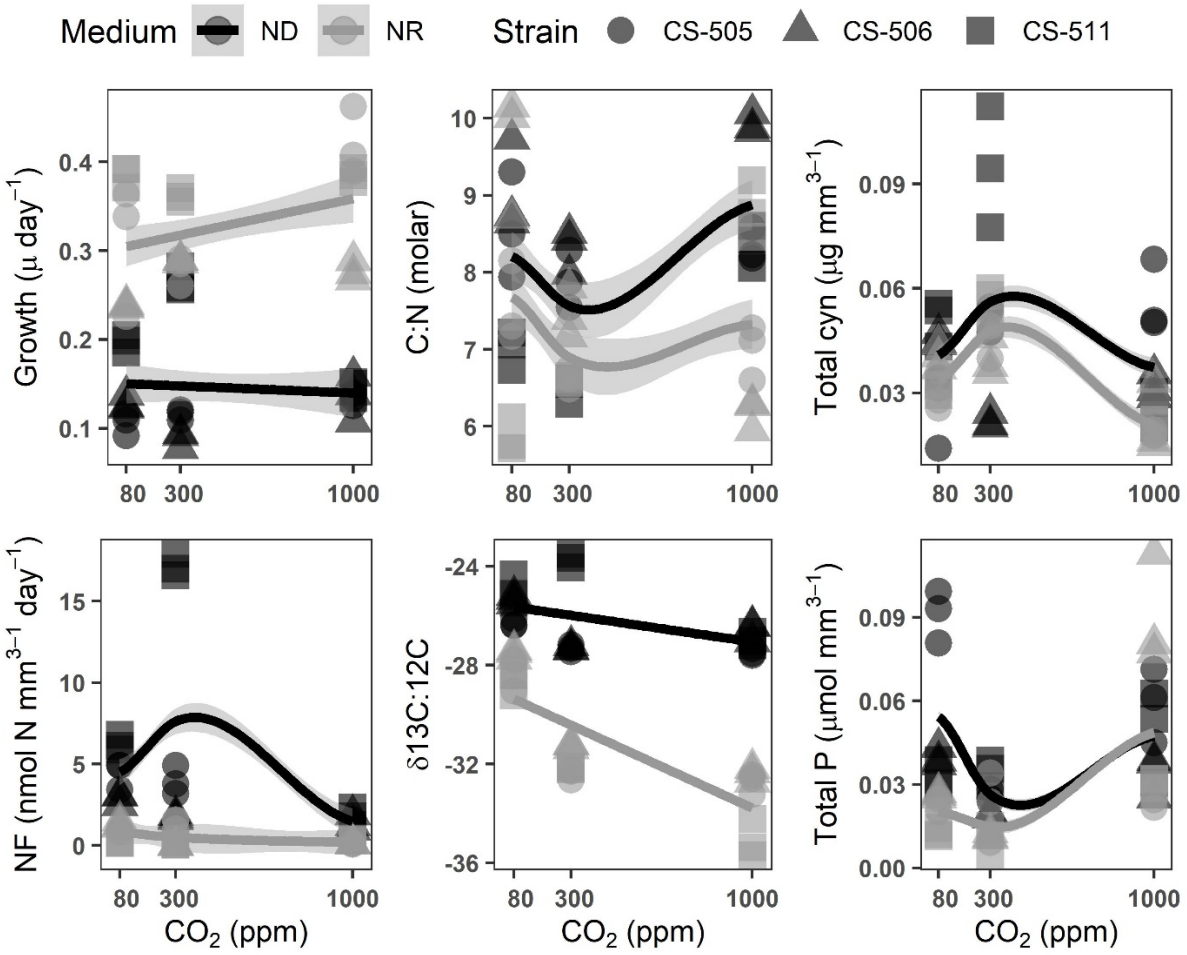

Figure 3.2: Modelled relationships between metabolic and stoichiometric responses of the cultures to CO_2 and N-availability. From left to right, top to bottom, with marginal and conditional r^2 in brackets: top-left: Growth (μ d^{-1}) (0.68, 0.87), top-middle: cellular C:N (molar) (0.25, 0.45), top-right: total intra and extracellular cylindrospermopsin (μg mm^{3-1}) (0.3, 0.38), bottom-left: N-fixation (nmol mm^{3-1} h^{-1}) (0.43, 0.55), bottom-middle: cellular δ13C (0.83, 0.83), and bottom-right: cellular P (μg mm^{3-1}) (0.38, 0.41). ND = nitrogen deficient medium, NR = nitrogen rich medium

In contrast to temperature, CO_2 exhibited mostly quadratic trends, particularly for the elemental composition data (Fig. 3.1 and Fig. 3.2). CO_2 had no significant effects on growth ($p = 0.57$), however DIN availability remained a significant driver under different CO_2 conditions ($p = 0$). Whilst CO_2 as an isolated term was insignificant, the interaction between CO_2 and growth medium was significant ($p = 0.016$). Both CO_2 and the quadratic CO_2 term had significant effects on N2-fixation ($p = 0.01$ and 0.002, respectively). Although DIN availability was insignificant, the interactive effects between CO_2 and growth medium were significant ($p = 0.02$). The δ13C term was significantly driven by both CO_2 ($p = 0.02$) and DIN availability ($p = 0$). For cellular C:N, all terms were insignificant, however marginal significance was found in the quadratic CO_2 term ($p = 0.056$). For the toxin data, total cylindrospermopsin was significantly affected by both CO_2 and the quadratic CO_2 term ($p = 0.02$ and 0.01, respectively).

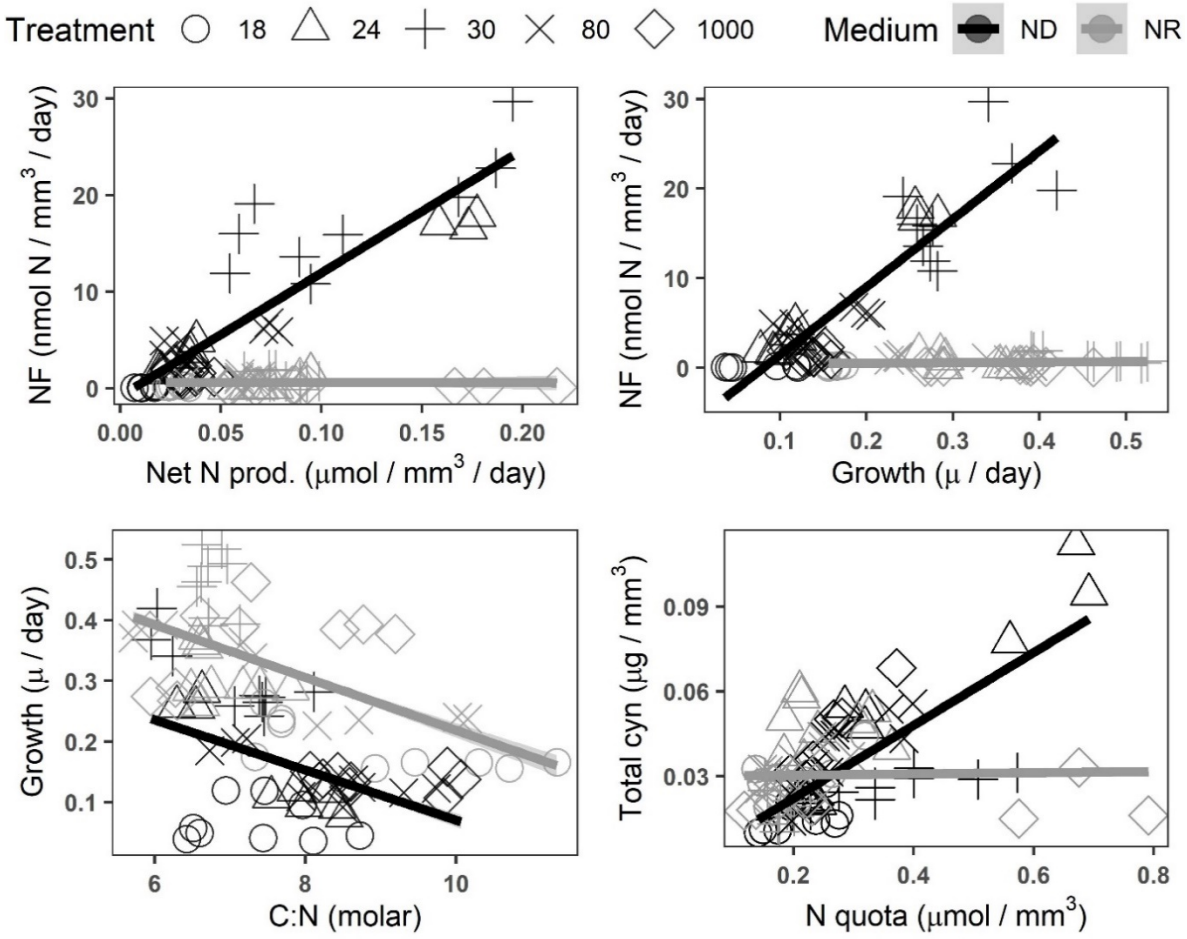

Figure 3.3: Modelled relationships between selected variables, with data taken from both temperature and CO_2 experiments. From top to bottom, left to right: top-left: N-fixation modelled against net N

production, top-right: N-fixation modelled against growth, bottom-left: growth modelled against intracellular C:N, and bottom-right: total cylindrospermopsin modelled against cellular N-quota.

Under N-depleted conditions, growth was correlated to C:N ($p = 0.01$), N-fixation was correlated to growth ($p = 0$) and net N production ($p = 0$), and total cylindrospermopsin quota was correlated to N quota ($p = 0$), across CO_2 and temperature treatments as well as strains. When inorganic nitrogen was supplied in the medium, most responses became insignificant, except for the negative correlation between growth and intracellular C:N (Fig. 3.3a).

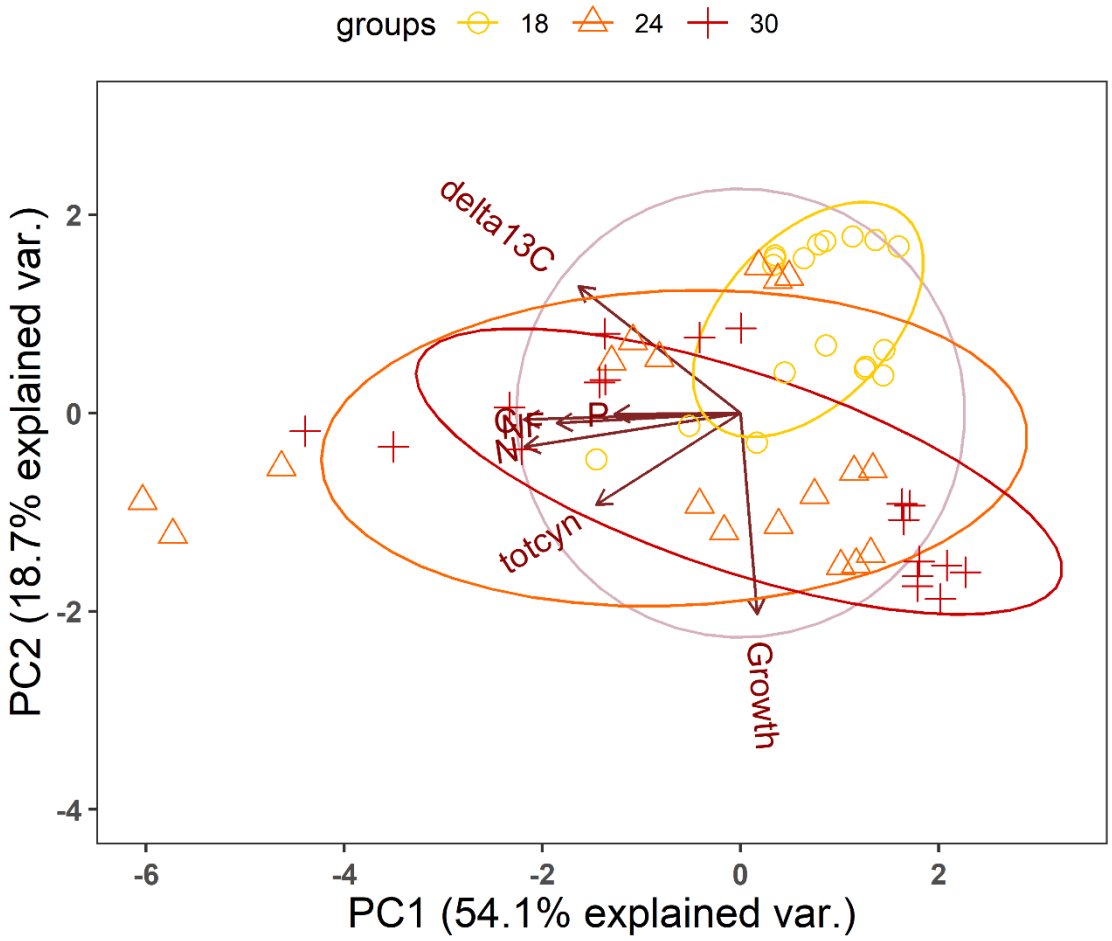

Figure 3.4: Biplot of a principal component analysis on data from the temperature experiments, with group based on temperature (°C). Terms are as follows: delta13C = $\delta^{13}C$:^{12}C, N = total intracellular N, P = total intracellular P, NF = nitrogen fixation, and totcyn = total cylinndrospermopsins.

Our results from the linear mixed models (Fig. 3.1) were supported by the principal component analyses. Under warming treatments, all metabolic and elemental terms depart

from the 18 °C ellipsoid to favor growth at higher temperatures. The first two components explained a total of 72.8% of the variation in the data (Fig. 3.4).

Under increasing CO_2 conditions, both N2-fixation and toxin quota depart away from higher CO_2 levels (Fig. 3.5), supporting the inhibition by elevated CO_2 levels found by the models (Fig. 3.2). Growth appears not to be driven by CO_2 levels at all, whereas low and high CO_2 concentrations result in elevated levels of P in the cells. The direction of the ellipsoids at low and high CO_2 levels is in direct conflict with the direction of the present-day CO_2 ellipsoid. The total variation explained by the first two components of the PCA was 69.6%.

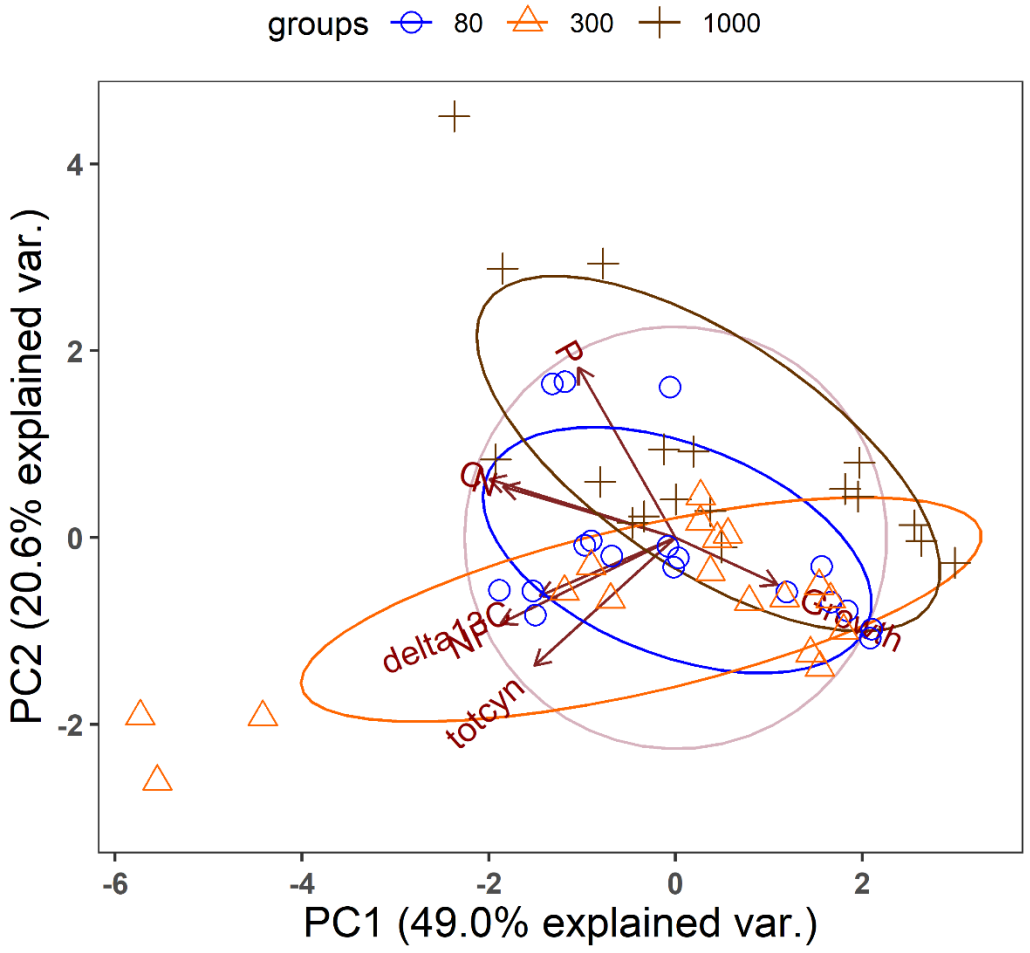

Figure 3.5: Biplot of a principal component analysis on data from the CO_2 experiments, with group based on CO_2 concentration (ppm). Terms are as follows: delta13C = $\delta^{13}C$:^{12}C, N = total intracellular N, P = total intracellular P, NF = nitrogen fixation, and totcyn = total cylinndrospermopsins.

3.5 Discussion

The positive association between growth and temperature in *R. raciborskii* reported here are well documented in other studies (Saker and Griffiths 2000, Yamamoto and Shiah 2012, Lürling et al. 2013). This association was complimented by a positive relationship between increases in diazotrophy under higher temperatures and N-depleted conditions (Fig. 3.1). Growth between cultures grown under N-rich and N-depleted conditions exhibited differences between the model means of 0.144 in the temperature experiments, and 0.168, 0.154, and 0.223 at low, medium and high CO_2 concentrations respectively (data not shown), suggesting that the cost of diazotrophy in growth remains relatively constant across temperatures and CO_2 levels, with the exception of high CO_2. Nonetheless, cultures sourcing N only from N-fixation maintained constant growth effectively, suggesting that *R. raciborskii* is physiologically flexible in surviving under nutrient poor conditions. Nitrogen fixation increasing under warmer conditions is to be expected since temperature increases the rate of enzymatic reactions, to a certain point (Tsygankov et al. 1991). Interestingly, the intracellular C:N of the cultures only differed significantly between mediums at 18 °C; at 24 and 30 °C, there was no significant difference reported, although C:N in N-rich cultures was lower. Therefore, cultures were able to maintain stoichiometric ratios even under completely N-free conditions, suggesting that diazotrophy in *Raphidiopsis* is a sufficient compensatory measure in place of no available N. Intracellular P exhibited little variation under our temperature treatments, however at 18 °C, P was significantly higher in N-rich cultures than in N-deprived. However, at 24 and 30 °C, significance was found in the opposite direction, with N-deprived cultures containing significantly more intracellular P. Since the only clear difference between ongoing processes between the mediums is diazotrophy, it is sensible to conclude that this plays some part in P-uptake, but this role remains unclear. Perhaps, due to N-limitation, protein synthesis is limited in cultures dependent on N-fixation, resulting in a surplus of unused intracellular P compared to cultures growing with available N. The significant effect temperature had on δ13C may also be linked with N-fixation, due to the differences in trend between N-replete and N-depleted conditions. The increase in δ13C as temperatures increase under N-starvation suggests that cultures dependent on diazotrophy seem to be experiencing ^{13}C enrichment. Gu et al. (2006) reports that the $\delta^{13}C$ of HCO^-_3 is ~ 8% higher than that of dissolved free CO_2, and thus phytoplankton are gradually enriched with ^{13}C as they take up HCO^-_3 as their source of C. N-fixation is an expensive process (Stam et al. 1987); perhaps cultures dependent on diazotrophy are preferentially taking up HCO^-_3,

whilst cultures with access to free N are utilizing both HCO^{-}_{3} and dissolved CO_2. This explains why cultures grown with available DIN show a negative trend in $\delta^{13}C$ (Fig. 3.1, Fig. 3.2), why δ13C declines as CO_2 concentration increases in our CO_2 experiments (Fig. 3.2), and why δ13C remains higher in the N deprived cultures compared to the N replete cultures in our CO_2 experiments (Fig. 3.2).

Our CO_2 results show a neutral to negative response in *R. raciborskii* to increasing CO_2, with no significant differences in growth, but significant reductions in cylindrospermopsin synthesis and N-fixation at both low and high levels of CO_2 (Fig. 3.2). Growth responses of cyanobacteria to elevated CO_2 in the literature have been mixed; Eichner et al. (2014) reported a significant decrease in growth in *Nodularia spumigena*, a species commonly found in estuaries, that, like *R. raciborskii*, is a filamentous, toxin producing, diazotrophic cyanobacteria. However, Wannickle et al. (2012) found significant increases in growth in *Nodularia* due to high CO_2. Furthermore, studies report increases in both growth and N-fixation in *Trichodesmium*, a filamentous marine cyanobacteria (Ramos et al. 2007, Hutchins et al. 2013). Holland et al (2012) reported on the effects of CO_2 on *R. raciborskii*, however their findings from a growth standpoint were inconclusive. The unusually high levels of cellular P at the low and high CO_2 levels perhaps suggests that, although P-uptake is functioning, processes that utilize this P are slowed or not taking place, such as ATP-driven intracellular transport or nucleic acid production (Reynolds 2006). However, Unger et al. (2013) found that *Nodularia* exposed to higher concentrations of CO_2 caused accelerated DIP and DOP uptake, due to enhanced C-fixation and growth. It is curious that we do not see this trend here.

The effects of elevated CO_2 levels on N2 fixation are more enigmatic. Our study is the first to report elevated CO_2 levels causing a reduction in nitrogenase activity, particularly for strain 511. This might be due to the heterocyst physiology of *R. raciborskii*. Unlike other diazotrophic cyanobacteria, *R. raciborskii* can only differentiate a maximum of two heterocysts, which are invariably positioned at the terminal points of the filament (Plominsky et al. 2013). Because of this, *R. raciborskii* relies on a nitrogen transport mechanism that can shunt fixed N from the atmosphere from the terminal heterocysts to the vegetative cells along the filament (Plominsky et al. 2015). The increases in CO_2 inhibit N-fixation may be because the carbon concentrating mechanism (CCM) of *R. raciborskii* is downregulated due to the excess available C, and thus the cyanobacteria loses the available electron sink that CCM provides. This may disrupt the pathway from the heterocysts to the vegetative cells (Tomar et

al. 2017). However, more experimental work is needed to explain this result. Under low CO_2 concentrations, it is likely that N-fixation will be limited by the reduced supply of CO_2 for photosynthesis, which would explain the rise in N-fixation from 80 to 300 ppm CO_2. The observed declines in cylindrospermopsin synthesis at the low and high CO_2 treatments may also be a function of C limitation at the low treatment and stalling of metabolic processes at the high treatment, but beyond this our data does not support any further argument. Similar to the $\delta^{13}C$ effects observed in our temperature treatments, our observed declines in $\delta^{13}C$ under our CO_2 enrichment experiment reinforce our experimental design, as this represents our cultures preferentially taking up free dissolved CO_2 and avoiding ^{13}C enrichment. This result has been reported in species of marine dinoflagellates in response to CO_2 enrichment (Hoins et al. 2016).

Principal component analysis results show that, whilst the eigenvectors for temperature effects are relatively aligned with each other, the eigenvectors for CO_2 effects are quite different between the values of today (~ 350 ppm) and our low and high CO_2 treatments. This result supports a hyperbolic relationship between metabolic variables, such as N-fixation and cylindrospermopsin production (Fig. 3.2) of *R. raciborskii* and CO_2 concentrations. This means that both low and high CO_2 levels act as a limiting agent, though not, curiously, on growth. Conversely, the levels of temperature we set in our experiments promoted *R. raciborskii* growth, although cylindrospermopsin quota significantly decreases at 30 °C. Possibly, investment in growth is prioritized over the synthesis of toxins. The parabolic relationship between cylindrospermopsin production and CO_2 seems strongly strain dependent and based on our results, CO_2 is unlikely to be a driving factor in cylindrospermopsin production, save when the consequential reduction in N-fixation results in lower amounts of N available for cylindrospermopsin synthesis. Such strain specific differences may relate to the operation of different CO2 and HCO3- transporters, as has been observed for *Microcystis* (Sandrini et al. 2015).

