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Combined effects of resources and warming on lake microbial communities across environmental gradients

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by

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The Dissertation of Marika Allison Schulhof is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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ABSTRACT OF THE DISSERTATION

Combined effects of resources and warming on lake microbial communities across environmental gradients

by

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Multiple aspects of the global environment are changing rapidly, including rising temperatures, altered biogeochemical cycles and a redistribution of biodiversity. Different aspects of environmental change may interact through synergistic processes or interference; however, how these processes magnify or dampen one another's effects on lakes is largely unknown. My dissertation research explores the independent and interactive effects of warming and resource supply on lake food webs from multiple perspectives. Chapter 1 investigates the independent and interactive effects of temperature, supply and origin of dissolved organic material, and atmospheric nitrogen deposition on prokaryotic and eukaryotic microbial community composition and diversity in Sierra Nevada lakes. Chapter 2 focuses on stoichiometry and growth of phytoplankton communities in response to warming, nutrient

addition and grazing in three Dutch lakes across a productivity gradient. Chapter 3 explores whether the competition-defense tradeoff regulates coexistence within or among members of phytoplankton communities across a productivity gradient, and how warming may alter this tradeoff. These studies collectively show that resource supply is more important than temperature in regulating microbial community composition and stoichiometry across a variety of lake ecosystems. Additionally, interactive effects of temperature, nutrient supply, and grazing on phytoplankton community stoichiometry, growth rates, biomass buildup and functional group composition depend on the trophic state and size structure of communities. Finally, turnover in communities along productivity gradients resulted in a positive correlation between nutrient and grazer limitation across taxa among lakes, but no relationship between top-down and bottom-up limitation within lakes. This result suggests that traits like small cell size that make phytoplankton more susceptible to grazing also confer strong responses to nutrient pulses in low-nutrient environments. Thus, my results provide no support for the hypothesis that costly defenses against grazing increase nutrient limitation, resulting in a tradeoff between nutrient and consumer limitation. In fact, the opposite pattern was found whereby the taxa that are most sensitive to grazing and nutrients are segregated in the least productive system, and the responses to both factors decline in more productive lakes due to increasing dominance by inedible forms. My thesis demonstrates functional associations among the traits of microbes that shape their responses to climate, resources and consumers, promote diversity at the local and regional scales, and determine how aquatic ecosystem productivity is controlled by multiple limiting factors.

INTRODUCTION

Microorganisms such as phytoplankton and bacterioplankton play critical roles in freshwater ecosystems by supplying energy to food webs, recycling nutrients and organic matter, and driving biogeochemical cycling of carbon, nitrogen, and phosphorus. Microbial communities are influenced by abiotic and biotic factors that can affect species composition, food web structure, and functioning of the entire ecosystem. Lakes are undergoing rapid environmental changes due to drivers at local and regional spatial scales including climate warming and nutrient pollution that may impact microbial communities. However, the interactive effects of multiple stressors on microbial communities in lakes remain poorly understood, in part because of the difficulty of assigning causality when several aspects of the environment are changing simultaneously. The goal of my dissertation is to understand how multiple environmental changes, including warming and changes in resource supply, may interact to affect microbial communities in lakes. I examine how these changes independently and interactively affect community composition of phytoplankton and bacterioplankton, stoichiometry and growth of phytoplankton communities, and tradeoffs in phytoplankton functional traits across environmental gradients.

Microorganisms are the most taxonomically and metabolically diverse organisms on earth, distributed globally in all habitat types including extreme environments that are inhospitable for many metazoans. Microorganisms, broadly defined, are members of the domains Bacteria, Archaea, and Eukarya (unicellular algae, fungi, protists) smaller than 500μm in size (reviewed in Martiny et al. 2006). Microbial taxa and community composition of microbes play critical roles in ecosystems and food webs, influencing

processes such as biogeochemical cycling, respiration, decomposition, and both autotrophic and heterotrophic production. In freshwater ecosystems, microorganisms such as phytoplankton and bacterioplankton play critical roles in supplying energy to food webs and recycling nutrients, thus influencing biogeochemical cycling of carbon, nitrogen, and phosphorus.

Phytoplankton occupy important ecological roles as primary producers in freshwater and marine systems, and changes to phytoplankton abundance and community traits have consequences for higher trophic levels and biogeochemical cycling. Phytoplankton consist of diverse photosynthetic unicellular or colonial pelagic microorganisms (algal eukaryotes and cyanobacteria) with great ecological importance, as they are responsible for approximately 50% of global primary production (Falkowski & Raven 2013) and the source of 70% of Earth's atmospheric oxygen supply (Reynolds 1984). Phytoplankton account for a large proportion of organic carbon available to pelagic foodwebs (Reynolds 2006), and play an important role in global biogeochemical cycling of biologically active elements such as carbon, nitrogen, and phosphorus (Falkowski et al. 2004).

Bacterioplankton also occupy central roles in aquatic food webs as drivers of biogeochemical cycling and ecosystem function. Pelagic heterotrophic bacteria are critical members of the microbial loop: they metabolize dissolved organic carbon (DOC) from both terrestrial and aquatic sources and are consumed by higher trophic levels such as heterotrophic flagellates, ciliates and metazoan zooplankton (Azam 1983, Tranvik 1992, Hessen 1992). Bacterioplankton respiration results in outgassing CO_2 from freshwater environments to the atmosphere and can exceed phytoplankton production

when terrestrial organic matter is abundant (Duarte & Prairie 2005, Battin et al. 2009). Bacterioplankton influence the flow of energy, carbon and the metabolism of freshwater ecosystems, and therefore determine whether lakes absorb or emit carbon to the atmosphere. Therefore, understanding how bacterial communities will respond to warming and resource addition has critical implications for food web dynamics and biogeochemical cycling.

Both temperature and nutrients are strong structuring forces in aquatic phytoplankton and bacterioplankton communities. The importance of temperature as a primary driver of microbial community composition has been established in numerous studies of lacustrine (Yanarell & Triplett 2004, Lindstrom et al. 2005) and marine (Sunagawa et al. 2015, Barton et al. 2016) ecosystems. However, responses to temperature may interact with and be mediated by resource supply for both bacterioplankton and phytoplankton. For example, higher temperatures can lead to increases in nutrient-use efficiency and growth rates in phytoplankton (de Senerpont Domis et al. 2014), and nutrients and warming together can synergistically increase phytoplankton abundance and biomass (Weidman et al. 2014, Thompson et al. 2008, de Senerpont Domis et al. 2014).

Global environmental change

Freshwater ecosystems are sensitive indicators of multiple global change stressors, including climate warming and nutrient pollution (eutrophication, Adrian et al. 2009). Multiple stressors when combined can interactively affect organisms and ecosystems, potentially causing antagonistic or synergistic effects (Folt et al. 1999).

Warming and eutrophication are two major stressors in freshwater systems that can have a large impact on top-down and bottom-up processes. Bottom-up control represents growth limitation via the supply of abiotic resources for autotrophs, such as nutrients and light availability, and the resulting availability of organic resources (including energy and nutrients) for heterotrophic consumers in higher trophic levels (DeAngelis 1992, Lindeman 1942, Strong & Frank 2010). Bottom-up processes are critical in structuring resource and energy flow in food webs and greatly influence the composition and functioning of ecological communities. Eutrophication is one symptom of the importance of bottom-up control, as fertilization with mineral nutrients results in dramatic changes in biomass and species composition, oxygenation, the quality of water resources and fisheries productivity. Because bottom-up control can have a large influence on the number of trophic levels and standing crop of organisms in ecosystems (DeAngelis 1992), changes to abiotic environments and resource supplies can have important implications for food web structure and community composition.

Global climate warming is unfolding at unprecedented rates, with an increase in globally averaged land and sea surface temperature of 0.85°C from 1880-2012 (IPCC 2013). Satellite data indicates that surface temperatures of lakes worldwide have warmed significantly since 1985, and in some areas, have increased more rapidly than regional air temperature (Schneider & Hook 2010, O'Reilly et al. 2015). Impacts of climate warming on lakes and ponds include changes in physical processes such as longer durations of ice-free and stratified seasons (McCormick 1990), increased vertical stratification, and reduced vertical mixing, with ecological consequences such as shifts in community structure and trophic interactions (Woodward et al. 2010). Warming temperatures and

associated changes in physical processes affect biogeochemical activity at the land-water interface, such as the quantity of allochthonous dissolved organic carbon (DOC) inputs entering aquatic systems from terrestrial sources (Carpenter et al. 1992, Van de Waal et al. 2010, Greig et al. 2012, Weidman et al. 2014). Allochthonous DOC inputs to inland waters in Europe and North America have shown increasing trends since the 1990s (Evans et al. 2005, Monteith et al. 2007) and climate warming has been proposed as one explanation. Temperature- driven biotic processes that increase allochthonous DOC inputs into alpine lakes include increased soil mineralization (Anderson et al. 1991, Schmidt et al. 2002) and increased vegetation growth in the surrounding watershed due to shifting plant distributions and advancing tree-lines at higher elevations (Walther et al. 2005, Lenoir et al. 2008). The quantity of allochthonous DOC entering lakes has important consequences for bottom-up control in lake food webs because of its role in carbon cycling and fueling microbial production in lakes.

In addition to climate warming, anthropogenic nutrient loading to freshwater ecosystems due to deposition from the atmosphere or watershed has implications for community structure and ecosystem function. Point and non-point sources of nutrients to water bodies can cause eutrophication due to excessive phosphorus (P) and nitrogen (N) inputs. Global N and P inputs to the biosphere have been amplified by human activities by approximately 100% and 400% respectively (Falkowski et al. 2000, Galloway et al. 2004). Ecological effects of eutrophication on aquatic ecosystems include increased productivity and blooms of algae and phytoplankton, including harmful dinoflagellates and cyanobacteria, and anoxia due to decomposition of algae biomass (reviewed in Carpenter et al. 1998). Additionally, variation in the amount of nutrients and DOC in

lakes can shift lake trophic status between oligotrophic (low nutrients, low productivity, low humic content, clear water), eutrophic (high nutrients, high primary productivity, low humic content, green water), and dystrophic (high DOC, low productivity, high humic content, brown water) conditions (reviewed in Carpenter and Pace 1997). Thus, understanding how resource supply, in concert with warming, may affect bottom-up control in lakes has important implications for trophic interactions, biogeochemical cycling, ecosystem function, and ecosystem services such as water quality.

Species sorting, stoichiometry, and trait-based approaches

Environmental selection plays a dominant role in shaping microbial composition, with repercussions for ecosystem-level processes. Species sorting, or shifting community structure is likely to be an important mechanism for microbial adaptation to climate change (Wallenstein and Hall 2012, Litchman et al. 2012, Barton et al. 2016). Microbial traits related to habitat preference, growth substrate and resource use are often phylogenetically conserved and associated with specific taxonomic groups, therefore shifting microbial taxonomic composition is likely to have an effect on ecosystem function via community-wide trait distributions (Wallenstein and Hall 2012, Krause et al. 2014, reviewed in Martiny et al. 2015).

Additionally, stoichiometric C:N:P ratios of autotrophic biomass are important for understanding relationships between environmental nutrient supply, uptake by autotrophs, interactions of algal species, and producer-consumer interactions. Phytoplankton cellular C:nutrient ratios depend upon the environmental supplies of inorganic nutrients, temperature, and availability of light for photosynthesis (Sterner and

Elser 2002). Phytoplankton species and taxonomic groups exhibit interspecific variation in nutrient acquisition and demand. Several physiological traits contribute to variation in nutrient uptake and utilization across phytoplankton species. Differences in nutrient utilization and uptake based on tradeoffs for growth (*r*) versus competitive ability (*K*) may determine the stoichiometric requirements of cellular machinery, as ribosomes needed for growth require P, while proteins needed for resource acquisition require N (reviewed in Litchman & Klausmeier 2008). Human enrichment of terrestrial and aquatic systems with N and P relative to C can cause stoichiometric imbalance (Sterner & Elser 2002, Van de Waal et al. 2010) and altered nutrient limitation (Sickman et al. 2003, Elser et al. 2009). These alterations can affect the quantity and quality of primary production, and consequently, secondary production. P-limitation can constrain nutritional quality of freshwater phytoplankton to consumers, causing limited growth for consumers with high P demands such as *Daphnia* (Anderson & Hessen 1991, Elser & Hassett 1994). C:P ratios in seston and the difference in C:P between producers and consumers is a key driver of trophic efficiency and energy transfer from autotrophs to higher trophic levels (Hessen 2008). Therefore, the nutritional quality of primary producers is critical in determining the trophic structure of aquatic ecosystems because higher nutritional quality of primary producers, expressed as high N or P relative to C, results in higher herbivore-to-producer biomass ratios in ecosystems (Cebrian et al. 2009). Poor stoichiometric quality can propagate up the food chain, potentially affecting secondary and tertiary consumers (reviewed by Hessen et al. 2013).

Cell size of phytoplankton is an important trait that influences key metabolic processes such as nutrient uptake and utilization strategies, in addition to trophic

interactions. Due to the allometric scaling relationship between cell size and volume, differences in size impact nutrient uptake rates and growth rates of phytoplankton (Litchman et al. 2007). Increasing cell size and volume are correlated with higher maximum uptake rates, half-saturation constants, and storage capacity (Litchman et al. 2007; Maranon et al. 2013), and such unimodal size scaling of phytoplankton growth is likely a result of size-related constraints in nutrient uptake, enzyme kinetics, and metabolic rates rather than taxonomic differences (Maranon et al. 2013). Small cells tend to have higher maximum growth rates and are able to acquire limiting nutrients more efficiently due to the high surface area to volume ratio and smaller diffusion boundary layer (Litchman & Klausmeier 2008), while large cells have greater maximum uptake rates per cell and may have larger storage capacity for nutrients (Edwards et al. 2011). The size of phytoplankton, in addition to having significance as an ecophysiological trait, plays an important role in trophic interactions by influencing susceptibility to grazing by zooplankton. Increasing cell size results in greater resistance to gape-limited grazers, creating a potential trade-off between nutrient competitively ability and grazing susceptibility (reviewed in Litchman et al. 2007, Litchman et al. 2010, Ward et al. 2014). Additionally, elemental stoichiometry of phytoplankton can be influenced by cell size and associated traits, including metabolic rate and degree of vacuolation (reviewed in Finkel et al. 2010). Trait-based approaches to phytoplankton community ecology, with a focus on ecophysiological traits such as cell size and elemental stoichiometry, hold promise for understanding and predicting responses of phytoplankton communities to global change (Litchman & Klausmeier 2008, Finkel et al. 2010, Litchman et al. 2010, Reynolds et al. 2002).

Study system

Changes in ecological communities along environmental gradients provide unique opportunities to understand how species distributions and traits change in relation to abiotic drivers across environments. Elevation gradients in mountain lakes provide a system in which to study the effects of temperature and associated vegetation changes on ecosystem structure and function. In the Sierra Nevada mountains of California, a regional (north-south) atmospheric nitrogen deposition gradient generates spatial contrasts in supply of at least one limiting nutrient. By comparison, shallow lakes of the Netherlands are on average much more productive, but also vary considerably in nutrient supply and therefore trophic status. Because of the flat topography, lakes in the Netherlands vary little spatially in temperature, but show strong seasonal cycles of warming and cooling. These two systems form the basis for my thesis research to ask how microbial communities change in nature and respond to experimental manipulations of nutrients, temperature and grazing.

Chapter summaries

My dissertation chapters aim to determine effects of environmental change on bottom-up control in lake food webs using both comparisons among lake ecosystems in the field and experimental manipulations of naturally occurring phytoplankton assemblages:

In **Chapter 1**, I ask how warming, the supply and origin of dissolved organic carbon (DOC), and nitrogen deposition independently and interactively affect the composition, richness, and evenness of communities of autotrophic eukaryotes

(phytoplankton) and heterotrophic prokaryotes (bacterioplankton) in mountain lakes. I sampled lakes along an elevation gradient in the Sierra Nevada mountains of California that varied in atmospheric nitrogen deposition and showed that compositions of prokaryotic and eukaryotic communities varied more in response to input of inorganic and organic resources than temperature. In addition, community composition of the prokaryotic and eukaryotic groups were more strongly correlated with one another than with any measured environmental variable, suggesting a role for species interactions in determining community membership. Increasing N availability reduced species richness of prokaryotic and eukaryotic communities. My results provide insight into abiotic and biotic processes structuring microbial communities across environmental gradients and show the unexpected result that temperature was less influential than resources in structuring both prokaryotic and eukaryotic communities.

In **Chapter 2**, I ask how the effect of warming on phytoplankton community growth, biomass and stoichiometry (N:P and C:P in two size classes, <30um and >30um) varies across a trophic state gradient in the presence and absence of grazing and nutrient addition. I conducted microcosm experiments using phytoplankton communities collected from three Dutch lakes varying in productivity and measured the response of each community to multifactorial combinations of nutrient, temperature, and grazing treatments. I found that nutrients elevated growth rates and biomass and reduced stoichiometric ratios of all three phytoplankton communities, but the main and interactive effects of temperature and grazing depended on lake productivity and cell size. My experiments indicate that stoichiometric responses to warming and interactions with nutrient addition and grazing are not universal but instead depend on lake productivity

and associated variation in composition and cell size. The effects of climate warming on phytoplankton stoichiometry therefore depend on interactions with resources, consumers and the size structure of the local community.

In **Chapter 3**, I ask whether the competition-defense tradeoff promotes coexistence within or among lakes across a productivity gradient and how this tradeoff may shift with warming. Using data from the experiments described in Chapter 2, I used a trait-based approach to classify functional groups based on information from the literature on grazing susceptibility. I found that nutrients and grazing both had the strongest effects in the least productive lake. Functional group turnover across productivity gradients results in assemblages that are both most grazing resistant and least nutrient limited in more eutrophic lakes. Additionally, the effect of warming on top-down and bottom-up control of the edible size fractions (<30um) differed among lakes. My results found no indication that tradeoffs between top-down and bottom-up control influenced differences in composition within or among lakes rather than promoting local diversity. Instead, there was a positive relationship between limitation by resources and consumers among lakes. Warming had idiosyncratic effects on the response of communities to grazing and nutrients among functional groups and lakes.

Together, my thesis identifies abiotic and biotic factors that regulate microbial community composition, traits, and diversity across resource supply and climatic gradients. Using both a field survey and experimental manipulations allows us to better understand processes and mechanisms driving changes in microbial community composition in different types of systems. My results suggest that the effect of warming and its interactions with resource supply depend on a complex interplay between top-

down and bottom-up factors. Therefore, the effects of global change on phytoplankton communities may not be easily generalizable but instead depend on several important aspects of lake ecosystems including trophic state, and composition of species and associated traits.

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CHAPTER 1

Microbial communities in mountain lakes are independently controlled by nutrients, detritus and biotic interactions

Abstract

Warming, eutrophication (nutrient fertilization) and brownification (increased loading of allochthonous organic matter) are three global trends impacting lake ecosystems. However, the independent and synergistic effects of resource addition and warming on autotrophic and heterotrophic microorganisms are largely unknown. In this study, we investigate the independent and interactive effects of temperature, dissolved organic carbon (DOC, both allochthonous and autochthonous), and atmospheric nitrogen (N) deposition on the composition, richness, and evenness of prokaryotic and eukaryotic microbial communities in lakes across elevation and N deposition gradients in the Sierra Nevada mountains of California. We found that prokaryotic communities are structured by DOC quantity and origin, while eukaryotic communities are shaped by nitrate, total nitrogen, and allochthonous DOC. Additionally, the abundances of prokaryotic and eukaryotic organisms were more strongly correlated with one another than with any measured abiotic factor, suggesting a role for biotic interactions in structuring microbial communities. Finally, increasing N availability was associated with reduced richness of prokaryotic and eukaryotic communities, and lower evenness of eukaryotes. Our data suggest that 1) organic and inorganic resources structure microbial communities independent of temperature across our elevation gradient; 2) autotrophic and heterotrophic microbial community assembly is highly correlated 3) atmospheric N deposition reduces richness of both prokaryotic and eukaryotic microbial communities,

likely by reducing niche dimensionality. Our survey provides insight into abiotic and biotic processes structuring microbial communities across environmental gradients and their potential roles in material and energy fluxes within and between ecosystems.

Introduction

A multitude of anthropogenic stressors are changing the composition of microbial communities of lakes, with consequences for the structure and function of aquatic food webs and ecosystems (Kratina et al. 2012, Shurin et al. 2012). Inland aquatic systems are highly connected to terrestrial and atmospheric processes via water transport, gas exchange and nutrient cycling, and thus are especially sensitive to changes occurring in surrounding environments (Williamson et al. 2009). The quantity and quality of inorganic and organic resources available to food webs are critical in structuring material and energy flow by bottom-up processes (Lindeman 1942, DeAngelis 1992, Strong & Frank 2010), which are being altered by global environmental change. Three pervasive global trends in lake ecosystems are warming, increased inputs of terrestrial dissolved organic carbon (DOC), and eutrophication from inorganic nutrient runoff. Multiple stressors can interactively affect organisms and ecosystems, potentially leading to antagonistic or synergistic effects (Folt et al. 1999). However, the interactive effects of multiple stressors on trophic interactions in natural systems remain poorly understood (Ormerod et al. 2010).

Eukaryotic and prokaryotic microorganisms play integral roles in aquatic food webs, influencing processes such as autotrophic and heterotrophic production, respiration, decomposition, and biogeochemical cycling (reviewed in Martiny et al.