It is challenging to discuss specifically what the implications of metabolic shifts in toxin producing cyanobacteria mean, since we do not yet fully understand what purpose toxin synthesis fulfils. A few studies report findings that imply a significance to toxin production; allelopathy (Rangel et al. 2016, M-Hamvas et al. 2017), defense against parasites (Rohrlack et el. 2013), and strategic inhibition of photosynthesis (Downing et al. 2015). Our data cannot be used to suggest any sort of role of cylindrospermopsin synthesis, but we see clearly that toxin synthesis has an optimal response, and is highly strain specific (Fig. 3.1, 3.2). It is also

clear that under nutritional stress, where *R. raciborskii* must supply itself with N through diazotrophy, that cylindrospermopsin production is still occurring (Fig. 3.1, 3.2, Rigamonti et al. 2018); indeed, at similar N quotas, N-rich and N-deprived cultures are synthesizing similar amounts of cylindrospermopsin despite the high energy cost of N-fixation (Fig. 3.3). We find that at higher temperatures, cylindrospermopsin production decreases (Fig. 3.1). For another toxic species, *Microcystis*, a recent study conducted in a eutrophic temperate lake found that elevated temperatures resulted in increased production of microcystins coupled with declines in biomass (Walls et al. 2018). However, the opposite was found in another study, where microcystin production declined in favour of growth at higher temperatures under eutrophic conditions (Lürling et al. 2017). This could perhaps be strain variability, or the difference could be between the communities the cyanobacteria were grown in, we do not know for certain. Our optima trend is supported by data reviewed by Cirés and Ballot (2016), who find that toxin production in filamentous *Chryosporum* spp. and other closely related species decreases at elevated temperatures. We can imagine a system where, at lower temperatures, toxin synthesis helps cyanobacteria 'clear the stage' of competitors for warmer temperatures and nutrient pulses to come later in the season, where there is more to be gained by simply shunting resources into vegetative cell development instead of toxins. Compared to other studies that have measured cylindrospermopsis cell quotas, our data illustrates toxin concentrations somewhat lower per unit of volume than Pierangelini et al. (2015), and significantly lower concentrations than were found by Willis et al. (2015). The reasons for this are unclear, however culture conditions may be put forward as a possible influencing factor: the results obtained by Willis et al (2015) were obtained from strains recently isolated from the wild, whereas our strains and the strain used by Pierangelini et al. (2015) were obtained from the Australian National Algae Culture Collection, CSIRO, Hobart, Tasmania, Australia. More data is needed to explain this observation. Variation between toxin production in strains of *Raphidiopsis* has been established but not explained; despite possessing the same *cyr* gene cluster responsible for the biosynthesis of cylindrospermopsin analogues (Mihali et al. 2008), there is a high level of intraspecific strain variability in the amounts of toxin produced, and the proportions of different cylindrospermopsin analogues (Willis et al. 2016). An examination of genomic differences between *Raphidiopsis* variants find that the only detectable genomic difference within populations of *Raphidiopsis* is the presence or absence of the *cyr* gene cluster, with some cryptic clusters also being identified in select strains (Sinha et al. 2014). Thus, the exact purpose of cylindrospermopsin remains unclear, and arguably a defining mystery for the characterisation of the genus.

The relationships established between metabolic variables highlight the importance of the presence or absence of available nitrogen (Fig. 3.3). The ability of *R. raciborskii* to achieve a comparable net nitrogen production in an environment completely free of nitrogen is remarkable (Fig. 3.3). Interestingly, this ability was only evident in CS-511, which achieved a significantly higher diazotrophic rates than CS-505 or CS-506 (Fig. 3.1 bottom-left). This is potentially significant; CS-511 is a mainland strain; could a difference in ecological pressures between Palm Island and mainland Queensland explain this strain discrepancy? Nonetheless, strains 505 and 506, despite exhibiting lower levels of N fixation, were able to maintain comparable levels of growth to strain 511 under N-depleted conditions (Fig. 3.1 top-left, 3.2 top-left). From an examination of cylindrospermopsin production, it appears that the excess N strain 511 fixed was shunted into production of large amounts of an alternate cylindrospermopsin variant, deoxy-cylindrospermopsin (Fig. 3.1 top-right). The reasons for this are not clear, as the strains were treated in exactly the same way; more research is needed here. Despite the demonstrated capabilities of diazotrophy in enabling growth under N-depleted conditions, our results nonetheless indicate that growth is on average ~ 0.15 μd^{-1} less than cultures growing in N-rich media, illustrating the growth costs of nitrogen fixation (Fig. 3.1, Fig. 3.2). Our results also indicate that under N-limited conditions, the intracellular C:N ratio of the cultures increases, as the cells are taking up more carbon relative to nitrogen. Despite this, cylindrospermopsin production was comparable between the N-replete and N-depleted conditions, suggesting that it is evolutionarily favourable to produce a certain amount of the toxin regardless of how much N is available, and at cost to growth (Fig. 3.1, Fig. 3.2). This is likely due to the competitive benefits of allelopathic suppression, however it is commonly acknowledged that more research is needed on the scope of cyanotoxin functionality (Sukenik et al. 2015). It should be noted that our growth, nitrogen fixation and toxin production were based on biovolume measurements without pairing between cell counts or heterocyst counts. Adopting such an approach would facilitate an easier comparison with other sources in the literature; for example, is the increase in nitrogen fixation displayed by our data due to higher levels of nitrogenase activity or higher numbers of heterocysts? Undertaking these measurements would be a worthwhile avenue for futher research.

Whilst strain was modelled as a random effect and not as a factor in our analyses, there were some interesting trends associated with strain-specific responses. The mainland strain, CS-511, exhibited higher rates of growth and N2-fixation than the two island strains, CS-505 and CS-506, especially under N-limited conditions (Tab. 3.2, Fig. 3.1). Additionally, CS-511

produced much more total cylindrospermopsin than CS-505 and CS-506 (Fig. 3.1 and Fig. 3.2). This was due to large levels of deoxy-cylindrospermopsin being detected from CS-511. No deoxy-cylindrospermopsin was detected from the two island strains, although they have been shown to produce deoxy-cylindrospermopsin in small quantities (Willis et al. 2015) (data not shown). Additionally, CS-505 and CS-506 behaved in remarkably similar ways in response to the temperature treatments (Fig. 3.1), however these strains started to exhibit differences in response to different levels of CO_2 (Fig. 3.2). These patterns could suggest that habitat may have influenced the evolution and metabolism in *Raphidiopsis*. Their different responses to CO_2 concentrations, for instance regarding their cellular C:N ratios and P levels, can potentially be explained with two theories. The first is that the strains have evolved sympatrically, where the difference in responses either represents a communal approach to dealing with pressures such as grazing, or an attempt by either strain to outcompete the other (van Gremberghe et al. 2009). The second theory is that these strains have not evolved under shifting CO_2 conditions, and unusual CO_2 levels favour different relic gene expression in individual strains (Sandrini et al. 2015). Significant levels of strain intraspecific variability in *Raphidiopsis* have been demonstrated in the literature, which includes coiled morphology as a separating factor between strains (Willis et al. 2016). Differences in strain morphology has been suggested as indicative of subpopulations (Willis et al. 2018), with differences in physiological responses to environmental conditions (Pierangelini et al. 2014). It could also be that our strains are expressing traits in connection with their culturing. Interestingly, there are no glaring differences between their respective habitats; both Soloman Dam and McKinlay Dam are subject to a tropical climate and low rainfall, suggesting the potential for severe stratification in these systems. However, we are not certain whether CS-511 was isolated from McKinlay Dam, or McKinlay river, which would potentially imply a difference in habitat conditions. Our data is not expansive enough to support an effective discussion of which of these theories is prevailing, and we stress the need for more research in this area.

The significant increases in growth due to warming reported here suggest that *R. raciborskii* would indeed respond strongly to an increase in temperature on a system-wide scale, such as lake warming caused by human activity (Paerl and Paul 2012, O'Reilly et al. 2015). Although research has indicated that chlorophytes are able to grow at a faster rate than cyanobacteria (Deblois et al. 2013, Lürling et al. 2013), they are not diazotrophic. Stratified systems typically exhibit a nutrient poor epilimnion, with greater concentrations of nutrients present in the hypolimnion (Kraemer et al. 2015), and increased phosphorus release from anoxic

sediments (Jeppesen et al. 2009). This potentially represents a substantial advantage for *R. raciborskii*, which can regulate its buoyancy to take advantage of this contrast in water layers, store luxury phosphorus, and fix atmospheric N in order to maintain growth in the epilimnion (Willis et al. 2015, Burford et al. 2016). In contrast to this, we found that elevated CO_2 levels had virtually no impact on growth, whereas the literature does demonstrate some evidence of other varieties of cyanobacteria able to take advantage of higher concentrations of CO_2 to increase their growth, such as the freshwater microcystin producer, *Microcystis aeruginosa* (Brito et al. 2018). As such, *R. raciborskii* could potentially be at a competitive disadvantage under CO_2 enrichment scenarios, provided the system contains adequate concentrations of nutrients to allow non-diazotrophic cyanobacteria to grow. It is worth noting that our light concentration of ~ 45 µmol photons m^{-2} sec^{-1} may play a significant role in our interpretation; Pierangelini et al. (2015) notes an advantage conveyed to *R. raciborskii* from higher CO_2 concentrations due to the down-regulation of the cyanobacterial CCM, however this was only observed at light concentrations of ~ 80 µmol photons m^{-2} sec^{-1}; it could be that our cultures were light limited and thus not able to take advantage of the higher concentration of CO_2.

Increases in N-fixation in response to warming has been confirmed as a significant driving force in phytoplankton communities (Straub et al. 2013). Our results demonstrate a substantial increase in N-fixation as temperatures rise, suggesting that under warming scenarios, *R. raciborskii* will benefit from an increased capacity to flourish under N-poor scenarios. In the past, eutrophication has been prioritized as a key water quality problem, due to its promotion of cyanobacterial growth, especially by the unicellular microcystin producer *Microcystis* (Carey et al. 2012). However, many subsequent catchment management strategies have since focused on the reduction of N loads into systems suffering from blooms. This strategy has been shown to help reduce *Microcystis* dominance, however research has suggested that P suppression, instead of N suppression, could potentially be a more effective approach, due to N suppression having negligible effects on diazotrophic cyanobacteria (Molot et al. 2017). The success of *R. raciborskii* to proliferate under N free conditions is concerning, as our strains have shown capability to not only fix N and achieve comparable growth rates to N-rich cultures, but also to synthesise toxins. Curiously, despite the reduction in N-fixation that was observed at low and high CO_2 concentrations, toxin production also showed decreases in N-rich cultures at low and high CO_2, suggesting that toxin production is independent from the two N scenarios.

Several studies have suggested that global warming trends could exacerbate the pressures freshwater systems are under from toxic blooms of cyanobacteria, not only because warmer temperatures cause increases in growth and facilitate bloom development (Pearl 2017), but also because warming may promote toxin synthesis (Mowe et al. 2015b). Another factor frequently posited as crucial in driving the increase in bloom formation is eutrophication, brought about by a combination of agricultural activity and heightened intensity of storm events that mobilise larger quantities of sediment than are historically typical (O'Neil et al. 2012). Whilst warming temperatures are certainly shown to promote *Raphidiopsis*, the species is not typically associated with eutrophication (Chapter 2, Chapter 5). Our data suggests that the increase from 18 °C to 24 °C facilitates increased cylindrospermopsin synthesis, however toxin production then decreased from 24 °C to 30 °C (Fig. 3.1). This may be for several reasons; the specific function of cyanobacterial toxins is poorly understood, and its history of production suggests factors of influence outside the scope of human activity (Waters 2016). Furthermore, several sources in the literature report cylindrospermopsin production as constitutive, and outside the influence of environmental factors (Pierangelini et al. 2015, Willis et al. 2016). Despite this, several studies also report the influence of environmental conditions on cylindrospermopsin synthesis (Kokocinski et al. 2013, Rigamonti et al. 2018, Nor et al. 2019, Fu et al. 2019), and our data does suggest the influence of our treatment structures on toxin synthesis. Although we see a reduction in cylindropermopsin quota at 30 ºC, total concentrations within a system will likely increase under warming scenarios as the increases in cell numbers will outweigh decreases in cell-specific toxin levels. Thus, even though the effect cylindrospermopsin may have on other competing microbes may help facilitate invasion (Briand et al. 2016), there may be a turning point where conditions favour growth instead of toxin synthesis. Despite this, cylindrospermopsins have further been implicated in zooplankton suppression (Rangel et al. 2016), and allelopathic impairment of other phytoplankton (Mello et al. 2012), which represents substantial ecological imbalance, in addition to economic and resource stress for society. Further stress on other varieties of phytoplankton will be imposed through climate change, which will promote warming and stratification of systems, reduction in flow, and more unstable nutrient regimes due to rainfall regimes becoming more erratic (Pittock and Finlayson 2011). Climate change scenarios also expose *R. raciborskii* to high irradiance levels, as low flowing systems typically exhibit low turbidity, allowing light to penetrate to deeper layers (Davis and Koop 2006). This will elicit increased growth and as a byproduct increased concentrations of cylindrospermopsin (Nor et al. 2019). Thus, the health of systems

is threatened through environmental changes that will favour the domination of filamentous, diazotrophic cyanobacteria such as *R. raciborskii*.

3.6 Conclusion

Here we have demonstrated broad laboratory responses from three strains of *R. raciborskii* to variable conditions, which may help inform how freshwater systems worldwide may be impacted by cyanobacterial dominance under climate change. We find unequivocal increases in growth and nitrogen fixation under warmer temperatures, with the species able to grow almost optimally under N depleted conditions. Under different CO_2 concentrations, the lack of growth response and nitrogen fixation by *Raphidiopsis* runs contrary to the current literature, which typically reports moderate increases in growth. Optima trends for toxin synthesis under both differing temperatures and CO_2 suggest a competitive trade-off when producing toxins depending on growth potential. These physiological responses confirm the metabolic plasticity of *Raphidiopsis* and its potential for success under shifting climatic conditions, especially in warm stratified water bodies. Australia experiences an already erratic rainfall regime, and a high rate of evapotranspiration, with a significant component of its freshwater systems being ephemeral. Shifts associated with climate change, such as warming and increasingly sporadic rainfall patterns, with an increased incidence of storms, will result in many systems losing flow and becoming more stratified, creating ideal conditions for *R. raciborskii* to dominate. There has been much research attributing the rising incidence of *R. raciborskii* blooms to the above-mentioned conditions, as well as its physiological advantages. Based on expected climate change, and our findings above, it seems feasible that *R. raciborskii* is well suited to expanding its range from Northern tropical Australia to the more temperature Southern and Eastern regions, where the cyanobacteria *Dolichospermum* and *Microcystis* have an extensive history of bloom formation due to eutrophic conditions and building of dams and weirs. This range expansion could have serious ecological consequences, and more research is needed here to assess what effects on Australia aquatic systems a *R. raciborskii* expansion could potentially have.

4. Urban freshwater laboratory ponds exhibit loss of stability in response to warming and the introduction of *Raphidiopsis raciborskii*

4.1 Abstract

Biological invasions are a commonly reported and keenly investigated ecological phenomenon, especially in the context of rapid environmental change. However, the interplay between these two factors, and their impacts on ecosystems, is difficult to examine, especially at the micro-scale. The cyanobacteria *Raphidiopsis raciborskii* is a focus of much research for its invasiveness, toxin production, and affinity to rising temperatures and oligotrophic conditions, but little is known on the effect the species has on communities in systems it invades. Here, we have generated a set of laboratory ponds from a eutrophic pond system in Sydney, Australia, and simulated an invasion by *R. raciborskii*, whilst also implementing an increase in temperature in half the systems. Weekly phytoplankton and zooplankton counts and measurement of physiochemical properties were taken from the systems, community DNA was extracted at the beginning and end of the experiment and sequenced, and measures of biodiversity were calculated. We hypothesised that warming would elicit significance differences in how the systems developed, however, our results indicate that time, rather than temperature, was the dominant driver of shifts in the systems. Bloom dynamics of our buckets mirrored an emerging inverse relationship between *Rahpdiopsis* and *Dolichspermum*, where blooms of *Dolichospermum* are closely followed by *Raphidiopsis* activity after their demise. Furthermore, zooplankton and crustacean declines through time and at warmer temperatures, combined with decreases in ecological diversity indices, demonstrate the destabilising effect of warming on freshwater ecosystems. Finally, proportions of prokaryotic sequences from our ponds are linked to recent material in the literature highlighting these prokaryotic ratios as potentially indicative of system health and stability. Our results hold implications for experimental design and system resilience and suggest that warming temperatures alone may not account for changes observed in freshwater systems worldwide.

4.2 Introduction

Biological invasions are distinguished by the rapid expansion of natural species ranges, often at detriment to the native species of the area being invaded. They have been described as the most significant ecological events arising from the unprecedented ecological disruptions caused by human transport and resource exploitation (Mack et al. 2000). Biological invasions can be examined from several standpoints, such as the characteristics of invaded communities (Lonsdale 1990), the demand and security of specific resources (Sousa et al 2011), and even the most eclectic of ideas such as the personalities of animals (Carere and Gherardi 2013). The effects of invasions may be further complicated by secondary dynamics including parasites (Tella and Carrete 2008), climate change (Neubert et al 2000), the differences between Anthropocene-generated invasions and natural colonisations (Wilson et al 2016), and landscape homogenization due to agricultural development of land (Reichard and White 2001). Biological invasions are seen overwhelmingly as a negative phenomenon, for the most part (Bellwood and Goatley 2017), as the prevailing recognition of biological invasions comes from negative consequences such as economic and ecosystem services losses and declines in biodiversity and ecological stability, not to mention the virtual impossibility of removing an alien species once they are established (Simberloff et al 2013). Although many invasions have been documented, the ambiguity surrounding cases where effects of invasion seem indeterminant or even positive may be due to a time-lag between the establishment of an invader population, and the significant reduction or extinction of resident keystone species (Gallardo et al 2016). Thus, as the incidence of biological invasions increase exponentially, an amassing effect may be taking place, the consequences of which we are yet to discern. This makes research surrounding the mechanisms and dynamics of invasion important.

Biological invasions are often considered in the context of the rapid environmental change we are currently observing. One of the key recognised consequences of anthropogenic climate change is a loss of water resource security (Xia 2012). Aquatic ecosystems are excellent at conveying invaders, particularly microbiological invaders, to new habitats and constitute one of the most crucial resources that humanity depends on, fresh water. In the past two decades, there has been a dramatic increase in research directed towards monitoring and maintaining water quality; its drivers and its effects on overall ecosystem health, due to the increases in extreme hydrological events such as droughts and flooding (Bakker and Hilt 2016). These events can worsen water quality due to mobilisation of sediments and nutrients or fertilisers and sewage, in the case of flooding, or through the exacerbation of column

stratification or cease of flows in the case of drought (Paerl and Paul 2012). Through either of these dynamics, conditions can become suitable for what has been put forward as 'the greatest inland water quality threat to public health and aquatic ecosystems': harmful algal blooms (Brooks et al 2016). Deleterious toxin production that can harm humans, livestock, fish and other phytoplankton and zooplankton, smothering and deoxygenating effects that can be devastating for ecosystem health, and severe reductions in community services such as swimming pools and commercial aquaculture operations, make harmful algal blooms a multi-faceted biological, economic and social problem (Elliott 2012). Due to the onset of significant worldwide lake warming and nutrient fluxes (O'Reilly et al. 2018) as well as the artificial transport of species to new locales (Angeler and Johnson et al. 2015), dramatic shifts in phytoplankton community structure are beginning to be observed (Zohary 2004, Hadas et al. 2012). These shifts have been characterized by the emergence of several invasive phytoplankton species (Chapter 2).

Of relevance to Australian water security is the invasive toxin-producing cyanobacterium *Raphidiopsis raciborskii*, which has an extensive history of bloom formation, and was responsible for the worst documented cyanobacterial poisoning event in Australia (Hawkins et al. 1985). The species carries a number of physiological traits that make it particularly suited to Australian waters, namely diazotrophy, buoyancy regulation, and an affinity to high temperatures and low phosphate concentrations (Burford et al. 2016). Australian water systems are characterised by small or even ephemeral flows due to limited rain regimes and heavy irrigation demands, severe stratification regimes due to high irradiance and hot Summers, and poor bank health and soil erosion due to the misguided and destructive habits of the first European settlers (Ryder and Boulton 2005). Australia has an intense history of drought, most recently embodied by the millennium drought (2003 – 2009), and climate projections suggest that Eastern Australia will continue to experience progressive drying through the 21st century (Cook et al. 2016), with temperatures predicted to rise by 0.1 – 0.4 ^{o}C per decade (Jacobs et al. 2013). The combination of these factors is placing an increasing amount of stress on the stability of our aquatic resources. It has been shown that, although more extreme inflow events reduce overall cyanobacterial biomass in the short-term, reservoirs and rivers consequentially exhibit higher vulnerability to cyanobacterial blooms in following seasons due to sediment remineralisation of ammonium and phosphates (Leigh et al. 2015). In the periods preceding extreme flow events, however, Australian systems will generally display a nutrient profile tending towards oligotrophy, due to stratification of the

water column. Whilst climatic shifts are established as key drivers of aquatic system vulnerability, it remains unclear how cyanobacterial shifts will affect ecosystem properties and stability. Microbial invaders are arguably the most potentially devastating invaders; the demographic features, spatial processes, and selective pressures that come with every ecosystem can cause the favouring of particular phenotypic traits that promote range expansion and resource competition (Chuang and Peterson 2016), and microbial organisms, such as *R. raciborskii*, enjoy the highest rate of genetic turnover and are primely placed to take advantage of this dynamic.

In this study, we generate laboratory ponds from a hypereutrophic freshwater system in urban Sydney, New South Wales, and maintain them for ten weeks. We simulate increases in water temperature, introduce *Raphidiopsis raciborskii* into all systems, and monitor the laboratory pond physiochemical and plankton community profiles. This approach is combined with Next Generation Sequencing for deeper community structure analysis. We hypothesise that *R. raciborskii* will dominate heated systems more readily than unheated systems, and that system diversity will drop in heated systems in contrast to unheated systems. The resulting data will help shed light on the interplay between warming and system stability, and on how the spread of invasive species may influence this dynamic.

4.3 Method

4.3.1 Experimental setup

Laboratory ponds were generated by filling twelve 10 L black buckets with 8 L of water from Duck Pond, Centennial Park, Sydney, Australia, and subjecting them to a 14:10 photoperiod with light concentration set at ~ 40 µmol photons $m^{2\,-1}$ sec^{-1}. Buckets were refreshed manually with 350 mL of freshly collected pond water from Tuesday to Friday, which was delivered via peristaltic pump and silicon tubing. 350 mL was chosen to represent ~ 4.5%, slightly lower than used elsewhere in the literature (Canavate et al. 2015). Pond water was collected every two weeks on Monday and stored in plastic reservoir tanks. Spill-over from the buckets was controlled via silicon tubing inserted into a hole which was drilled into the buckets ~ 5 cm from the top. Spill-over was treated as system run-off and was discarded.

4.3.2 Treatment structure

The laboratory ponds were maintained for 3 months under control conditions, which consisted of no invasion or heating. The relatively long maintenance time was observed to ensure the buckets had reached an ecological steady state relative to one another before the treatments were implemented. The temperature of the bucket water was controlled using aquarium heaters (JBA 25W DZ-3025), with 6 of the buckets set to a constant 20 ºC, and the other 6 set to a constant 24 ºC. Buckets were inoculated with *R. raciborskii* to make up a density of 37 cells/mL at the start of the experiment. The *R. raciborskii* inoculum was comprised of a 1:1 ratio of *R. raciborskii* strains CS-506 (coiled trichomes) and CS-511 (straight trichomes) (CSIRO, Chapter 3). We followed the approach adopted by Sperfeld et al. (2010) for judging the density of the invader inoculum to introduce into the treatments. The experiment was run for 10 weeks, from the 14th of September to the 16th of November 2016, to capture an exclusively Spring phytoplankton community in the pond system. Our experimental design and philosophy were loosely based on Rasconi et al. (2015), in its implementation of temperature as a treatment structure, and its focus on biodiversity indices and proportions of phytoplankton type as representative of system state.

4.3.3 Sampling approach and analysis

Samples were taken weekly from each laboratory pond. For phytoplankton counts, two samples of 250 ul were extracted via pipette at ~ 1 cm and 10 cm depths, fixed with Lugols iodine, and allowed to settle for 24 – 48 hours following the Utermöl method (Lund et al. 1958). Phytoplankton were counted on an inverted microscope (Nikon Eclipse TE200) and identified to the genus level. Phytoplankton biovolumes were assigned using reference data (Kremer et al. 2014). Zooplankton were counted by gently filtering 100 mL of laboratory pond water through a 0.2 um filter, and taxonomically identifying specimens caught on the filter using a stereo microscope at 40x magnification (Olympus BX51). Laboratory pond physiochemical profiles and productivity were assessed using a YSI EXO^2 multiparameter sonde probe, which measured chlorophyll-a and phycocyanin concentration (μg l^{-1}), dissolved oxygen (%), temperature (°C), electrical conductivity (μS cm^{-1}), and pH. Prior to the experiment beginning, the probe was calibrated using commercial standards for each of the above variables.

Community structure was assessed through Next Generation Sequencing DNA. DNA was extracted using an adapted protocol from Patyshakulieva et al. (2014). Briefly, filters were dissolved in a 1.7 mL tube with chloroform:isoamyl alcohol solution (24:1) combined with a

GuHCl lysis buffer. Tubes were then centrifuged at 10,000 rcf for 2 minutes in order to separate phases. The top phase was combined with 100% EtOH and the entire sample was then filtered through pipette columns, made according to the process outlined in Tagliavia et al. (2009). The columns were then washed with 70% EtOH and the bound DNA was eluted using MQ water, with the samples being frozen at -80 ºC. Samples were sent to RTL Genomics (http://rtlgenomics.com/) for 16S and 18S amplicon sequencing for diversity. For 16S, the Earth Microbiome Project primer set 515yF-806bR was used, and for 18S, the default TAReuk set from RTL Genomics was used. Resultant data took the form of fraction of hits for each of our 24 samples and was provided in 'full' and 'trimmed' versions, where 'full' did not consider confidence levels surrounding classification, and 'trimmed' did; only trimmed data was carried forward into our analysis.

4.3.4 Data analysis

Data were split into separate sets for each taxonomic classification level, including phylum, class, order, family, genus, and species. Where classification resulted in no hit, data was omitted before analysis. Physiochemical data was displayed as boxplots by day to illustrate temporal trends and variability. Planktonic count and biovolume data were assessed using generalised least-squares (GLS) regression using the package "nlme". Despite patterns in nature generally being non-linear, our two-level treatment structure warrants the use of a linear modelling method. The GLS method allowed this approach to be taken whilst simultaneously providing a more robust estimation of the regression coefficients given that there was likely to be correlation between residuals due to the relatively low number of experimental units (buckets), as well as autocorrelation through time. Models were run with all variables, and took the following structure:

$$\begin{aligned} log_{10}(\gamma) = {} & \beta_0 + \beta_1 week + \beta_2 temperature + \beta_3 week.temperature + \beta_4 conductivity + \beta_5 pH \\ & + \beta_6 DO + \beta_7 log_{10}(Diatoms) + \beta_8 log_{10}(Cryptophytes) \\ & + \beta_9 log_{10}(Cyanobacteria) + \beta_{10} log_{10}(Chlorophyte) + \beta_{11} Zooplankton \\ & + \alpha_1 log_{10}(\gamma)_{week-1} \end{aligned}$$

Where γ is the response variable and included cyanobacteria, chlorophyte, cryptophyte, and diatom biovolumes, zooplankton individual counts, key species abundances including *Raphidiopsis, Dolichosperumum*¸Cladoceran and Ostracod zooplankton counts, and diversity indices. β indicates coefficients of the covariates, and α is a correlation term for the covariates. A random effect to account for within bucket variation was included as an inverse variance weighting. Temperature is a binary factor to reflect the treatment structure (20 and

24 C°). Models were first run with a week by temperature interaction term, however if this term was not found to be significant, models were then run with week and temperature without an interaction. Additionally, autocorrelation between weeks was accounted for by adding an AR1 autocorrelation structure to the model and the variance was weighted by bucket as it was shown to not be equal between the treatments. A likelihood ratio test was used to test whether the additional autocorrelation term was needed by comparing the with and without models and it was shown to be a significant term in the model. Model means were calculated and presented where significant results were obtained for the treatment interaction, or either of the main treatment effects (week or temperature). For visualization, the estimated model means, lower confidence limits, and upper confidence limits were calculated using the package *emmeans* (Lenth 2018). Additionally, key genera for our experiment were identified as *Raphidiopsis* and *Dolichospermum*, due to the introduction of *R. raciborskii*, and to the blooming behaviour of both. These were also modelled according to the methodology above.

To establish whether significant differences were present between either week and/or temperature groupings in the community taxa data, Bray-Curtis similarity matrices were generated, and ANOSIMs (analysis of similarity) were run on each taxonomic level of the prokaryotic and eukaryotic data. Where significant dissimilarity was found, differences between significance levels for each treatment group and the treatment interaction were identified using a pairwise permutational analysis of variance (PERMANOVA), with p-values adjusted with a Bonferroni correction. Separate models run for prokaryotic and eukaryotic data, and at each taxonomic classification, to ensure that results were not skewed towards variation at any single taxonomic level. Where a significant result was obtained for either week and/or temperature or the interaction of week and temperature, a similarity percentage (SIMPER) analysis was used to calculate the percent-similarity between classifications at the different levels of the significant treatment and to test for significance between the contributions of each classification at different treatment levels. For assessments of community diversity, three separate indices were calculated based on our count and community taxa data. These were species richness (1), Shannon's Index (2), and evenness (3). Species richness is simply the number of unique genera present in a sample, and discounts relative abundances. Shannon's Index was calculated as:

$$S = -\sum_{i=1}^{R} p_i \ln(p_i)$$

Where p_i is the proportion of individuals of species i within the community. Evenness was calculated as:

$$\varepsilon = S/S_{Max}$$

Where S is the Shannon Index and S_{max} is the maximum diversity possible, which is equal to $\ln(richness)$. Diversity indices were then modelled using the approach outlined above for the count data, but with the plankton terms omitted due to autocorrelation. Data were log-transformed where necessary to satisfy assumptions of normality and constant variance. All statistical analysis was done using the R project (address), with all visualization performed using the package "ggplot2".

4.4 Results

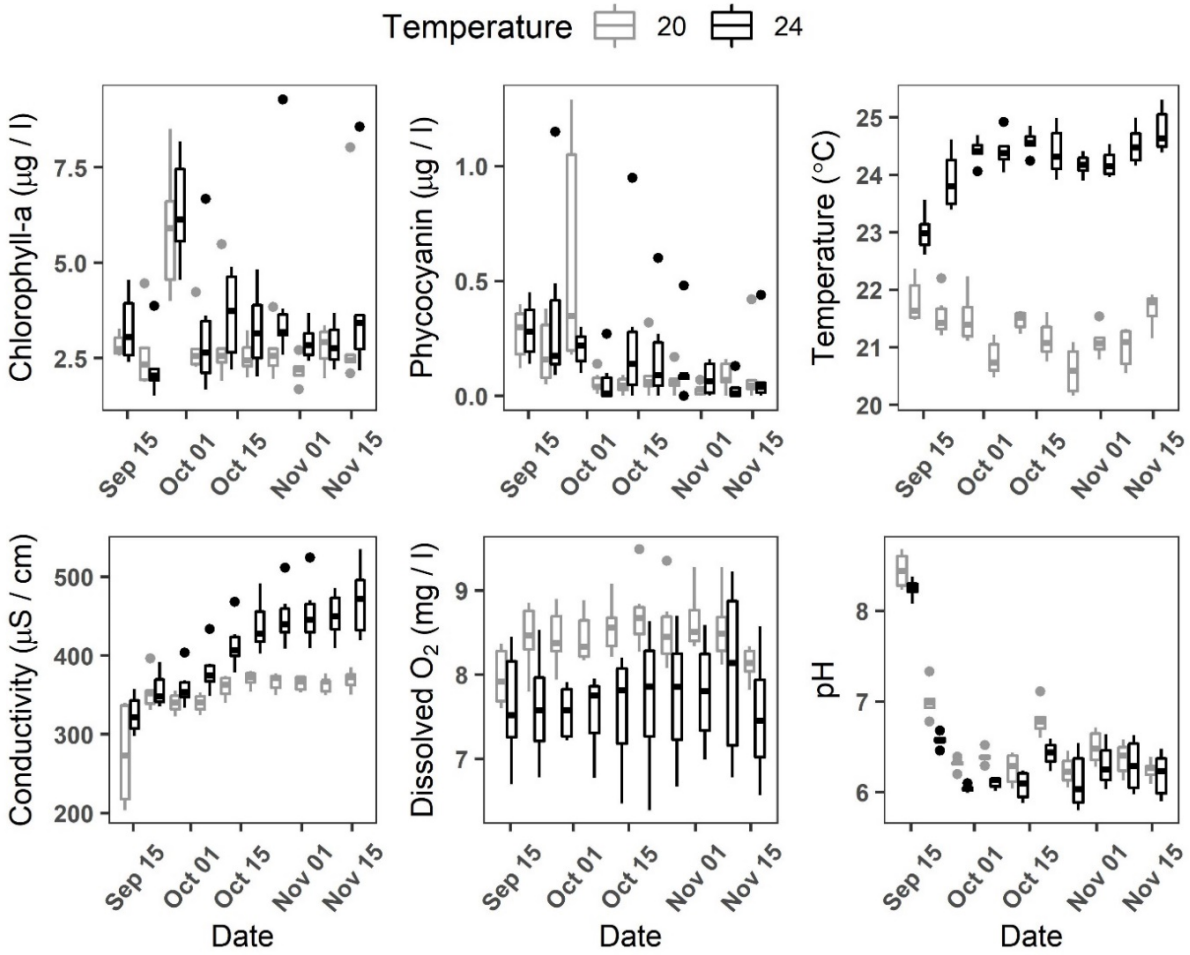

Figure 4.1: Boxplots of physiochemical measurements over the course of the experiment.

Chlorophyll-a increased dramatically in late September in conjunction with a *Dolichospermum* bloom and increased again in mid-October as a result of a smaller *Raphidiopsis* bloom. Phycocyanin levels remained relatively constant throughout the experimental period, with small increases showing in mid-October, likely due to the *Raphidiopsis* bloom. Conductivity increased steadily from the start of the experiment until mid-October; at this point, it levelled out for the cooler laboratory ponds, but kept increasing for the warmer systems. Dissolved oxygen remained constant over the entire course of the experiment, relative to time, however there were differences evident between the warmer and cooler buckets. The warmer laboratory ponds had consistently lower pH than the cooler laboratory ponds. System pH responded to the two bloom events due to the uptake of dissolved inorganic carbon; pH rose in late September due to the *Dolichospermum* bloom and rose again in mid-October due to the *Raphidiopsis* bloom (Fig. 4.1).

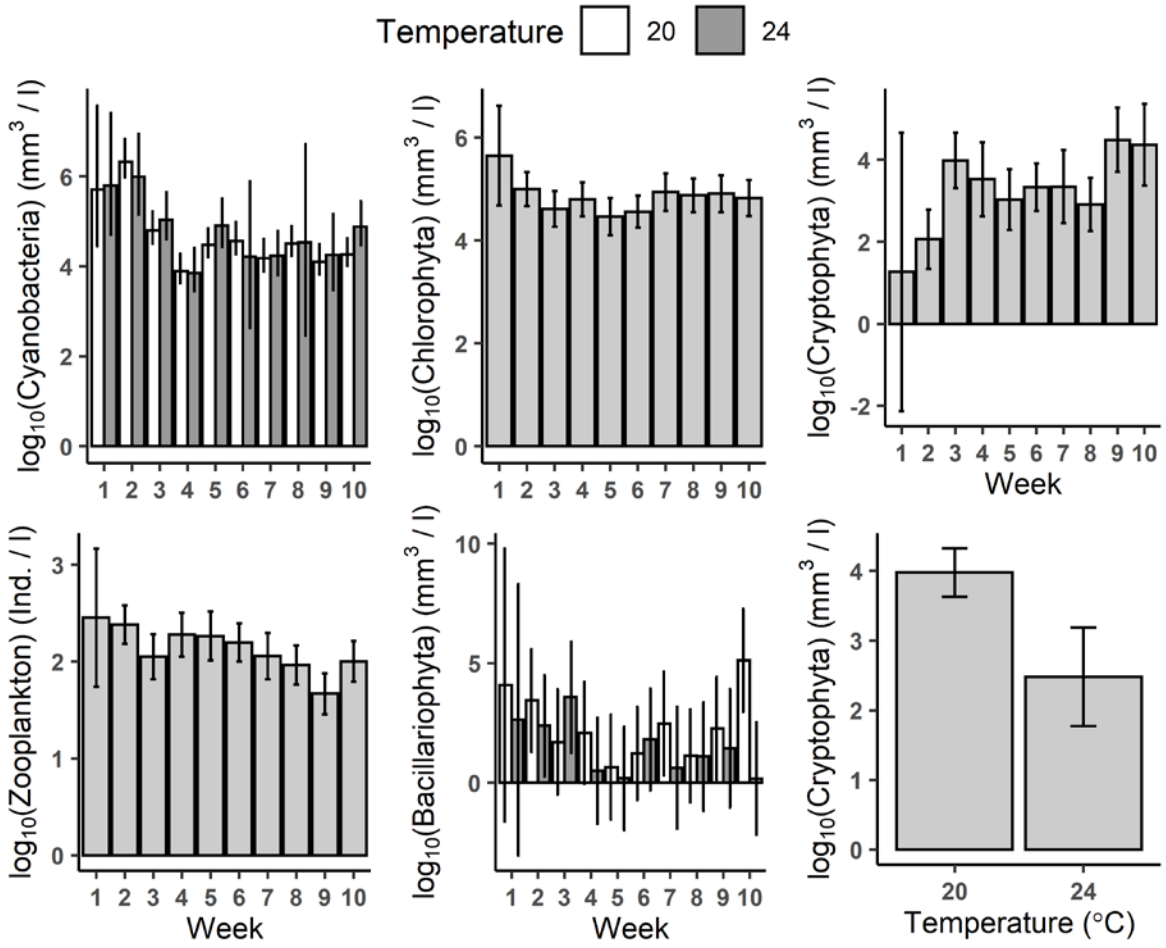

Figure 4.2: Model means of biovolumes of cyanobacteria (blue-green algae), chlorophyta (green algae), cryptophyte, bacillariophyte (diatoms), and counts of zooplankton individuals. Plots were produced based on whether a significant result was obtained; either over time, or at different temperatures.

The model run for cyanobacterial biomass found week ($p < 0.0001$), the temperature×week interaction ($p = 0.03$), conductivity ($p < 0.05$), pH ($p < 0.001$), temperature ($p < 0.05$), chlorophyte biovolume ($p = 0.0001$) and cryptophyte biovolume ($p = 0.05$) as significant drivers, and the model achieved an r-squared of 0.51. Cyanobacteria biomass seemed to be decreasing through time, with consistently negative coefficients for the week terms, however warming had a slight positive effect. Cyanobacteria abundance was also positively correlated with chlorophyte and cryptophyte abundance. Chlorophyte abundance was negatively correlated with week ($p = 0.05$), positively correlated with cyanobacteria ($p = 0.001$), and negatively correlated with diatoms ($p < 0.05$), with the model achieving an r-squared of 0.26.

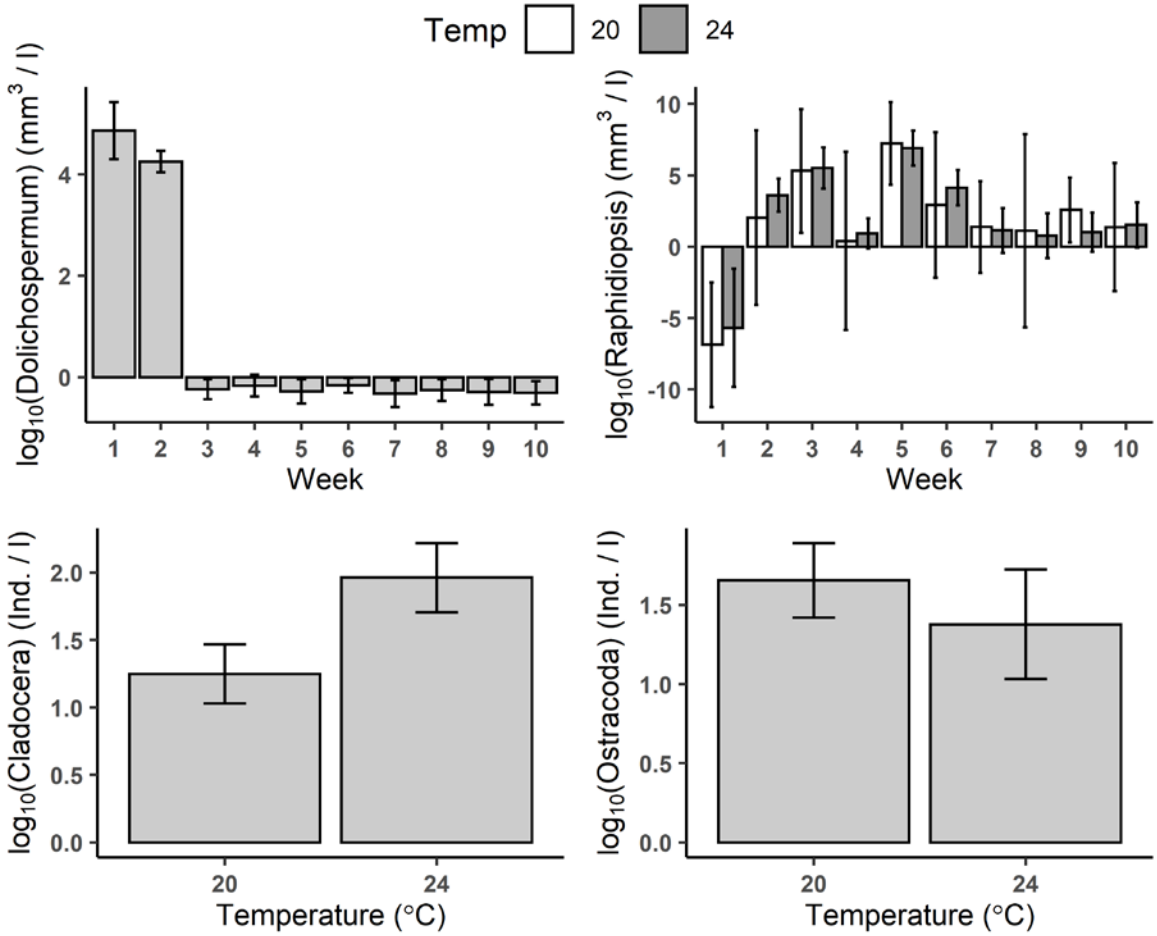

Figure 4.3: Model means of *Dolichospermum*, *Raphidiopsis* biovolumes, and individual counts of Cladocera and Ostracoda zooplankton, with error bars representing lower and upper 95% confidence limits. Plots were produced based on whether a significant result was obtained; either with the treatment interaction, or with the main effects of temperature and/or week.

Diatom abundance was negatively correlated with temperature ($p = 0.0085$), and positively correlated with dissolved oxygen ($p = 0.014$); additionally, the interaction between temperature and week was significant ($p < 0.05$). The significant interaction revealed a tendency for biomass to increase in conjunction with the bloom events at weeks 2 and 6, especially at the warmer temperature, however outside these times diatoms were more abundant in the cooler buckets ($p = 0.03$). The model achieved an r-squared of 0.34. Cryptophyte biovolume was positively driven by week ($p < 0.0001$), and negatively driven by temperature ($p < 0.0001$), with the model achieving an r-squared of 0.35. Finally, zooplankton counts were negatively correlated with week ($p < 0.0001$), and positively correlated with dissolved oxygen ($p < 0.01$), with the model achieving an r-squared of 0.32 (Fig. 4.2).

Dolichospermum abundance was correlated with week ($p < 0.0001$), with significant declines through time evident (Fig. 4.3). *Dolichospermum* was also negatively correlated with dissolved oxygen ($p = 0.01$), and negatively correlated with the warmer temperature ($p < 0.01$). The model for *Dolichospermum* achieved an r-squared of 0.53. *Raphidiopsis* was negatively correlated with week ($p < 0.0001$), positively correlated with pH ($p < 0.001$), negatively correlated with diatom biovolume ($p = 0.0017$), positively correlated with chlorophyte biovolume ($p = 0.0001$) and interaction between temperature and week ($p < 0.05$), with lower biomass predicted in warmer weeks than in colder weeks. The model achieved an r-squared of 0.54. A depiction of the model means suggests the possibility of an inverse relationship between the two, however neither the genera were found to be significant in explaining the abundance of the other. Counts of Cladocerans were predicted to increase under warmer conditions ($p < 0.001$) and with time ($p < 0.0001$). Furthermore, Cladocerans were positively correlated with dissolved oxygen ($p < 0.0001$), and the model achieved an r-squared of 0.49. Ostracods however were negatively correlated with temperature ($p < 0.01$) and cryptophyte biovolume ($p < 0.05$), with the model achieving an r-squared of 0.18.

Species richness was found to be significantly decreasing over the course of the experiment and under warmer conditions based on the count data (Fig. 4.4, $p < 0.0001$, r-squared = 0.63). This trend was also observed within the prokaryotic sequenced data ($p < 0.0001$, r-squared = 0.34), and the eukaryotic sequenced data ($p < 0.0001$, r-squared = 0.24). The interaction between richness and temperature for the count data suggested that system recovery from the *Dolichospermum* bloom in week 2 was slower for the warmer systems than the cooler systems (Fig. 4.4). Richness was also significantly driven by temperature within the

eukaryotic data, with a lower richness observed at 24 ºC than at 20 ºC (Fig. 4.5, $p < 0.0001$, r-squared = 0.24). Within the count data, Shannon's index was reported to be increasing overall through time, however decreasing with temperature, according to the significant interaction ($p < 0.05$, r-squared = 0.443. Shannon's Index was also found to have decreased within the taxonomic data over the course of the experiment in conjunction with the warming effect. At the prokaryotic level, this decrease was accompanied by a stronger decrease in the warmer buckets ($p = 0.0001$, r-squared = 0.43). At the eukaryotic level, Shannon's Index decreased with time and temperature ($p = 0.0002$, r-squared = 0.30, fig. 4.5). Evenness was difficult to interpret; the count data exhibited what seemed to be a fluctuating evenness that increased with bloom events until week 7; beyond this point a significant decline was evident in both treatment groups ($p < 0.0001$, r-squared = 0.44). Trends were ambiguous in the sequenced prokaryotic data, with litte change over time or with temperature (r-squared = 0.38), however in the eukaryotic data evenness decreased over both time and temperature ($p < 0.0001$, r-squared = 0.27, fig. 4.5).

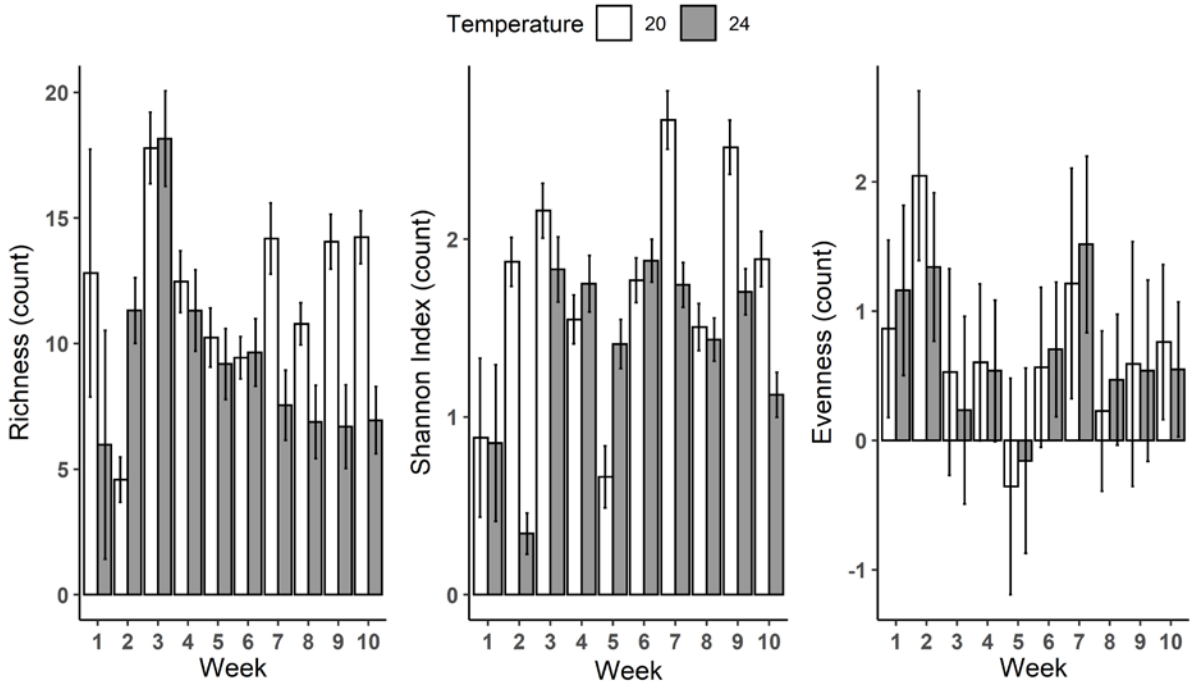

Figure 4.4: Model means with lower and upper confidence levels (error bars) for diversity parameters calculated on the microscopic count data. Plots were produced depending on whether the interaction between week and time was significant, or whether only the main effects of temperature and/or week were significant. Plots here demonstrate significant interactions between temperature and week for the three diversity parameters (Richness, Shannon Index, and Evenness).