2006). Phytoplankton (both eukaryotes and prokaryotes) are primary producers in freshwater and marine systems, accounting for the majority of organic carbon available to pelagic foodwebs (Reynolds 2006) and play important roles in global biogeochemical cycling of elements such as carbon, nitrogen, and phosphorus (Falkowski et al. 2004, Worden et al. 2015). Bacterioplankton (prokaryotes) also occupy important roles in aquatic food webs and biogeochemical cycling as decomposers that mineralize dissolved organic carbon (DOC) from terrestrial and aquatic sources, releasing CO_2 from freshwater environments to the atmosphere (Duarte & Prairie 2005, Battin et al. 2008) and recycling nutrients through the microbial loop (Azam et al. 1983, Hessen 1992, Tranvik 1992). In oligotrophic lakes, bacterial respiration often exceeds photosynthetic carbon fixation by phytoplankton, resulting in net heterotrophic systems and frequent CO_2 supersaturation (Del Giorgio 1997, Biddanda et al. 2001, Sobek et al. 2005). Inorganic and organic substrate availability play important roles in controlling the balance between productivity of bacteria and phytoplankton, (Sanders et al.1992, Biddanda et al. 2001, Lennon & Cottingham 2008). Thus, understanding how phytoplankton and bacterioplankton respond to the addition of organic and inorganic resources in a warming environment has critical implications for food web dynamics and biogeochemical cycling.

Warming temperatures have both direct physical effects on lakes and indirect effects at the land-water interface by increasing terrestrial sources of nutrients and detritus entering lakes (Carpenter et al. 1992, Van de Waal et al. 2010, Greig et al. 2012, Weidman et al. 2014). Warming increases terrestrial DOC inputs into alpine lakes through more rapid soil mineralization (Anderson et al. 1991, Schmidt et al. 2002) and

increased vegetation growth in the surrounding watershed due to lengthened growing seasons and shifting plant distributions that advance tree-line to higher elevations and latitudes (Walther et al. 2005, Lenoir et al. 2008). These processes are collectively referred to as 'brownification'. Terrestrial (allochthonous) DOC is derived primarily from decomposition of vegetation and plays an important role in carbon cycling by sustaining bacterial production. Terrestrial (allochthonous) and aquatic (i.e., autochthonous, from algal and microbial sources) dissolved organic matter differ in that the former contains more aromatic, humic, colored compounds (Wetzel 1983, Thurman 1985, McKnight 1994), that attenuate UV light, reduce water transparency and mixing depth, and suppress phytoplankton production (reviewed in Williamson et al. 1999, Solomon et al. 2015, Carpenter et al. 2016). Variations in DOC quantity and quality can drive biogeographic (Ruiz-Gonzalez et al. 2015), seasonal (Crump et al. 2003), and diel (Sadro et al. 2011b) patterns in bacterial metabolism and community composition and thus are strong predictors of bacterial production and lake ecosystem metabolism. Terrestrial DOC can also affect eukaryotic microbial communities, as brownification may select for shade-tolerant and mixotrophic phytoplankton species that employ heterotrophic feeding strategies when autotrophy is limited by light or nutrients, or when bacterial concentrations are high (Jones et al. 1997, Urrutia-Cordero et al. 2017, Wilken et al. 2018).

In addition to climate warming and brownification, anthropogenic nutrient loading due to deposition from the atmosphere or watershed has implications for the structure and function of aquatic systems. Anthropogenic nitrogen production has increased more than tenfold in the last century (Galloway 2004), and both point and non-

point sources of nutrients to water bodies can cause eutrophication due to excessive phosphorus (P) and nitrogen (N) inputs. High elevation lakes may be less susceptible to watershed runoff but more impacted by atmospheric deposition of nutrients, resulting in eutrophication, acidification, and shifting nutrient limitation from N to P (Sickman et al. 2001, Fenn et al. 2003a&b, Elser 2009a&b). Nitrate and ammonium are key sources of N for phytoplankton, and different taxonomic groups may differ in traits such as competitive abilities and uptake affinities for different N sources (Litchman et al. 2007, Litchman & Klausmeier 2008, Edwards et al. 2012). As a result, shifting nutrient regimes due to N deposition may drive shifts in community composition (Litchman et al. 2012), but how these dynamics are influenced by concurrent stressors across an elevational gradient remains unknown.

Temperature, nutrients and detritus may exert both independent and interactive effects on phytoplankton and bacterioplankton communities. Temperature is a primary driver of microbial community composition in lacustrine (Yanarell & Triplett 2004, Lindstrom et al. 2005) and marine (Sunagawa et al. 2015, Barton et al. 2016) ecosystems. However, responses to temperature may be mediated by resource supply for microorganisms. In bacteria, the effect of temperature on growth is resource-dependent (Hall et al. 2008), with different substrate-temperature interactions for different taxa (Pomeroy and Weibe 2001) and shifts in competitive abilities and nutrient use efficiencies of different taxa along thermal gradients (Hall et al. 2008, 2009). Similarly, in phytoplankton, nutrients and warming together can synergistically increase phytoplankton abundance and biomass due to temperature-driven increases in nutrient assimilation (Thompson et al. 2008, de Senerpont Domis et al. 2014, Weidman et al.

2014). Elevated nutrients and temperature may also synergistically increase the abundance of harmful cyanobacteria that often dominate warm, eutrophic lakes (Paerl and Huisman 2008, Kosten et al. 2012). Additionally, warming may increase bacterivory in mixotrophic phytoplankton (Wilken et al. 2013), and the combination of warming and brownification could interactively decrease phytoplankton diversity and increase the abundance of mixotrophs (Urrutia-Cordero et al. 2017, Wilken et al. 2018).

In addition to abiotic factors, biotic interactions between microbes in aquatic environments can have a large influence on community composition. Bacteria, viruses, archaea and protists are linked in complex interaction networks characterized by mutualism, commensalism, predator-prey and host-parasite relationships (reviewed in Faust and Raes 2012, Worden et al. 2015). A study of the global marine plankton 'interactome' (Lima-Mendez et al. 2015) found that community composition of plankton was more accurately predicted by models containing only operational taxonomic unit (OTU) data, than those containing environmental data or the combination of both, suggesting that biotic interactions can explain as much or more of the variation in microbial community composition than abiotic conditions. Thus, biotic interactions between microbes are likely to influence the presence-absence patterns and species abundances in mountain lakes, but to date such biotic associations have not been explored in these habitats.

Our study aims to identify the independent and interactive effects of warming, DOC quantity and quality, and atmospheric N deposition (measured as NO_3^- supply) on prokaryotic and eukaryotic community structure and OTU richness and evenness in lakes of the Sierra Nevada Mountains in California. Sierra Nevada lakes vary in temperature

and DOC quality and quantity along an elevation gradient, and in NO_3^- concentrations along a latitudinal N deposition gradient, as there are higher rates (15kg N/ha-year) of deposition in southwestern Sierra Nevada sites (Sickman et al. 2001, Clow et al. 2002, Fenn et al. 2003a&b), as compared to 3kg N/ha-year on average in high-elevation alpine and subalpine Sierra Nevada catchments. Lakes in the alpine and sub-alpine zones are situated upon substrates of granite and granodiorite rock, and are naturally oligotrophic (Sickman et al. 2001). The major sources of anthropogenic N emissions include transportation, agriculture, and industry, with emissions from burning fossil fuels mostly found as NO_X forms and from agriculture primarily as NH_X forms (Sickman et al. 2001). Because Sierra Nevada mountain lakes are oligotrophic with low acid neutralizing capacities, they are especially sensitive to eutrophication and acidification as a result of atmospheric N deposition (Clow et al. 2002, Fenn et al. 2003b, Sickman et al. 2003, Shaw et al. 2014). Additionally, ecosystems at high altitudes and latitudes are warming more rapidly than other regions (reviewed in Woodward et al. 2010). Thus, mountain lakes provide a fitting system in which to investigate how temperature and both inorganic and organic resource addition independently and interactively affect lake microbial communities.

We hypothesized that the origin and quantity of DOC would be significant drivers of changes in bacterial community composition, because differences in DOC origin (aquatic vs. terrestrial) may select for bacteria that utilize different carbon sources (Fierer et al. 2007). DOC quantity and origin may also affect phytoplankton species composition by selecting for mixotrophic species as DOC concentrations increase, especially in combination with warming (Urrutia-Cordero 2017, Wilken et al. 2013, Wilken et al.

2018). We also hypothesized that phytoplankton community composition would change along NO_3^- gradients, as in previous studies of mountain lakes (LaFrancois & Nydick 2003, Nydick et al. 2004). Additionally, temperature is an important structuring force of microbial communities in aquatic environments and therefore is likely to impact community composition of prokaryotic and eukaryotic communities (Yanarell & Triplett 2004, Lindstrom et al. 2005, Shade et al. 2007, Fuhrman et al. 2008). Finally, we hypothesized that correlations between eukaryotic and prokaryotic communities may result from facilitative or antagonistic biotic interactions between the two communities (Faust & Raes 2012, Lima-Mendez et al. 2015).

Materials & Methods

Field data collection

Between June and August 2015, thirty-four Sierra Nevada lakes (Appendix Table 1) containing fish were sampled across a 904m elevation gradient (2433-3337m), encompassing lakes in the montane (below 2450m), subalpine (2450-2900m) and alpine zones (2900m and above). The lakes were also sampled across a regional north-south N deposition gradient across Yosemite National Park, Inyo National Forest, and Sequoia National Park. The lakes encompassed a 10.2°C temperature gradient (8.7-18.9°C), and 805.7 μM DOC gradient (68.8-874.5 μM) and 2.6 uM NO_3^- gradient (0.0044-2.6 μM). The lakes ranged 0.5–21 hectares in surface area and 1.8 to 40.8m in depth. All lakes were oligotrophic (chl-*a* $\leq$ 3.33 μg l^{-1}) and have similar geologic and chemical characteristics as they are situated on granite and granodiorite bedrock (Sickman et al. 2001).

Water samples from the epilimnion (surface to 1m depth) were collected at each lake for biological and chemical analyses. At the deepest point in each lake, in situ depth profiles of temperature, conductivity, salinity, dissolved oxygen (DO) and pH were taken using a YSI probe (YSI Incorporated, Yellow Springs, Ohio, USA). Water samples were collected with a 1m integrated tube sampler and filtered through 63-μm mesh to remove zooplankton and processed for chlorophyll-a (chl-a), particulate organic matter (POM), total nitrogen (TN), total phosphorus (TP), particulate nitrogen, particulate phosphorus, dissolved nitrate (NO_3^-), and dissolved organic carbon (DOC). Chl-a and POM samples were kept frozen in the dark until sample processing. For chl-a quantification, a known volume of water was filtered through 0.45 μm glass fiber filters (GF/F Fisher Scientific) and frozen. Chl-a, a proxy for phytoplankton biomass, was measured using a fluorometer after a 24 hr cold methanol extraction. For calculation of C:N in particulate organic matter (POM), a known volume of water was filtered through pre-weighed pre-combusted (7 hours, 500°C) 0.45μm glass fiber filters.

Water was collected for microbial DNA extraction in presterilized containers (washed with 5% bleach) and filtered through 0.22 μm Sterivex filters (Millipore) using a sterile filtration setup. Up to 2L of water were filtered at each lake, and filters were kept cold during transport back to the Sierra Nevada Aquatic Research Laboratory (SNARL) and frozen immediately at -80°C for later DNA extraction.

Laboratory analyses

Total nitrogen (TN) and phosphorus (TP) samples were collected in HDPE vials and preserved with H_2SO_4 to pH<2 and stored at ~4°C until analysis. TN, TP, and NO_3^-

were measured using an autoanalyzer (LaChat QuikChem 8500) via persulfate digestions (TN, TP) and hydrazine reduction (NO_3^-). Upon returning to the lab, POM samples were dried for 24 hours at 60°C, weighed and packaged in tin capsules for stable isotope and elemental analysis of C and N at the University of California, Davis analytical laboratory (http://anlab.ucdavis.edu/). Quantification of P in POM was carried out by combustion of filters at 500°C followed by persulfate digest and subsequent measurements using an autoanalyzer (LaChat QuikChem 8500).

To quantify DOC, water samples were filtered through precombusted glass fiber filters (Whatman GF/F, pore size 0.7μm) into triple-rinsed 20 mL glass vials and preserved with HCl to pH<2. DOC was measured using a total organic carbon analyzer (TOC-V CSN, Shimadzu Scientific Instruments, Japan). To characterize DOC quality, we used UV-vis absorbance and fluorescence spectroscopy, which reflect several aspects of the molecules comprising the light absorbing and fluorescing DOM pool, respectively. We used excitation emission matrices (EEMs) as a 3-dimensional representation of fluorescence intensities scanned over a range of excitation/emission wavelengths (Coble 1996, Chen et al. 2003). EEMs were collected with a JY-Horiba Aqualog spectrophotometer (HORIBA, Japan) at room temperature using 5nm excitation and emission slit widths and an integration time of 1.0s. The Aqualog spectrophotometer simultaneously collects both fluorescence and absorbance spectra on a sample. All fluorescence spectra were collected in signal-to-reference (S:R) mode with instrumental bias correction. Instrument-specific corrections, Raman area normalization, and Milli-Q blank subtraction were conducted with Matlab (version 2014). From the UV-vis absorbance and EEMs data, we calculated two indices of DOC quality: the freshness

index (β:α) and specific UV absorption (SUVA). Freshness Index is a ratio of emission intensity at 380 nm to that of the region between 420 and 435 nm at an excitation of 310 nm and is reflective of recently produced algal organic matter (Parlanti et al. 2000). SUVA is a DOC-normalized index of aromaticity calculated as UV absorbance at 254nm/[(DOC(mg/L) x Path length (0.01m)] (Weishaar et al. 2003). Freshness Index increases with autochthonous carbon production whereas SUVA increases with allochthonous carbon production.

DNA extraction and 16S and 18S rRNA gene amplicon sequencing

DNA was extracted from Sterivex filters using a PowerWater DNA Isolation Kit (MOBIO, Carlsbad, CA, USA) and DNA concentrations were quantified using Qubit fluorometric quantitation (Life Technologies). Approximately 500 bp of the v4v5 region of 16S rRNA gene was PCR amplified using the primers 515F (5'-GTGYCAGCMGCCGCGGTAA -3') and 926R (5'-CCGYCAATTCMTTTRAGT-3') (Parada et al. 2015), and approximately 500 bp of the v4 region of the eukaryotic 18S rRNA gene was amplified with the nearly universal primers TAReuk454FWD1 (5′-CCAGCASCYGCGGTAATTCC-3′) and TAReukREV3 (5′-ACTTTCGTTCTTGATYRA-3′) (Bertrand et al. 2015). 16s and 18s PCR products were prepared using AccuPrime high fidelity Taq DNA polymerase (Life Technologies) and BioRad PTC0200 thermocycler. The PCR products were purified using Agencourt AMPure XP beads (Beckman Coulter), quantified using Quant-iT PicoGreen dsDNA assay kit (Life Technologies), quality-screened and equimolar-mixed at the J. Craig Venter Institute (La Jolla, CA) and sequenced at the UC San Diego Institute for Genomic

Medicine (La Jolla, CA) using 600 cycle paired-end sequencing (Miseq V3, Illumina, San Diego, CA, USA). The 16S library contained data for 34 lakes with an average read depth of 599353 reads per sample, while the 18S library contained data for 32 lakes (due a failure to PCR amplify the 18S region in two samples) with an average read depth of 437278 reads per sample.

Bioinformatic methods

Illumina sequencing reads were processed to remove primer and adapter fragments and FASTQ files were input into the rRNA pipeline written by J.P. McCrow (https://github.com/allenlab/rRNA_pipeline). Paired-end reads were combined using PEAR (Zhang et al. 2013) with a -t parameter for minimum trim length of 50bp. Chimeric sequences were identified and removed using USEARCH (Edgar 2010), with the -strand plus parameter set. Reads were then quality trimmed to Q25 average quality across a window of 2 bases, for 16S and Q10 for 18S, using the fastq_filter.py script. Amplicons were clustered into OTUs using SWARM (Mahé et al. 2014) with default parameters and further filtered to require at least 3 reads across at least 2 samples. Taxonomic best hits were assigned by GLSEARCH36 (Pearson 2016), with default parameters. The SILVA database was used as a reference for 16S rRNA sequences (Quast 2013, v128). A modified PR2 database was used for 18S rRNA sequences, with updates from Tara Oceans W2 (de Vargas et al. 2015).

Statistical methods

All statistical analyses were performed using the statistical program R version

3.4.2 (R Development Core Team 2017). All variables were normalized using z-score transformation by subtracting the mean and dividing by the standard deviation. Environmental variables that were non-normally distributed were $\log_e$ transformed prior to normalization. Principal Components Analysis (PCA) was used to examine patterns of correlation among environmental variables. A one-way analysis of variance (ANOVA) was used to determine differences in mean nitrate concentrations in lakes between park jurisdictions. Linear regression was used to determine the association between temperature and lake elevation.

Ecological community analyses were conducted using the Vegan package version 2.3-5 (Oksanen et al. 2016). To analyze species richness, the OTU richness for each lake was rarefied to the smallest sample size (minimum number of individual reads in a lake community) using the 'rarefy' function in the vegan package. OTU evenness in each lake was calculated in the vegan package using the Pielou's evenness ($J = -\Sigma_i p_i \log_b p_i$/log(species richness)).

Multiple linear regression was used in conjunction with model simplification procedures to identify environmental variables that best predict variation in OTU richness and evenness across lakes. Model simplification procedures included Akaike's Information Criterion (AIC) using backward stepwise procedure with the stepAIC function in the MASS package (Venables and Ripley 2002) and model averaging using the dredge function in the MuMin package, which performs automated model selection with subsets of the supplied 'global' model including all predictor variables.

To visualize dissimilarities in community composition across lakes, we generated a Nonmetric Multidimensional Scaling plot with a 2D solution based on Bray-Curtis

dissimilarity of OTU relative abundance data ('metaMDS' function in vegan) for both prokaryotic [*n*=34, stress=0.16] and eukaryotic [*n*=32, stress= 0.21] communities. As nMDS axis scores represent rank orders of community similarity, we extracted axis 1 and 2 scores and ran linear regressions of these axis scores against major taxonomic groups in both prokaryotic and eukaryotic communities to visualize which taxonomic groups increase or decrease across each nMDS axis (SI Figs. S3 & S4)

To test the effects of environmental variables on dissimilarities in community structure across lakes, we used distance-based redundancy analysis (dbRDA) (Legendre & Anderson, 1999) using the 'capscale' function in vegan, ordinated with Bray-Curtis distances calculated from OTU relative abundance data in each lake. The vegan 'ordiR2step' function was used to carry out forward selection of explanatory variables from a global model while maximizing adjusted R^2 values at each step (Blanchet et al. 2008) to construct the most parsimonious dbRDA model. The global model before model selection procedures included the following environmental variables: surface temperature, TN, SUVA, log DOC, log Freshness Index, and log NO_3^-. TP was below detection in a majority of lakes and therefore was not included as an explanatory variable in models. Variables that were found to be significant in the global model were included in the dbRDA model, along with terms that tested for interactions between those resource variables and temperature. Additionally, nMDS axis 1 and 2 scores from the microbial community in each lake were in included in the model (that is, the first and second nMDS axis for eukaryotic composition was included as an explanatory variable in the prokaryotic model and vice versa) to account for biotic interactions between the two communities. Thirty-two lakes were included in the model for prokaryotes but only

twenty-eight lakes were included in the model for eukaryotes due to missing NO_3^- data in four lakes. P-values were calculated using 10,000 random permutations. Relative abundances of major taxonomic groups were examined in relation to variables identified as significant in models. We examined phylum-level shifts for prokaryotes along elevation gradients using linear regression and Welch's two-sample t-tests that compared lakes above and below treeline (2900m).

Mantel tests were used to test for correlations between prokaryotic and eukaryotic relative abundance (Bray-Curtis dissimilarity matrices) and presence-absence data (Sorenson distance matrices); between Bray-Curtis dissimilarity matrices and environmental data (using a Euclidean distance matrix containing environmental variables included in the aforementioned dbRDA global model); and for spatial autocorrelation between each Bray-Curtis dissimilarity matrix and site using a Euclidean distance matrix containing the latitude and longitude of each lake. P-values were calculated using 10,000 random permutations. Finally, a Pearson correlation matrix (n=28) was computed to show correlations between relative abundance data for dominant prokaryotic (Alphaproteobacteria, Betaproteobacteria, Cytophagia, Opitutae, Sphingobacteriia, Flavobacteria, Actinobacteria) and photosynthetic eukaryotic (Dinophyceae, Chrysophyceae-Synurophyceae, Cryptophyceae, Chlorophyceae) classes and environmental data.

Random forest regression models were also constructed to predict the relative abundance of the dominant taxonomic groups described above (see description of Pearson correlation matrix) in the prokaryotic and eukaryotic data sets. The models predicted the number of reads recorded for each dominant taxonomic group based either

only on the other taxonomic groups (both prokaryotes and eukaryotes), only the environmental data, or both. The analysis constructed 400 trees (ntree=400 in the R package "randomForest") and randomly added 3 variables (mtry=3) at each step. The models were compared by the mean squared error (mse) of the 400th model. The mse generally stabilized after about 50-100 trees (ie. adding new variables to the model did not change the mse beyond the first 50-100).

Results

Environmental and spatial gradients

Lake surface temperature was inversely correlated with elevation (adj.R^2= 0.2347, p<0.01). Mean lake nitrate concentrations ranged from 0.0044 to 2.59 μM and were significantly higher in Sequoia National Park (the most southerly lakes) than in Yosemite National Park (p<0.001) or Inyo National Forest (p<0.001) to the north. DOC quantity and quality varied among lakes (DOC ranged from 68.8 to 874.5 μM, SUVA ranged from 0.063 to 2.68 L $mg^{-1}m^{-1}$, and Freshness Index ranged from 0.34 to 0.77), but neither DOC quantity nor quality were significantly correlated with elevation (Fig. 1A.1).

16S and 18S abundant taxa

A total of 8810 unique OTUs were identified in the 16S library, the majority of which belonged to the following taxa: HgcI clade (24.5%), Flavobacterium (12.5%), Sediminibacterium (9.9%), Arcicella (5.8%), Polynucleobacter (4.2%), Var.78 (3.2%) Ferruginibacter (2.4%) and uncultured varieties (Fig. 1A.2 shows relative abundances of genera by lake). At the class level, the most abundant groups in the library were

Actinobacteria (24.9%), Betaproteobacteria (20.5%), Sphingobacteriia (17.6%), Flavobacteria (14.5%), Alphaproteobacteria (9.2%), Opitutae (3.1%), Cytophagia (2.7%) and Gammaproteobacteria (2.1%).