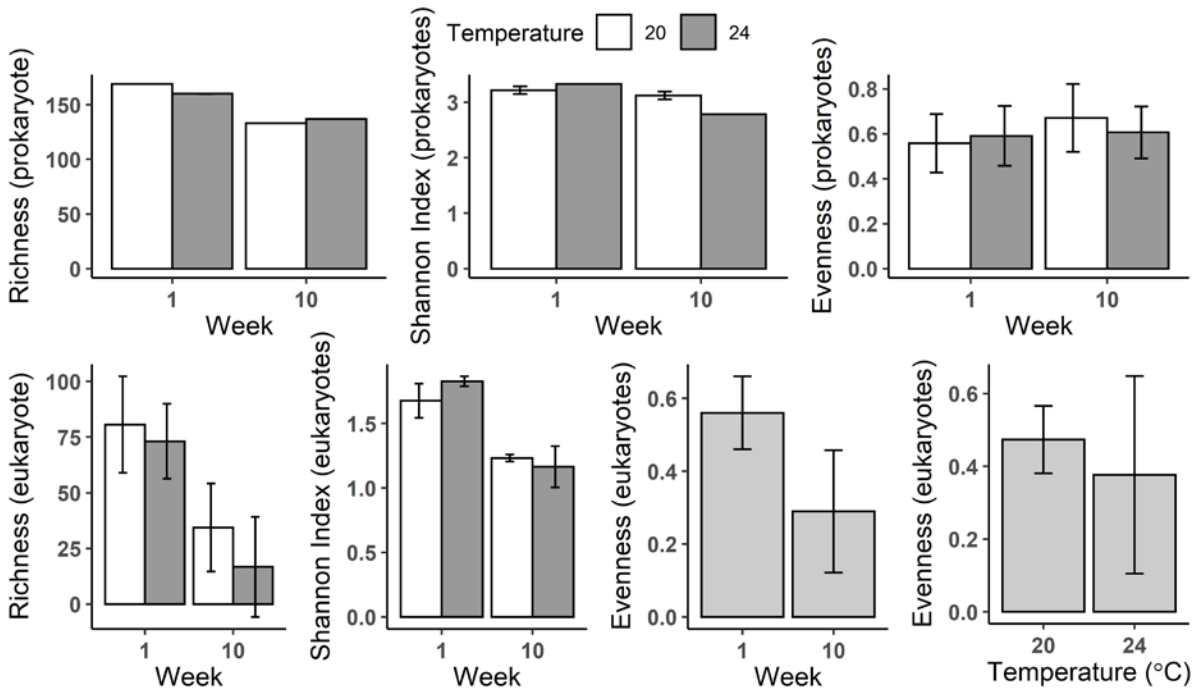

Figure 4.5: Model means with lower and upper confidence levels (error bars) for diversity parameters calculated on the prokaryotic and eukaryotic sequenced data. Plots were produced depending on whether the interaction between week and time was significant, or whether only the main effects of temperature and/or week were significant. Plots here demonstrate significant interactions between temperature and week for all diversity parameters calculated on the prokaryotic data (top row), and significant interactions between temperature and week for Richness and Shannon Index calculated on the eukaryotic data (bottom row).

For the eukaryotic data, ANOSIMs on the taxonomic levels revealed no significant dissimilarities between or within the temperature and week groups (data not shown). For the prokaryotic data, ANOSIMs revealed significant dissimilarities within the data, at each taxonomic level (Fig. 4.6). Pairwise PERMANOVAs revealed that whilst temperature and the interaction between temperature were not driving the significant dissimilarities, there were significant differences between weeks 1 and 10 across all taxonomic levels ($p = 0.001$). SIMPERs illustrated the contributions made by each component of the community to the dissimilarity, with significance levels illustrating the probability of similar proportions of the component being identified in a random sample from each group. Two of the top five contributing species were cyanobacteria; *Dolichospermum flos-aquae* ($p = 0.001$), and

Synecoccus (p = 0.362), with significantly more *Dolichospermum* being found at week 1 compared to week 10 (Supp. Tab. 4.1).

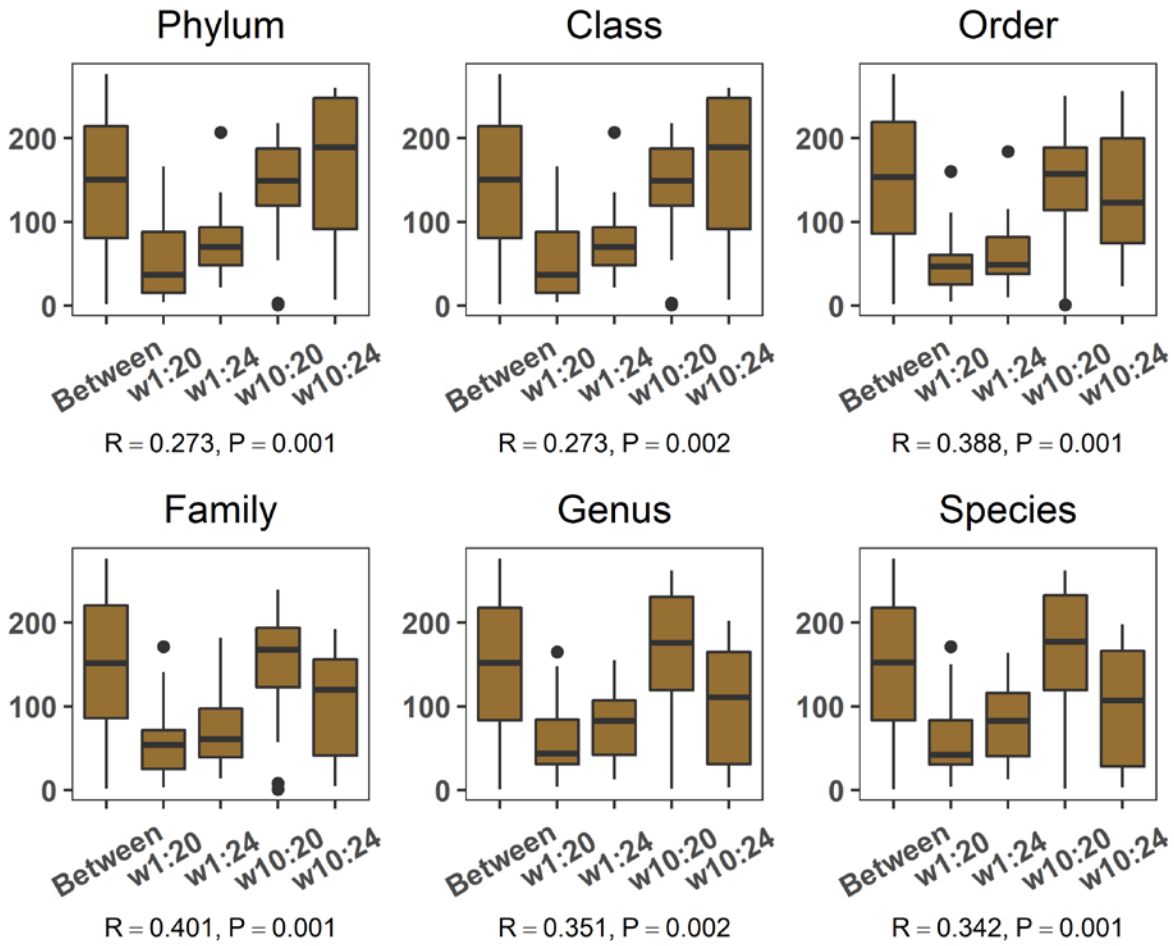

Figure 4.6: Visualisation of ANOSIMs run on the prokaryotic data, at each taxonomic level, with p-values displayed.

4.5 Discussion

Despite warming being a key element of our experimental design, few of our models found temperature to be a significant, with time being the driver of most the shifts we observed. Cyanobacterial dominance, and the spread of *R. raciborskii* has been historically associated with warming temperatures (Chapter 2) and warming has demonstrated clear system shifts in experimental freshwater plankton systems (Rasconi et al. 2017, Urrutia-Cordero et al. 2017, Hao et al. 2018), however it must be acknowledged here that the duration of our experiment was relatively short compared to other contemporary examples (Rasconi et al. 2015). This limitation notwithstanding, there were some significant associations with temperature,

namely a recovering cyanobacterial population in the warmer buckets against the overall cyanobacterial declines through time, declining diatom populations in the warmer buckets against a rising population in the cooler buckets, and less cryptophytes at the warmer temperatures (Fig. 4.2). The recovery of cyanobacteria in the warmer buckets may suggest either promotion of cyanobacterial growth, suppression of other organisms responsible for limiting cyanobacterial growth, or a combination of both. There is evidence that the growth of both *Raphidiopsis* and *Dolichospermum* can have negative effects on the abundance of Cladoceran zooplankton (Soares et al. 2009, Hochmuth JD and De Schamphelaere 2014). Of the zooplankton individuals counted here, a significant component was the Cladocerans *Daphnia* and *Bosmina* (data not shown) and these were found to be decreasing through time (Fig. 4.2) and increasing at warmer temperatures (Fig. 4.3). This reduction in time would theoretically lead to a reduction in grazing pressure, and potential for further development of cyanobacterial populations. The declines in the Ostracods under warmer conditions are particularly significant as Ostracods are recognised for being able to survive and reproduce in poor quality systems rich in detritus and cyanobacteria, such as shallow urban ponds with low flushing rates, of which Duck Pond is an example (Fernandez et al. 2016). Furthermore, it appears that declines in zooplankton were centred around the two blooms during the experimental period, in weeks 2 and 5 (Fig. 4.2); although neither of the genera were directly correlated with zooplankton abundances, both varieties are known to be toxic, and the temporal lag between cyanobacterial abundance and toxin release and persistence in water bodies is still poorly understood (Dong et al. 2016). The loss of diatom abundance under the warmer conditions can be expected; these populations were established during the three-month acclimation period before the treatments were implemented, and diatoms are sensitive to shifts in temperature (Reynolds et al. 2002). Evidence on the drivers of Cryptophytes in freshwater systems is scant, however the literature suggests that some cyanobacterial toxins may have a negligible effect on their survival (B-Beres et al. 2012). Furthermore, they are cited as a preferred food source for zooplankton grazers (Stutzman 2006). Thus, the higher proportions of Cladocerans at warmer temperatures (Fig. 4.3) may be linked with the lower proportions of cryptophytes at warmer temperatures (Fig. 4.2), however our modelling results do not indicate this, with no significant relationship detected between Cladocerans and Cryptohytes, and a significant but negative relationship detected between Ostracods and Cryptophytes. Food web dynamics could explain this observation. Recent research has highlighted Ostracod pellets as a rich food source that can sustain populations of filter feeder zooplankton (Fernandez and Alcocer 2018); high Ostracod populations in the early phases of

the experiment could have been responsible for facilitating healthy populations of filter feeders, which in turn pressured the Cryptophyte populations. However, declines in Ostracods over time could have contributed to the declines in zooplankton over time (Fig. 4.2), and thus fed into the increasing populations of cryptophytes through time (Fig. 4.2).

The bloom dynamics exhibited by the laboratory ponds reinforce an emerging trend of an inverse relationship between *Raphidiopsis* and *Dolichospermum*, which has also been found in the literature (Everson et al. 2011, Fig. 4.3, Chapter 5). Why would one species precede the other? Physiologically, the two genera are similar; they are both filamentous, toxin producing, nitrogen fixing, freshwater cyanobacteria. Their geographical distribution, however, is different; whereas *Dolichospermum* tends to proliferate in temperate, nutrient rich environments, *Raphidiopsis* is most commonly reported in tropical, nutrient poor environments. This discrepancy may represent a difference in the N-fixing capabilities of the genera. A potentially crucial difference between the two is that *Dolichospermum* is unrestricted in the number of heterocysts it can generate, whereas *Raphidiopsis* is restricted to a maximum of two per filament (Plominsky et al. 2013). Whilst *Raphidiopsis* is capable of sustained growth in N-free medium (Chapter 3), *Dolichospermum* is not due to overexpression of superoxide dismutase production under nitrogen starvation which can lead to oxidative stress (Raghavan et al. 2011). Thus, in our laboratory ponds, the most likely scenario to explain the switch in dominance from *Dolichospermum* to *Raphidiopsis* is nutrient starvation, although we have no nutrient data to confirm or deny this. Perhaps, post a bloom of *Dolichospermum*, nutrient starvation of the water body provides suitable conditions for *Raphidiopsis* to develop relatively free of competition. Interestingly, studies have shown *Dolichospermum* to develop the largest numbers of akinetes under heavily stratified conditions with little to no flow, which is a response in N-fixing cyanobacteria to compensate for little available N (Everson et al. 2011). However, the collapse of the *Dolichospermum* populations suggests that nitrogen levels remained at a level that was too low for continual growth. Based on the above reasoning, together with the declining zooplankton, the profile of our laboratory ponds seems to fit a stressed system with low flushing levels. Contrary to this conclusion, field studies have illustrated that bloom collapse or shift in bloom composition is often attributable to biological factors that are difficult to isolate and identify, such as viral lysis or bacterial predation (Martin et al. 2019). Furthermore, *D. circinalis* is most often associated with conditions involving sudden nutrient pulses that cause a switch from oligotrophic to mesotrophic or eutrophic conditions (Callieri et al. 2014, Wood et al. 2017).

Recent research has suggested that cyanobacterial species form niche 'clusters' that are suited to occupy certain conditions; for example *Chryopsporum* and *Dolichospermum* have been identified as a dominant cluster, with opportunistic clusters emerging in response to increases in temperature (Bertos-Fortis et al. 2016), or the decline of the original bloom (Bowling et al. 2013a). Interestingly, the dominance of *R. raciborskii* can be subverted under the right conditions; the invasive dinoflagellate *Ceratium* sp. has replaced *R. raciborskii* as the dominant variety of phytoplankton due to intermittent mixing periods in a South American reservoir, which significantly increase nitrogen availabilities (Crossetti et al. 2019). Thus, although environmental changes towards stratified low-flow conditions may promote the dominance of *R. raciborskii*¸ alternative change regimes in other regions may promote a switch in dominance away from the invader.

Cylindrospermopsin production in *R. raciborskii* has been strongly correlated with high conductivity levels, ~ 450 – 500 µS cm^{-1} (Everson et al. 2011). It could be that the declines in Cladocerans, Ostracods (Fig. 4.3), and cryptophytes (Fig 4.2) could be a function of heightened toxin production by the *R. raciborskii* bloom. Toxin production in *R. raciborskii* has also been linked to stress by lack of N-availability (Yang et al. 2018), and reached a peak in optima from 18 – 24 °C (Chapter 3); based on this we may conclude that toxin production by *R. raciborskii* was likely higher in the warmer buckets, leading to elevated levels of stress on other varieties of plankton, and perhaps contributing to exacerbated declines in diversity (Fig. 4.4). However, the drivers of toxin production remain unclear in the literature, with several studies reporting constitutive cylindrospermopsin synthesis that is not influenced by environmental conditions (Pierangelini et al. 2015, Willis et al. 2015), thus more data is needed to reach a definitive conclusion on the environmental influence on toxin concentrations. Previous studies have also found that *R. raciborskii* produces high numbers of cells under high pH levels, typically around 8 or higher (McGregor and Fabbro 2000), whereas the systems in this study showed a pH of around 6.5 – 7; this may have played a factor in the disappearance of *R. raciborskii* from the systems. In order to more closely examine the bounds of the relationship between *D. circinalis* and *R. raciborskii*, a future avenue of research could focus on monitoring their relative population fluctuations under more controlled conditions, such as within chemostat vessels (Liu et al. 2016). This approach would allow either the maintenance of a constant pH, or incremental adjustment of pH, to determine what environmental conditions might instigate a switch from one species to the other.

The most significant effect of temperature was observed within the biodiversity indices, with temperature seeming to exacerbate overall losses in diversity through time under the warming treatment. Our results demonstrate loss of richness in the heated buckets at the count level, more consistent losses in Shannon's Index at the count level, and larger declines in Shannon's Index and evenness within the prokaryotic and eukaryotic data at warmer temperatures (Fig. 4.4). Declines in phytoplankton richness and diversity in freshwater systems due to increases in temperature have been observed in other studies, together with an observed dominance in small phytoplankton varieties (Rasconi et al. 2015). Bloom activity has also been linked to losses in both phytoplankton and zooplankton diversity, and further to this, losses of phytoplankton diversity have been linked to losses in zooplankton diversity, due to a reduction in niche availability (Bockwoldt et al. 2017). If this theory is upheld, then the blooming activity observed in our results could be linked to both the declines in chlorophytes and zooplankton (Fig. 4.2). The decreasing species richness and Shannon's Index within the count data was supported by similar declines in the diversity of unique hits within the sequenced prokaryote data, suggesting that the community effects of blooming behaviour extend beyond phytoplankton assemblages and can be applied to heterotrophic bacteria. Indeed, recent research has illustrated a link between different varieties of blooming cyanobacteria and heterotrophic bacteria (Imen et al. 2015). Changes in the composition of phytoplankton communities can have important bottom-up effects on food webs, as they represent a significant component of primary production in freshwater systems, and directly influence the quality and diversity of food resources for higher trophic levels (Yvon-Durocher et al. 2011).

Our bacterial community mostly resembles that reported as 'typical' of freshwater systems, with contributions from the Actinobacteria, Cytophaga and Flaviobacteria within the Bacteroidetes, Verrucomicrobia, Cyanobacteria, and alpha-, beta-, and gamma-Proteobacteria (Zwart et al. 2002). The Actinobacteria phylum are recognized as being the most overall abundant bacterial group in freshwater habitats (Lemke et al. 2009) and have been reported to usually make up 20 – 60% of sequences in freshwater lakes (Humbert et al. 2009). Here, although substantial in our laboratory ponds, they contributed only ~ 10% of sequences; the contributions from the Proteobacteria were much larger, with the phyla making up ~ 23% of total hits (Supp. Tab. 4.1). Alongside these two were contributions from the Verrumicrobia, Bacteroidetes, and Cyanobacteria. The dominance of the Proteobacteria was also found by Imen et al. (2015), who examined bacterial communities in a lake suffering *Dolishospermum*

and *Microcystis* blooms, and additionally reported the unusual result of a relatively small contribution of the Actinobacteria to the community; our study further supports the emerging inverse correlation between the Actinobacteria phylum and cyanobacterial proliferation (Parvenn et al. 2013, Ghai et al. 2014). Recently, Actinobacteria have been associated with the degradation of ligneous organic detritus, a process responsible for the recycling of dead plant matter into soil constituents (Ghai et al. 2014). As such, their ecological value may be crucial, and their suppression disruptive on a fundamental level. Given that the incidence of harmful freshwater blooms is increasing worldwide (Chapter 1), this association may be an additional facet of ecological decline associated with excessive cyanobacterial growth. Woodhouse et al. (2016) found similar cyanobacterial dynamics in sampling undertaken in Kensington Pond, a neighbouring system to Duck Pond, in Centennial Park, however dominance was heavily in favour of *Microcystis*, with a secondary population of *Dolichospermum* present, and strong fractions of Actinobacteria, Alphaproteobacteria, and Betaproteobacteria, compared to Gammaproteobacteria and Bacteroidetes. Interestingly, no hits were reported for Verrucomicrobia, which distinguishes it from our study (Woodhouse et al. 2016). The contributions of the Verrucomicrobia, at ~ 11%, is higher than has been reported in some studies; ~ 4% in a mesotrophic lake (Parveen et al. 2013), however is in alignment with others; ~ 10% in the Murray-Darling Basin, Australia, a large agricultural catchment currently experiencing loss of flow and elevated nutrient loads, and consequent cyanobacterial blooms (Bowling et al. 2018). Information on the ecological role of Verrucomicrobia is scant, however interestingly, higher than usual proportions of the phylum has been associated with blooms of *R. raciborskii* (Bagatini et al. 2014). As such, its relatively large contribution may have been a function of the *R. raciborskii* bloom observed midway through the experimental period. The lack of significance of dissimilarity between the Eukaryotic fractions here is interesting; although we have noted several differences in Eukaryotic organisms within the count data, non-significance of our sequences across treatments means the contribution of these classifications to the overall community is negligible. It is worth bearing in mind however that our DNA sequences only capture the dynamics at the beginning and end of the experiment, so it is perhaps feasible that differences we have noted originated from the bulk of the weeks that were not sequenced. Furthermore, although our community data is enlightening, it has a limitation in that, in the case of bacteria, we are unable to determine what fraction of a certain phyla were metabolically active at the time of sampling.

Several limitations exist within the present study. The first is that no nutrient or toxin dynamics were captured using our methodology; thus we cannot connect our community data with relatable conditions from other systems, classify the buckets as either oligotrophic, mesotrophic, or eutrophic, or relate our results to nutrient use strategies across different varieties of phytoplankton. Secondly, the open nature of our systems could theoretically lead to contamination by bacteria present in the laboratory environment, which could potentially distort the applicability of our results to freshwater systems. Despite this, it is worth noting that Duck pond is a closed system with no river or stream feeding it; as such 'laboratory ponds' are an appropriate physical replication of the system. A final limitation is that use of the YSI EXO2 probe introduces several accuracy and interference issues reported in the literature. Significant variation between fluorometric phycoyanin and calculated cyanobacterial biovolume across different sites has been reported, with the variation larger as lower cyanobacterial biovolumes were reported (Bowling et al. 2013b). Furthermore, the presence of picoplanktonic cyanobacteria has been flagged as a potential factor that reduces the accuracy of phycocyanin readings (Bowling et al. 2016), and the presence of such cyanobacterial varieties as *Synechococcus* in our systems could have led to such errors in accuracy being present in our measurements. Finally, the use of chlorophyll-a has been shown to be a poor estimate of cyanobacterial biovolume, especially where multiple varieties of cyanobacteria are present (Bowling et al. 2016). Despite this, chlorophyll-a and phycocyanin measurements were not used extensively in the present study and thus these errors do not detract significantly from our main findings.

Given the right conditions, how could the increasing activity of *R. raciborskii* in our waterways affect our aquatic ecosystems? Because zooplankton tend to avoid consuming *R. raciborskii* where possible, the presence of *R. racoborskii* indirectly places an elevated amount of pressure on other species cohabiting in the same region, due to preferential grazing from zooplankton (Hong et al. 2015). Although the current study has shown some effects of elevated temperature and the presence of blooming cyanobacteria on freshwater phytoplankton, zooplankton, and bacterial communities, distinguishing between the effects of temperature and blooming, given our experimental structure, is not possible. It is well established that temperature fluctuations facilitate the range expansion of invasive species, not only by promoting competitive species that may flourish under warmer temperatures, but by reducing the likelihood that native species will successfully become established (Rahel and Olden 2008, Chapter 2). A natural conclusion to draw is that the more the growth of an

invasive species is promoted, the more their effects on ecosystem functioning will be exacerbated. This is certainly the case with toxin producing phytoplankton; although evidence may suggest that toxin production does not necessarily increase under warmer temperatures (Chapter 3), the dramatic increases in biomass brought on by warmer temperatures may compensate for this.

4.6 Conclusion

Our hypothesis that warming would influence the composition of our freshwater laboratory ponds was confirmed by the declines in zooplankton and crustacean populations, and diversity indices, over time and at warmer temperatures. The bloom patterns exhibited an inverse relationship between *Raphidiopsis* and *Dolichospermum*, the reasons for which are not clear. These communities are also potentially reflective of freshwater systems globally due to Actinobacteria fractions lower than are typically reported, and an emerging link between cyanobacterial blooms and declines in Actinobacteria. The presence of invaders in aquatic systems, coupled with dramatic environmental change, will potentially cause significant changes to the plankton and bacterial community structures of Australian freshwater systems. *R. raciborskii* exhibits a flexible physiology and a trait-set suitable to take advantage of the high temperatures, erratic flow events, and severe stratification regimes of Australia's lakes and water reservoirs. Our data has revealed complex relationships between blooming cyanobacteria, other plankton, and diversity indices, that warrant further research, so that we may better understand and manage our freshwater systems.

5. How do drivers of abundance in *Raphidiopsis raciborskii* differ in tropical and temperate regions of Australia?

5.1 Abstract

Studying the distribution of invasive species can reveal much information about what ecological niche these species are utilising to facilitate their migration. Large monitoring data sets that span over many years are very useful for exploring long-term trends in system properties, however such data is difficult to pair with species populations. An example of this pairing is aquatic monitoring data taken from freshwater catchments, as this data typically includes counts of phytoplankton. In recent years, the harmful cyanobacteria *Raphidiopsis raciborskii* has drawn much attention and research due to its bloom formation, toxin production, and invasive behaviour, however because of the latter two factors, it has been counted in many monitoring datasets for ~ 20 years. The sudden emergence of the species as a successful invader provides the opportunity to review such data in order to elucidate factors of the mechanism behind its spread. In this study, we take historical water quality and phytoplankton count data from the Australian states of Queensland and South Australia, which represent two contrasting climates and hydrological regimes. We collate this monitoring data into two datasets that span 14 and 17 years respectively. We construct generalised additive mixed models for the abundance of *R. raciborskii* in each state, and critically examine the variables involved. Our findings demonstrate significant differences between the climatic and cyanobacterial dynamics of the two states, with significantly warmer temperatures in Queensland playing a key role in promoting cyanobacterial growth. Dominant seasons for cyanobacterial activity also differed between the states, with Summer providing being most closely linked to cyanobacteria in Queensland, and Spring being most closely linked to cyanobacteria in South Australia. We also find a significant inverse relationship between *Raphidiopsis* and *Dolichospermum*, as has been reported elsewhere in the literature. Finally, negative relationships between *Raphidiopsis* and physiochemical parameters including phosphorus and turbidity reinforce the ecological potential of the species under stratified, nutrient poor conditions, whilst slightly declining densities at temperatures typically reported as optimal for the species represent an unusual finding. The present study illustrates the value in historic monitoring data, and its use as a tool to link findings obtained in contemporary field and laboratory studies.