The 18S library contained 453 unique OTUs (after removing metazoan reads), of which the majority belonged to photosynthetic classes (Dinophyceae (49.5%), Chrysophyceae-Synurophyceae (10.7%), unclassified Alveolata (4.4%), Cryptophyceae (3.5%), Chlorophyceae (1.0%)) and a smaller percentage in non-photosynthetic classes, including Ichthyosporea (8.5%), Bicoecea (7.9%), Chytridiomycota (7.0%), Spirotrichea (3.5%) (Fig. 1A.3 shows relative abundances of classes by lake).

nMDS analysis

The nMDS plot of prokaryotic communities at the OTU level (Fig. 1.1) shows that lake communities cluster broadly by elevation and vegetation zone along axis 1, with lakes below treeline and above treeline clustering separately. Several bacterial classes showed significant shifts in relative abundance along the nMDS axes: relative abundances of Cytophagia and Opitutae are greatest at positive values of nMDS1, while negative values correlated with higher relative abundances of Betaproteobacteria and Alphaproteobacteria (Fig. 1.1 & Fig. 1A.4). On axis 2, positive values are correlated with higher relative abundances of Flavobacteria and negative values are correlated with higher abundances of Actinobacteria (Fig. 1.1 & Fig. 1A.4).

Eukaryotic communities do not segregate as clearly by elevation/vegetation zone in the nMDS ordination (Fig. 1.2). However, several photosynthetic classes do show patterns along each axis: Cryptophyceae have positive loadings on nMDS axis 1, while

Chrysophyceae-Synurophyceae have negative loadings (Fig. 1.2 & Fig. 1A.5). On axis 2, positive values are correlated with higher abundances of Dinophyceae, while Cryptophyceae have negative loadings (Fig. 1.2 & Fig. 1A.5).

Distance-based RDA (dbRDA)

We found that the strongest predictors of prokaryotic OTU community composition were eukaryotic nMDS axis 1 scores. Specifically, as the abundance of Cryptophyceae, and Chrysophyceae-Synurophyceae changed significantly, the composition of prokaryotes also changed concordantly (Table 1.1, p=0.001). Microbial resources were also important for structuring prokaryote communities. Total DOC concentration (Table 1.1, p<0.01), freshness index (Table 1.1, p<0.01) and SUVA (Table 1.1, p<0.05) all impacted prokaryotic community composition. The best predictors of eukaryotic OTU community composition were prokaryotic nMDS axis 1 scores (associated with significant shifts in Alphaproteobacteria, Betaproteobacteria, Cytophagia, and Opitutae abundance; Table 1.1, p<0.01). We found that SUVA was also an important determinant of eukaryotic community composition (Table 1.1, p<0.01), but that in addition two different resources impacted community dissimilarity in this group: nitrate (Table 1.1, p<0.01) and TN (Table 1.1, p<0.05). Surprisingly, for both types of microbial communities, temperature did not alter how resources impacted community composition (i.e., no interaction terms between temperature and resources were significant).

We found that the relative abundance of Proteobacteria was negatively correlated with elevation (n=34, adj.R^2=0.29, p<0.001), and was lower above than below treeline

(Welch's two-sample t-test, t=3.68, p<0.01). Verrucomicrobia exhibited the opposite relationship with higher abundance above treeline (Welch's two-sample t-test, t=-3.04, p<0.01).

Mantel tests, Pearson correlation matrix, and Random Forest Models

The degree of spatial autocorrelation varied for prokaryotic and eukaryotic communities. Mantel tests for Bray-Curtis distance matrices and a Euclidean distance matrix containing latitude and longitude coordinates for each lake showed that the prokaryotic communities did not show significant spatial autocorrelation (n=34, r=0.063, p>0.05) while eukaryotic communities were spatially autocorrelated (n=32, r=0.23, p<0.01). However, despite the lack of correlation between prokaryotic community composition and space, and consistent with eukaryotes being a significant predictor in our dbRDA models, prokaryotic and eukaryotic communities are correlated with one another (n=32, r=0.39, p<0.0001, Fig. 1.3A). Furthermore, using presence-absence data instead of read abundance produced the same result (n=32, r=0.68 p<0.0001, Fig. 1.3A). Additionally, Mantel tests applied to the Bray-Curtis distance matrices and a Euclidean distance matrix with environmental variables of interest indicated that eukaryotic community matrices were significantly associated with environmental variables (n=32, r=0.27 p<0.01, Fig. 1.3A), while prokaryotic communities were not (n=34, r=0.12, p>0.05, Fig. 1.3A). Together, these results suggest that prokaryotic and eukaryotic communities are more strongly correlated with each other than with the measured spatial and environmental variables.

A correlation matrix in Fig. 3B shows Pearson correlations between dominant

prokaryotic and photosynthetic eukaryotic classes, and environmental variables. Strong correlations are evident among bacterial taxa (e.g., Cytophagia vs. Sphingobacteriia (r=0.39) and Alphaproteobacteria (r=-0.42); Flavobacteria vs. Actinobacteria (r=-0.54); Sphingobacteriia vs. Alphaproteobacteria (r=-0.43) and Betaproteobacteria (r=-0.41)), algal taxa (e.g., Cryptophyceae vs. Dinophyceae (r =-0.47)), and between bacteria and algae (e.g, Cryptophyceae vs. Opitutae (r=0.50) and Sphingobacteriia (r=0.46); Dinophyceae vs. Sphingobacteriia (r=-0.40), in addition to correlations between taxa and environmental variables (e.g., NO_3^- vs. Cryptophyceae (r=-0.44), Betaproteobacteria (r=0.39), and Opitutae (r=-0.39)).

For 3/4 of the eukaryotic groups (Chrysophyceae-Synurophyceae, Dinophyceae, Cryptophyceae), and for 5/7 of the prokaryotic groups (Actinobacteria, Cytophagia, Flavobacteria, Sphingobacteriia, Opitutae), the random forest model with just the abiotic environment had the highest mean square error, suggesting the abiotic data alone was not the best predictor of abundance of microbial groups (Figs. 1A.6 & 1A.7). In most cases, both the model with both abiotic and biotic predictors, and with just biotic predictors, had lower mse than the model with only abiotic predictors (Figs. 1A.6 & 1A.7). This supports the argument that correlations among groups were as strong or stronger than the correlations between taxonomic groups and the environment.

OTU richness and evenness

Before rarefaction, the total number of 16S reads across all lakes ranged from 244 to 1449 OTUs. After rarefaction, 16S OTU richness ranged from 40 to 79 across all lakes sampled, and both stepwise model selection by AIC and model averaging indicated that

the best explanatory variable of rarefied 16S OTU richness in lakes was N:P of POM. 16S richness was inversely correlated with N:P ratios (Fig. 1.4A, n=34, adj.R^2 =0.18, p<0.01), indicating that bacterial richness is higher in lakes with low N:P ratios (more P-rich POM). Stable isotope analysis of ^{13}C and C:N ratios indicated that the source of organic matter in POM is primarily phytoplankton rather than terrestrial detritus. 16S evenness was not correlated with N:P ratios or other environmental variables (Fig. 1.4C, n=34, adj.R^2 =-0.0002, p>0.05). The total number of 18S reads across all lakes ranged from 23 to 107 OTUs. After rarefaction, 18S OTU richness ranged from 4 to 15 OTUs, and both model simplification methods indicated that TN (uM) was the best explanatory variable for eukaryotic richness patterns, where richness is inversely related to TN (Fig. 4B, n=32, adj.R^2=0.26, p< 0.01). 18S evenness also declined significantly with increasing TN (Fig. 1.4D, n=32, adj.R^2=0.15, p<0.05).

Table 1.1: Results of dbRDA for prokaryotic and eukaryotic communities

Prokaryotic dbRDA	**Df**	**Sum of Squares**	**F value**	**Pr(>F)**
SUVA	1	0.4091	2.0403	**<0.05 ***
log DOC (uM)	1	0.4860	2.4238	**<0.01 ****
log Freshness Index	1	0.4425	2.2064	**<0.01 ****
18S nMDS axis 1	1	0.7073	3.5274	**0.001 *****
18S nMDS axis 2	1	0.2629	1.3110	0.158
Temperature	1	0.3254	1.6226	0.068
SUVA:Temperature	1	0.1996	0.9952	0.449
Temperature:log DOC	1	0.2167	1.0807	0.339
Temperature:log Freshness Index	1	0.2909	1.4506	0.085
Eukaryotic dbRDA				
SUVA	1	0.6181	2.0311	**<0.01 ****
log NO_3^- (uM)	1	0.6288	2.0663	**<0.01 ****
TN (uM)	1	0.5087	1.6715	**<0.05***
16S nMDS axis 1	1	0.6276	2.0623	**<0.01 ****
16S nMDS axis 2	1	0.3765	1.2371	0.220978
Temperature	1	0.4383	1.4404	0.100690
SUVA:Temperature	1	0.3074	1.0100	0.454255
Temperature:TN	1	0.2081	0.6839	0.860614
Temperature: log NO_3^-	1	0.2726	0.8958	0.585841
Residual	18	5.4777		

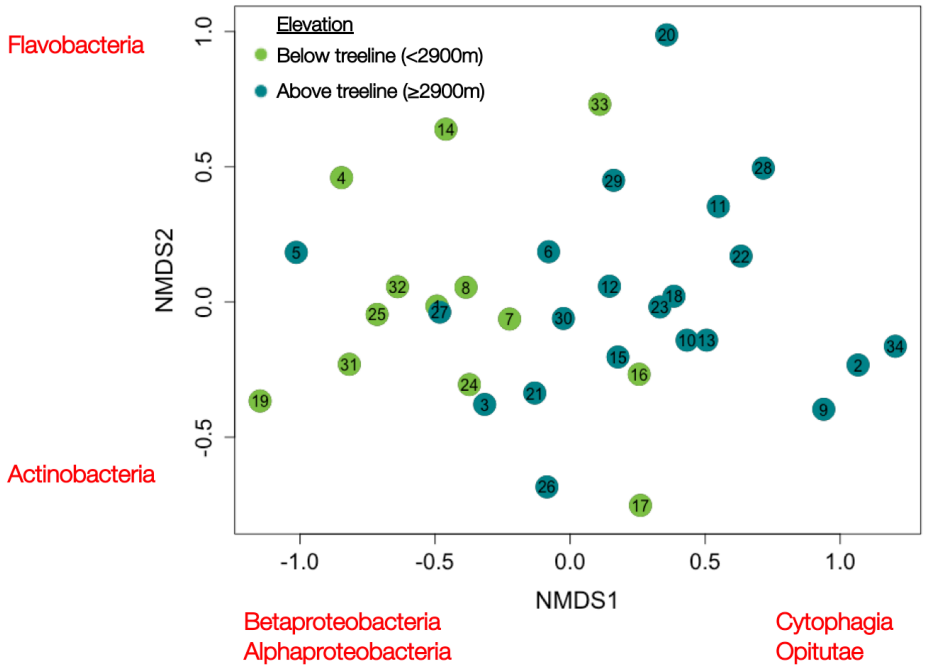

Figure 1.1: nMDS ordination of prokaryotic communities (*n*=34, stress=0.16) using Bray-Curtis distances at the OTU level is shown. Each point represents a lake community and is colored by vegetation zone: below treeline (<2900m) and above treeline (2900m and above). Numbers correspond to lake identity (SI Table S1), and bacterial classes that increase significantly along each ordination axis are shown in red.

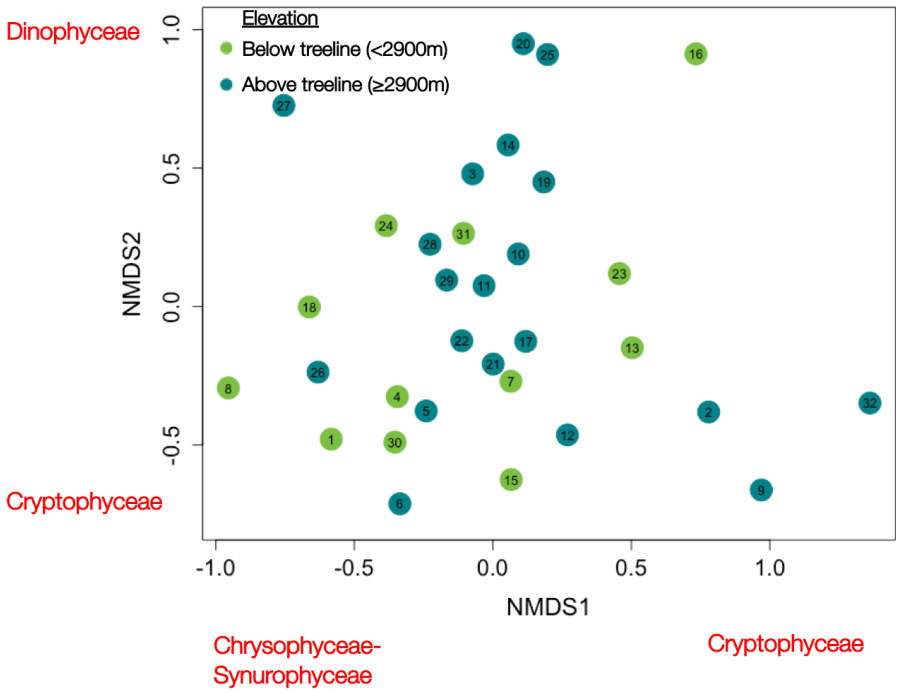

Figure 1.2: nMDS ordination of eukaryotic communities (*n*=32, stress= 0.21) using Bray-Curtis distances at the OTU level is shown. Each point represents a lake community and is colored by vegetation zone: below treeline (<2900m) and above treeline (2900m and above). Numbers correspond to lake identity (SI Table S1), and taxonomic groups that increase significantly along each ordination axis are shown in red.

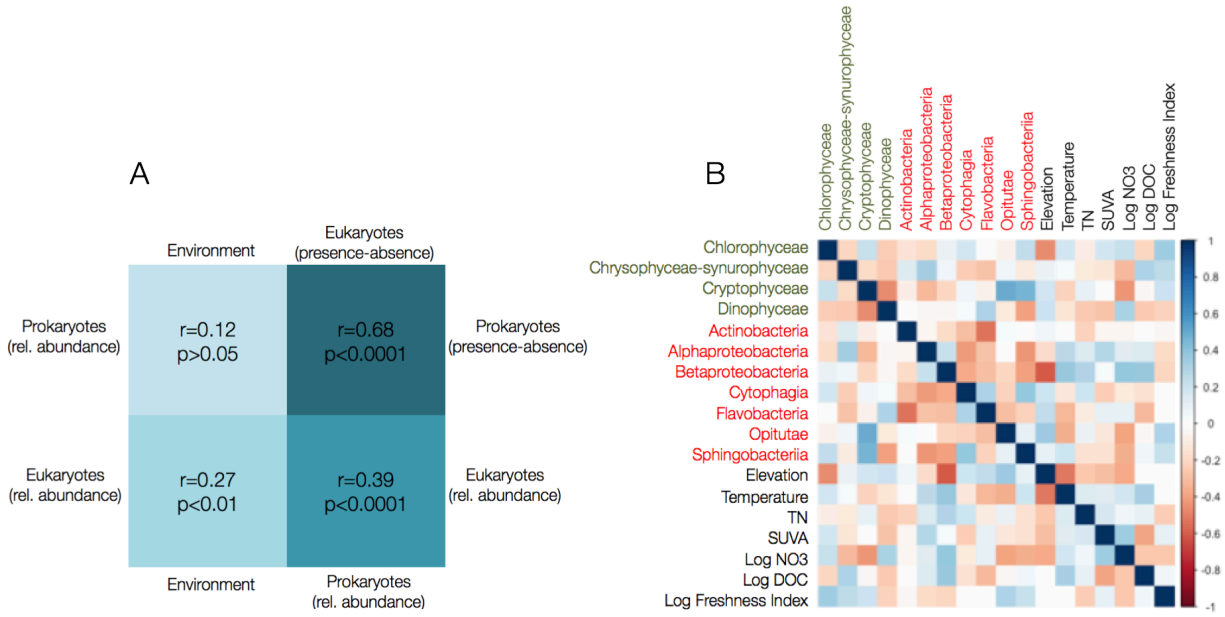

Figure 1.3: (A) Correlation matrix showing results from Mantel tests, with correlation (*r*) values and *p*-values. Labels adjacent to each square indicate which two distance matrices are being compared in each square, and colors indicate the strength of the correlation (darker shades indicate higher correlations). (B) Pearson correlation matrix between dominant photosynthetic eukaryotic classes (green text), dominant bacterial classes (red text) and environmental variables (black text). Positive correlations are shown in blue, and negative correlations are shown in red; stronger correlations are indicated by darker shades.

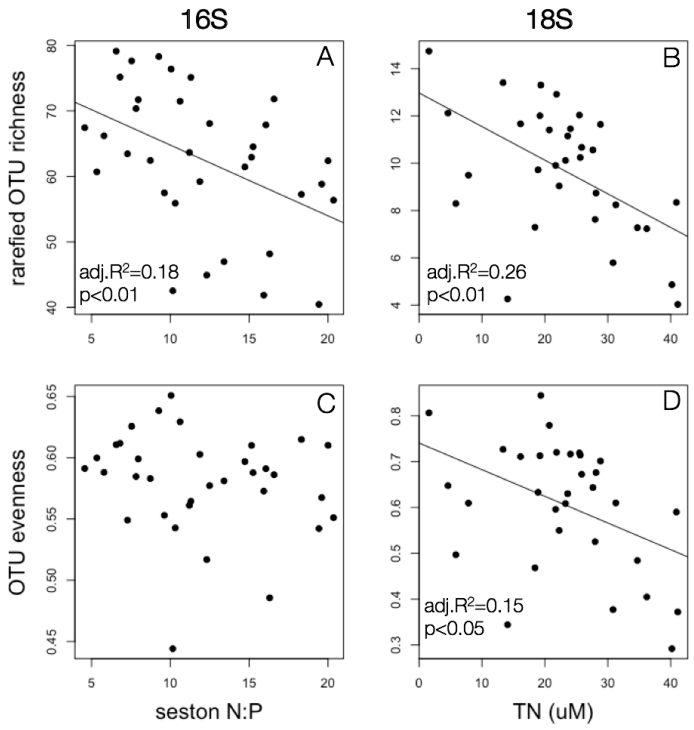

Figure 1.4: Richness (A) and evenness (B) of 16S OTUs are plotted against seston N:P, and richness (C) and evenness (D) of 18S OTUs plotted against TN. Significant relationships at $p<0.05$ are denoted with a regression line.

Discussion

Our survey of Sierra Nevada mountain lakes across broad elevational gradients showed independent roles for biotic interactions, organic, and inorganic resources in structuring prokaryotic and eukaryotic microbial communities. We also found that different organic and inorganic resources were associated with the community composition of prokaryotes and eukaryotes. For prokaryotes, DOC quantity and origin (the supplies of autochthonous and allochthonous DOC) predicted community composition (Table 1.1), while for eukaryotic communities, nitrate, total nitrogen and allochthonous DOC best explained variation in community composition (Table 1.1). Although the lakes showed substantial independent variation along the main

environmental axes, we found no indication of synergy or interference between temperature and the supplies of organic or inorganic resources. Thus, warmer lake temperatures do not appear to alter the impacts of nitrogen deposited from the atmosphere or organic detritus from the watershed. Additionally, the composition of prokaryotic and eukaryotic microbial communities (measured either as abundance or presence-absence) were strongly correlated among lakes (Figs. 1.3A, 1.3B), suggesting that interactions and feedbacks between prokaryotic and eukaryotic microbes may influence community assembly across environmental gradients.

Contrary to our expectations, temperature was not a primary force structuring prokaryotic or eukaryotic community composition along our elevation gradient. Previous studies have found that temperature is a strong explanatory variable in lake epilimnial bacterial communities across different geographic regions (Yanarell & Triplett 2004, Lindstrom et al. 2005, Shade et al. 2007). Temperature has also been found to be an important determinant of eukaryotic microbial communities, as it drives physical and phenological processes such as mixing and bloom dynamics in aquatic systems (Sommer et al. 1986, Falkowski & Oliver 2007). While we hypothesized that temperature would be an important determinant of prokaryotic and eukaryotic microbial communities, we found no significant association between community composition and temperature after accounting for the roles of nutrients, DOC, and taxonomic groups. The lack of association between temperature and community composition across lakes was surprising given the 10°C range among our lakes, but seasonal phenology and thermal stability of lakes may be more important for community composition than temperature *per se,* as Sierra Nevada lake bacterioplankton communities exhibit strong interannual phenological

patterns associated with snowmelt, ice-off and mixing during spring and fall (Nelson 2009). During the summer when we sampled, shifts in communities were better predicted by organic and inorganic resources and the identities and abundances of other microbial groups, indicating that temperature may play a secondary role to resource supply in structuring these lake microbial communities, consistent with previous findings that the effect of warming depends on trophic state and has a smaller effect on microbes in oligotrophic environments (Elliott et al. 2006, Huber et al. 2008, Tadonléké 2010 , Rigosi et al. 2014).

We found that DOC quantity and origin – specifically, the balance between organic compounds of aquatic and terrestrial origin, as indicated by Freshness Index and SUVA – were important determinants of prokaryotic community composition (Table 1.1), consistent with our hypotheses and previous studies in aquatic ecosystems. We expected DOC origin to shift along elevation, as allochthonous, plant-derived DOC inputs are highest below treeline in subalpine lakes, and DOC is more autochthonous above treeline in alpine lakes (Rose et al. 2015). However, we found no significant relationship between DOC quantity or origin and elevation (Fig. 1A.1), potentially due to seasonal atmospheric deposition of allochthonous carbon dust to high elevation lakes (Mladenov et al. 2011, Oldani et al. 2017). DOC source and quantity are important drivers of spatial and temporal shifts in bacterial community structure and metabolism in freshwater ecosystems, including Sierra Nevada lakes (Yanarell and Triplett 2004, Nelson 2009, Nelson et al. 2009, Sadro et al. 2011a, Lennon & Pfaff 2005). Previous studies have found that in most clear-water lakes, DOC exudates released by phytoplankton production fuel 50% or more of bacterial production (Cole et al. 1982,

reviewed in Hessen 1992) and can drive shifts in community composition (Perez and Sommaruga 2006, Nelson 2009). Our results support the importance of DOC origin as a predictor of both eukaryotic and prokaryotic microbial communities.