5.2 Introduction

Algal blooms are an internationally recognised threat to water quality, with severe blooms being documented across regions of Europe, Asia, America and Africa (Davis and Koop 2006; Gobler et al. 2012; Wu and Xu 2011). Agricultural and urban run-off (Carey et al. 2012) and climate change (Salmaso et al. 2018) create ideal conditions for the growth and proliferation of blue-green algae (BGA). Proliferation of BGA in water systems can lead to blooms, which take the form of physical mats or scunges that rest on the surface of water bodies. Made up of large concentrations of algal cells, blooms can cause reductions in water quality, poisoning and death of humans and livestock who ingest contaminated water, and loss of biodiversity (Hadas et al. 2015). Follow-on effects from these include social and economic consequences. A reduction in available water for use in society, the deficit from loss of productivity within agricultural and industrial sectors, and maintenance costs associated with the clogging of pipes and drains all indicate a need for effective algal management strategies (Hudnell et al. 2008). Historically, Australia has experienced many severe algal bloom events, due to its colonisation history and erratic hydrological regimes (Chapter 1). Clearly, research is needed in this field for the benefit of the Australian water supply.

Australia is hydrologically dynamic, with different regions displaying different water quality patterns, rainfall regimes, and geological structures (Chapter 1). For example, Queensland is a tropical region prone to high temperatures, with increases in daily minimum temperatures evident over the past 80 years (Smith et al. 2015). Rainfall is strongly seasonal; precipitation clears 2000 mm $year^{-1}$ in the North, to ~ 1000 – 1500 mm $year^{-1}$ in the South, however the state is expansive, and the vast swathes of Western landmass are semi-arid. Catchment flow in the Western regions of the state is ephemeral, and catchments are weathered, contributing to severe stratification regimes (McNeil et al., 2008). Rainfall mostly occurs as rare isolated intense storm events, and tropical cyclones are semi-regular. Furthermore, pan evaporation exceeds precipitation in most areas (Gentilli 1971). Catchment land areas are used for various agricultural purposes, including rangeland beef grazing, sugarcane cultivation, horticulture, grain and cotton, and native forests (Brodie et al. 2011). Suspended sediments and nutrient loads being delivered to southern catchments in lower Queensland and New South Wales have increased as a result of erratic and heavy rainfall events, high pan evaporation, extensive agricultural land-use, soil erosion brought on by beef grazing, and land-clearing. Furthermore, the use of synthetic fertilisers has increase nutrient loading in waterways and

the inorganic component are bioavailable to phytoplankton (Brodie and Mitchell 2005). Consequently, these tropical and sub-tropical systems experience longer growing seasons and more prevalent thermal stratification, with more efficient nutrient recycling, and heavily weathered soils have led to systems being regularly P-limited (Townsend 2000). In contrast, the South Australian region has a cooler, more temperate climate and the state is the driest state in Australia based on rainfall. Therefore, water security has become a primary concern post the Millenium drought of 1997 – 2011 (Cox et al. 2016). The state relies primarily on the Murray-Darling for its freshwater, with up to 90% of freshwater being sourced from the river in times of drought. The projected decreases in rainfall and increases in temperature that apply to both Queensland and South Australia will have follow-on effects on the chemistry of freshwater systems. These include depleted dissolved oxygen, lowered pH, elevated salinity levels, and potentially larger loads of nutrients due to reduced intermittent rainfall and more extreme flooding events (Hobday and Lough 2011). Furthermore, due to the heavy irrigation demands already placed on the river and surrounding wetlands for irrigation purposes, and the predicted recurrence of drought periods, the Murray-Darling and associated reservoirs in South Australia could return to extreme low-flow states. This may promote shifting assemblages of cyanobacteria (Shand et al. 2017).

R. raciborskii is a toxin producing cyanobacterium that is purported to be invasive, and that in Australia has a history of forming extensive blooms throughout the tropical reservoirs and weirs of Queensland, and to a less extent through the Northern areas of New South Wales. Its range expansion success is often linked to its preference for currently observed warming and associated intensification of stratification, its high affinity for inorganic phosphates and diazotrophic nature, and in a more ambiguous sense its toxin production (Chapter 3). It has recently been reported as expanding its range in North and South America, Europe, Asia and South-East Asia, and New Zealand (Sinha et al. 2012). Due to its extensive activity in Queensland, and the serious water security follow-on effects of its proliferation, it is important to establish what conditions, environmental events, or management actions are most relevant to its success, from a risk management and ecological perspective for Australian water systems. In the present study, we compare two large data sets comprising physiochemical variables and cyanobacterial cell counts within water systems in Queensland and South Australia, Australia. We summarise the differences between the two states and employ a matrix reconstruction algorithm to facilitate the presentation of principal component analyses to examine variability in the data. We then model *Raphidiopsis* against

multiple environmental variables, and compare how the abundance of the genus can be explained within the two states.

5.3 Method

5.3.1 Data acquisition

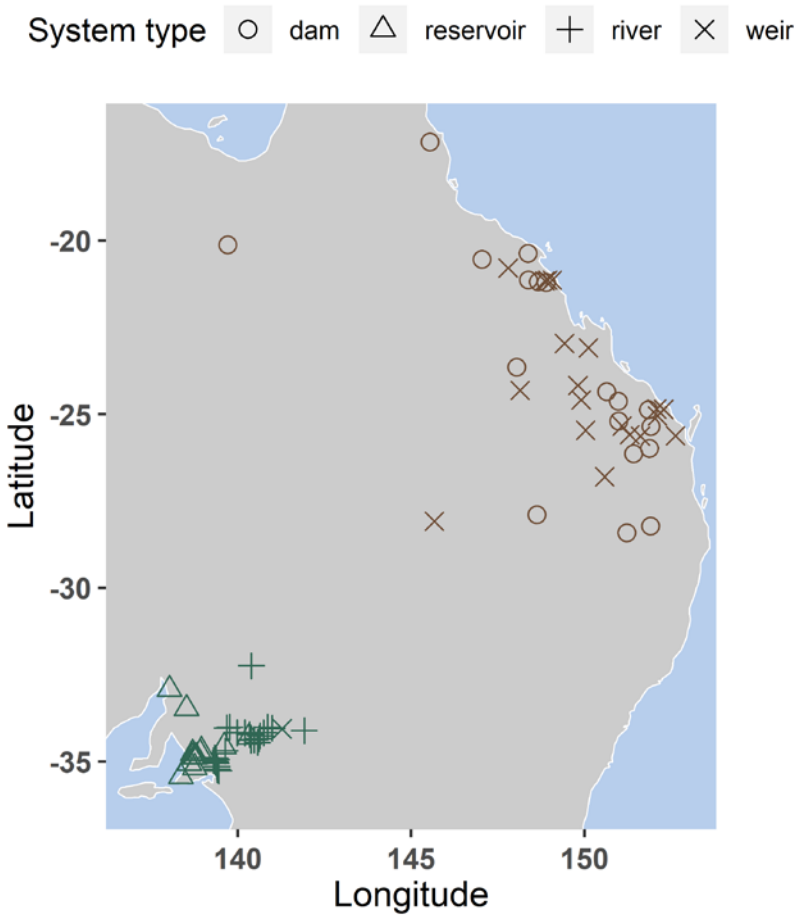

Figure 5.1: Map of East Australia showing the locations of the sampling sites for Queensland (brown) and South Australia (green). Sampling sites have been divided into type of site.

Data was requested from the Department for Environment and Water, South Australia, and the Queensland department of Environment, Land, and Water. The South Australian government responded with a dataset spanning from 1997 – 2016, and contained a very large number of variables, including inorganic nutrients, genus-level phytoplankton counts

including chlorophytes, cyanobacteria, diatoms, and dinoflagellates, pesticide and herbicide concentrations, heavy metals, and other parameters. Due to a shift in monitoring practice, genus-level phytoplankton counts changed to total cyanobacterial counts in 2015, and so the data set was disregarded beyond this point. The monitoring sites were predominantly sampling sites on the Murray-Darling river, however several reservoirs in the South and North-West of the state were included (Fig. 5.1). The Queensland government responded by requesting that we contact SunWater, a public/private company that manages much of Queenslands water supply. A data set spanning 15 years was subsequently purchased and released to us, containing counts for four species of common bloom-forming cyanobacteria, including *Anabaena* (*Dolichospermum*) *circinalis*, *Microcystis aeruginosa*, *Chryosporum ovalisporum*, and *Raphidiopsis raciborskii*. Physiochemical variables were less comprehensive than the South Australian data set, comprising total N and P, conductivity, turbidity, dissolved oxygen, and pH. For the sake of an effective comparison, only these physiochemical variables were examined for the South Australia data set, despite the availability of others. For both data sets, the latitude and longitude of each monitoring site was found using Google Earth and logged in the data sets to be used as predictor variables.

5.3.2 Data organisation and analysis

Data was originally organised in bulk by site for Queensland, and by year for South Australia, with variables listed under one column and organised by unit of measurement. Data were reorganised using R software (https://www.r-project.org/) into separate files for each site, and parameters were listed under separate columns. Data were then bound to a master date column using the aggregate function in combination with the “Zoo” package. The resulting data files were organised as a daily time series, with single day increments, resulting in very ‘gappy’ data sets, that would make analysis difficult. To overcome this, all data was averaged by month. The resulting data sets were then stacked for each state, resulting in two distinct data sets for South Australia, and Queensland. Physiochemical parameters were compared between the states through calculation of summary statistics including number of data points, mean, median, standard deviation, skewness, and the coefficient of variation.

As a precursor to analysis, we decided to run Principal Component Analyses (PCAs) on each data set. This approach was met with the obstacle of the aforementioned ‘gappy’ data. To solve this, a DINEOF matrix reconstruction algorithm was applied to the data, following the methods outlined by Beckers and Rixen (2003), through use of the function provided in the

"sinkr" package. Small randomly selected portions of data (n = 110 and n = 128 for Queensland and South Australia, respectively) were removed from the original data sets prior to reconstruction, and then compared with the reconstructed values to assess the effectiveness of the reconstruction. PCA was then undertaken to assess correlation patterns and variance between all variables.

The non-linear relationships between the abundance of *R. raciborskii* and environmental parameters were assessed using generalised additive models (GAMs), which were run on the original data without DINEOF application, using the MGCV package (Wood 2017). Separate models were run for the Queensland and South Australian data sets, with variables including temperature, total nitrogen, total phosphorus, turbidity, pH, dissolved oxygen, conductivity, and the abundance of the other cyanobacteria *Dolichospermum*, *Microcystis*, and *Chryosporum*. All variables were modelled as smoothed terms, using thin plate regression splines, apart from month, which was smoothed as a cyclic cubic regression spline, and constrained in the formation of knots (k = 3). Briefly, the use of thin plate regression splines is advantageous as they are low rank, meaning they have fewer coefficients than there is data to smooth, and because they may be applied in a way that allows terms to be shrunk to zero. In this sense, the model may be run and terms automatically selected based on those which are not penalised to zero (Wood 2017). Finally, the month term in the model was smoothed using a cyclic cubic regression spline, applied to allow the ends of the term to match, therefore replicating the cyclic nature of a calendar year (Wood 2018). Data were analysed using R software (https://www.r-project.org/) including the packages "psych" for calculation of summary statistics, "ggplot" and "ggbiplot" for visualisation.

5.4 Results

There were several marked differences in the physiochemical and environmental profiles of the two data sets (Tab. 5.1 and Tab. 5.3). Queensland sites were consistently warmer than South Australian sites, and exhibited greater seasonal variability, with the greatest difference being in the cooler seasons in South Australia, which dropped compared to the cooler seasons in Queensland. Total nitrogen and total phosphorus, which included inorganic and organic forms, was greater in South Australian systems than in Queensland systems, with means of 0.5 and 0.94 mg l^{-1} respectively. For South Australia, these nutrients tended to peak in the Summer, although nitrogen in South Australia also remained high throughout Spring and

Autumn. Conductivity remained constant throughout the year for South Australia, however varied for Queensland, with reductions through Winter with a mean of 223.6, and increases through Spring with a mean of 389 µS cm^{-1}. Dissolved oxygen peaked through Winter in South Australia with a mean of 10.1 mg l^{-1} and lowered through the Summer with a mean of 7.65 mg l^{-1}. A similar trend was observed for Queensland, which exhibited generally lower dissolved oxygen levels than South Australia. Waters were more turbid through Summer and Autumn for both states, however turbidity was consistently higher in South Australia than in Queensland. Finally, on average waters had similar pH across both regions (Tab. 5.1, Tab. 5.2).

Cyanobacterial counts between the states exhibited several differences (Tab. 5.2 and 5.4). In Queensland, the dominant genus was consistently *Raphidiopsis*, with means at least double those of the other genera. Standard deviations for *Raphidiopsis* were very large, and together with skewness suggested a heavily positively skewed distribution. *Raphidiopsis* counts also exhibited variability between seasons, with the highest counts observed in Summer, and decreases taking place in Spring and Autumn, with lower numbers observed in Winter. *Microcystis* and *Dolichospermum* showed more consistency in their abundances, having comparable counts in Spring, Summer, and Autumn, with decreases then observed in Winter. *Microcystis*, unlike the other genera, had the highest mean counts in Winter, however the standard deviation here was high, suggesting counts populations in Winter were more erratic. Counts of *Chryosporum* were relatively low, and not comparable with the other genera.

Cyanobacterial counts in South Australia were considerably lower on average than in Queensland (Tab. 5.2, 5.4). The dominant genera were *Microcystis* and *Chryosporum*, with *Dolichospermum* exhibiting middling abundances, and *Raphidiopsis* counts being low. The highest counts for both *Microcystis* and *Chryosporum* were in Spring, with *Microcystis* maintaining relatively high abundances through Sumer and Autumn. *Dolichospermum* achieved moderate concentrations in Spring, and *Raphidiopsis* displayed moderate concentrations in the Summer.

Table 5.1: Summary statistics computed for physiochemical parameters for the Queensland data set. Parameters include temperature (temp), dissolved oxygen (DO), conductivity (cond), total nitrogen (total N), total phosphorus (total P), turbidity (turb), and pH. Statistics reported are number of data points (n), mean ($\bar{\mu}$), median ($\hat{\mu}$), standard deviation (sd), skewness (skew), and the coefficient of variation.

Parameter	Spring						Summer						Autumn						Winter					
	n	$\bar{\mu}$	$\hat{\mu}$	sd	skew	CV	n	$\bar{\mu}$	$\hat{\mu}$	sd	skew	CV	n	$\bar{\mu}$	$\hat{\mu}$	sd	skew	CV	n	$\bar{\mu}$	$\hat{\mu}$	sd	skew	CV
Temp	335	22.62	22.5	3.2	0.05	0.14	414	25.27	25.62	3.01	-0.91	0.12	291	22.6	23	2.77	-0.27	0.12	94	18.1	18.6	2.65	-0.59	0.15
DO	307	7	7.15	2.63	0.44	0.38	379	5.99	6.27	2.59	0.03	0.43	261	6.25	6.47	2.57	-0.11	0.41	84	7.32	7.4	2.35	-0.34	0.32
Cond	335	389	290	349	2.24	0.90	418	372.3	282.8	325	2.32	0.87	294	315.9	267.7	248	1.97	0.79	94	223.6	196.8	165.8	1.15	0.74
Total N	351	0.55	0.5	0.3	0.97	0.55	470	0.62	0.57	0.39	0.82	0.63	327	0.62	0.59	0.4	0.71	0.65	98	0.66	0.5	0.41	1.03	0.62
Total P	345	0.05	0.04	0.05	4.9	1.00	434	0.07	0.05	0.05	1.96	0.71	309	0.06	0.05	0.06	3.37	1.00	97	0.04	0.03	0.02	1.5	0.50
Turb	327	13.5	5.2	28.6	5.83	2.12	402	28.1	7.7	70	5.13	2.49	284	22.2	8.14	50	4.83	2.25	88	17	6.15	32.8	4.7	1.93
pH	335	7.84	7.78	0.58	0.86	0.07	414	7.71	7.7	0.61	0.3	0.08	291	7.67	7.6	0.66	0.86	0.09	94	7.83	7.74	0.75	1.71	0.10

Table 5.2: Summary statistics computed for cyanobacterial counts for the Queensland data set. Parameters include temperature (temp), dissolved oxygen (DO), conductivity (cond), total nitrogen (total N), total phosphorus (total P), turbidity (turb), and pH. Statistics reported are number of data points (n), mean ($\bar{\mu}$), median ($\hat{\mu}$), standard deviation (sd), skewness (skew), and the coefficient of variation.

Genus	Spring						Summer						Autumn						Winter					
	n	$\bar{\mu}$	$\hat{\mu}$	sd	skew	CV	n	$\bar{\mu}$	$\hat{\mu}$	sd	skew	CV	n	$\bar{\mu}$	$\hat{\mu}$	sd	skew	CV	n	$\bar{\mu}$	$\hat{\mu}$	sd	skew	CV
Dol.	645	7886	0	35255	8.1	4.47	820	7711	0	42558	8.3	5.52	613	7316	0	31164.5	5.7	4.26	331	2814	0	12344	8.38	4.39
Cyn.	687	29830	2800	72160	4.38	2.42	874	47100	7400	109000	5.17	2.31	637	25311	3237.5	65950	5.09	2.61	364	11091	910	42337	7.08	3.82
Mic.	650	2024	0	10868	7.77	5.37	821	1127	0	8060.7	11.4	7.15	615	589.2	0	3219.5	11.6	5.46	339	2077	0	11063	8.06	5.33
Aph.	630	146.6	0	1035.8	9.78	7.07	810	224.4	0	1474.5	9.94	6.57	612	117	0	1414.4	21.2	12.09	330	11.3	0	138.9	15.8	12.29

Table 5.3: Summary statistics computed for physiochemical parameters for the South Australia data set. Parameters include temperature (temp), dissolved oxygen (DO), conductivity (cond), total nitrogen (total N), total phosphorus (total P), turbidity (turb), and pH. Statistics reported are number of data points (n), mean ($\bar{\mu}$), median ($\hat{\mu}$), standard deviation (sd), skewness (skew), and the coefficient of variation.

Parameter	Spring						Summer						Autumn						Winter					
	n	$\bar{\mu}$	$\hat{\mu}$	sd	skew	CV	n	$\bar{\mu}$	$\hat{\mu}$	sd	skew	CV	n	$\bar{\mu}$	$\hat{\mu}$	sd	skew	CV	n	$\bar{\mu}$	$\hat{\mu}$	sd	skew	CV
Temp	1057	17.4	17.1	3.67	1.45	0.21	1140	23.2	23.3	2.52	-0.69	0.11	1109	19.007	19	2.8583	0.089	0.15	1052	11.9	12	1.27	0.18	0.11
DO	453	9.12	9.13	1.58	-0.19	0.17	487	7.65	8.05	1.83	-0.74	0.24	466	8.4	8.62	1.61	-0.78	0.19	460	10.1	10.2	1.41	-0.58	0.14
Cond	837	468.3	513	198.7	-0.03	0.42	854	470	451	199.7	0.37	0.42	846	471.1	448.2	192	0.56	0.41	852	479.4	486.5	194	0.4	0.40
Total N	587	0.99	0.94	0.38	0.74	0.38	642	1.04	1.01	0.38	0.67	0.37	596	0.98	0.94	0.35	1.31	0.36	579	0.91	0.88	0.32	0.77	0.35
Total P	724	0.092	0.07	0.076	2.71	0.83	793	0.11	0.08	0.088	1.27	0.80	742	0.11	0.08	0.08	1.15	0.73	710	0.082	0.068	0.059	2.44	0.72
Turb	1062	38.4	28	42.4	2.36	1.10	1126	40.05	32.8	37	1.25	0.92	1078	40.5	31.4	38.5	1.77	0.95	1042	29.5	25	24	1.26	0.81
pH	514	7.69	7.68	0.29	0.29	0.04	556	7.73	7.7	0.48	-7.24	0.06	577	7.81	7.78	0.32	-0.1	0.04	546	7.8	7.8	0.24	0.057	0.03

Table 5.4: Summary statistics computed for cyanobacterial counts for the South Australia data set. Parameters include temperature (temp), dissolved oxygen (DO), conductivity (cond), total nitrogen (total N), total phosphorus (total P), turbidity (turb), and pH. Statistics reported are number of data points (n), mean ($\bar{\mu}$), median ($\hat{\mu}$), standard deviation (sd), skewness (skew), and the coefficient of variation.

Genus	Spring						Summer						Autumn						Winter					
	n	$\bar{\mu}$	$\hat{\mu}$	sd	skew	CV	n	$\bar{\mu}$	$\hat{\mu}$	sd	skew	CV	n	$\bar{\mu}$	$\hat{\mu}$	sd	skew	CV	n	$\bar{\mu}$	$\hat{\mu}$	sd	skew	CV
Dol.	699	675.7	50.8	10893	25.5	16.12	948	279.8	80.6	1172.6	12.2	4.19	817	186.7	58.3	638.4	11.6	3.42	651	123.2	55.8	315.6	11.2	2.56
Cyn.	5	65.2	31	78.5	0.71	1.20	159	600	51	2314.1	7.25	3.86	263	432.1	58	1132.4	4.92	2.62	78	63.2	41.5	63.9	2.6	1.01
Mic.	85	2229	94	17858	8.9	8.01	340	1041	142	4674.3	9.9	4.49	236	866.6	147	3481.1	7.2	4.02	99	234.9	116	420.9	4.3	1.79
Aph.	201	1594	44	18277	13.8	11.47	514	241.4	69.5	574.9	5	2.38	477	242.9	54.5	985.4	13.1	4.06	328	202.5	40	743.8	8.7	3.67

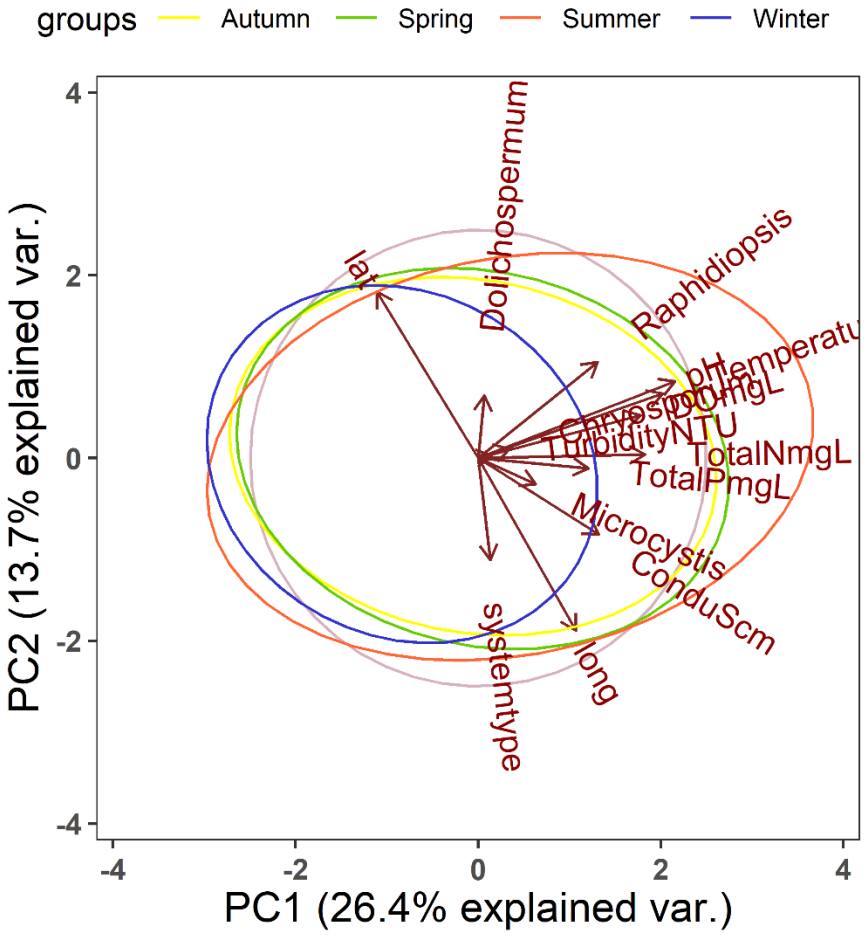

Figure 5.2: Biplot of a PCA run on the Queensland data set, with the first two components explaining a combined total of 40.1% of the variation within the data.

The first two principle components from the PCA run on the Queensland data set explain a total of 40.1% of the variability within the reconstructed data (Fig. 5.2). Here, temperature, pH, dissolved oxygen, and to a lesser extent, total nitrogen and phosphorus, conductivity, and turbidity, are all correlated with *Raphidiopsis*¸ and all reach a maximum through the summer, with a minimum through the winter. Both *Microcystis* and *Dolichospermum* are aligned away from the bulk of the data; there appears to be a slight correlation between *Dolichospermum* and turbidity. If the next two principle components are examined, they explain a combined 17.1 % of the variation within the data. Here, phosphorus is aligned with turbidity and to a lesser extent *Chryosporum*, and *Dolichospermum* is aligned with dissolved oxygen. Additionally, *Microcystis* is moderately aligned with nitrogen (data not shown).