While we did not observe significant changes in DOC concentration or origin across elevation, we observed distinct clustering of bacterial communities by lakes above and below treeline (Fig. 1.1) and significant shifts in relative abundances of some bacterial taxa at treeline elevation, possibly due to decreased watershed vegetation above treeline in alpine lakes. We found that Proteobacteria (especially Betaproteobacteria) are more abundant at lower elevations in subalpine lakes and decrease at higher elevations above treeline. Betaproteobacteria have previously been found to be associated with increased terrestrial DOC fluxes in Sierra Nevada lakes, seasonally associated with snow-melt and ice-off (Nelson 2009), consistent with our results. Betaproteobacteria may therefore be particularly reliant on organic substrates of terrestrial origin. In contrast, Verrucomicrobia were more abundant above treeline. Previous findings have indicated that that some types of Verrucomicrobia may be copiotrophic, associated with algal blooms or high nutrient environments, and possible symbionts of eukaryotes (Newton et al. 2011). Verrucomicrobia may therefore decline in abundance at lower elevation if aquatic production is lower than allochthonous inputs.

Allochthonous carbon (measured as SUVA) affected the composition of both prokaryotic and eukaryotic communities. Whether allochthonous DOC affects eukaryotic microbes (which are mainly phytoplankton) primarily by suppressing photosynthesis (Carpenter et al. 1998, 2016), or fueling heterotrophic production (Jones et al. 1997) in our lakes remains uncertain, but warming and brownification together can favor

conditions for mixotrophy and increase the abundance of mixotrophs in the community (Urrutia-Cordero et al. 2017, Wilken et al. 2018). Our eukaryotic communities are dominated by dinoflagellates, chrysophytes and cryptophytes (Fig. 1A.2), which are often mixotrophic (Jones et al. 1997, Stoecker 1999). Research has shown that mixotrophy is favored in oligotrophic environments (Sanders 1991) such as mountain lakes, as it allows phototrophic species to employ phagotrophy to supplement energetic requirements when conditions for autotrophy become limiting due to low inorganic nutrient availability (Jones et al. 1997, Stoecker 1999).

The impact of TN and NO_3^- on eukaryotic communities likely relates to phytoplankton taxonomic shifts along the N deposition gradient, selecting for more nitrophilous species as N increases in lakes. Previous studies on N deposition in mountain lakes have shown that chrysophytes favor low-N lakes while cyanophytes and chlorophytes favor high-N lakes (LaFrancois and Nydick 2003). While we did not observe these same taxonomic shifts along our N deposition gradient, the greater importance of organic resources relative to NO_3^- on the prokaryotic community indicates that the north-south gradient in N supply associated with atmospheric deposition plays a greater role in shaping the eukaryotic component of the microbial assemblage than the prokaryotes.

Microbial community composition, and the abundances of particular taxonomic groups, were often better predicted by the identities and numbers of other groups present than measured aspects of the physical environment. The correlations among taxonomic components of the prokaryotic and eukaryotic microbial communities, and the importance of the first nMDS axis in dbRDA models for both microbial communities

(Table 1.1), suggest either that unmeasured environmental variables are driving the correlation, or that interaction networks link the two communities (reviewed in Faust & Raes 2012). For instance, heterotrophic bacteria rely on phytoplankton for extracellular exudates, and phytoplankton utilize nutrients that are remineralized by bacterioplankton via the microbial loop (Azam et al. 1983, Hessen 1992, Tranvik 1992). Nutrient recycling loops may cause tight associations between groups of heterotrophic and autotrophic microbes through community assembly, leading to the strong associations in composition that we observed between bacteria and eukaryotes independent of the environmental parameters we measured. Studies have shown correlated patterns of succession between bacterial and phytoplankton communities in lakes where changes in phytoplankton communities over time are driven by climate and meteorological variability, and bacterial communities shift synchronously as the phytoplankton community changes (Newton et al. 2006, Kent et al. 2007). Strong interactions among microbes may therefore determine community composition in addition to water chemistry, temperature and the availability of organic and inorganic resources.

In the oligotrophic lakes of the Sierra Nevada, the bacteria and phytoplankton communities may be tightly coupled due to low nutrient availability, especially for dissolved phosphorus (P), which was below detection in most of our lakes. In low nutrient environments, bacteria are competitively superior to phytoplankton for limiting nutrients such as PO_4^{3-} because of their small cell size and high surface area to volume ratio, and can take up at least 50% of P in lakes (Currie and Kalff 1984, Cotner and Wetzel 1992, reviewed in Kirchman 1994). Bacterioplankton in a Sierra Nevada lake respond rapidly to P additions, indicating likely P-limitation and P-induced increases in

growth and productivity of the bacterial community (Nelson and Carlson 2011). The relationship between bacterioplankton and phytoplankton may be mutualistic at steady state conditions, because bacterial growth rates are ultimately limited by the availability of DOC and thus the rate of CO_2 fixation by phytoplankton, while phytoplankton rely on bacterioplankton to remineralize P to bioavailable forms (Currie 1990). Regardless of specific taxonomic group patterns, the strong correlations between our phytoplankton and bacterioplankton communities may be centered on a mutualism involving the cycling of DOC to bacteria and nutrients (especially P) to phytoplankton.

The finding that richness (prokaryotes and eukaryotes) and evenness (eukaryotes) decrease as N enrichment increases is consistent with previous studies in terrestrial ecosystems, in communities of both terrestrial plants and soil microbes. In terrestrial plant communities, several studies have found that atmospheric N deposition causes loss of rare species and reduced richness and evenness (Harpole & Tilman 2007, reviewed in Cleland & Harpole 2010). Some soil microbial communities show similar patterns of species loss with N enrichment (Ramirez et al. 2010), with consequences for functional potential of those communities. Reduced niche dimension may be driving species loss with N enrichment, due to an imbalanced supply of limiting resources and subsequent loss of species diversity and productivity (Harpole & Tilman 2007). Increasing N inputs are likely to exacerbate P-limitation and eliminate all but the strongest P-competitors in the phytoplankton community (Elser et al. 2009a). The decreasing richness of prokaryotic communities with increasing seston N:P (Fig. 4A) is also consistent with this explanation. Our findings suggest that increasing N deposition may reduce the taxonomic and functional diversity of prokaryotic and eukaryotic microbes in aquatic ecosystems.

Our study shows that inorganic and organic resources structure prokaryotic and eukaryotic microbial communities along natural climatic gradients in mountain lakes, but showed no indication of synergies or interference despite substantial independent variation between resources and temperature. Climate and bottom-up factors may therefore operate independently in structuring some aspects of microbial assemblages. We also found that relative abundances and membership in the two communities were strongly correlated, suggesting that biotic interactions between certain taxonomic groups may place constraints on community membership and abundance. Finally, the reduction of OTU richness for both prokaryotes and eukaryotes as N increases is consistent with other findings in plant and soil microbial communities and may indicate a universal response to N deposition of decreasing richness and increasing dominance of taxa across a diverse range of ecosystems. As micro-organisms are central to biogeochemical cycling and trophic interactions, their response to environmental changes are likely to have a large effect on ecosystems.

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Appendix 1A

Table 1A.1: List of lake names, numbers corresponding to points in nMDS plots, elevation, latitude, longitude, and park jurisdiction (SEKI=Sequoia-King's Canyon National Park; INYO=Inyo National Forest; YOSE=Yosemite National Park)

16S NMDS	18S NMDS	Lake	Elevation (m)	Latitude	Longitude	Park Jurisdiction
1	1	Aster	2774	36.6026149	-118.679035	SEKI
2	2	Blue	3015	38.0511336	-119.2703313	INYO
3	3	Cascade	3147	37.9900446	-119.3065766	INYO
4	4	Cathedral 1	2832	37.8450836	-119.4243486	YOSE
5	5	Cathedral 2	2924	37.8393589	-119.4155802	YOSE
6	6	Crystal	2932	37.5938711	-119.018434	INYO
7	7	Elizabeth	2897	37.8451855	-119.3699099	YOSE
8	8	Emerald	2814	36.5974591	-118.6759219	SEKI
9	9	Gardisky	3198	37.9562813	-119.2515007	INYO
10	10	Gaylor 1	3151	37.9126276	-119.2688475	YOSE
11	--	Gaylor 2	3205	37.9226947	-119.2673837	YOSE
12	11	Granite 1	3167	37.9217949	-119.2758264	YOSE
13	12	Granite 2	3178	37.9260157	-119.2791797	YOSE
14	13	Grant 1	2826	37.8865939	-119.5391612	YOSE
15	14	Greenstone	3092	37.9798401	-119.2904565	INYO
16	15	Hamilton 1	2433	36.5669904	-118.5818421	SEKI
17	16	Hamilton 2	2512	36.5619759	-118.5757782	SEKI
18	17	Heart	3294	37.4185378	-118.7544477	INYO
19	18	Heather	2825	36.6010482	-118.6879484	SEKI
20	19	Helen	3337	37.8306612	-119.2287315	YOSE
21	20	Little Steelhead	3143	37.993143	-119.3002142	INYO
22	21	Long	3279	37.4056012	-118.7590982	INYO
23	22	Mack	3156	37.4272646	-118.7507729	INYO
24	23	May	2845	37.8474768	-119.4930758	YOSE
25	24	Mcleod	2832	37.6079948	-119.0306013	INYO
26	25	Nine Lakes 1	3187	36.5607234	-118.5464254	SEKI
27	26	Pear	2909	36.6012316	-118.6675004	SEKI
28	27	Serene	3120	37.43842	-118.7444485	INYO
29	28	Spillway	3186	37.8415211	-119.2324424	YOSE
30	29	Steelhead	3137	37.993199	-119.3026364	INYO
31	--	Sunrise 1	2801	37.8041514	-119.4521835	YOSE
32	30	Sunrise 3	2882	37.8062159	-119.443259	YOSE
33	31	TJ	2832	37.5916442	-119.007691	INYO
34	32	Virginia	2993	38.0469747	-119.2650757	INYO

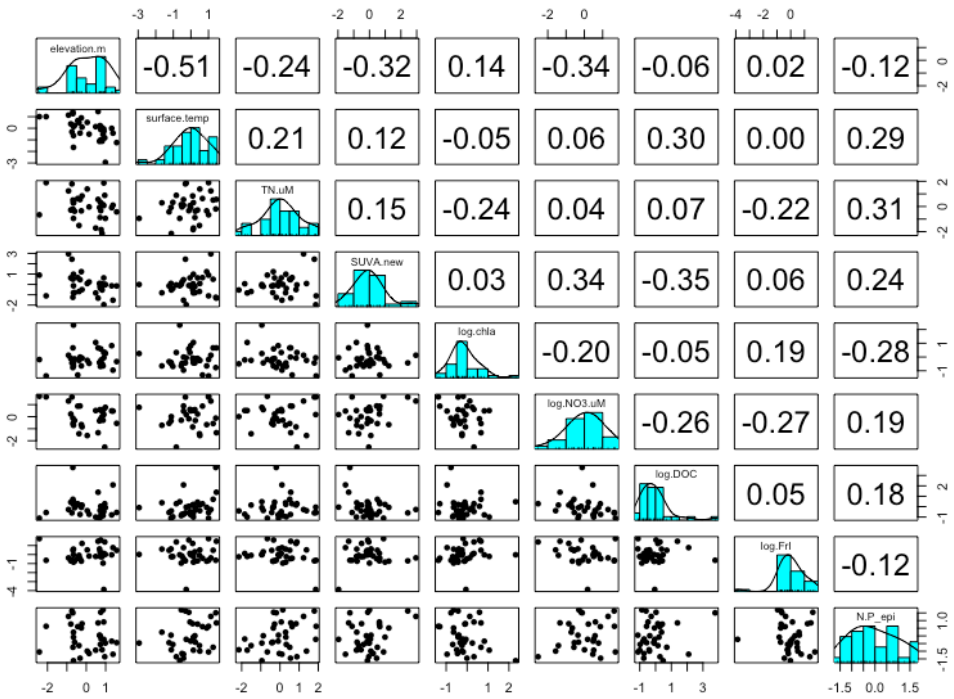

Figure 1A.1: Pairs panel showing pairwise correlations between z-score transformed environmental variables, and histograms for each variable in blue (n=28 due to missing nitrate data in 6 lakes). The scatterplot or correlation value for any two variables occurs at the intersection of the row and column for those two variables from the diagonal blue histogram panel. The numbers in the panels above the diagonal are the Pearson correlation coefficients between the two variables in the same row and column.

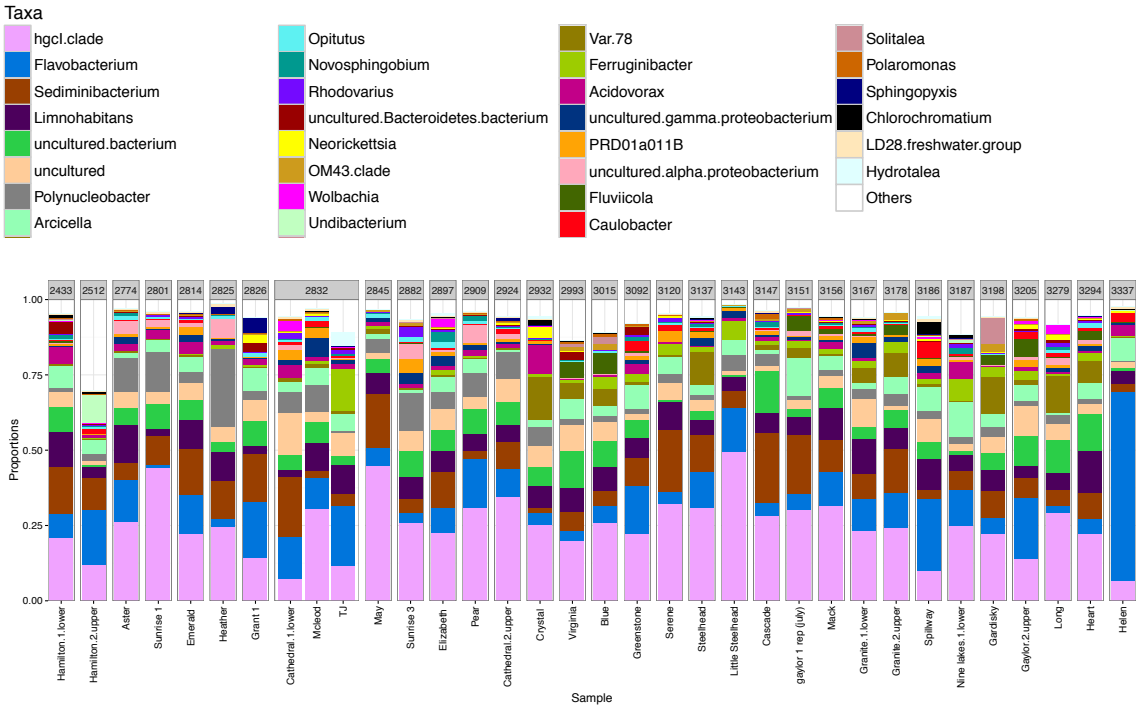

Figure 1A.2: Relative abundances of prokaryotic genera in each lake shown from low (left) to high (right) elevation, with the grey panel displaying the elevation in meters ASL.

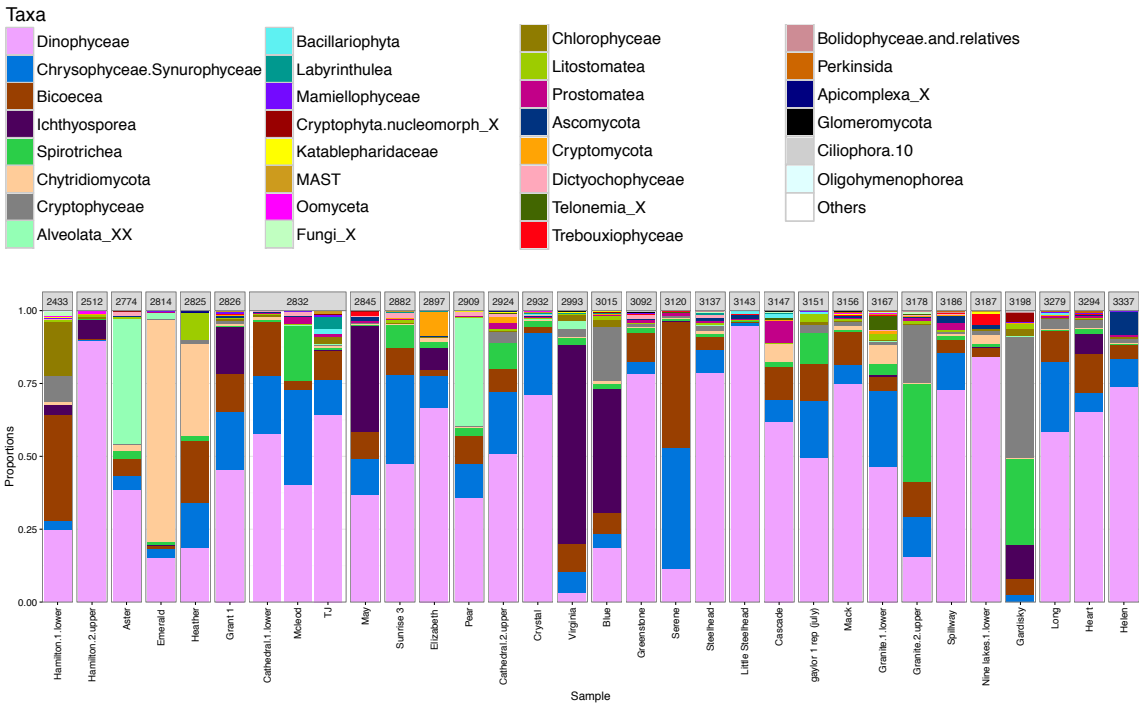

Figure 1A.3: Relative abundances of eukaryotic classes in each lake shown from low (left) to high (right) elevation. with the grey panel displaying the elevation in meters ASL.

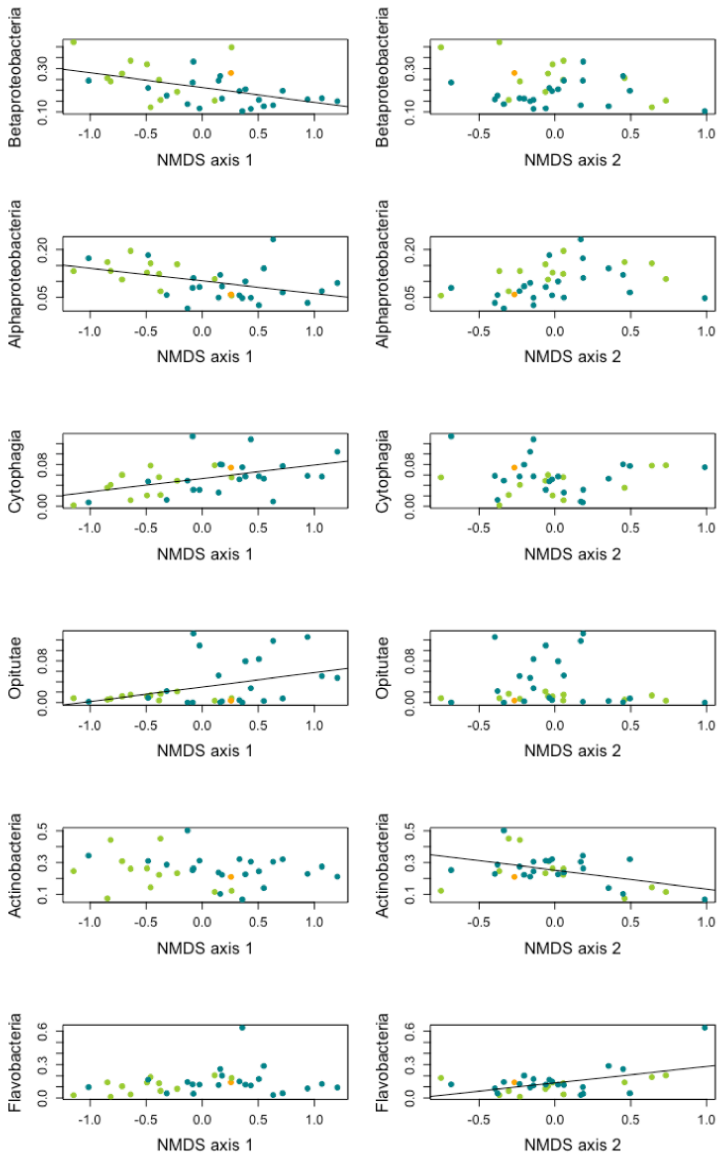

Figure 1A.4: Plots show the relationship between relative abundances of dominant prokaryotic classes and nMDS axis 1 and 2 scores (ordinated at OTU level), with points colored by elevation (orange: montane (below 2450m); green: subalpine (2450-2900m); turquoise: alpine (2900m and above)). Significant relationships at $p<0.05$ are denoted with a regression line.

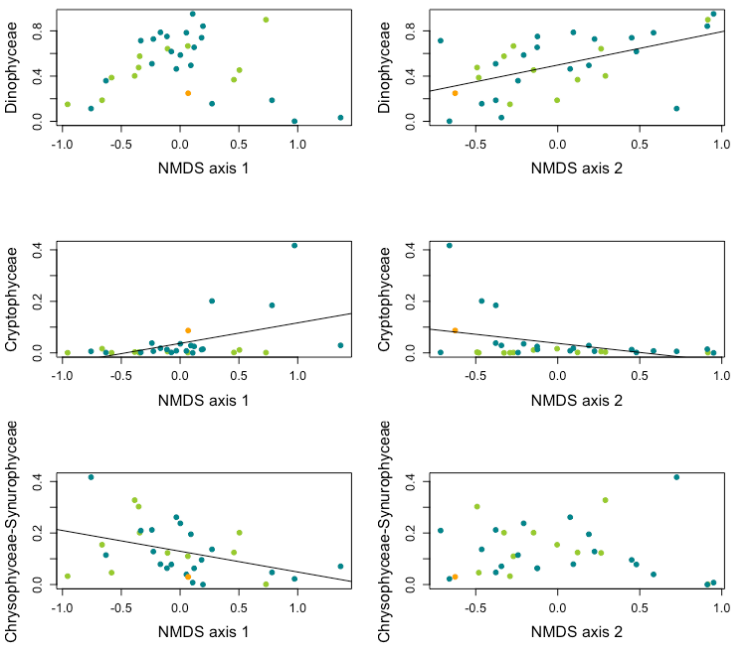

Figure 1A.5: Plots show the relationship between relative abundances of dominant photosynthetic eukaryotic classes and nMDS axis 1 and 2 scores (ordinated at OTU level), with points colored by elevation (orange: montane (below 2450m); green: subalpine (2450-2900m); turquoise: alpine (2900m and above)). Significant relationships at $p<0.05$ are denoted with a regression line.

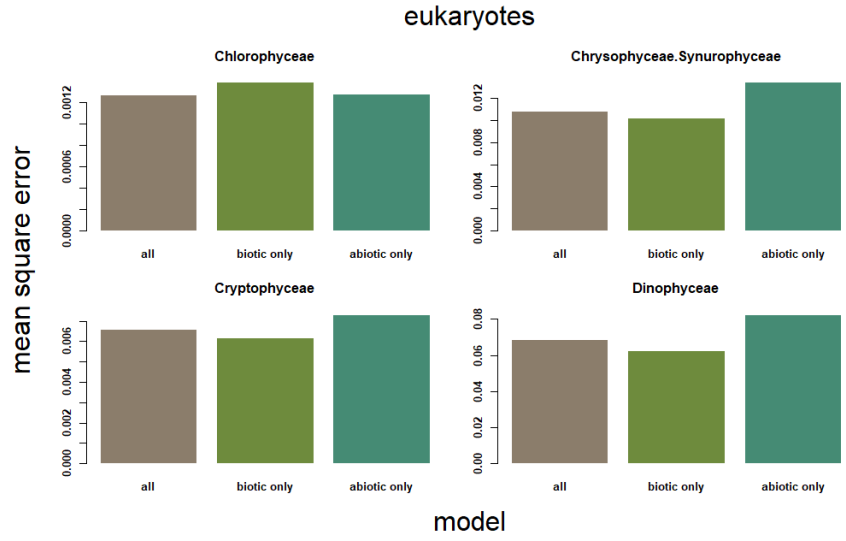

Figure 1A.6: Mean squared errors from random forest models that include biotic, abiotic, and both predictors in modeling relative abundance of dominant eukaryotic taxa. For ¾ of taxa (all except Chlorophytes), the "abiotic only" model had the highest MSE (worst prediction). For chlorophytes, the best model was "all" with both environment and other organismal groups), so in no case was the "abiotic only" the best model.

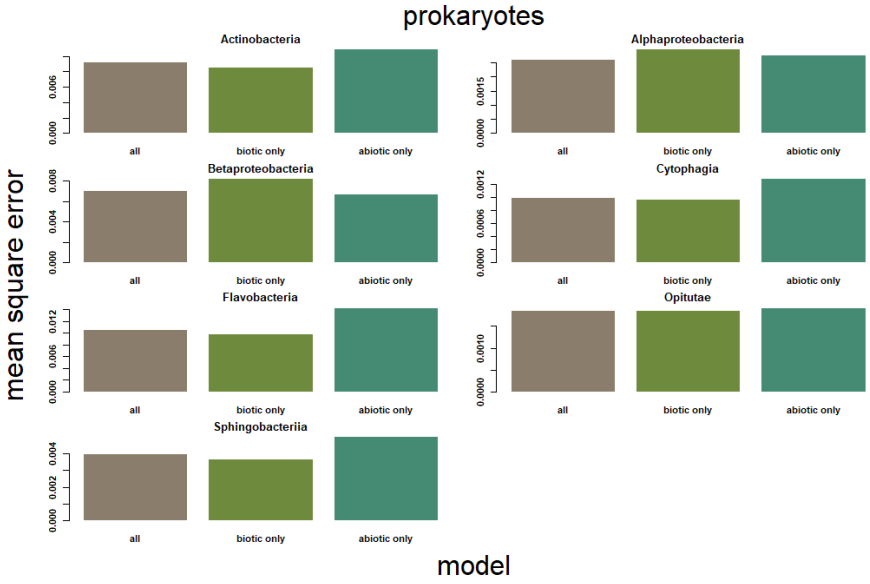

Figure 1A.7: Mean squared errors from random forest models that include biotic, abiotic, and both predictors in modeling relative abundance of dominant prokaryotic taxa. The "abiotic only" model was the worst for 5/7 groups (all but Betaproteobacteria and Alphaproteobacteria). For Alphaproteobacteria, the best model included both abiotic and biotic predictors, and for Betaproteobacteria, the best model was the abiotic only.

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CHAPTER 2

Phytoplankton stoichiometric responses to warming, nutrient addition and grazing depend on lake productivity and cell size

Abstract

Phytoplankton stoichiometry is important for nutrient cycling and food web dynamics and may be affected by temperature, grazing and nutrient supply. The generality of and interactions among these effects in lakes of varying trophic status, or among phytoplankton of variable cell sizes, are poorly understood. In order to test for the effects of warming on phytoplankton net growth rate, biomass buildup, and C:N:P stoichiometry in the presence and absence of grazing and nutrient addition, we conducted microcosm experiments using phytoplankton communities from three Dutch lakes across a trophic gradient and measured the response of each community to multifactorial combinations of nutrient, temperature, and grazing treatments. We found that nutrients elevated growth rates and biomass build-up but reduced C:nutrient ratios of all three phytoplankton communities, but the main and interactive effects of temperature and grazing depended on lake productivity. Grazing had the strongest effect on the lowest productivity community while temperature most impacted the highest productivity community. Additionally, C:P of small cell size classes (the <30 μm size fraction) were more sensitive to warming and grazing than cells >30 μm. Our experiments indicate that stoichiometric responses to warming and interactions with nutrient addition and grazing are not universal but instead depend on lake productivity and cell size distribution. The effects of climate warming on phytoplankton stoichiometry therefore depend on interactions between resources, consumers and the size structure of the local community.

Introduction

Global environmental change involves shifts in nutrient fluxes and alterations to the climate that affect the structure and functioning of food webs. Shifts in nutrient supply due to eutrophication and climate warming may alter the elemental composition of primary producers with consequences for higher trophic levels. Surface temperatures of lakes worldwide have warmed significantly since 1985, and in some areas, have increased more rapidly than air temperature (O'Reilly et al. 2015, Schneider & Hook 2010). Additionally, point and non-point sources of nutrients to water bodies cause eutrophication due to excessive phosphorus (P) and nitrogen (N) inputs (Carpenter et al. 1998). Human enrichment of terrestrial and aquatic systems with N and P also changes biogeochemical cycling processes and results in stoichiometrically imbalanced systems (Sterner & Elser 2002, Van de Waal et al. 2010) and altered nutrient limitation patterns (Elser et al. 2009, Sickman et al. 2003) with attendant consequences for food webs (Sterner & Elser 2002).

Ecological stoichiometry describes the balance of energy (as carbon (C)) and nutrients between organisms and their environment, and can support our understanding of the impacts of environmental change on food webs. Elemental ratios of autotrophic biomass are important for understanding relationships between environmental nutrient supply, uptake by autotrophs, species composition, producer-consumer interactions, and biogeochemical cycling. Elemental stoichiometry of phytoplankton, which are predominant autotrophs in aquatic systems, is primarily influenced by environmental supply of inorganic nutrients, most notably N and P (Sterner and Elser 2002). Moreover, phytoplankton stoichiometry depends on various traits, such as cell size and growth rate (reviewed in Finkel et al. 2010).

Cell size of phytoplankton influences key cellular processes such as nutrient uptake and utilization strategies, in addition to trophic interactions. Size affects nutrient uptake and growth rates according to allometric scaling relationships (Litchman et al. 2007). Small cells tend to have higher maximum growth rates and are able to acquire limiting nutrients more efficiently due to the high surface area to volume ratio and smaller diffusion boundary layer, while large cells have greater maximum uptake rates per cell and may have larger storage capacity for nutrients (reviewed in Litchman and Klausmeier 2008). Low-nutrient environments should therefore favor small cells that are strong nutrient competitors, while high and fluctuating nutrient environments should be dominated by large-celled species (Irwin et al. 2006, Litchman et al. 2007, Litchman et al. 2010, Edwards et al. 2011, Cloern et al. 2018,). In addition, the size of phytoplankton has an important role in trophic interactions and susceptibility to grazing by zooplankton, as increasing cell size results in greater resistance to gape-limited grazers (reviewed in Litchman and Klausmeier 2008, Litchman et al. 2010, Ward et al. 2014).

Elemental ratios are important for understanding how resources are allocated in cells to support cellular functions and overall metabolism. For instance, investment in P-rich ribosomes is required for growth, while N-rich proteins are required for resource acquisition. The growth rate hypothesis posits that allocation of P to ribosomes increases as growth rates increase, resulting in reduced N:P ratios (Elser et al. 2003; but see Flynn et al. 2010). Optimal N:P ratios vary across phytoplankton taxa and reflect nutrient requirements determined by their cellular machinery (Klausmeier et al. 2004), as well as the degree of stoichiometric plasticity, which is influenced by nutrient storage capacity (Hall et al. 2005). Phytoplankton N:P ratios are more constrained at high growth rates when nutrients are not limiting, while vary substantially when nutrients become limiting (Hillebrand et al. 2013).

Eutrophication is expected to reduce C:nutrient stoichiometry of phytoplankton due to increased nutrient availability (Sterner and Elser 2002), while shifts in N:P ratios will depend on the prevailing limiting nutrient, as well as on the N:P ratio of the nutrient loads. The effect of warming on phytoplankton stoichiometry, however, is less understood. Warming can increase N:P in eukaryotic phytoplankton due to increased rates of protein synthesis and a reduction in the quantity of ribosomes required to produce proteins (Toseland et al. 2013) and due to species shifts that favor higher C:P and N:P in warmer environments (Yvon-Durocher et al. 2017). Warming has also been shown to enhance C:nutrient stoichiometry of a phytoplankton community, particularly under oligotrophic (low P) conditions, which was presumably caused by enhanced nutrient use efficiencies (De Senerpont Domis et al. 2014). However, warming has also been found to reduce C:P and N:P ratios, due to increased nutrient availability as a result of nutrient recycling by consumers or heterotrophic microbes (Velthuis et al. 2017). The presence of grazers can also alter phytoplankton stoichiometry due to consumer-driven nutrient recycling (Elser and Urabe 1999). As zooplankton tend to have higher nutrient demands and exhibit lower stoichiometric plasticity as compared to phytoplankton (reviewed in Meunier et al. 2017), they retain more limiting nutrients and thereby further enhance nutrient limitation for phytoplankton (Elser and Urabe 1999). The effects of warming on phytoplankton stoichiometry is thus likely interact with nutrient loading as well as the abundance of zooplankton.

Here, we test the effects of warming, eutrophication and grazing on phytoplankton growth and stoichiometry. To this end, we conducted multifactorial experiments on three phytoplankton communities from Dutch lakes distributed across a productivity gradient. We measured net growth rates, maximum biomass buildup, N:P, and C:P for two size fractions (<30 μm and >30 μm) for all three communities and tested for the independent and interactive effects

of nutrient addition, warming, and grazing on each phytoplankton community. We hypothesized that C:P and N:P will decrease as a result of nutrient addition, and that the effect size of warming and grazing varies by lake due to differences in phytoplankton community composition. Additionally, we expected that the effect of warming on biomass will be strongest in conditions with ample nutrient availability supporting enhanced production (Tadonléké 2010), while the effects on stoichiometry will be strongest in conditions with low nutrient supply constraining phytoplankton growth leading to accumulation of excess elements.

Methods

Field sampling and experimental setup

Spring phytoplankton communities were collected from three lakes, sampled one month apart: Maarsseveen (52.144402, 5.080691; March 2017), Tjeukemeer (52.890225, 5.802871; April 2017) and Loosdrecht (52.196582, 5.080495; May 2017). The community from Maarsseveen was comprised primarily of small flagellated green algae, diatoms (*Aulacoseira, Asterionella*) and mucilaginous cyanobacterial colonies. Tjeukemeer was dominated by filamentous cyanobacteria, but also contained medium-sized green algae (*Scenedesmus, Pediastrum*), pennate diatoms and mucilaginous cyanobacterial colonies. Loosdrecht was also dominated by filamentous cyanobacteria, mucilaginous cyanobacterial colonies, and small-sized green algae such as *Scenedesmus.*

At each lake, 340L of water from 0.5–1.0 m depth was collected in 10L containers and brought back to the laboratory to inoculate experiments. Additionally, depth profiles of water temperature and pH were recorded using HydroLab sensors (OTT Hydromet, USA), and water samples were collected for dissolved nutrient analyses (DIN, DIP) and seston samples were collected in triplicate for chlorophyll-a (chl-a) analysis and C, N, P elemental analysis. Plankton inoculum were stored in the laboratory in the dark overnight and experiments were inoculated the next morning. All inocula were pre-screened through a 200µm mesh to remove large zooplankton grazers, and gently mixed in a large cattle tank before filling equal 10L volumes into transparent Nalgene containers.

Using a fully factorial design, the culture containers were subjected to two temperature, nutrient, and grazing treatments, for a total of eight factorial treatment combinations. Each of the eight treatments were replicated four times, resulting in thirty-two experimental units for each of three experiments. The temperature treatments consisted of an ambient treatment, chosen based on the lake water temperature at the time of sampling, and a +4°C warming treatment based on global change scenarios. However, due to problems with temperature control in the incubation system, there were differences between the magnitude of warming for each experiment. The mean ambient and elevated temperatures, respectively, for each experiment were as follows: 9.6±0.5 and 11.0±0.2°C for Maarseveen, 12.0±0.4 and 15.0±0.5°C for Tjeukemeer and 15.8±0.3 and 20.0±0.2 for Loosdrecht. For the nutrient addition treatment nitrogen and phosphorus were added in concentrations of 1 mM NO_3^- and 0.0625 mM PO_4^{3-}, respectively. For the grazing treatment, Daphnia was added to a final population density of 5 *Daphnia magna* individuals/L. Daphnia were purchased and cultured in the laboratory, and for each experiment, adult individuals of a standardized size were selected. Culture vessels were randomly positioned and

submerged in temperature-controlled aquaria using the Farex SR minisystem (RKC Instruments, Tokyo, Japan) and subjected to controlled light conditions (120 umol photons m^{-2} s^{-1}) with a day-night cycle of 16:8 that simulated the spring light conditions in The Netherlands. Every two days, chl-a samples were collected from each culture vessel by gentle mixing and using a depth-integrated tube sampler. Chl-a concentrations were quantified using a Phyto-PAM fluorometer (Walz, Germany). Each experiment ran for a duration of 6 days, when phytoplankton communities started to enter the stationary phase of growth.

On day 6 of the experiment, the experiments were harvested. Samples from each culture vessel were collected for chl-a analyses, elemental analyses (particulate C, N, P), dissolved nutrient analyses, flow cytometry, and microscopy. Additionally, temperature and pH inside of each culture vessel were recorded. For chl-a analyses, samples were analyzed in two ways: fluorometrically (Phyto-PAM, Walz, Germany) and using high performance liquid chromatography (HPLC). For the chl-a (HPLC) and elemental analyses, seston samples were filtered onto pre-rinsed glass microfiber filters (Whatman GF/F, Maidstone, UK) in two size fractions: for the whole community and <30µm fraction (separated using 30µm mesh). Molar elemental quantities for the smaller size fraction were subtracted from the whole fraction to calculate measurements for >30µm size fraction.

Samples for chlorophyll-a were collected on GF/F filters (Whatman) and stored in Eppendorf tubes at -20°C. Prior to extraction, filters were thawed for 30 min at room temperature, and 1.5 mL of 80% ethanol was added. The tubes were subsequently placed in a water bath at 80°C for 10 min in the dark. After manual mixing, 1 mL of sample was syringe filtered (0.45 um) and immediately analyzed on an HPLC UltiMate 3000 (Thermo Scientific)

equipped with a Hypersil ODS column (25 cm, 5 um, 4.6x250 mm; Agilent) and an RF 2000 fluorescence detector (Dionex/Thermo Scientific).

Filtrate samples were collected in polyethylene containers and stored at -20°C for analyses of dissolve inorganic nitrogen (DIN, including NO_3^-, NO_2^- and NH_4^+) and phosphorus (including soluble reactive phosphate SRP), and seston samples on filters were dried at 60°C for 24 hours and stored in a desiccator until further analysis. Soluble reactive phosphate was determined by absorption at 715 nm following Murphy and Riley (1962). Ammonium ($NH4^+$) and nitrate ($NO3^-$) were determined using a Technicon TV AAcs 800 autoanalyzer (Technicon, Tarrytown, New York, USA). P content in seston retentate was assessed by incinerating the samples for 30 min at 500°C, followed by a 2% persulphate digestion step in the autoclave for 30 min at 121°C. The digested samples were analyzed using a QuAAtro segmented flow analyzer (Seal Analytical Incorporated, Beun de Ronde, Abcoude, The Netherlands). C and N content in seston retentate was determined using a FLASH 2000 organic elemental analyzer (Brechbueler Incorporated, Interscience B.V., Breda, The Netherlands).

Data analysis

All statistical analyses were performed using the statistical program R version 3.4.2 (R Development Core Team 2017). We tested for a productivity gradient in the initial field data from lakes by testing for differences in the mean values of TN (µM), TP (µM) and chl-a (µg/L) measured in the three lakes using one-way Anova and Tukey's post-hoc test. Net growth rates in each treatment were calculated by dividing the difference between the log-transformed chl-a values from the beginning and end of the experiment by the duration of the experiment: [ln(chl-a_{day0})-ln(chl-a_{day6})]/6. Biomass buildup for each treatment was determined by calculating the

difference between chl-a concentrations on the final and initial day of each experiment.

We fit generalized linear models ('glm' function in lme4 package) with Gaussian distributions to determine the main and interactive effects, and effect sizes, of experimental treatments on response variables (net growth rate, biomass buildup, N:P, C:P <30 and C:P > 30, dissolved nutrients) in each experiment and assessed statistical significance using a chi-squared test. Parameter estimates and standard error values from the models were used to represent effect sizes of treatments on response variables. We also tested for differences between C:P in the two size fractions and nutrient treatments for each lake using one-way Anova and Tukey's post-hoc test.

Results

Lake data

Mean chl-a (μg/L), fraction of chl-a <30μm, TN (μM) and TP (μM) were significantly different in the three sampled lakes (P<0.01). Highest chlorophyll-a (chl-a) concentrations occurred in Lake Tjeukemeer (35.8±0.2 μg/L), followed by Lake Loosdrecht (19.5±0.2 μg/L) and Lake Maarsseveen (2.37±0.007 μg/L) (Fig. 2.1). The fraction of chl-a <30μm showed the opposite pattern: the highest fraction occurred in Lake Maarsseveen (0.86±0.001), followed by Loosdrecht (0.71±0.02) and Tjeukemeer (0.60±0.02) (Fig. 2.1). TN and TP were highest in Lake Tjeukemeer (79.1±1.4 μM TN, 3.15±0.03 μM TP) followed by Loosdrecht (46.2±3.3 μM TN, 1.15±0.03 μM TP) and Maarsseveen (25.5±1.2 μM TN, 0.34±0.03 μM TP) (Fig. 2.1). Additionally, ambient mean dissolved inorganic nitrogen (DIN) concentrations were 15.9 μM, 2.8 μM, and 9.9 μM for Maarsseveen, Loosdrecht and Tjeukemeer respectively, while dissolved inorganic phosphorus (DIP) concentrations were below detection for Maarsseveen and

Loosdrecht and 0.53 uM for Tjeukemeer.

Therefore, phytoplankton communities from lakes Maarsseveen, Loosdrecht, Tjeukemeer will be referred to as the "low", "medium" and "high" productivity communities, respectively.

Biomass buildup

Nutrient addition increased biomass buildup for all three communities ($P<0.001$, Fig. 2.2), while grazing, temperature and interactions differed by community. Biomass of only the low productivity community was reduced by grazing ($P<0.001$, Fig. 2.2) even under elevated nutrient conditions (nutrients x grazing, $P<0.001$, Fig. 2.2). In the high productivity community, warming increased biomass ($P<0.001$, Fig. 2.2), and magnified the effect of nutrient addition on biomass buildup (nutrients x temperature, $P<0.001$, Fig. 2.2). Warming also impacted biomass of the lowest productivity community ($P<0.05$, Fig. 2.2), as it increased growth interactively with nutrient addition (nutrients x temperature, $P<0.05$, Fig. 2.2) similar to the high productivity community. Biomass buildup of the medium productivity was only affected by nutrients but not temperature or grazing ($P<0.001$, Fig. 2.2).

Net growth rate

For all three communities, nutrient addition significantly increased net growth rates ($P<0.001$, Fig. 2.3), while the effects of temperature and grazing varied by community. Nutrients increased growth rate most for the medium productivity community, followed by the low and high productivity communities, respectively. The low productivity community was the only one that experienced reduced growth rates as a result of grazing ($P<0.001$, Fig. 2.3), across all temperature and nutrient treatments. In the high productivity community, warming alone

elevated growth rates ($P<0.001$, Fig. 2.3), and in the medium and high productivity communities, temperature and nutrients interacted significantly ($P<0.05$, Fig. 2.3). However, the direction of the interactive effect differed by community. In the medium productivity community, warming had a positive effect at ambient nutrient levels, but at elevated nutrient levels, the effect of warming was negative ($P<0.05$, Fig. 2.3). The opposite result, a positive nutrient-by-warming interaction, was found in the most eutrophic community where nutrient addition stimulated growth more at high temperature. In the low productivity community, the interaction between warming and nutrients was positive but not significant.