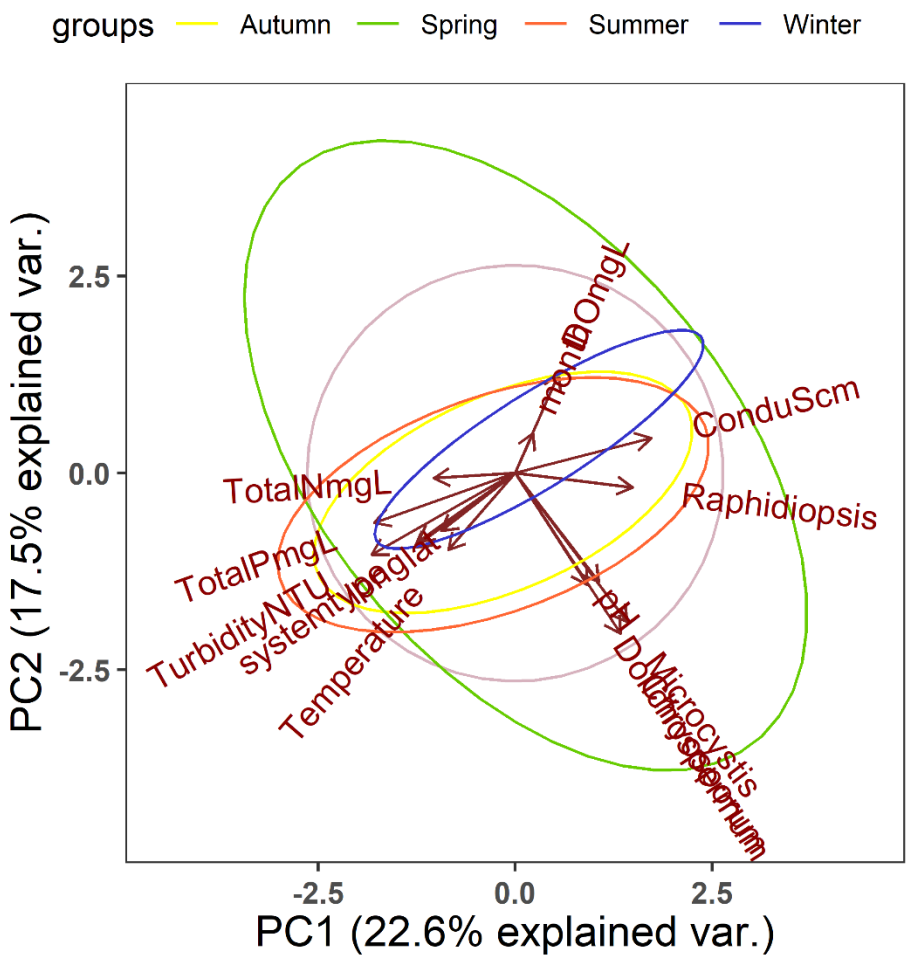

Figure 5.3: Biplot of a PCA run on the South Australian data set, with the first two components explaining a combined total of 40.1% of the variation within the data.

The first two principle components from the PCA run on the South Australian data set explained a combined 40.5% of the variation within the reconstructed data (Fig. 5.3). Three of the cyanobacterial genera, *Microcystis*, *Dolichopsermum*, and *Chryosporum*, are clearly correlated, and are most prevalent in the spring, which matches earlier results (Tab. 5.4). *Raphidiopsis* departs from this trend and is only loosely correlated with conductivity. Nitrogen, phosphorus, turbidity, and temperature are correlated, and values are close across Spring, Summer, and Autumn. Dissolved oxygen is correlated with a numeric month term and is highest during Spring and Autumn. If the next two principal components are examined, their combined variation explained is 20.6 %. *Dolichospermum* and *Microcystis* remain correlated, however here *Raphidiopsis* departs from the other two cyanobacteria and begins to show some correlation with turbidity (data not shown). Here, turbidity is correlated with

Microcystis and *Dolichospermum*, nitrogen is correlated with phosphorus, *Chryosporum* is correlated with dissolved oxygen, and *Raphidiopsis* is correlated with pH.

For modelling the abundance of *Raphidiopsis* in Queensland, model selection yielded a GAM with temperature ($p < 0.001$), total nitrogen ($p < 0.001$), conductivity ($p < 0.01$), total phosphorus ($p = 0.01$), dissolved oxygen ($p < 0.01$), turbidity ($p < 0.01$), the combined latitude and longitude term ($p < 0.001$), the month term ($p < 0.001$), and the transformed counts of *Dolichospermum* ($p < 0.001$), and *Microcystis* ($p < 0.001$). System type was also incorporated as a factor, and was found to be significant, with weirs exhibiting significantly less *Raphidiopsis* abundance ($p < 0.001$, $x = -1.3429$). The model achieved an adjusted r-squared of 0.67 and an explained deviance of 67.7%. *Raphidiopsis* displayed an optimal response to temperature, with increases in concentration from 15 to 20 °C, but steady decreases as temperatures rose above 20 °C. Despite this, the bulk of the data is grouped around ~ 25 °C.

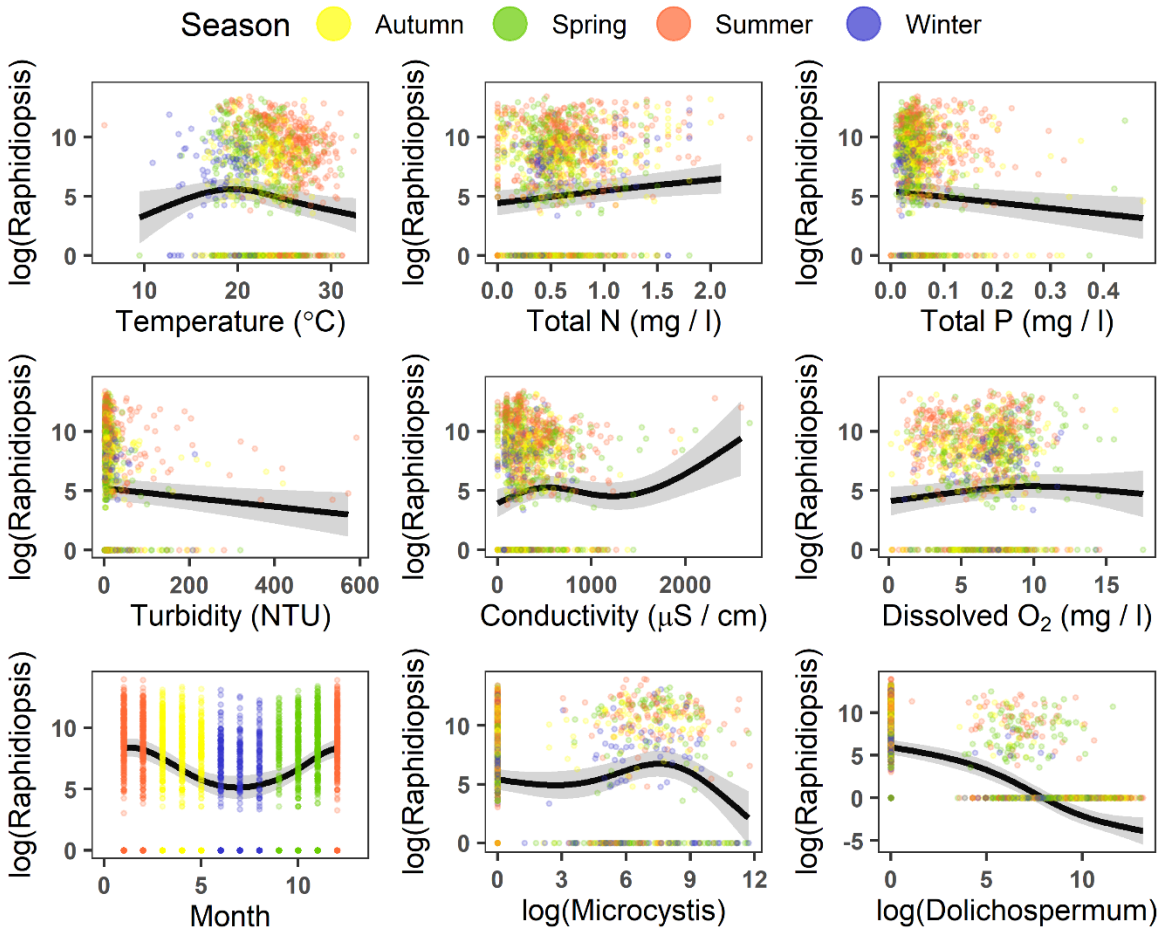

Fig. 5.4: Smooth term plots of the relationship between *Raphidiopsis* and environmental variables from the Queensland data set, with grey areas representing model standard errors.

A positive relationship was found between *Raphidiopsis* and total nitrogen, however the majority of the *Raphidiopsis* data was clustered around the lower nitrogen concentrations. Negative relationships between *Raphidiopsis* and total phosphorus and turbidity were found, with data clustering around the low ends of both variables. *Raphidiopsis* responded positively to conductivity, however the majority of the data was centred around the lower range, ~ 400 µS cm^{-1}. Similar to temperature, the response shape to dissolved oxygen resembled an optima, with higher *Raphidiopsis* concentrations towards the middle of the distribution (Fig. 5.4). A clear negative relationship between *Raphidiopsis* and *Dolichospermum* was found, whereas with *Microcystis* the relationship is quite even until *Microcystis* reaches a threshold population, at which point *Raphidiopsis* abundance declines. There was also a clear negative relationship with turbidity. The modelled relationship between *Raphidiopsis* and total nitrogen revealed little, however the bulk of the data clearly rests in the lower half of the nitrogen distribution, and abundances appear to rise slightly at the extreme lows of nitrogen. A negative relationship was found with total phosphorus, and dissolved oxygen revealed little other than a sharp drop at either end of the data distribution. Finally, there appeared to be a positive relationship between *Raphidiopsis* and conductivity, although the bulk of the abundance data rests in the lower half of the conductivity distribution, suggesting that the positive relationship may be the product of outliers (Fig. 5.4).

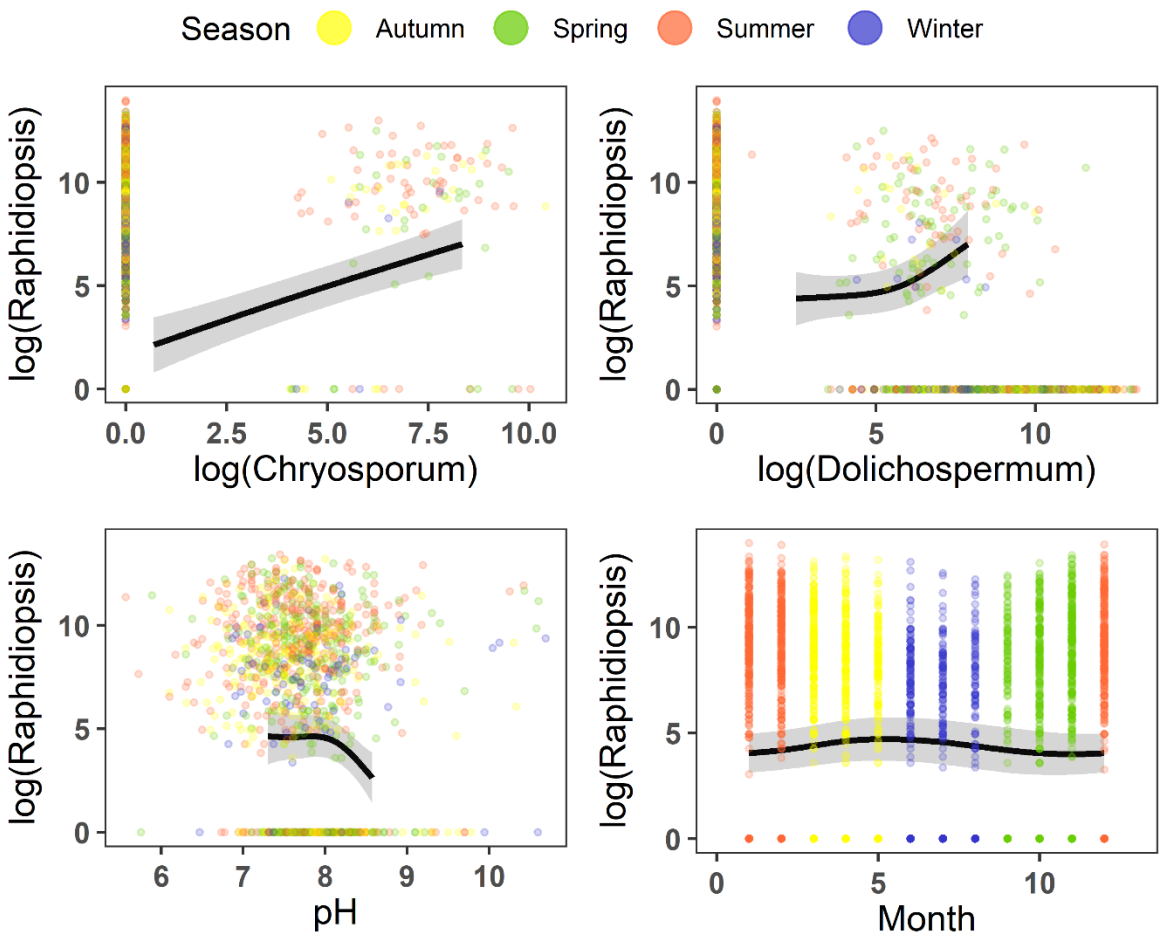

Figure 5.5: Smooth term plots of the relationship between *Raphidiopsis* and environmental variables from the South Australia data set, with grey areas representing model standard errors.

For modelling the abundance of *Raphidiopsis* in South Australia, model selection yielded a GAM with smoothed *Chryosporum* counts ($p < 0.001$), *Dolichospermum* counts ($p < 0.001$), pH ($p < 0.01$), and the month term ($p < 0.05$). System type was incorporated as a factor but was not found to be significant ($p = 0.87$). The model achieved an adjusted r-squared of 0.65 and an explained deviance of 70.1%. Relationships between *Raphidiopsis* and the abundance of *Chryosporum* and *Dolichospermum* were both positive, with data appearing to be normally clustered around the distributions of both genera. The pH term showed steady counts from pH ~ 7 to pH ~ 8, however beyond this point *Cylindrospermpsis* abundance decreased. Data also appeared normally distributed around the range of pH values. The month term displayed some evidence of a seasonal trend however it exhibited a less clear pattern than the month term from the Queensland model (Fig. 5.4, Fig. 5.5).

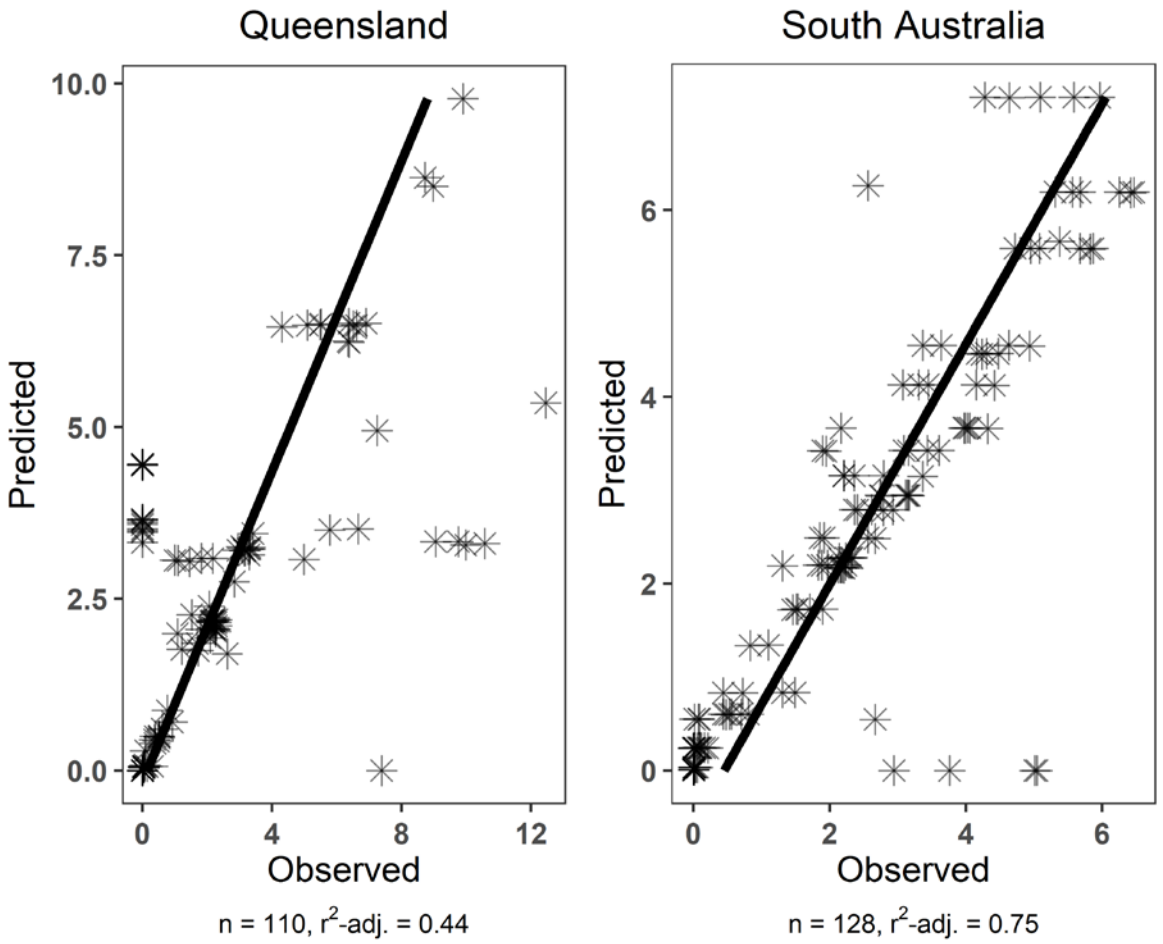

Figure 5.6: Regression of the observed data and the predicted data from the DINEOF algorithm applied to both datasets, with adjusted r-squared and number of data points used for the validation both displayed.

The validation of the DINEOF matrix reconstruction revealed that the algorithm performance was stronger in the South Australian data than in the Queensland data (Fig. 5.6).

5.5 Discussion

Our results indicate that the potential for the build-up of cyanobacterial biomass is significantly higher in the systems of Queensland than the systems of South Australia. This is based on several interrelated factors, which are predominantly climatic in nature. Firstly, and most simply, higher temperatures promote elevated growth, and more consistently high temperatures across seasons facilitate the maintenance of substantial cyanobacterial populations in cooler seasons, which allow for easier development of typically dense

populations in warmer seasons (Fabbro and Duivenvoorden 2000). Conversely, the South Australian systems exhibit cooler Spring and Summer temperatures, and substantially cooler Autumn and Winter temperatures. At the height of Summer, in January, the difference between the average temperatures is 2.3 ºC, however the difference between the averages of the depth of Winter in August was 6.9 ºC. Similar differences were also found for May and June (Tab. 5.1). These colder winter waters in South Australia assist in resetting phytoplankton cycles on an annual basis, however the warmer winter waters of Queensland, which do not experience drastic cooling, are still viable for cyanobacterial growth (Chester and Robson 2014). Globally, these regional temperature dynamics are observed elsewhere, both naturally and artificially. For example, land-clearing in Europe has been shown to cause an intensifying of temperature extremes, with warmer Summers and colder Winters (Hu et al. 2019). Land use change of this nature would theoretically facilitate *Raphidiopsis* during the Summer, but perhaps suppress it during the Winter; whether the species would be able to 'make the difference' depends on other factors such as irradiance, nutrient loading (Burford et al. 2016), community dynamics and parasitism (Jephcott et al. 2017). In conjunction with this, European temperatures are warming due to greenhouse gas emissions, with greater intensity to heatwaves (Schar et al. 2004). Similar observations can be made for other continents including Asia (Xu et al. 2009), and the Americas (Barkhordarian et al. 2018). Thus, the concept of synergistic warming can be introduced, with multiple factors contributing to conditions that are driving ecosystem change. Consistently elevated water temperatures with sporadic rainfall patterns lead to stratification, where cooler water remains set in a deeper layer, called the hypolimnion, and warmer water remains at the surface, and is referred to as the epilimnion. The hypolimnion is nutrient rich but typically light poor, which means most varieties of algae cannot utilise these nutrients, whereas the epilimnion is usually nutrient poor, but subject to high irradiance (Smith and Lester 2007). Queensland's sporadic rainfall patterns and high temperatures result in many of its systems, especially those with typically low flow such as weirs and dams, exhibiting stratified layers (Bormans et al. 2004). These conditions favour the cyanobacteria *Dolichospermum* and *Raphidiopsis*, as they are able to both fix atmospheric nitrogen and regulate their buoyancy to take optimal advantage of the hypolimnion for nutrient supply and the epilimnion for photosynthesis (Mitrovic et al. 2011, Burford at al. 2016), and warmer temperatures facilitate higher levels of nitrogen fixation, allowing these genera to flourish in nutrient poor conditions (Chapter 3). *Microcystis*, however, possesses neither of these physiological traits, which may explain why it does not reach the population densities of *Dolichospermum* or *Raphidiopsis* in Queensland

waters (Tab. 5.2, Tab. 5.4). Alternatively, the combination of a temporal climate with regular annual rainfall means the systems in South Australia are generally less stratified, with higher levels of flow. This is also reflected in turbidity, which is a measure of how much suspended material is present in water bodies at the time of measurement (Tab. 5.3). These factors typically reduce cyanobacterial growth through shading and filament or colony dispersal through water agitation (Elliott et al. 2012).

The discrepancies in nutrient profiles are likely a combination of the more stratified conditions in Queensland systems reducing surface level nutrients, and the heavily farmed river and reservoir sites of South Australia delivering regular nutrient laden runoff into the catchment (Gell et al. 2007). Furthermore, the strength of rainfall events between the states differs; Queensland is known for its strong tropical storms, which, although mobilising nutrients through runoff, flush very large amounts of water (Harris 2001). Consequently, these nutrient loads are transported further downstream, leaving these systems relatively nutrient poor with little or no flow for most of the year (Leigh et al. 2013). These conditions give *Raphidiopsis* a competitive advantage, as *Dolichospermum* cannot thrive under nitrogen deficiency (Loaiza et al. 2016), and *Microcystis* cannot fix atmospheric nitrogen. Whilst South Australia has a mostly consistent annual rainfall regime (Evans et al. 2009), the region has been subjected to several severe storms and drought episodes, which have caused dramatic changes in the phytoplankton composition of the surrounding waters, especially of the Murray-Darling Basin (Bowling et al. 2013a) It has been posited that the incidence of droughts and storms will increase in both frequency and intensity, and the combination of this natural dynamic coupled with human intervention in the system has caused the system to be identified as particularly vulnerable to climate change (Williams and Souter 2015). Although our data suggest the South Australian systems exhibit a significantly higher conductivity than the Queensland systems (Tab. 5.1, Tab. 5.3) caution must be applied here as the sampling sites lie close to the mouth of the Murray, and salts from the Indian ocean are likely contributing to this reading.

The consistently lower dissolved oxygen in Queensland than in South Australia points to systems in generally poorer health, with less refreshment. Lower levels of dissolved oxygen in water bodies can be caused by elevated cyanobacterial biomass. Cells that perish at the water surface float to the bottom of systems, where, in sufficient quantities, their metabolism by heterotrophic bacteria, and the resultant respiration, causes a reduction in available O_2 in water bodies. In extreme instances, this deoxygenation may create anoxic conditions that

have been known to kill other organisms such as fish and crustaceans (Mallin et al. 2016, Svircev et al. 2017). This is typically caused by chronic cyanobacterial blooms, however mass mobilisation of sediment, caused by flooding events, can also trigger hypoxic blackwater events, where unusually elevated levels of dissolved organic carbon (DOC) cause microbial digestion and oxygen uptake (Zhu et al. 2017). Such an event occurred in 2010 – 2011 in the Southern Murray-Darling basin, as a result of an extensive drought followed by significant rainfall, which caused floodplain inundation (Whitworth et al. 2012). Black water events place significant levels of stress on freshwater heterotrophs, including zooplankton populations, which are suppressed by oxygen deprivation (Doubek et al. 2018, Chapter 4). Additionally, zooplankton typically exhibit reluctant grazing of *Raphidiopsis* in Queensland, preferentially targeting other varieties of phytoplankton (Hong et al. 2013) and express metabolic indicators of stress when exposed to sufficient concentrations of cyanobacteria (Dao et al. 2013). Grazing by zooplankton represents a critical phytoplankton population control mechanism and facilitates the transfer of energy to higher trophic levels (Matveev 2003). An examination of our results would suggest that the significant build-up of cyanobacterial biomass and resultant toxin concentrations would cause significant stress to zooplankton concentrations in Queensland waters. In contrast to cyanobacterial levels in Queensland, South Australian waters exhibit reduced cyanobacterial biomass, likely due to their elevated flow and dissolved oxygen levels (Tab. 5.3, Tab. 5.4), which will promote a healthier zooplankton population and facilitate higher grazing levels. This may be a significant factor in explaining why the levels of biomass are different between the two regions.