Stoichiometry

N:P ratios at ambient temperature, ambient nutrients and without added grazers were lowest for the low productivity community, intermediate for the medium productivity community, and highest for the high productivity community. Nutrient addition reduced N:P for all three communities ($P<0.001$, Fig. 2.4), with the largest effect size on the medium productivity community, followed by high and low productivity communities respectively. Grazing reduced N:P in the low productivity ($P<0.01$, Fig. 2.4) community and increased N:P in the medium productivity community ($P<0.05$, Fig. 2.4), with no significant effect in the high productivity community. Warming had a negative interaction with grazing at ambient nutrient levels in the medium productivity community, indicating that grazers increased N:P more at ambient than warmed temperature (temperature x nutrients x grazing, $P<0.05$, Fig. 2.4). The high productivity community showed an interactive effect of nutrients and warming, indicating that warming increased N:P at ambient nutrient levels but not when fertilized ($P<0.05$, Fig. 2.4), while the

lower productivity community showed the opposite, with negative temperature effects on N:P at ambient nutrient levels ($P<0.05$, Fig. 2.4).

Ambient phytoplankton C:P ratios were higher in the larger size fraction (i.e. >30 um) as compared to the smaller size fraction (i.e. <30 um) for the lowest ($P<0.001$) and highest productivity systems ($P<0.001$). Conversely, in the medium productivity system, C:P ratios were higher in the smaller size fraction ($P<0.001$). Nutrient addition reduced C:P ratios in all treatments, irrespective of the size fraction (Fig. 2.5), yet remained higher in the larger size fraction for the medium ($P<0.001$) and high ($P<0.01$) productivity communities. With nutrient addition, both size fractions show significant reductions in C:P for all three communities ($P<0.001$, Fig. 2.5), but for the large size fraction, only nutrient additions affected C:P. In contrast, grazing at ambient nutrient levels lowered C:P in the smaller size fraction in the low productivity community (grazing x nutrient interaction, $P<0.001$, Fig. 2.5) indicating a possible nutrient recycling effect by grazers. Warming affected the smaller size fraction in the high productivity lake, as C:P increased at warmed temperatures ($P<0.001$, Fig. 2.5), especially when combined with ambient nutrients and grazing (temperature x nutrient x grazing interaction, $P<0.05$, Fig. 2.5).

Dissolved nutrient data

The effects of each treatment on DIN and DIP (μM) at the end of the experiments varied by community. In the low productivity community, DIN and DIP increased in response to nutrient addition and grazing ($P<0.001$, Fig. 2A.1 & Table 2A.1), and were highest in treatments that combined grazing and nutrient addition, indicating an interaction between the two treatments (nutrients x grazing, $P<0.05$ for DIN, $P<0.001$ for DIP, Fig. 2A.1 & Table 2A.1). Additionally,

there was a significant three-way interaction between nutrients, temperature and grazing for DIN, whereby warming resulted in a slight decrease in DIN in the treatment with elevated nutrients and no grazers, and in the treatment with ambient nutrients and presence of grazers. In the medium productivity community, DIN was significantly higher in nutrient addition treatments (P<0.001, Fig. 2A.1 & Table 2A.1). There were no significant differences among treatments for DIP. In the high productivity community, nutrient addition significant increased DIN (P<0.001, Fig. 2A.1 & Table 2A.1) and warming significantly reduced DIN (P<0.001, Fig. 2A.1 & Table 2A.1), while DIP remained the same (~0.3uM) in all treatments (Fig. 2A.1 & Table 2A.1).

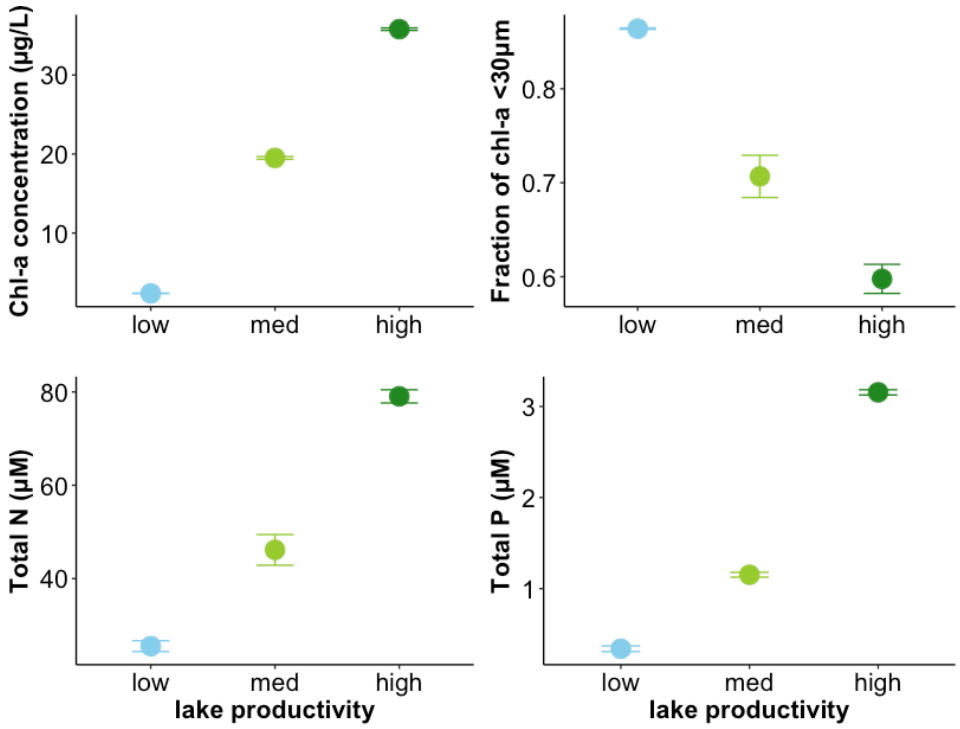

Figure 2.1: The top panel shows mean (±SE) values of total chl-a and fraction of chl-a in the <30um size fraction across a lake productivity gradient, while the bottom panel shows mean (±SE) values of total nitrogen (TN) and total phosphous (TP) across a lake productivity gradient. TN, TP, and chl-a increase significantly as lake productivity increases, while the fraction of chl-a in the <30um size fraction decreases along the productivity gradient (P<0.001).

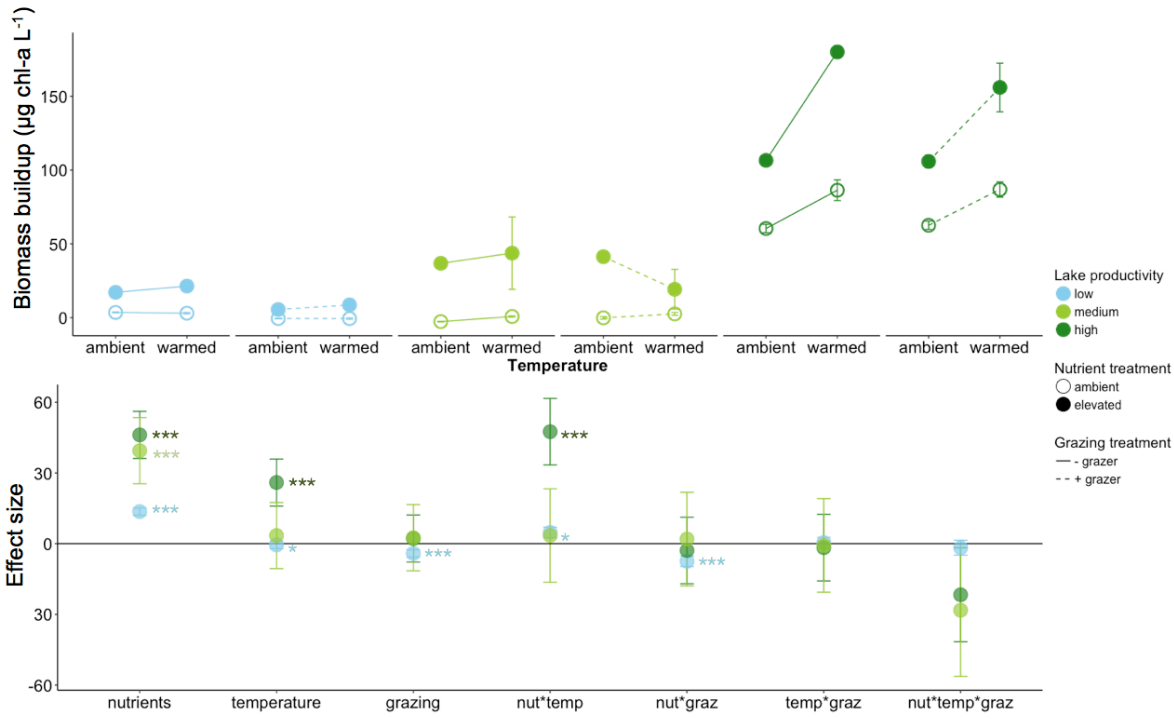

Figure 2.2: The top panel shows mean (±SE) biomass buildup in each treatment for each lake community, with colors indicating lake productivity, circle fill indicating nutrient treatment, line type indicating grazing treatment, and x-axis indicating temperature treatment. The parameter estimates (effect sizes) and standard error values from generalized linear models for each community are shown on the bottom panel, with asterisks indicating significance values and x-axis labels indicating the treatment effect (*P<0.5, **P<0.01, ***P<0.001).

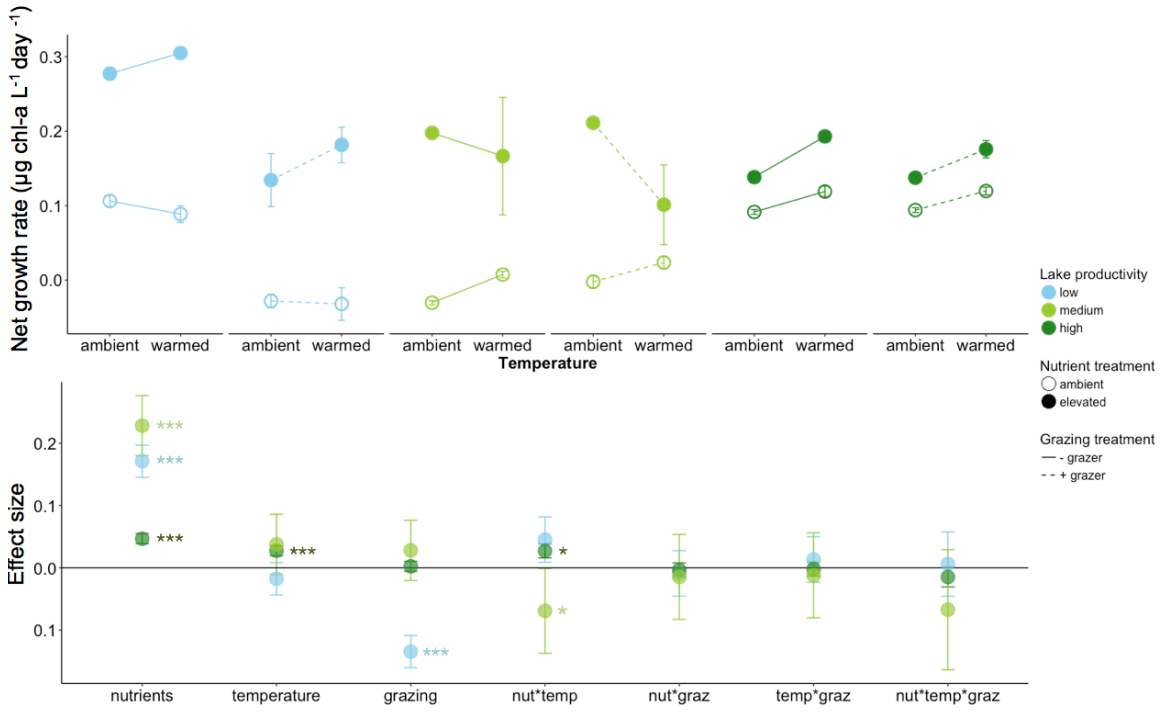

Figure 2.3: The top panel shows mean (±SE) net growth rates in each treatment for each lake community, with colors indicating lake productivity, circle fill indicating nutrient treatment, line type indicating grazing treatment, and x-axis indicating temperature treatment. The parameter estimates (effect sizes) and standard error values from generalized linear models for each community are shown on the bottom panel, with asterisks indicating significance values and x-axis labels indicating the treatment effect (*P<0.5, **P<0.01, ***P<0.001).

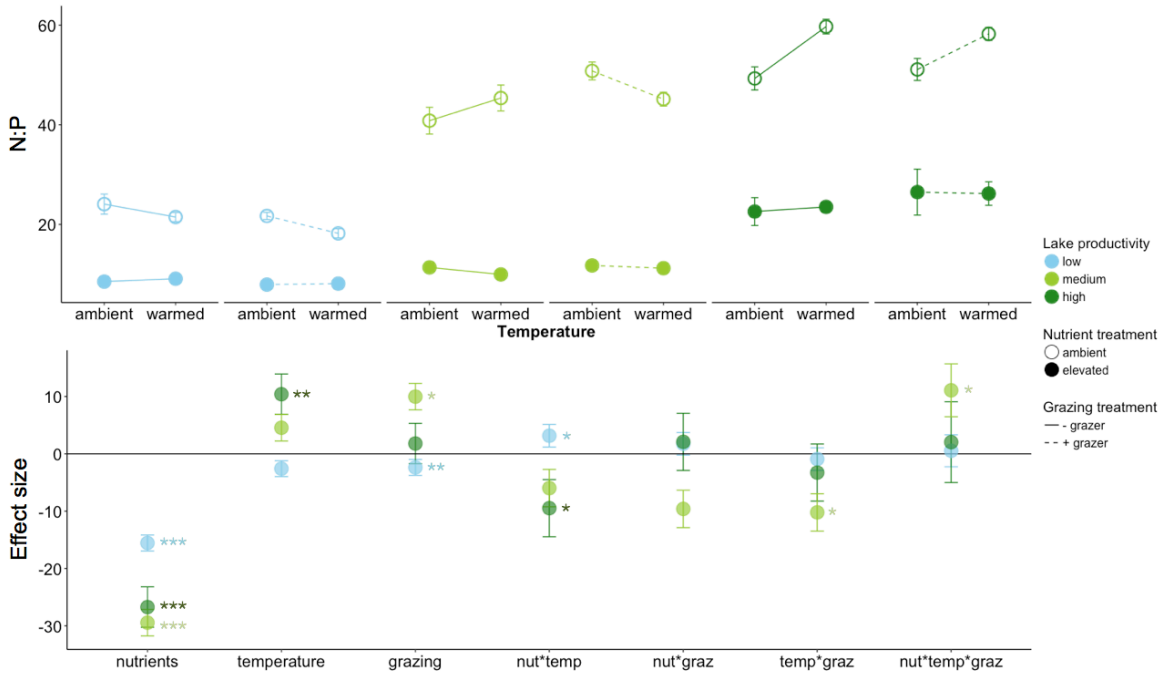

Figure 2.4: The top panel shows mean (±SE) N:P in each treatment for each lake community, with colors indicating lake productivity, circle fill indicating nutrient treatment, line type indicating grazing treatment, and x-axis indicating temperature treatment. The parameter estimates (effect sizes) and standard error values from generalized linear models for each community are shown on the bottom panel, with asterisks indicating significance values and x-axis labels indicating the treatment effect (*P<0.5, **P<0.01, ***P<0.001).

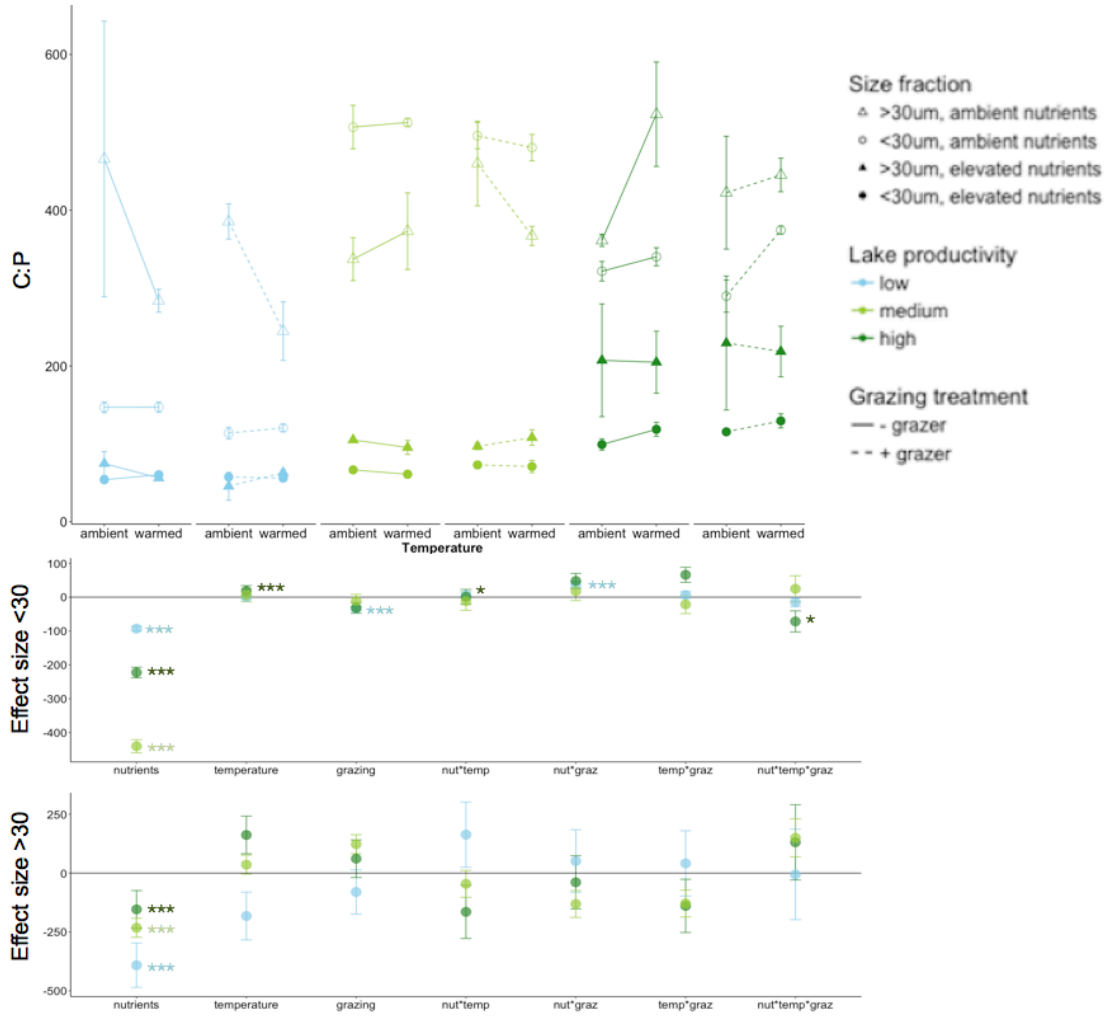

Figure 2.5: The top panel shows mean (±SE) C:P for two size fractions (<30um and >30um) in each treatment for each lake community, with shape indicating size fraction, colors indicating lake productivity, circle fill indicating nutrient treatment, line type indicating grazing treatment, and x-axis indicating temperature treatment. The parameter estimates (effect sizes) and standard error values from generalized linear models for each community and size fraction are shown on the bottom two panels, with asterisks indicating significance values and x-axis labels indicating the treatment effect (*P<0.5, **P<0.01, ***P<0.001).

Discussion

Our results show that across phytoplankton communities from lakes of different trophic status, warming and its interactions with eutrophication and grazing had effects that varied in both size and magnitude on growth, biomass, and stoichiometry of phytoplankton communities, while eutrophication had positive effects on all communities. Specifically, nutrient additions

consistently elevated growth, biomass and nutrient content of phytoplankton cells in all communities, with the greatest magnitude in the medium productivity system. Thus, while the effects of eutrophication on phytoplankton stoichiometry can be reliably predicted across a range of communities, changes in temperature and grazing have context-dependent effects on growth, biomass and stoichiometry that will help predict variation among lake responses which might experience similar levels of warming but have differing nutrient inputs.

In addition, interactions were prevalent but variable among communities, indicating that synergies and interference occurred frequently but depended on the trophic status and productivity of the lake from which communities originated. Thus, predicting the effects of warming on phytoplankton stoichiometry, and therefore biogeochemical cycles, requires further understanding of local environmental conditions.

Nutrient addition strongly influenced biomass buildup, growth rates and stoichiometry of all three communities. Decreases in N:P and C:P ratios with increased nutrient supply may reflect increased P-storage (reviewed in Meunier et al. 2017), or possibly increased growth rates following the Growth Rate Hypothesis (Elser et al. 2003). The low productivity community achieved the highest growth rates under nutrient addition. This community was strongest limited by nutrients and contained highest contributions of smaller cells that are better in acquiring nutrients (Maranon et al. 2013, Litchman et al. 2007). Also, smaller cells exhibit higher maximum growth rates (Banse 1976, Litchman et al. 2007), explaining the highest growth rates in the low productivity community. Differences in stoichiometric responses across the communities may have been constrained by the amount of stoichiometric flexibility associated with the species composition of each community, as nutrient requirements for functional machinery is species-specific (Klausmeier 2004a). The low productivity community had the

lowest N:P of the three communities at ambient nutrient levels, which may have occurred because this community is comprised of diatoms as well as fast-growing, small-celled species that tend to have higher P content and more constrained elemental composition (Elser et al. 2003, Martiny et al. 2013). In contrast, the high productivity system had highest N:P at both ambient and elevated nutrient treatments (Fig. 2.3). This community is dominated by filamentous cyanobacteria, and the relatively high N:P ratios are consistent with findings that cyanobacteria tend to have higher N:P (Klausmeier et al. 2004a, Hillebrand et al. 2013) and lower growth rates (Edwards et al. 2012) than diatoms. While the medium productivity community was also dominated by filamentous cyanobacteria, it had intermediate N:P values as compared to the low and high productivity lakes, perhaps due to differences in species composition.

The effects of warming on stoichiometry were variable by community, consistent with findings in previous studies. While warming decreased N:P in the low productivity community, it increased N:P in the high productivity community. Velthuis et al. (2018) also found that warming reduced seston C:P and N:P, likely as a result of nutrient recycling by heterotrophic microbes, although such shifts can also be caused by changes in species or size composition of phytoplankton (Klausmeier et al. 2004a, Hillebrand et al. 2013, Toseland et al. 2017). For the high productivity lake, DIN was significantly lower in all warming treatments while DIP concentrations were the same across all treatments (Fig. 2A.1 & Table 2A.1). This may suggest that N uptake increased relative to P in warmed treatments, causing N:P to increase (Fig. 2.3). This pattern is consistent with the finding that warming caused eukaryotic phytoplankton to increase rates of protein synthesis while reducing the density of P-rich ribosomes to necessary to produce cellular proteins, resulting in higher N demand and higher N:P (Toseland et al. 2013). In

general, it appears that the effect of temperature on stoichiometry is greater at low rather than high nutrient loads (De Senerpont-Domis et al. 2014).

C:P responses differed by size fraction, indicating that overall community responses to warming, nutrients, and grazing are mediated by the size structure and taxonomic composition of communities. There were significant differences between C:P for the two size fractions at ambient and elevated nutrient levels (Fig. 2.5), and the effects of each treatment on C:P ratios in the three lakes differed according to the size fraction examined (<30μm vs. >30μm), suggesting that traits associated with size, such as nutrient uptake affinities and grazer susceptibility, influence responses to each treatment. The higher C:P ratios in the larger size fraction suggest that the larger cells were more nutrient-limited than smaller cells, consistent with expectations for nutrient uptake traits associated with size (Maranon et al. 2013, Litchman et al. 2007). While the larger size fraction was only affected by nutrients, the smaller size fraction responded to nutrients, grazing, temperature, and their interactions. In the low productivity lake, C:P ratios of the smaller size fraction were reduced by grazing at ambient nutrient levels, suggesting that smaller cells can effectively take up recycled P from grazing (Fig. 2.5; Elser and Urabe 1999). Warming increased C:P in the smaller size fraction in the high productivity lake and especially when grazers were present. Warming has also been shown to increase phytoplankton C:P ratios, but only under P-limited conditions (De Senerpont Domis et al. 2014). Such differences in combined effects of warming and nutrient availabilities are possibly caused by the community structure and size distribution. Overall, we demonstrate that the smaller size fraction of all three communities is more sensitive to the effects of temperature and grazing, suggesting that small cells are generally more responsive to warming and shifts in grazing pressure. Possibly, smaller

cells, as better nutrient competitors, may respond more effectively to small perturbations in nutrient availability indirectly caused by warming and grazing.

The effect of grazing on all measured response variables was most pronounced in the lowest productivity community. Grazing significantly reduced biomass and growth rates in the low productivity lake, likely because it had the highest proportion of cells in the edible size fraction (<30μm; Fig. 2.1). This result is consistent with the expectation that small cells, as better nutrient competitors, are most likely to inhabit low nutrient environments but are also more susceptible to grazers (reviewed in Litchman & Klausmeier 2008; Grover 1995; Leibold 1989, 1996).

Warming had a positive effect on biomass buildup and net growth rate only in the high productivity community. These results are in line with previous studies indicating that the effect of warming on phytoplankton communities depends on trophic state and species composition, with more positive effects on growth in systems with high P supply (Tadonléké 2010, Rigosi et al. 2014, Elliott et al. 2006, Huber et al. 2008). Additionally, the high productivity community was dominated by filamentous cyanobacteria, and cyanobacterial growth tends to be favored under warm conditions (Paerl and Huisman 2008, Kosten et al. 2012, Reynolds 1984, Sommer et al. 1986). Interactions between temperature, nutrients and grazing were idiosyncratic for each lake. Most notably, the interaction between nutrient addition and warming showed opposite effects for biomass and net growth rates in the medium and high productivity communities. While warming stimulated growth for both communities at ambient nutrient levels, the interaction with nutrient addition had a positive effect in the high productivity community but a negative effect in the medium productivity community, despite filamentous cyanobacterial dominance in both communities. This contrasting response might have been caused by the

temperature of the warming treatments which were determined relative to the ambient lake temperature at the time of sampling (see methods). The medium productivity community experienced the highest temperatures (20ºC in warmed treatments; see methods) and therefore the warming treatment might have surpassed optimal temperatures for growth (Litchman et al. 2010), a condition that can be exacerbated by nutrient addition (Rigosi et al. 2014).

We observed strongest impacts of warming on growth, biomass buildup, and stoichiometry in the most productive community. Warming under ambient nutrient levels caused increased C:nutrient ratios, and these effects were dampened when nutrients were added. These findings are comparable to nutrient dependent effects of elevated pCO_2 on cyanobacterial stoichiometry and biomass buildup (Verspagen et al. 2014). It is conceivable that when nutrients are in ample supply, enhanced metabolic rates from warming or higher carbon availabilities through elevated pCO_2 can be invested in growth, leading to enhanced biomass buildup. Under nutrient limitation, however, growth is constrained and elements may instead accumulate in the cell, leading to stoichiometric shifts. These findings thus suggest that climate change may lead to higher phytoplankton biomass, particularly cyanobacteria, when nutrients are available in excess, while it will lead to stoichiometric shifts with higher C:nutrient ratios when nutrients are limiting.

Together, our results indicate that the effects of climate warming, eutrophication, and grazing may elicit distinct responses in lake phytoplankton communities depending on the trophic state, community composition and size structure. Across a gradient of increasing productivity, we showed that the fraction of small cells in communities decrease, resulting in strongest impacts of grazing and consumer-driven nutrient recycling on C:P and N:P in communities from the lowest productivity lake with the highest fraction of small cells.

Additionally, C:P responses differ by size fraction for all three communities examined, indicating that traits associated with cell size will mediate community stoichiometry in response to various stressors. The variable effects of warming and its interactions with nutrient addition in each community across our productivity gradient is consistent with numerous studies indicating that the effect of warming on phytoplankton communities in lakes depends on nutrient supply and species composition, with stronger effects in eutrophic systems (Tadonléké 2010, Rigosi et al. 2014, Elliott et al. 2006, Huber et al. 2008). Here we demonstrate that integrating trait-based ecology and ecological stoichiometry (Meunier et al. 2017) may help in assessing the impacts of global environmental change on growth, biomass buildup and elemental composition of phytoplankton communities.

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Chapter 2, in full, is currently being prepared for submission for publication of the material. Schulhof, M.A., J.B. Shurin, S.A.J. Declerck, D.B. Van de Waal. The dissertation author is the primary investigator and author of this paper.

Appendix 2A

Table 2A.1: P-values from generalized linear models

Growth rate							
productivity	temp	nutrients	grazing	temp*nut	temp*grz	nut*grz	temp*nut*grz
Low	0.30	**<0.001*** **	**<0.001*** **	0.061	0.52	0.83	0.91
Medium	0.42	**<0.001*** **	0.94	**<0.05***	0.35	0.32	0.49
High	**<0.001*** **	**<0.001*** **	0.36	**<0.05***	0.25	0.19	0.36
Biomass buildup							
productivity	temp	nutrients	grazing	temp*nut	temp*grz	nut*grz	temp*nut*grz
Low	**<0.05***	**<0.001*** **	**<0.001*** **	**<0.05***	0.82	**<0.001*** **	0.59
Medium	0.75	**<0.001*** **	0.58	0.45	0.29	0.39	0.31
High	**<0.001*** **	**<0.001*** **	0.27	**<0.001*** **	0.21	0.17	0.28
N:P							
productivity	temp	nutrients	grazing	temp*nut	temp*grz	nut*grz	temp*nut*grz
Low	0.056	**<0.001*** **	**<0.01 ** **	**<0.05***	0.64	0.14	0.85
Medium	0.51	**<0.001*** **	**<0.05***	0.85	**<0.05***	0.078	**<0.05***
High	**<0.01 ** **	**<0.001*** **	0.33	**<0.05***	0.52	0.38	0.77
C:P <30um							
productivity	temp	nutrients	grazing	temp*nut	temp*grz	nut*grz	temp*nut*grz
Low	0.39	**<0.001*** **	**<0.001*** **	0.89	0.92	**<0.001*** **	0.29
Medium	0.66	**<0.001*** **	0.48	0.96	0.64	0.12	0.52
High	**<0.001*** **	**<0.001*** **	0.35	**<0.05***	0.052	0.43	**<0.05***
C:P >30um							
productivity	temp	nutrients	grazing	temp*nut	temp*grz	nut*grz	temp*nut*grz
Low	0.062	**<0.001*** **	0.43	0.093	0.67	0.61	0.98
Medium	0.49	**<0.001*** **	0.13	0.47	0.18	0.16	0.063
High	0.28	**<0.001*** **	0.90	0.22	0.36	0.74	0.41
DIN (uM)							
productivity	temp	nutrients	grazing	temp*nut	temp*grz	nut*grz	temp*nut*grz
Low	0.089	**<0.001*** **	**<0.001*** **	0.26	0.60	**<0.05***	**<0.05***
Medium	0.30	**<0.001*** **	0.64	0.12	0.81	0.33	0.47
High	**<0.001*** **	**<0.001*** **	0.88	0.99	0.59	0.24	0.86
DIP (uM)							
productivity	temp	nutrients	grazing	temp*nut	temp*grz	nut*grz	temp*nut*grz
Low	0.96	**<0.001*** **	**<0.001*** **	0.94	0.59	**<0.001*** **	0.61
Medium	0.053	0.053	0.35	0.053	0.35	0.35	0.35
High	0.27	0.81	0.27	0.46	0.54	1	0.81

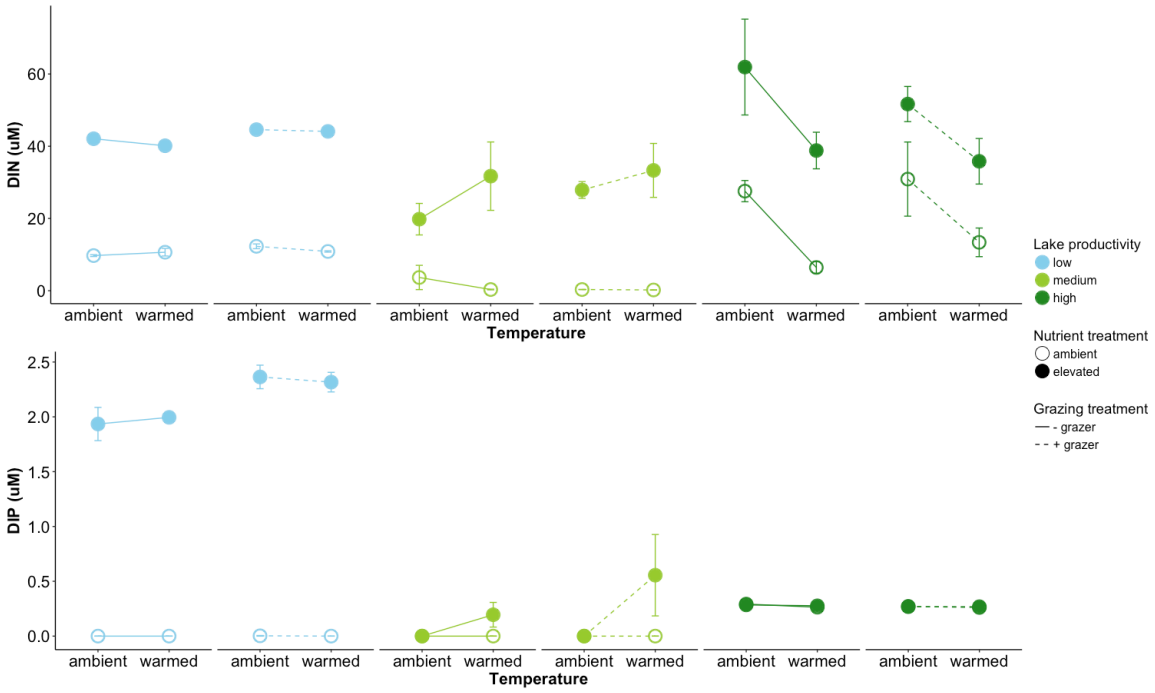

Figure 2A.1: The top panel shows mean (±SE) dissolved inorganic nitrogen (DIN) in each treatment and the bottom panel shows mean (±SE) dissolved inorganic phosphorus (DIP) for each lake community, with colors indicating lake productivity, circle fill indicating nutrient treatment, line type indicating grazing treatment, and x-axis indicating temperature treatment.

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CHAPTER 3

Functional turnover reduces both grazing and nutrient limitation of lake phytoplankton with increasing productivity

Abstract:

Functional tradeoffs among ecologically important traits govern the diversity of communities and changes in species composition along environmental gradients. A tradeoff between predator defense and resource competitive ability has been invoked as a mechanism that may maintain diversity in lake phytoplankton. Tradeoffs may promote diversity within lakes if grazing and resource-limited taxa coexist locally, or among lakes if shifts from resource to consumer control occur across productivity gradients. In addition, changes in temperature may alter nutrient demands and grazer activity, changing the balance between the two regulating factors. Our study aims to understand whether the competition-defense tradeoff promotes coexistence within or among lakes across a productivity gradient, and how this tradeoff may shift in a warmer world. We conducted multifactorial experiments manipulating grazing, nutrients, and temperature in phytoplankton communities from three Dutch lakes varying in productivity and used a trait-based approach to classify functional groups based on grazing susceptibility. We found that nutrient and grazing both had the strongest effects in the least productive lake. Functional groups turned over across productivity gradients such that groups that are both more grazing resistant and less nutrient limited dominated more eutrophic lakes. Additionally, the effect of warming on top-down and bottom-up control on the edible size fractions (<30um) differed among lakes: warming increased top-down control on small cells in the low and high productivity lakes, amplified positive effects of fertilization in the high productivity lake and had an antagonistic effect on fertilization in the medium productivity lake. Our results indicate that 1)

the strength of both top-down and bottom-up control on phytoplankton communities weakened with increasing productivity, 2) tradeoffs between top-down and bottom-up control influenced differences in functional composition among lakes rather than promoting diversity within lakes and 3) warming had idiosyncratic effects on the response of communities to grazing and nutrients among functional groups and lakes.

Introduction

An interspecific tradeoff between predator defense and resource competitive ability, whereby strong resource competitors are more susceptible to predation and poor resource competitors are more defended against predators, has been invoked as a mechanism that may maintain coexistence and diversity in ecological communities (Paine 1966, Lubchenco 1978, Leibold 1996, McCauley & Briand 1979) at different spatial scales (Kneitel and Chase 2004). Such tradeoffs may arise from constraints on allocation of resources to different organismal functions such as morphological or chemical defenses vs. structures involved in resource acquisition (Kneitel and Chase 2004). In phytoplankton communities, the coexistence of many phytoplankton species despite competition for the same limiting nutrients has been described as the "Paradox of the Plankton" because it seemingly defies the competitive exclusion principle (Hutchinson 1961).

Tradeoffs between nutrient competition and predation could be one mechanism maintaining the diversity and coexistence of species in phytoplankton communities. Competition-defense tradeoffs can be detected based on the response of co-occurring species to manipulation of different potentially limiting factors. For example, the species most limited by resources (poor competitors) should respond most positively to resource addition, while species

that are strongly limited by their consumers should increase the most in response to predator removal. An inter-specific correlation in the response to manipulation of multiple limiting factors indicates the presence of a tradeoff. A meta-analysis of terrestrial plant communities found that the association between competition and defense was highly variable across studies, indicating that weaker nutrient limitation is not generally associated with stronger grazing effects (Viola et al. 2010). However, a globally distributed grassland experiment showed that a growth-defense trade-off, rather than a competition-defense tradeoff, appeared to be the prevailing tradeoff maintaining grassland diversity (Lind et al. 2013). Grazing resistance may therefore come at a cost of slower growth rather than reduced resource acquisition or competitive ability.

The association between resource competition and grazer resistance strategies in phytoplankton communities, and their role as regulators of diversity within and among lakes, remains to be tested. Functional tradeoffs among ecologically important traits govern the diversity of algae and changes in species composition along environmental gradients. Cell size and morphology of phytoplankton are important traits that influence key metabolic processes such as nutrient uptake and utilization strategies, in addition to trophic interactions. Small cells are able to acquire limiting nutrients more efficiently due to their high surface area to volume ratio but increasing cell size and volume are correlated with higher maximum uptake rates, half-saturation constants, and storage capacity (Litchman et al. 2007; Maranon 2013, Edwards et al. 2011). The size of phytoplankton also plays an important role in trophic interactions by influencing susceptibility to grazing by zooplankton. Larger cell size results in greater resistance to gape-limited grazers, creating a trade-off between nutrient competitively ability and grazing susceptibility (reviewed in Litchman et al. 2007, Litchman et al. 2010). In addition to cell size, morphological traits such as shape and cell composition (eg. silica) influence this tradeoff by

influencing susceptibility to grazing (Colina et al. 2016). Additionally, the environment has a large influence on cell size and morphology, as small cells that are strong nutrient competitors are favored in low-nutrient environments, while large celled species dominate high and fluctuating nutrient environments (Cloern et al. 2017, Irwin et al. 2006, Litchman et al. 2007; Litchman et al. 2010; Edwards et al. 2011). Thus, mean cell size increases across productivity gradients as phytoplankton biomass increases due to increasing availability of nutrients (Kiørboe 1993, Chisholm 1992). As a result, the competition – defense tradeoff may determine composition at a regional rather than local scale, whereby mean size of species increases along productivity gradients and relationships between nutrient competitive ability and grazer resistance are more observable between communities than within communities.

As the climate warms and anthropogenic nutrient inputs to water bodies increase, it is important to understand how functional tradeoffs operate among algal taxonomic groups in order to predict responses to elevated temperatures, nutrient addition, and changes in grazing pressure (Litchman et al. 2012). Trait-based approaches to phytoplankton community ecology, with a focus on ecophysiological traits such as cell size and elemental stoichiometry, hold promise for better understanding and predicting responses of phytoplankton to simultaneous global change (Litchman & Klausmeier 2008, Finkel et al. 2010, Litchman et al. 2010, Reynolds et al. 2002, Litchman et al. 2007). Warming temperatures are expected to alter the structure and functioning of ecological communities, including trophic interactions and size structure of organisms, and therefore may alter the competition-defense tradeoff. Higher temperatures result in increased metabolic rates, resource requirements and flux rates for organisms, and therefore affect population dynamics and interspecific interactions (Brown et al. 2004). Empirical studies have demonstrated that warming alters trophic interaction strengths by enhancing top-down,

consumer-driven control, causing increased grazing and thus reduced primary producer biomass (O'Connor et al. 2009, Shurin et al. 2012, Kratina et al. 2012). Additionally, nutrient limitation and resource availability directly affect producer biomass and interact with warming to affect trophic dynamics and structure. Smaller sized species may be competitively superior in warmed environments because temperature increases metabolic rates and therefore demand for limiting nutrients (Yvon-Durocher 2012) and a higher surface area to volume ratio is advantageous for limiting nutrient acquisition. Thus, warmer environments may be dominated by smaller sized species (Yvon-Durocher et al. 2011) and increase nutrient use efficiencies of some species (de Senerpont Domis et al. 2014). Temperature is therefore expected to alter the balance between resource and grazer limitation among taxa.

Our study aims to determine whether a competition-defense tradeoff is apparent within or between phytoplankton communities across a productivity gradient, and whether the shape of the function relating grazer and nutrient limitation is affected by temperature. We conducted multifactorial microcosm experiments on phytoplankton communities from three Dutch lakes across a productivity gradient and manipulated temperature, grazing pressure, and nutrient load. We determined the effect sizes of nutrient addition and grazing on phytoplankton functional group biovolumes using a trait-based framework (from Kruk et al. 2010) and compared the association between grazing and nutrient limitation among functional groups within and between lakes to test for the presence of a tradeoff. We hypothesized that more edible functional groups would show strong negative effects of grazing but weak positive responses to nutrient addition, whereas larger inedible functional groups would show stronger positive responses to nutrient addition and weak negative responses to grazing in all three lakes. Additionally, we hypothesized that warming would shift the tradeoff surface by changing the strength of top-down and bottom-

up control due to increased grazing pressure and nutrient uptake efficiency at higher temperatures for each functional group.

Materials and Methods

Experimental setup

Spring phytoplankton communities were collected from three lakes, sampled one month apart: Maarsseveen (52.144402, 5.080691; March 2017), Tjeukemeer (52.890225, 5.802871; April 2017) and Loosdrecht (52.196582, 5.080495; May 2017). At each lake, 340L of water from 0.5–1.0 m depth was collected in 10L containers and brought back to the laboratory to inoculate experiments. Plankton inoculum were stored in the laboratory in the dark overnight and experiments were inoculated the next morning. All inoculum was pre-screened through a 200um mesh to remove large zooplankton grazers, and thoroughly mixed in a large cattle tank before filling equal 10L volumes into transparent Nalgene containers. Mean chl-a (μg/L), fraction of chl-a <30μm, TN (μM) and TP (μM) were significantly different in the three sampled lakes ($P<0.01$). Highest chlorophyll-a (chl-a) concentrations occurred in Lake Tjeukemeer (35.8±0.2 μg/L), followed by Lake Loosdrecht (19.5±0.2 μg/L) and Lake Maarsseveen (2.37±0.007 μg/L) (Fig. 3.1). The fraction of chl-a <30μm showed the opposite pattern: the highest fraction occurred in Lake Maarsseveen (0.86±0.001), followed by Loosdrecht (0.71±0.02) and Tjeukemeer (0.60±0.02) (Fig. 3.1). TN and TP were highest in Lake Tjeukemeer (79.1±1.4 μM TN, 3.15±0.03 μM TP) followed by Loosdrecht (46.2±3.3 μM TN, 1.15±0.03 μM TP) and Maarsseveen (25.5±1.2 μM TN, 0.34±0.03 μM TP) (Fig. 3.1). Additionally, ambient mean dissolved inorganic nitrogen (DIN) concentrations were 15.9 μM, 2.8 μM, and 9.9 μM for Maarsseveen, Loosdrecht and Tjeukemeer respectively, while dissolved inorganic phosphorus

(DIP) concentrations were below detection for Maarsseveen and Loosdrecht and 0.53 uM for Tjeukemeer. Therefore, these communities from these lakes will be referred to as the "low", "medium" and "high" productivity communities, respectively.