The DINEOF reconstruction of the datasets, and the running of principal component analyses, revealed further differences between the two regions. The variation within the Queensland dataset appeared to be less driven by seasonality than by the South Australian dataset. For Queensland, the correlation of nitrogen, phosphorus, conductivity, dissolved oxygen, temperature, pH, and *Raphidiopsis* are suggestive of *Raphidiopsis* dominance under a specific set of conditions (Burford et al. 2016). These conditions are, as discussed, met by Queensland systems, that exhibit stratified layers and consistently warm temperatures. The departure of *Dolichospermum* from these correlations, and its alignment with turbidity, suggests that storm events, and mobilisation of sediments, favour *Dolichospermum* over *Raphidiopsis*, most likely due to the periodic increases in nutrient availability (Loaiza et al. 2016). The seasonal groupings and ellipsoids indicate that, with the modest exception of

Winter, the data exhibits seasonal homogeneity, which reflects the consistently warm temperatures and dry conditions typical of the region. Similarly, the departure of *Microcystis* from the bulk of the data, and its positive correlation with longitude, suggests that as you move further east, into more densely populated coastal regions, the incidence of *Microcystis* increases, possibly due to urban run-off (Atkins et al. 2001). Finally, the slight negative-positive correlation between *Dolichospermum* and latitude suggests that as you move further south into more temporal regions, the incidence of *Dolichospermum* increases (Fig. 5.2). In contrast, the biplot for the South Australian data illustrates stark differences between seasonal dynamics. Interestingly, *Microcystis*, *Dolichospermum*, and *Chryosporum* are all correlated, suggesting that cyanobacterial dynamics are somewhat independent of species; where one succeeds, the others do as well. Contrary to this is *Raphidiopsis*, which is moderately correlated with conductivity, suggesting either that *Raphidiopsis* favours the saltier water from oceanic influxes, or more extreme rainfall events; neither is in line with current literature (McGregor and Fabbro 2000, Engstrom-Ost et al. 2015). From the comparison of the means of the cyanobacterial counts, it is evident that *Raphidiopsis* is sub-dominant here, with *Chryosporum* and *Dolichospermum* making up the majority of cyanobacterial abundance (Tab. 5.4). An exception to this is late Spring, in November, where *Microsystis* and *Chryosporum* achieve relatively high abundances, whilst *Raphidiopsis* is negligible. This period is associated with a lower average turbidity and higher dissolved oxygen than either Summer or Autumn, and could represent the beginning of the wetter season, where moderate rainfall refreshes the system but not to the point where turbidity becomes limiting, or where excessive organic matter begins to deplete dissolved oxygen, as may be seen in Summer and Autumn (Tab. 5.4). Total nitrogen, temperature, total phosphorus, and turbidity are all correlated, which was also the case for the Queensland PCA. However, no clear cyanobacterial associations with physiochemical properties were evident.

There were stark differences between the findings of our GAMs fit to the data for the two states. The abundance of *Raphidiopsis* in Queensland was driven by many variables. From the literature, we expected temperature to be significant, with *Raphidiopsis* found to respond strongly to warmer temperatures, 24 – 30 ºC (Chapter 3). Our modelling illustrates that whilst the abundance of *Raphidiopsis* increases from 15 – 20 ºC, it then drops consistently as temperatures rise further. This is strange, considering that our summary statistics report the highest mean abundances of *Raphidiopsis* during the summer. However, the maximum value reported during Summer for *Raphidiopsis* is 1178800 cells ml^{-1}, almost double the next

highest maximum value for any other season (data not shown). It could be that these large cell counts are skewing our summary statistics; this is shown as the skewness for Summer is higher than for Spring or Autumn (Tab. 5.1). We can also see that during the Summer in Queensland, turbidity is, on average, at its highest, as is phosphorus, and our model reported negative relationships between *Raphidiopsis* and these variables. The shading effect of turbidity inhibits phytoplankton photosynthesis, and in more turbid waters cyanobacteria are often outcompeted by mixotrophic algae that can take advantage of the elevated organic matter (Chapter 2). The literature typically reports that *R. raciborskii* is competitive at low phosphorus concentrations, due to its high affinity for phosphates and ability to store luxury P for later use (Burford et al. 2016). Although Summer represents temperatures at which *Raphidiopsis* would be expected to dominate, Queensland also experiences Summer storms, and the resultant influxes may raise phosphorus and turbidity to a point that the overall abundances of *R. raciborskii* decrease. Furthermore, *Raphidiopsis* exhibits higher levels of growth, and higher resultant toxin concentrations, under high irradiance levels (Nor et al. 2019); the reduction in light exposure through higher turbidity could decrease cylindrospermopsin levels and the competitive advantages they bring through allelopathic suppression of competitors and grazers (Leao and Vasconcelos 2009). If accurate, our result here could suggest that event-driven processes are more important in explaining organism trends than physiological capabilities reported in laboratory conditions. Not only does *R. raciborskii* exhibit a negative relationship with phosphorus and turbidity, but the majority of counts are clustered at the low end of both variable distributions. Because of its diazotrophy and ability to compete at low P concentrations, it has been posited that *R. raciborskii* is an oligotrophic organism that thrives in nutrient poor conditions (Burford et al. 2016). However, our modelling suggests a positive association with nitrogen concentrations. This is not unusual; although *R. raciborskii* is capable of significant growth under nitrogen starvation, its growth is also higher under nitrogen replete conditions in laboratory situations (Chapter 3). A positive association with nitrogen hints at phenotypic flexibility, where the genus can dominate under either N-limiting or N-replete conditions. It is worth noting, however, that the majority of the count data is clustered at the lower half of the nitrogen distribution. The positive relationship with conductivity is also unusual here; *R. raciborskii* does not have a high salt tolerance (Engstrom-Ost et al. 2015), and we can see that the majority of its counts lie at the low end of the conductivity distribution.

The relationships reported between *Raphidiopsis* and other varieties of dominant phytoplankton are cryptic. We reported significant negative relationships between *Raphidiopsis* and both *Microcystis* and *Dolichospermum*. It could be that each genus proliferates in a specific hydrological niche which does not favour the others; *Microcystis* tends to dominate under eutrophic conditions (Davis and Koop 2006), and *Dolichospermum* may dominate under middling nutrient concentrations, as it can overexpress superoxide dismutase production under nitrogen starvation which can lead to oxidative stress (Raghavan et al. 2011). Under eutrophic conditions, *Microcystis* can cause stress to *Dolichospermum* through allelopathic suppression of diazotrophy and growth (Chia et al. 2018). The relationships observed in the South Australia data set were different; there were positive associations with *Dolichospermum* and *Chryosporum*, and a negative association with pH. If the abundances are compared between South Australia and Queensland, the levels of cyanobacterial biomass are vastly reduced in South Australia, and *Raphidiopsis* is the lowest contributor to this biomass of the four genera, both in concentration and number of data points (Tab. 5.4). The positive association therefore seems to represent *Raphidiopsis* being found in conjunction with other varieties of cyanobacteria as conditions favour their growth. There is no clear dominance of any genera in South Australia, with concentrations generally being higher in *Microcystis*, but *Dolichospermum* and *Chryosporum* being more commonly observed (Tab. 5.4). This would suggest nutrient profiles alternating between the middling levels that would promote *Dolichospermum*, and the eutrophic levels that would promote *Microcystis*. Of probable significance too is system type; the majority of sampling sites in our South Australia data set are river sampling sites (Fig. 5.1); these can be expected to exhibit higher levels of flow than the reservoirs of Queensland, reflected in the consistently higher levels of turbidity observed (Tab. 5.1, Tab. 5.3). Both of these factors will contribute to suppression of cyanobacterial growth through colony dispersal and filament agitation. Recently, bloom dynamics have switched in the Murray-Darling system from mostly *Dolichospermum*-dominated blooms, to *Chryosporum*-dominated blooms (Crawford et al. 2017). The reasons for this shift have not been clearly elucidated, however low salinity and the movement of cold water through the system channel that caused the vertical migration and distribution of nutrients in the water column are thought to have played a role (Bowling et al. 2018). Because our dataset ends at 2015 due to a change in approach of the monitoring body from genus-specific phytoplankton counts to amalgamation of cyanobacterial counts, we unfortunately no not capture this dynamic shift in our data. The need for robust monitoring data to support ecological research cannot be overstated here.

It must be acknowledged that in comparison with *Dolichospermum* and *Chryosporum* in the South Australia dataset, there were few data points for *Raphidiopsis*; our results may not be capturing dynamics that would be reliably observed in the field, and additionally the lack of historic presence of *Raphidiopsis* in South Australia could lead to it being overlooked or alternately classified. However, our lack of data expresses that *Raphidiopsis*, whilst highly successful in Queensland, is not successful in South Australia. Given the environmental change predicted for the region, could *Raphidiopsis* invade and become the dominant genus in the systems of South Australia examined here? Based on our results, it seems unlikely, however there are considerations that may change this. Typical management strategies employed to combat excessive nutrient loads in freshwater systems with high agricultural activity, such as the Murray-Darling basin, have focused mostly on the reduction of inorganic nitrogen, which promotes *Microcystis*. However, contemporary approaches are now examining more closely the potential that phosphorus reduction could hold for the suppression of blooms (Schindler et al. 2008). *Raphidiopsis* has an association of dominance under phosphorus poor conditions, and so this change in management strategies, combined with increasing temperatures to intensify stratification, could create conditions more suitable for the genus to proliferate. To more effectively answer this question, it could be worthwhile to include data from the state of NSW here; this would provide a geographic and climatic continuum, as opposed to the direct contrast between the two regions of Queensland and South Australia.

5.6 Conclusion

Although the physiological characteristics of *R. raciborskii* have allowed it to become the dominant cyanobacteria in the subtropical regions of North Australia, the more temperate zones in South Australia exhibit a combination of conditions that are not suitable for it to compete. However, prospective conditions presented by anthropogenic climate change, coupled with increasing demand placed on freshwater systems, could facilitate the expansion of *R. raciborskii* to South Australian waters. Our results here demonstrate that the conditions in reservoirs in Queensland over the past 15 years align well with conditions described in the literature as being conducive to proliferation in *R. raciborskii*, however several results, such as declining abundance of *R. raciborskii* at ~ 23 ºC, a positive association with nitrogen, and most particularly an inverse relationship with *Dolichospermum*¸ remain unanswered within

this study. Seasonal variation between the states was also significant, with Summer presenting the highest levels of cyanobacterial activity in Queensland, whilst Spring was the dominant season in South Australia. We found this was reflected in the differences between dominant cyanobacteria; the history of blooms in South Australia are most often connected with Spring storm events that lead to transport of nutrient rich sediments along channel systems like the Murray-Darling, which suits mesotrophic cyanobacteria like *Dolichospermum*, and eutrophic cyanobacteria like *Microcystis*. Summer in Queensland however presents significantly warmer temperatures that contribute to low-flowing, stratified systems; these suit *Raphidiopsis* which is suited to oligotropic conditions. Despite the dramatic differences between the concentrations of harmful cyanobacteria between the surveyed systems in Queensland and South Australia, toxin production continues to present a serious issue for South Australian systems during sporadic but intense bloom events, which are becoming more frequent. The present chapter serves as an example of the potential value in relic data, and the need to continually monitor our systems to capture long-term field dynamics and extend our mechanistic understanding of freshwater systems.

Thesis Conclusion

The above collection of studies addresses the question ‘is the cyanobacterium *Raphidiopsis raciborskii* on the path to ecological dominance in Australia?’ from several standpoints; literature review, lab-based experiments and data-driven investigations. The paradox quoted by Albert Einstein ‘the more I learn, the more I realise how much I don’t know’ is certainly applicable here. Although we have discovered several mechanisms that indicate that *C. raciborski* should have significant advantages over other cyanobacterial competitors under certain scenarios, our results have yielded several further questions that could be promising avenues of further research.

In Chapter 1, it was established that Australia has a unique hydrological profile and it is critical to understand this in order to truly address the questions posed in this thesis. Expected shifts in Australian hydrological regimes were discussed, Australia’s issues with invasive species were introduced, and literary evidence that biological invasions have disrupted the ecosystems of Australia was presented. Australia’s experiences with cyanobacterial blooms was also explored, and key systems vulnerable to the above dynamics were put forward. This Chapter posited two central questions, and has answered them as follows:

1) *How are Australian aquatic systems changing?*

Rises in temperature, and less frequent but more intense rainfall and storm events, are resulting in low-flow conditions for greater portions of the year. Water extraction from fresh flowing systems for various purposes is compounding this issue, and shallower systems are especially at risk. Losses in flow and warming lead to stratification, the separation of the epilimnion and hypolimnion, and the resultant shift in nutrient dynamic has a significant impact on the microbial communities present in the system. More intense rainfall events are mobilising larger quantities of sediment in shorter periods of time and are also delivering larger loads of terrestrial run-off. This, combined with land clearing, causes elevated stream channel degradation. The mobilised sediment represents a significant store of nutrients, which phytoplankton are well-positioned to take advantage of post rainfall, however with antecedent conditions matching the profile of stratified and low-flowing, diazotrophic cyanobacteria will most likely dominate due to their ability to grow through low-nutrient conditions. This includes *Raphidiopsis raciborskii*.

2) *Why are Australian aquatic systems vulnerable to change?*

Australia already experiences an ephemeral rainfall regime, high temperatures, and high levels of evaporation. The projected changes in climate are impact environmental dynamics that are already under pressure. Australian colonisers have caused significant to aquatic systems through inappropriate management strategies and pollution, and whilst recovery in some systems is ongoing, growing populations, increasing water extraction, and dam construction, are placing further stress on these systems.

In Chapter 2, four species of invasive phytoplankton were examined, and key environmental variables that drive their distribution were analysed. The four species of phytoplankton that we outline are quite distinct from one another, physiologically as well as biogeographically; whilst an Australian focus is maintained through this thesis, Chapter 2 suggests to the reader that there is potential in applying the concepts and approaches of the thesis to other regions.

The approach we took in this chapter was also valuable in using the physiological properties of these phytoplankton as a proxy for environmental change in their respective habitats. For example, the raphidophyte does very well under turbid conditions because it is mixotrophic, and this highlights the water brownification taking place in the Scandinavian systems it is succeeding in. Extending this approach to Australia, *R. raciborskii* performs well under high temperatures and stratified conditions, and these changes were linked to Australian systems in Chapter 1. This Chapter posited two central questions, and has answered them as follows:

1) *Are specific environmental parameters more important in influencing invasive phytoplankton than others?*

Using a relative significant difference measure, an assessment of the parameters responsible for the abundance of these species revealed that temperature was the most consistently significant driver; it was also the only parameter found to be overall significant for all four species. The presence of other species of phytoplankton was the next most important, suggesting that allelopathy was a significance driver, or effect, of phytoplankton invasion. Finally, P was significant for both the golden alga and the diatom, but insignificant for the cyanobacteria, and the data was sparse for the raphidophyte. N, a parameter we traditionally associate with phytoplankton success, was highly insignificant for the raphidophyte, and only mildly significant for *R. raciborskii*; it did not play as large a role in our results as one might expect.

2) *Does parameter significance link with physiological traits possessed by invasive phytoplankton?*

Temperature is a universally important parameter because it is a key driver of growth. From Chapter 1 temperature has been established as a primary factor influencing the distribution of *R. raciborskii*¸ which traditionally is most common in tropical regions and succeeds in warm stratified conditions. The physiologies of the other three invaders are different to that of *R. raciborskii*¸ yet temperature remained consistently significant for these species too. This is interesting because two of the invaders, *P. parvum* and *D. geminata*, are slow-growing species, and under nutrient rich conditions these species will get outcompeted by other varieties of phytoplankton. Thus, their significant association with phosphorus is due to their presence in systems that are phosphorus-poor. The association of the mixotrophic *G. semen* with water colour supports its association with systems containing high levels of organic matter, which acts as a food source.

In Chapter 3, a laboratory-based assessment of metabolic responses under a variety of conditions was used to validate theoretical responses of the species established by literary accounts in Chapter 1 and synthesis of field data in Chapter 2. This Chapter was also designed to examine the phenotypic plasticity of *R. raciborskii*, which is commonly raised as a key feature of the species that facilitates its invasive potential. Much literature addresses cyanobacterial growth under warmer temperatures, or with and without N, but very few studies also examine intracellular stoichiometry, nitrogen fixation, and toxin production. Assessing all these variables at once, and all under nitrogen replete or nitrogen depleted, warm or cold, CO_2 rich or poor conditions, gives a picture of how the organism might respond to a change in conditions. This Chapter posited two central questions, and has answered them as follows:

1) *How does R. raciborskii respond to shifts in temperature and CO_2?*

Many of our results here were expected; growth increased dramatically from 18 to 30 °C, as did nitrogen fixation in N-depleted cultures, but in N-rich cultures N-fixation was negligible. The intracellular carbon-to-nitrogen ratio decreased in warmer N-rich cultures and was highest in colder N-depleted cultures. However, other results were surprising; N-fixation decreased significantly at low or high levels of CO_2, and toxin production followed an optima pattern where the highest concentrations were found at 24 °C, and decreased at 18 or 30 °C.

Our results have illustrated a combination of linear and optimal responses, where *R. raciborskii* will generally continue to increase its growth and nitrogen fixation as temperatures increase but reduce production of toxins at low and high temperatures. At low temperatures the species may be experiencing stress causing loss of toxin production, and at high temperatures there may be a competitive advantage to shunt more resources into growth instead of toxins. There was no growth response to CO_2, however nitrogen fixation decreased, and P storage increased, at low and high CO_2 concentrations, suggesting metabolic impairment. Thus, whilst temperature clearly promotes *R. raciborskii*, it may not compete effectively under increasing CO_2 scenarios compared to other phytoplankton. The reason for the response of *R. raciborskii* here is not clear and should be an area of priority for future work.

2) *How successful is R. raciborskii at maintaining its population densities under nitrogen poor scenarios compared to nitrogen rich scenarios?*

R. raciborskii demonstrated effective regulation of N-fixation, with negligible fixation under N-rich conditions, and maximum N-fixation under N-replete conditions and warmer temperatures. Despite the complete absence of nitrogen from the growth medium, cultures were still able to enter the exponential phase and produce concentrations of toxins comparable to that of the N-rich cultures. This lends support to the literary findings in Chapters 1 and 2 that *R. raciborskii* is a successful competitor in nutrient limited systems.

In Chapter 4 the intention was to moderate the results of Chapter 3, a monocultural study, with a complex community study, and maintain temperature as a treatment. Artificial microbial community studies are becoming more common in the literature as the need for results in a systemic context becomes apparent. As the topic of invasion success was discussed in Chapter 1, Chapter 4 allows an experimental glimpse into an invasion example, with the success or failure of the invasion contributing to a theoretical picture of the expansion potential held by *R. raciborskii*. This Chapter posited two central questions, and has answered them as follows:

1) *How do freshwater microbial communities respond to warming conditions?*

Warming in the buckets had a noticeable effect most particularly on the proportions of prokaryotic and eukaryotic phyla, and on the system diversity indices; their progressive decrease suggests that, if we were to have continued the experiment beyond its 10 weeks, the

warmer buckets may have eventually collapsed. Certain taxa also responded to the temperature treatments, namely diatoms, cryptophytes, and Cladoceran zooplankton grazers decreased with increasing temperature. Our choice in system reflects many common urban freshwater systems; eutrophic, with little flow, and frequented by blooms of mostly *Dolichospermum*, thus these effects may be expected across a large number of systems. Associations with cyanobacteria and certain prokaryotic fractions in the literature were accurately reproduced by our sequencing data, and contributes a further piece of evidence that unchecked cyanobacterial proliferation is potentially damaging to ecosystem health.

2) *Do freshwater microbial communities exhibit changes in structure because of the presence of an alien invasive cyanobacterium?*

Bloom formation in the systems was observed twice; a large bloom of *Dolichospermum* hit from week 2 to week 3, and then petered off. Following this, a secondary bloom of *R. raciborskii* appeared in week 5, and again petered off to almost nothing, according to our cell counts. It is therefore debatable how successful invasion here was. Perhaps *R. raciborskii* is favoured by a bloom preceding its own activity, this would make sense given its physiological capabilities to thrive under low nutrient conditions, which were confirmed in Chapter 3.

In Chapter 5, large monitoring data sets were analysed to try and draw conclusions that could be linked back to the previous chapters. Chiefly, the chapter attempted to establish what parameters were most responsible in driving the abundance of *R. raciborskii*, and how these parameters differed between two very different systems, the tropical/sub-tropical stratified reservoirs of Queensland and the predominantly Murray River located temperate sampling sites of South Australia. We expected significant differences between the cyanobacterial profiles in the regions due to a number of factors, namely the temperature differences, however we established that the nutrient and physiochemical profiles were also significantly different, with the South Australian sites exhibiting higher total nitrogen, total phosphorus, turbidity, and conductivity. These findings are again consistent with the difference in system type; the Murray being a flowing system that is in close proximity to zones used for agricultural purposes. This Chapter posited two central questions, and has answered them as follows:

1) *How do the key drivers of R. raciborskii in two regions with different climates?*

Whilst *R. raciborskii* was prolific and dominant in Queensland, it achieved negligible concentrations in the systems of South Australia. This was linked to cooler temperatures and higher levels of turbidity in South Australia creating unfavourable conditions for growth in *R. raciborskii*, although it did maintain a consistent low-concentration presence in the system. Higher levels of nitrogen and phosphorus also suggest that the South Australia environment is more suited to *Dolichospermum* and *Microcystis*. Our modelling yielded some curious results; for instance, despite growth continuing to increase at 30 ºC in Chapter 3, the abundance of *R. raciborskii* increased up until ~ 23 ºC, at which point it began to decline. Despite an association with nutrient poor conditions, our model found a positive relationship between *R. raciborskii* and nitrogen. However, other results were consistent with our findings in Chapters 1 and 2, with negative relationships found between *R. raciborskii* and phosphorus, and with turbidity. We may conclude here that conditions in South Australia would need to undergo further climatic changes in order to facilitate the dominance of *R. raciborskii*, which they may do according to climate forecasts.

2) *Does R. raciborskii exhibit any relationships with other varieties of harmful algae over long temporal and spatial scales?*

An inverse relationship between *R. raciborskii* and *D. circinalis* was confirmed in the Queensland data set, which supports literature findings and our experimental observations in Chapter 4. This is a curious recurring feature that has received little attention and is a promising avenue for future research. Allelopathy is a dynamic that this thesis has not explored, however recent research indicates that it may play a critical role in freshwater community dynamics.

In conclusion, theoretically our chapters do suggest that the combination of traits possessed by *R. raciborskii*, and the nature of hydrological systems in Australia, would support the range expansion and ecological domination of the species. Despite this, our results are mixed in expressing the potential of *R. raciborskii* to become established, due to its questionable dominance under higher CO_2 conditions compared with the benefits other species can receive (Chapter 3) its somewhat ephemeral nature when paired with strong populations of competitor phytoplankton (Chapter 4), and its disassociation with storm events, which are predicated to increase in frequency and intensity in the future (Chapter 5). Our thesis is broad, and as such has much to offer and suggest for future research direction, both locally in Australia and internationally. We challenge the notion of ‘biological invasions’ with respect

to novel range expansion of microbial agents. Vectors of spread for microbial organisms are extremely difficult to identify and may not exist for cyanobacteria, as they are capable of hibernating in a state of dormancy for many years in sediments before favourable conditions trigger their germination. Thus, these invasive organisms may be simply environmental symptoms of change processes. We encourage other researchers to consider this perspective when examining novel dynamics of phytoplankton blooms. The plethora of published field studies can be difficult and time-consuming to process in a meaningful way. We provide an example of an easy-to-use methodology to synthesise such data in an examination of key environmental parameters and physiological traits of invasive species. The results from this methodology are readily paired with environmental change at different spatial and temporal scales. We emphasise the need for more research in strain variation of key species, especially in community settings. Variability in strain behaviour can be extremely high; what drivers this variation? This is an important biological and hydrological question that will help managing bodies better adjust to the specific nature of blooms they are faced with. We suggest that a system stability perspective is valuable when considering the many impact facets of cyanobacterial blooms. How does food web stability correlate with cyanobacterial activity? Does cyanobacterial correlate with management decisions? Answering these questions will create more readily interpretable links between our activities globally and the stability of our systems and justify expenditure on environmental protection measures. In conjunction with this, we also encourage more work in translating ecosystem services in an interpretable way so that action on these issues can be linked to direct impact on every-day living. Relic data sets provide opportunities to pair recent field and laboratory work with robust numeric insights, and help strengthen links between monitoring and management bodies, and research universities. Finally, a combination of these varying perspectives will help distil potential philosophical and paradigmatic directions in the use of Earth's resources, as we become more aware that our current decision-making lacks longevity and equity for future generations.

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Appendix

Chapter 2

Supplementary Table 2.1: A list of papers from which data was extracted for Chapter 2, together with the invader the paper focused on, and the method of analysis they employed.

Reference	Invader	Method of analysis
Anderson IJ, Saiki MK, Sellheim K, Merz JE (2014) Differences in benthic macroinvertebrate assembalges associated with a bloom of *Didymosphenia geminata* in the Lower American River, California. Southwestern Naturalist 59, 3, 389 - 195.	*D. geminata*	ANOVA (α = 0.05)
Sivarajah B, Kurek J, Ruhland KM, Smol JP (2015) Effects of *Didymosphenia geminata* blooms on benthic diatom assemblages in the Restigouche River Watershed, eastern Canada. Botany 5, 317 - 323.	*D. geminata*	ANOSIM (α = 0.05)
Novis P, Schallenberg M, Smissen R (2016) Aquatic nitrogen-fixing cyanobacteria associated with blooms of *Didymosphenia geminata*: insights from a field study. Hydrobiologia 770, 1, 37 - 52.	*D. geminata*	Chi-squared tests (α = 0.05), UniFrac analysis
Gillis C-A, Chalifour M (2010) Changes in the macrobenthic community structure following the introduction of the invasive algae *Didymosphenia geminata* in the Matapedia River (Qubec, Canada). Hydrobiologia 647, 1, 63 - 70.	*D. geminata*	Chi-squared tests (α = 0.05)
Bray J, O'Brien J, Harding JS (2017) Production of phosphatase and extracellular stalks as adaptations to phosphorus limitation in *Didymosphenia geminata* (Bacillariophyceae). Hydrobiologia 784, 1, 51 - 63.	*D. geminata*	ANOVA (α = 0.05)

Sanmiguel A, Blanco S, Alvarez-Blanco I, Cejudo-Figueiras C, Escudero A, Perez ME, Noyon G, Becares E (2016) Recovery of the algae and macroinvertebrate benthic community after *Didymosphenia geminata* mass growths in Spanish rivers. Biological Invasions 18, 5, 1467 - 1484.	*D. geminata*	GLM (α = 0.05)
Bray J, Harding J, Kilroy C, Broady P, Gerbeaux P (2016) Physiochemical predictors of the invasive diatom *Didymosphenia geminata* at multiple spatial scales in New Zealand Rivers. Aquatic Ecology 50, 1 - 14.	*D. geminata*	GLM (α = 0.05)
Ellwood NTW, Whitton BA (2007) Importance of organic phosphate hydrolyzed in stalks of the lotic diatom *Didymosphenia geminata* and the possible impact of atmospheric and climatic changes. Hydrobiologia 592, 121 - 133.	*D. geminata*	GLM (α = 0.05) (interpolated)
Rost AL, Fristen CH, Davis CJ (2011) Distribution of freshwater diatom *Didymosphenia geminata* in streams in the Sierra Nevada, USA, in relation to water chemistry and bedrock geology. Hydrobiologia 665, 1, 157 - 167.	*D. geminata*	Paired t-test / Mann-Whitney U test
Roat AL, Fristen CH (2014) Influence of a tributary stream on benthic communities in a *Didymosphenia geminata* impacted stream in the Sierra Nevada, USA. Diatom Research 29, 3, SI 249 - 257.	*D. geminata*	NMDS
Richardson DC, Olesky IA, Hoellein TJ, Arscott DB, Gibson CA, Root SM (2014) Habitat characteristics, temporal variability, and macroinvertebrate communities associated with a mat-forming nuisance diatom (*Didymosphenia geminata*) in Catskill mountain streams, New York. Aquatic Sciences 76, 553 - 564.	*D. geminata*	ANOVA (α = 0.05)
Montecino V, Molina X, Kumar S, Castillo MLC, Bustamante RO (2014) Niche dynamics and potential geographic distribution of *Didymosphenia geminata* (Lyngbye) M.	*D. geminata*	PCA, pearson correlations

Schmidt, an invasive freshwater diatom in Southern Chile. Aquatic Invasions 9, 4, 507 - 519.		
Miller MP, McKnight DM, Cullis JD, Greene A, Vietti K, Liptzin (2009) Factors controlling streambed coverage of *Didymosphenia geminata* in two regulated streams in the Colorado Front Range. Hydrobiologia 630, 207 - 218.	*D. geminata*	Linear regression (α = 0.05)
Lavery JM, Kurek J, Ruhland KM, Gillis CA, Pisaric MFJ, Smol JP (2014) Exploring the environmental context of recent *Didymosphenia geminata* proliferation in Gaspesie, Quebec, using paleolimnology. Canadian Journal of Fisheries and Aquatic Sciences 71, 4, 616 - 626.	*D. geminata*	PCA, Pearson correlations
Ladrera R, Rieradevall M, Prat N (2015) Massive growth of the invasive algae *Didymosphenia geminata* associated with discharges from a mountain reservoir alters the taxonomic and functional structure of macroinvertebrate community. River Research and Applications 31, 216 - 227.	*D. geminata*	DISTLM (Distance-base Linear Model)
Kumar S, Spaulding SA, Stohlgren TJ, Hermann KA, Schmidt TS, Bahls LL (2009) Potential habitat distribution for the freshwater diatom *Didymosphenia geminata* in the continental US. Frontiers in Ecology and the Environment 7, 8, 415 - 420.	*D. geminata*	MAXENT (Maximum entropy model)
Kirkwood AE, Shea T, Jackson LJ, McCauley E (2009) Are dams hotspots for *Didymosphenia geminata* bloomsα Freshwater Biology 54, 1856 - 1863.	*D. geminata*	Linear regression (α = 0.05)
Kirkwood AE, Shea T, Jackson LJ, McCauley E (2007) *Didymosphenia geminata* in two Alberta headwater rivers: an emerging invasive species that challenges conventional views on algal bloom development. Canadian Journal of Fisheries and Aquatic Sciences 64, 12, 1703 - 1709.	*D. geminata*	ANCOVA (α = 0.05)

Kilroy C, Larned ST, Biggs BJF (2009) The non-indigenous diatom *Didymosphenia geminata* alters benthic communities in New Zealand rivers. Freshwater Biology 54, 1990 - 2002.	*D. geminata*	Paired t-test / ANOSIM (α = 0.05)
Kilroy C, Bothwell ML (2012) *Didymosphenia geminata* growth rates and bloom formation in relation to ambient dissolved phosphorus concentration. Freshtwater Biology 57, 4, 631 - 653.	*D. geminata*	ANOVA (α = 0.05)
James DA, Model K, Chipps SR (2014) The influence of light, stream gradient, and iron on *Didymosphenia geminata* bloom development in the Black Hills, South Dakota. Hydrobiologia 721, 1, 117 - 127.	*D. geminata*	Logistic regression / linear regression (= 0.05)
James DA, Chipps SR (2016) Influence of *Didymosphenia geminata* Blooms on Prey Composition and Associated Diet and Growth of Brown Trout. Transactions of the American Fisheries Society 145, 1, 195 - 205.	*D. geminata*	MANOVA / ANOVA (= 0.05)
Jackson LJ, Corbett L, Scrimgeour G (2016) Environmental constraints on *Didymosphenia geminata* occurrence and bloom formation in Canadian Rocky Mountain lotic systems. Canadian Journal of Fisheries and Aquatic Sciences 73, 6, 964 - 972.	*D. geminata*	GLM / ANCOVA (= 0.05)
Gillis C-A, Lavoie I (2014) A preliminary assessment of the effects of *Didymosphenia geminata* nuisance growths on the structure and diversity of diatom assemblages of the Restigouche River basin, Quebec, Canada. Diatom Research 29, 3, SI 281 - 292.	*D. geminata*	NMDA / ANOVA (= 0.05)
Yamamoto Y, Shiah FK (2012) Factors related to the dominance of *Cylindrospermopsis raciborskii* (Cyanobacteria) in a shallow pond in Northern Taiwan. Journal of Phycology 48, 4, 984 - 991.	*R. raciborskii*	Linear regression (α = 0.05)
Wiedner C, Rucker J, Bruggemann R, Nixdorf B (2007) Climate change affects timing and size of populations of an	*R. raciborskii*	Linear regression (α = 0.05)

invasive cyanobacterium in temperate regions. Oecologia 152, 473 - 484.		
Saker ML, Griffiths DJ (2001) Occurrence of blooms of the cyanobacterium *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya and Subba Raju in a north Queensland domestic water supply. Marine and Freshwater Research 52, 6, 907 - 915.	*R. raciborskii*	Linear regression (= 0.05) (interpolated)
Mehnert G, Rucker J, Wiedner C (2014) Population dynamics and akinete formation of an invasive and a native cyanobacterium in temperate lakes. Journal of Plankton Research 36, 2, 378 - 387.	*R. raciborskii*	Mann-Whitney U test / Spearman-Rho correlation (= 0.05)
Bormans M, Ford PW, Fabbro L, Hancock G (2004) Onset and persistence of cyanobacterial blooms in a large impounded tropical river, Australia. Marine and Freshwater Research 55, 1, 1 - 15.	*R. raciborskii*	Linear regression (= 0.05) (interpolated)
Bouvy M, Ba N, Ka S, Sane S, Pagano M, Arfi R (2006) Phytoplankton community structure and species assemblage succession in a shallow tropical lake (Lake Guiers, Senegal). Aquatic Microbial Ecology 45, 2, 147 - 161.	*R. raciborskii*	ANOVA ($\alpha = 0.05$)
Briand JF, Robillot C, Quiblier-Lloberas C, Humbert JF, Coute A, Bernard C (2002) Environmental context of *Cylindrospermopsis raciborskii* (Cyanobacteria) blooms in a shallow pond in France. Water Research 36, 13, 3183 - 3192.	*R. raciborskii*	PCA
Dokulil MT (2016) Vegetative survival of *Cylindrospermopsis raciborskii* (Cyanobacteria) at low temperature and low light. Hydrobiologia 764, 241 - 247.	*R. raciborskii*	Linear regression (= 0.05)
Fabbro LD, Duivenvoorden LJ (1997) Profile of a Bloom of the Cyanobacterium *Cylindrospermopsis raciborskii* (Woloszynska) Seenaya and Subba Raju in the Fitzroy River in Tropical Central Queensland. Marine and Freshwater Research 47, 685 - 694.	*R. raciborskii*	Linear regression (= 0.05) (interpolated)

Figueredo CC, Giani A (2009) Phytoplankton community in the tropical lake of Lagoa Santa (Brazil): Conditions favoring a persistent bloom of *Cylindrospermopsis raciborskii*. Limnologica 39, 264 - 272.	*R. raciborskii*	PCA
Figueredo CC, von Ruckert G, Cupertino A, Pontes MA, Fernandes LA, Ribeiro SG, Maran NRC (2013) Lack of nitrogen as a causing agent of *Cylindrospermopsis raciborskii* intermittent blooms in a small tropical reservoir. FEMS Microbial Ecology 87, 557 - 567.	*R. raciborskii*	Spearman correlation (= 0.05)
Figueredo CC, von Ruckert G, Giani A (2016) The necessity of management in a lake of the Atlantic Forest biodiversity hotspot: nitrogen levels connected to a persistent bloom of *Cylindrospermopsis raciborskii*. Acta Botanica Brasilica 30, 2, 222 - 231.	*R. raciborskii*	PCA / Pearson correlation (= 0.05)
Fuentes MS, Rick RJ, Hasenstein KH (2010) Occurrence of a *Cylindrospermopsis* bloom in Louisiana. Journal of Great Lakes Research 36, 458 - 464.	*R. raciborskii*	ARIMA analysis (= 0.05)
Hadas O, Pinkas R, Malinsky-Rushansky N, Nishri A, Kaplan A, Rimmer A, Sukenik A (2012) Appearance and establishment of diazotrophic cyanobacteria in Lake Kinneret, Israel. Freshwater Biology 57, 6, 1214 - 1227.	*R. raciborskii*	Linear regression (= 0.05) (interpolated)
Hong Y, Steinman A, Biddanda B, Rediske R, Fahnenstiel G (2009) Occurrence of the Toxin-producing Cyanobacterium *Cylindrospermopsis raciborskii* in Mona and Muskegon Lakes, Michigan. Journal of Great Lakes Research 32, 645 - 652.	*R. raciborskii*	ANOVA ($\alpha = 0.05$)
Kokocinski M, Soininen J (2012) Environmental factors related to the occurrence of *Cylindrospermopsis raciborskii* (Nostocales, Cyanophyta) at the north-eastern limit of its geographical range. European Journal of Phycology 47, 1, 12 - 21.	*R. raciborskii*	t-tests, GLM (= 0.05)

Kokocinski M, Stefaniak K, Mankiewicz-Boczek J, Izydorczyk K, Soininen J (2010) The ecology of the invasive cyanobacterium *Cylindrospermopsis raciborskii* (Nostocales, Cyanophyta) in two hypereutrophic lakes dominated by Planktothrix agardhii (Oscillatoriales, Cyanophyta). European Journal of Phycology 45, 4, 365 - 374.	*R. raciborskii*	GLM (= 0.05)
Kovacs AW, Toth TR, Voros L (2012) Light-dependent germination and subsequent proliferation of N2-fixing cyanobacteria in a large shallow lake. International Journal of Limnology 48, 177 - 185.	*R. raciborskii*	Linear regression (interpolated)
Lei L, Peng L, Huang X, Han B-P (2014) Occurrence and dominance of *Cylindrospermopsis raciborskii* and dissolved cylindrospermopsin in urban reservoirs used for drinking water supply, South China. Environmental Monitoring and Assessment 186, 3079 - 3090.	*R. raciborskii*	Spearman correlation (= 0.01)
de Castro Medeiros L, Mattos A, Lurling M, Becker V (2015) Is the future blue-green or brownα The effects of extreme events on phytoplankton dynamics in a semi-arid man-made lake. Aquatic Ecology 49, 293 - 307.	*R. raciborskii*	Redundancy analysis (RDA)
Trigal C, Hallstan S, Johansson KSL, Johnson RK (2013) Factors affecting occurrence and bloom formation of the nuisance flagellate Gonyostomum semen in boreal lakes. Harmful Algae 27, 60 - 67.	G. semen	Random Forest
Hagman CHC, Ballot A, Hjermann DO, Skjelbred B, Brettum P, Ptacnik R (2015) The occurrence and spread of Gonyostomum semen (Ehr.) Diesing (Raphidophyceae) in Norwegian lakes. Hydrobiologia 744, 1 - 14.	G. semen	Linear regression (= 0.05)
Trigal C, Goedkoop W, Johnson RK (2011) Changes in phytoplankton, benthic invertebrate and fish assemblages of boreal lakes following invasion by Gonyostomum semen. Freshwater Biology 56, 1937 - 1948.	G. semen	NDMS / ANOSIM (= 0.05)

Peltomaa E, Ojala A, Holopainen A-L, Salonen K (2013) Changes in phytoplankton in a boreal lake during a 14-year period. Boreal Environment Research 18, 387 - 400.	G. semen	Pearson correlation / Partial least-squares (PLS) (= 0.05)
Rengefors K, Weyhenmeyer GA, Bloch I (2012) Temperature as a driver for the expansion of the microalga Gonyostomum semen in Swedish lakes. Harmful Algae 18, 65 - 73.	G. semen	Non-parametric Wilcoxon-test / Mann-Kendall test (= 0.05)
Peczula W, Poniewozik M, Szczurowska A (2013) Gonyostomum semen (Ehr.) Diesing bloom formation in nine lakes of Polesie region (Central–Eastern Poland). International Journal of Limnology 49, 301 - 308.	G. semen	ANOVA / Pearson correlation (= 0.05)
Sassenhagen I, Sefbom J, Sall T, Godhe A, Rengefors K (2015) Freshwater protists do not go with the flow: population structure in Gonyostomum semen independent of connectivity among lakes. Environmental Microbiology 17, 12, 5063 - 5072.	G. semen	AMOVA (analysis of molecular variance) (= 0.05)
Peczula W, Mencfel R, Kowalczyk-Pecka D (2014) Among-lake variation in vertical distribution of invasive, bloom-forming algal species Gonyostomum semen (Raphidophyceae) in stratified humic lakes of eastern Poland. International Review of Hydrobiology 99, 317 - 325.	G. semen	Spearman correlation (= 0.05)
Angeler DG, Johnson RK (2013) Algal invasions, blooms and biodiversity in lakes: Accounting for habitat-specific responses. Harmful Algae 23, 60 - 69.	G. semen	ANOVA (= 0.05)
Findlay DL, Paterson MJ, Hendzel LL, Kling HJ (2005) Factors influencing Gonyostomum semen blooms in a small boreal reservoir lake. Hydrobiologia 533, 243 - 252.	G. semen	Linear regression (= 0.05)
Karosiene J, Kasperoviciene J, Koreiviene J, Vitonyte I (2014) Assessment of the vulnerability of Lithuanian lakes to expansion ofGonyostomum semen (Raphidophyceae). Limnologica 45, 7 - 15.	G. semen	Pearson correalation (= 0.05)

Johansson KSL, Trigal C, Vrede T, Johnson RK (2013) Community structure in boreal lakes with recurring blooms of the nuisance flagellate Gonyostomum semen. Aquatic Sciences 75, 447 - 455.	G. semen	t-tests (= 0.05)
Angeler DG, Allen CR, Johnson RK (2012) insight on invasions and resilience derived from spatiotemporal discontinuities of biomass at local and regional scales. Ecology and Society 17, 2, 32.	G. semen	Discontinuity analysis
Angeler DG, Trigal C, Drakare S, Johnson RK, Goedkoop W (2010) Identifying resilience mechanisms to recurrent ecosystem perturbations. Oecologia 164, 231 - 241.	G. semen	Pearson correlation / ANOVA (= 0.05)
Hambright KD, Zamor RM, Easton JD, Glenn KL, Remmel EJ, Easton AC (2010) Temporal and spatial variability of an invasive toxigenic protist in a North American subtropical reservoir. Harmful Algae 9, 568 - 577.	P. parvum	Linear regression / Pearson correlation (= 0.05)
VanLandeghem MM, Farooqi M, Southward GM, Patino R (2015) Associations between water physiochemistry and *Prymnesium parvum* presence, abundance, and toxicity in West Texas reservoirs. Journal of the American Water Resources 51, 2, 471 - 486.	P. parvum	Zero-inflated Poisson regression (ZIP) / nonparametric classification and regression trees (CART) / Cubic regression
VanLandeghem MM, Farooqi M, Southward GM, Patino R (2015) Spatiotemporal associations of reservoir nutrient characteristics and the invasive, harmful alga *Prymnesium parvum* in West Texas. Journal of the American Water Resources Association 51, 2, 487 - 501.	P. parvum	Nonparametric regression and classification trees (CART)
VanLandeghem MM, Farooqi M, Farquhar B, Patino R (2013) Impacts of Golden Alga Prymnesium parvum on Fish Populations in Reservoirs of the Upper Colorado River and Brazos River Basins, Texas. Transactions of the American Fisheries Society 142, 581 - 595.	P. parvum	Mixed-ANOVA (= 0.05)

Roelke DL, Brooks BW, Grover JP, Gable GM, Schwierzke-Wade L, Hewitt NC (2012) Anticipated human population and climate change effects on algal blooms of a toxic haptophyte in the south-central USA. Canadian Journal of Fisheries and Aquatic Sciences 69, 8, 1389 - 1404.	P. parvum	PCA
Patino R, Dawson D, VanLandeghem MM (2014) Retrospective analysis of associations between water quality and toxic blooms of golden alga (Prymnesium parvum) in Texas reservoirs: Implications for understanding dispersal mechanisms and impacts of climate change. Harmful Algae 33, 1 - 11.	P. parvum	Classification trees (CART)
Michaloudi E, Moustaka-Gouni M, Gkelis S, Pantelidakis K (2009) Plankton community structure during an ecosystem disruptive algal bloom of Prymnesium parvum. Journal of Plankton Research 31, 3, 301 - 309.	P. parvum	Non-parametric multidimensional scaling (NMDS)
Hambright KD, Beyer JE, Easton JD, Zamor RM, Easton AC, Hallidayschult TC (2015) The niche of an invasive marine microbe in a subtropical freshwater impoundment. The ISME Journal 9, 256 - 264.	P. parvum	Logistic regression (= 0.05) / Classification trees
Jones AC, Liao TSV, Najar FZ, Roe BA, Hambright KD, Caron DA (2013) Seasonality and disturbance: annual pattern and response of the bacterial and microbial eukaryotic assemblages in a freshwater ecosystem. Environmental Microbiology 15, 9, 2557 - 2572.	P. parvum	ANOSIM ($\alpha = 0.05$)
Roelke DL, Gable GM, Valenti TW, Grover JP, Brooks BW, Pinckney JL (2010) Hydraulic flushing as a Prymnesium parvum bloom-terminating mechanism in a subtropical lake. Harmful Algae 9, 3, 323 - 332.	P. parvum	Non-metric multidimenstional scaling

Chapter 4

Supplementary Table 4.1: Simper output for prokaryotic analysis, with output displayed up to the cumulative contribution ('cumsum') of 50%. Results are separated according to phylogenetic classification. 'ava' stands for average proportion of hits in group a (week 10), and 'avb' stands for average proportion of hits in group b (week 1). P values represent the probability of randomly sampling both groups for the same classification and observing similar proportions.

Phylum	average	sd	ratio	ava	avb	cumsum	p	
Verrucomicrobia.6	0.050	0.052	0.9601	8.508877	3.188326	0.08014	0.109	
Actinobacteria.4	0.033	0.024	1.4085	7.219782	9.595522	0.1336	0.105	
Proteobacteria.122	0.032	0.054	0.5997	5.101966	2.457271	0.18518	0.333	
Proteobacteria.167	0.032	0.074	0.4342	5.153483	0.00042	0.23637	0.087	.
Proteobacteria.117	0.031	0.027	1.1575	7.496556	3.392296	0.28664	0.013	*
Proteobacteria.112	0.029	0.035	0.8309	2.254002	5.367986	0.33341	0.147	
Cyanobacteria.13	0.029	0.021	1.3675	0.006823	4.8052	0.3799	0.001	***
Bacteroidetes	0.023	0.018	1.2677	5.554825	3.18168	0.41714	0.065	.
Actinobacteria.15	0.022	0.028	0.795	2.269909	2.728737	0.4526	0.141	
Verrucomicrobia.8	0.021	0.031	0.6866	4.080826	2.206203	0.48657	0.101	
Proteobacteria.101	0.020	0.019	1.0069	1.64873	4.61191	0.51779	0.021	*
Class	average	sd	ratio	ava	avb	cumsum	p	
Verrucomicrobiae.6	0.062	0.064	0.9687	8.508877	3.188326	0.1005	0.089	.
Actinobacteria	0.041	0.030	1.38	7.219782	9.595522	0.1672	0.097	.
Betaproteobacteria.50	0.039	0.035	1.1149	7.496556	3.392296	0.2306	0.008	**
Alphaproteobacteria	0.038	0.060	0.6252	5.101966	2.457271	0.2916	0.366	
Alphaproteobacteria.45	0.037	0.084	0.4349	5.153483	0.00042	0.3507	0.167	
Betaproteobacteria.45	0.036	0.042	0.8487	2.254002	5.367986	0.4083	0.143	
Actinobacteria.11	0.027	0.032	0.8376	2.269909	2.728737	0.4512	0.141	
Betaproteobacteria.34	0.024	0.024	0.9971	1.64873	4.61191	0.4902	0.022	*
Betaproteobacteria	0.023	0.018	1.26	5.069441	2.73622	0.5269	0.027	*
Order	average	sd	ratio	ava	avb	cumsum	p	
Burkholderiales.30	0.059	0.053	1.1016	7.496556	3.392296	0.08349	0.022	*

Nostocales	0.053	0.039	1.3555	0.006823	4.8052	0.15812	0.001	***
Burkholderiales.25	0.051	0.058	0.8857	2.254002	5.367986	0.23109	0.071	.
Rhizobiales.8	0.046	0.105	0.4363	5.153483	0.00042	0.29606	0.48	
Actinomycetales	0.041	0.053	0.7693	2.269909	2.728737	0.35391	0.209	
Burkholderiales.14	0.036	0.037	0.9702	1.64873	4.61191	0.40517	0.009	**
Methylophilales	0.034	0.046	0.7368	3.230207	0.431847	0.45278	0.015	*
Chroococcales.1	0.023	0.035	0.6609	1.884846	0.47216	0.48544	0.394	
Sphingobacteriales.1	0.021	0.021	0.9903	1.449187	1.632098	0.51463	0.678	
Family	average	sd	ratio	ava	avb	cumsum	p	
Burkholderiaceae.3	0.085	0.077	1.101	7.496556	3.392296	0.121	0.055	.
Nostocaceae	0.076	0.058	1.3207	0.006823	4.8052	0.2292	0.001	***
Comamonadaceae.12	0.072	0.079	0.9135	2.254002	5.367986	0.3314	0.052	.
Rhizobiaceae.4	0.056	0.127	0.4363	5.153483	0.00042	0.4103	0.499	
Comamonadaceae.1	0.053	0.059	0.9093	1.64873	4.61191	0.4862	0.007	**
Methylophilaceae	0.028	0.038	0.735	1.629467	0.524151	0.5255	0.242	
Genus	average	sd	ratio	ava	avb	cumsum	p	
Polynucleobacter.1	0.100	0.093	1.079	7.496556	3.392296	0.1388	0.032	*
Dolichospermum	0.086	0.065	1.3342	0.006823	4.8052	0.2582	0.001	***
Comamonas	0.085	0.095	0.8952	2.254002	5.367986	0.3759	0.063	.
Rhizobium.2	0.062	0.143	0.4364	5.153483	0.00042	0.4621	0.476	
Synechococcus	0.041	0.060	0.6743	1.884846	0.47216	0.5183	0.395	
Species	average	sd	ratio	ava	avb	cumsum	p	
Polynucleobacter.sp	0.105	0.097	1.0799	7.496556	3.392296	0.1453	0.024	*
Dolichospermum.flos.aquae	0.090	0.067	1.3482	0.006823	4.8052	0.2701	0.001	***
Comamonas.sp	0.088	0.099	0.8924	2.254002	5.367986	0.3931	0.069	.
Rhizobium.sp	0.064	0.146	0.437	5.153483	0.00042	0.4819	0.441	
Synechococcus.sp	0.042	0.062	0.6763	1.884846	0.47216	0.5405	0.362