Using a fully factorial design, the culture containers were incubated at two temperatures, crossed with nutrient, and grazing treatments, for a total of eight factorial treatment combinations. Each of the eight treatments were quadruple replicated, resulting in thirty-two experimental units for each of three experiments. The temperature treatments consisted of an ambient treatment reflecting the lake temperature at the time of sampling, and a +4°C warming treatment based on global change scenarios. However, due to problems with temperature control in the incubation system, there were slight differences between the magnitude of warming for each experiment. The mean ambient and elevated temperatures, respectively, for each experiment were as follows: 9.56±0.54 and 11.04±0.24°C for Maarseveen, 12.04±0.44 and 15.04±0.52°C for Tjeukemeer and 15.76±0.27 and 20.04±0.20 for Loosdrecht. Nutrient treatments included an ambient lake water treatment and a nutrient addition treatment (1 mM NO_3^- and .0625mM PO_4^{3-}).

Grazing treatments consisted of a large grazer (>200um) removal treatment and large grazer addition treatment (5 *Daphnia magna* individuals/L). Daphnia were chosen because they are nonselective grazers and consume a wide spectrum of prey sizes (Hansen 1994, Reynolds 2006). Daphnia were purchased and cultured in the laboratory, and for each experiment, adult individuals of a standardized size were selected for grazing treatments.

Culture vessels were randomized and submerged in temperature-controlled aquaria using the Farex SR minisystem (RKC Instruments, Tokyo, Japan) and subjected to controlled light conditions (120 umol photons $m^{-2}s^{-1}$) with a day-night cycle that simulated the spring light

conditions in The Netherlands. Every two days, chl-a samples were collected from each culture vessel by thoroughly mixing and using a depth-integrated tube sampler. Chl-a concentrations were quantified using a Phyto-PAM fluorometer (Walz, Germany). Each experiment ran for a duration of 6 days, when phytoplankton communities started to enter the stationary phase of growth. On day 6 of the experiment, the experiments were harvested. Samples from each culture vessel were collected for chl-a analyses, flow cytometry, and three of four replicates for each treatment were analyzed using microscopy. Samples for microscopy were fixed in Lugol's iodine until enumeration.

To assess the size distribution of cells within the smaller size fraction (<30 um), samples were analyzed by flow cytometry (Influx Cell Sorter, BD Biosciences) equipped with a 488nm and 640nm laser. From the smaller size fraction samples (<30 um), 4 ml was fixed with a paraformaldehyde–glutaraldehyde solution (6.75/1) to a final concentration of 1% (v/v) and stored at 5°C for a maximum period of 6 weeks prior to analysis. Size fractions were calibrated based on the particle time of flight and side scatter using 2, 10 and 30 um polymer microspheres (Duke Standards, Thermo scientific), and counts were clustered accordingly into the size classes <2, 2-10, and 10-30 um.

Microscopy and image analysis

Phytoplankton communities were counted via microscopy using the Utermöhl method (Utermöhl 1958). Depending on the density of samples, 0.5-6 mL of water were allowed to settle overnight in Utermöhl chambers and counted using an inverted microscope. For small taxa (<30um), fields of view were counted at 400x magnification until approximately 400 units were counted. Cells were binned into categories based on size and morphology. Large cells (>30um)

were counted at 100x magnification: for samples from lake Maarseveen, 100 transects were counted by microscopy, while for lakes Tjeukemeer and Loosdrecht, cells were counted from images taken at 100x magnification because these communities were dominated by dense filamentous cyanobacteria that could more accurately be counted from images. For these lakes, 10 images equally spaced along a transect were taken at 100x magnification and 2-4 images chosen from the edges and center of the transect were counted until at least 100 filaments were counted.

Cell dimensions were measured from microscope images using ImageJ (version 1.50i, https://imagej.nih.gov/ij) and biovolumes were calculated based on cell geometry according to Hillebrand (1999). For taxa at 400x and 100x magnification that exhibited a consistent size range across all treatments, mean measurements taken across all treatments were used to calculate biovolumes. However, for cells that varied in their length (ie. chainforming diatoms), treatment-specific means were calculated from length measurements taken in each treatment. Additionally, for filamentous cyanobacterial cells that were counted via images for lakes Tjeukemeer and Loosdrecht, cell dimensions were measured on all counted cells in each image such that the total biovolume was calculated for each image.

Functional groupings

After all cell biovolumes were calculated and normalized by sample volume (um^3/mL), they were binned into seven morphology-based functional groups developed by Kruk et al. (2010) as follows: small cells with high surface area to volume ratio (FG I), small flagellated organisms with siliceous exoskeletons (FG II), large filaments with aerotopes (FG III), organisms of medium size lacking specialized traits (FG IV), unicellar flagellates of medium to

large size (FG V), non-flagellated organisms with siliceous exoskeletons (FG VI), and large mucilaginous colonies (FG VII). This functional group scheme was chosen because it can be well-predicted from environmental conditions (Kruk & Segura 2012, Kruk et al. 2011) and can be used to analyze zooplankton-phytoplankton interactions (Colina et al. 2016).

For all three communities, FG I consisted of cells with maximum linear dimensions (MLD) of 5um, including picocyanobacteria, and small round flagellated and nonflagellated cells. FG II was found only in the medium productivity community and consisted of *Dinobryon* sp. FG III, prevalent in the medium and high productivity lakes, consisted of filamentous cyanobacteria including *Planktothrix* sp., *Limnothrix* sp., *Anabaena* sp., and *Pseudanabaena* sp. FG IV consisted primarily of unicellular round cells with MLD of 9um in the low productivity lake, and larger colonial forms in the medium and high productivity lakes such as *Scenedesmus* sp., *Cosmocladium* sp., *Pediastrum* sp., *Actinastrum* sp., *Tetrastrum* sp., *Tetraedron* sp., *Ankistrodesmus* sp. FG V was present in the low productivity lake and consisted of *Plagioselmis* sp. and dinoflagellates such as *Gymnodinium* sp. and *Ceratium* sp. The medium productivity lake also contained dinoflagellates, but they were rare and found in small quantities in only four samples, so they were not counted as a separate functional group and were instead included in FG IV. FG VI consisted entirely of diatoms, which in the low productivity lake included pennate colonies including *Asterionella* sp. and *Fragilaria* sp., and centric unicells and colonies such as *Aulacoseira* sp. In the medium and high productivity lakes, FG VI was composed primarily of colonial (*Tabellaria* sp.) and single celled pennate diatoms such as *Synedra* sp. and *Ceratoneis* sp. FG VII for all three communities was composed of mucilaginous cyanobacterial colonies, including *Microcystis* sp., *Aphanocapsa* sp, and *Aphanothece* sp.

Statistical methods

All statistical analyses were performed using the statistical program R version 3.4.2 (R Development Core Team 2017). We fit generalized linear models ('glm' function in lme4 package) with gaussian distributions to determine the main and interactive effects of experimental treatments on response variables (functional group biovolume, size abundance from flow cytometry) in each experiment and assessed statistical significance using a chi-squared test. Prior to running models, data were tested for normality using the Shapiro-Wilk test, and were log-transformed if doing so improved normality.

Effect sizes of grazing and nutrient addition, separated by ambient and warmed temperatures, were calculated for functional group biovolumes and size abundances using the ln-transformed response ratio (Hedges et al. 1999) calculated as $RR = \ln(\overline{X}_E/\overline{X}_C)$, where $\overline{X}_E$ is the mean value in enriched treatments (nutrient addition or grazing), and $\overline{X}_C$ is the mean of the analogous control treatment lacking enrichment. The variance of each effect size was calculated using the following equation: $\frac{(SD_E)^2}{n_E\overline{X}_E{}^2}+\frac{(SD_C)^2}{n_C\overline{X}_C{}^2}$ where SD is the standard deviation and n is the sample size (Hedges et al. 1999).

Additionally, the mean percentages of each functional group biovolume and standard errors were calculated from the control treatments to understand the functional composition of each community.

Results

Percentage of biovolume by functional type

In the control treatments, the lowest productivity community included groups of varying grazing susceptibility, including groups I, IV, V, VI, and VII, while the medium and high

productivity communities were dominated by genera belonging to functional group III with low grazing susceptibility (Fig. 3.1). Specifically, percentages of each functional group by biovolume (±SE) in control treatments were as follows for the low productivity community: small cells with high S:V (I): 5.8±0.70%; medium cells with no specializations (IV): 33.5±11.6%; medium to large flagellates (V): 0.73±0.48%; nonflagellated with siliceous exoskeleton (VI): 23.3±5.3%; large mucilaginous colonies (VII): 36.6±8.1% (Fig. 3.1). The medium productivity community showed a different functional composition: functional groups I, IV, VI and VII were present in lower percentages (0.49±0.19% ,1.6±0.83%, 4.7±2.3% and 0.054±0.043% respectively; Fig. 3.1). Additionally, group II (small siliceous flagellates) was present in low abundances 0.033±0.016% whereas group III (large filaments with aerotopes) dominated the community (93.1±1.5%; Fig. 3.1). The high productivity community was also dominated by group III (71.6±3.0%), followed by group VII (11.8%±3.2%), while groups I (2.2±0.48%), IV (7.8±0.70%), and VI (6.7±1.0%) were more abundant than in the medium productivity community. Approximately 63% of the low productivity community is composed of groups with high or intermediate grazing susceptibility, whereas these groups represent only 7% and 17% in the medium and high productivity communities, respectively.

Treatment effects on functional group biovolumes

In the low productivity community, all groups increased in response to nutrient addition (Table 1), and all groups except VII showed losses due to grazing (Table 1, Fig. 3.2). There were no significant main effects of warming or interactions with nutrients or grazing on any group.

In the medium productivity community, nutrient addition significantly increased biovolumes of groups I ($P<0.001$), IV ($P<0.001$), and VII ($P<0.05$) but decreased biovolume of

group III ($P<0.001$) (Table 1, Fig. 3.2). In addition to nutrient addition, grazing also had a positive effect on the biovolume of group I ($P<0.05$), and the interaction of nutrient addition and grazing had a positive effect on group VI ($P<0.05$) (Table 1, Fig. 3.2). Similar to the low productivity community, there were no significant effects of warming or its interaction with nutrients or grazing on the medium productivity community.

In the high productivity community, only functional groups IV and VI biovolumes showed differences among treatments. For group IV, grazing had a positive effect on biovolume ($P<0.05$), and nutrient addition had a positive effect when warmed (warming*nutrients, $P<0.01$) (Table 1, Fig. 3.2). For FG VI, temperature had a negative effect on biovolume ($P<0.001$) and nutrients had a positive effect ($P<0.05$) (Table 1, Fig. 3.2).

When the effect size of grazing and nutrient addition on functional groups are compared across all three lakes (Fig. 3.2), it is evident that functional groups within lakes respond similarly, resulting in a pattern among lakes in which both the effect size of nutrients and grazing decrease with productivity. Nearly all groups in the low productivity lake show a positive response to nutrient addition and negative response to grazing at both temperatures; the medium productivity lake shows an intermediate response whereby functional groups show positive or negative responses to nutrient addition and some show positive responses to grazing; while in the high productivity lake, all functional groups show little to no effect of grazing and nutrient addition at both temperatures.

Treatment effects on cell size abundances from flow cytometry

In all three communities, all three edible size fractions (<2um, 2-10um, 10-30um) responded positively or negatively to nutrient addition. However, the effects of grazing,

warming, and interactions differed by community (Table 3.2, Fig. 3.2).

Cells <2um in the low productivity community responded positively to nutrients ($P<0.001$) and were affected by a three-way interaction between the treatments ($P<0.05$; Table 3.2, Fig. 3). Nutrient addition elevated<2um cell abundances at ambient temperature with grazers and at elevated temperatures with and without grazers, although the magnitude of increase was much greater at warmed temperature without grazers (Table 3.2, Fig. 3.3). Cells in the 2-10um and 10-30um fractions responded positively to nutrients ($P<0.001$; Table 3.2, Fig. 3.3) and negatively to grazing ($P<0.001$; Table 3.2, Fig. 3.3). There was also a significant three-way interaction between warming, nutrients and grazing in the 10-30um fraction, ($P<0.05$; Table 3.2, Fig. 3.3). Nutrient addition without grazers had no effect at ambient temperature, but warming in the equivalent treatment caused an increase in cell abundances ($P<0.05$; Table 3.2, Fig. 3.3).

In the medium productivity community, all three size fractions were affected by the interaction between temperature and nutrients. For cells <2um, nutrient addition had no effect at ambient temperature, but had a negative effect at warm temperature ($P<0.001$) and warming elevated abundances at ambient nutrient levels ($P<0.05$) (Table 3.2, Fig. 3.3). Additionally, nutrient addition had a positive effect on the 2-10um and 10-30um size fractions,at ambient temperature but not when warmed ($P<0.01$ and $P<0.001$ respectively; Table 3.2, Fig. 3.3).

In the high productivity community, temperature showed significant two-way interactions with both grazing and nutrients in the <2um and 2-10um size fractions but not in the 10-30um fraction. In the <2um size fraction, both nutrient addition and warming independently elevated cell counts ($P<0.001$) (Table 3.2, Fig. 3.3). Warming interacted with nutrients such that the effect of nutrient addition was greater in the warmed treatment ($P<0.05$), and also interacted with grazing such that there was no effect of grazing at ambient temperature, but a slight negative

effect of grazing when warmed ($P<0.05$) (Table 3.2, Fig. 3.3). In the 2-10um size fraction, nutrient addition elevated abundances ($P<0.001$), and warming amplified this effect ($P<0.001$) (Table 3.2, Fig. 3.3). Warming increased cell abundances at ambient nutrients without grazing, but caused a negative effect of grazing that was only significant when nutrients were added ($P<0.001$) (Table 3.2, Fig. 3.3). In the 10-30um fraction, there was a positive effect of nutrient addition ($P<0.001$) and warming increased abundances in the absence of grazers, especially when nutrients were added, although only the main effect of warming was significant and not the interactions ($P<0.01$) (Table 3.2, Fig. 3.3).

Similar to the functional groups, size class abundances also show responses that cluster by lake (Fig. 3.3). However, there were more interactions with warming on top-down and bottom-up control for size abundances than for functional groups. At ambient temperature, two size classes (2-10um and 10-30um) in the low productivity community show negative responses to grazing, while no size classes responded significantly to grazing in the medium and high productivity lakes. However, warming strengthened top-down control on the <2um fraction in the low productivity community and on the <2um and 2-10um fractions in the high productivity community (Fig. 3.3). At ambient temperature, nutrient addition had a positive effect on size classes in all communities (except <2um in medium productivity community), but warming had variable effects on bottom-up control, depending on the size fraction and community. Warming amplified the positive effect of nutrient addition on the 10-30um size fraction in the low productivity community and on all three size fractions in the high productivity community, whereas warming had an antagonistic effect with nutrient addition on all size classes in the medium productivity community (Fig. 3.3).

Table 3.1: P-values from generalized linear models for functional groups

FG I: small cells with high S:V							
productivity	temp	nutrients	grazing	tmp*nut	tmp*grz	nut*grz	tmp*nut*grz
Low	0.58	**<0.001*** **	**<0.001*** **	0.42	0.56	0.42	0.18
Medium	0.82	**<0.001*** **	**<0.05***	0.83	0.30	0.58	0.70
High	0.71	0.13	0.54	0.31	0.59	0.84	0.43
FG II: small siliceous flagellates							
productivity	temp	nutrients	grazing	tmp*nut	tmp*grz	nut*grz	tmp*nut*grz
Medium	0.57	0.49	0.38	0.97	0.67	0.14	0.55
FG III: large filaments with aerotopes							
productivity	temp	nutrients	grazing	tmp*nut	tmp*grz	nut*grz	tmp*nut*grz
Medium	0.34	**<0.001*** **	0.14	0.074	0.51	0.67	0.82
High	0.48	0.82	0.94	0.68	0.91	0.81	0.24
FG IV: organisms of medium size lacking specialized traits							
productivity	temp	nutrients	grazing	tmp*nut	tmp*grz	nut*grz	tmp*nut*grz
Low	0.22	**<0.01** **	**<0.001*** **	0.092	0.22	0.75	0.084
Medium	0.55	**<0.001*** **	0.78	0.14	0.79	0.98	0.54
High	0.72	0.72	**<0.05***	**<0.01** **	0.92	0.30	0.77
FG V: flagellates of medium to large size							
productivity	temp	nutrients	grazing	tmp*nut	tmp*grz	nut*grz	tmp*nut*grz
Low	0.22	**<0.01** **	**<0.01** **	0.77	0.85	0.86	0.48
FG VI: siliceous, non-flagellated organisms							
productivity	temp	nutrients	grazing	tmp*nut	tmp*grz	nut*grz	tmp*nut*grz
Low	0.60	**<0.001*** **	**<0.01** **	0.81	0.18	0.57	0.59
Medium	0.35	0.94	0.15	0.78	0.15	**<0.05***	0.48
High	**<0.001*** **	**<0.05***	0.17	0.74	0.24	0.23	0.96
FG VII: large mucilaginous colonies							
productivity	temp	nutrients	grazing	tmp*nut	tmp*grz	nut*grz	tmp*nut*grz
Low	0.19	**<0.05***	0.47	0.74	0.95	0.45	0.36
Medium	0.084	**<0.05***	0.73	0.15	0.44	0.72	0.61
High	0.52	0.91	0.47	0.69	0.32	0.92	0.22

Table 3.2: P-values from generalized linear models for cell size abundances

<2 um cell abundance							
productivity	temp	nutrients	grazing	tmp*nut	tmp*grz	nut*grz	tmp*nut*grz
Low	0.72	**<0.001*** **	0.14	0.30	0.30	0.13	**<0.05***
Medium	**<0.05***	**<0.01****	0.78	**<0.001*** **	0.38	0.30	0.70
High	**<0.001*** **	**<0.001*** **	0.18	**<0.05***	**<0.05***	0.91	0.86
2-10um cell abundance							
productivity	temp	nutrients	grazing	tmp*nut	tmp*grz	nut*grz	tmp*nut*grz
Low	0.20	**<0.001*** **	**<0.001*** **	0.22	0.98	0.34	0.45
Medium	0.86	**<0.001*** **	0.99	**<0.01****	0.12	0.12	0.90
High	**<0.001*** **	**<0.001*** **	0.13	**<0.001*** **	**<0.001*** **	0.37	0.37
10-30um cell abundance							
productivity	temp	nutrients	grazing	tmp*nut	tmp*grz	nut*grz	tmp*nut*grz
Low	**<0.05***	**<0.001*** **	**<0.001*** **	0.46	0.85	0.11	**<0.05***
Medium	**<0.001*** **	**<0.001*** **	0.44	**<0.001*** **	0.64	0.66	0.11
High	**<0.01****	**<0.001*** **	0.52	0.082	0.061	0.20	0.37

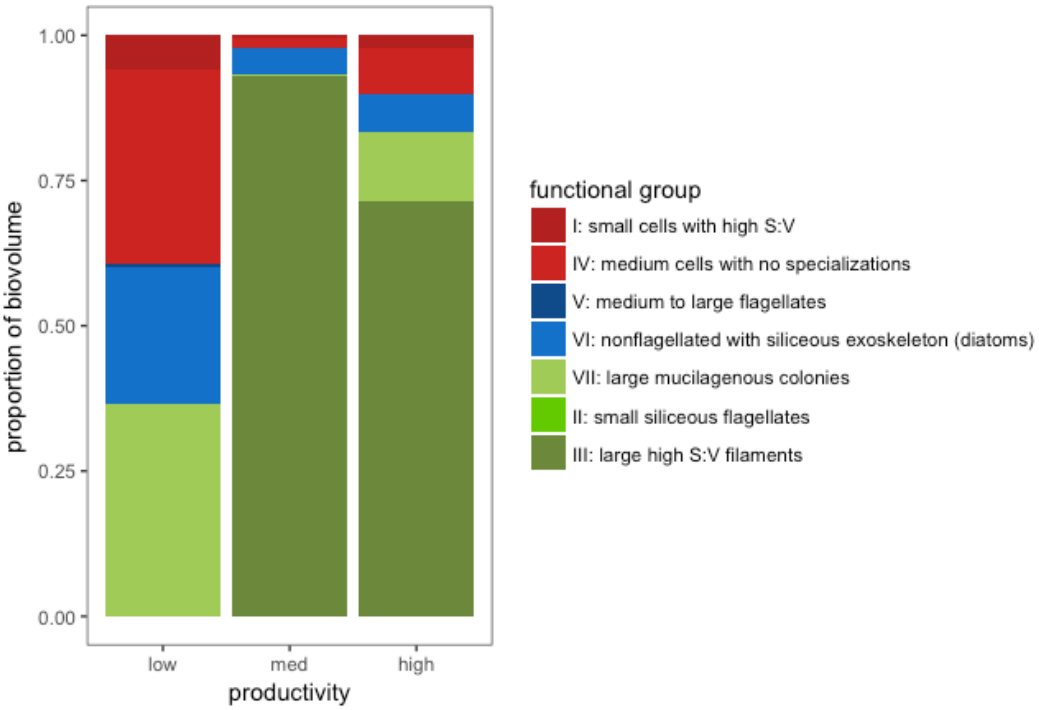

Figure 3.1: Mean relative abundances of each functional group in the control treatment are shown for each community along the productivity gradient. Colors correspond with functional groups, but shades of red indicate high grazing susceptibility, shades of blue indicate medium grazing susceptibility, and shades of green indicate low grazing susceptibility.

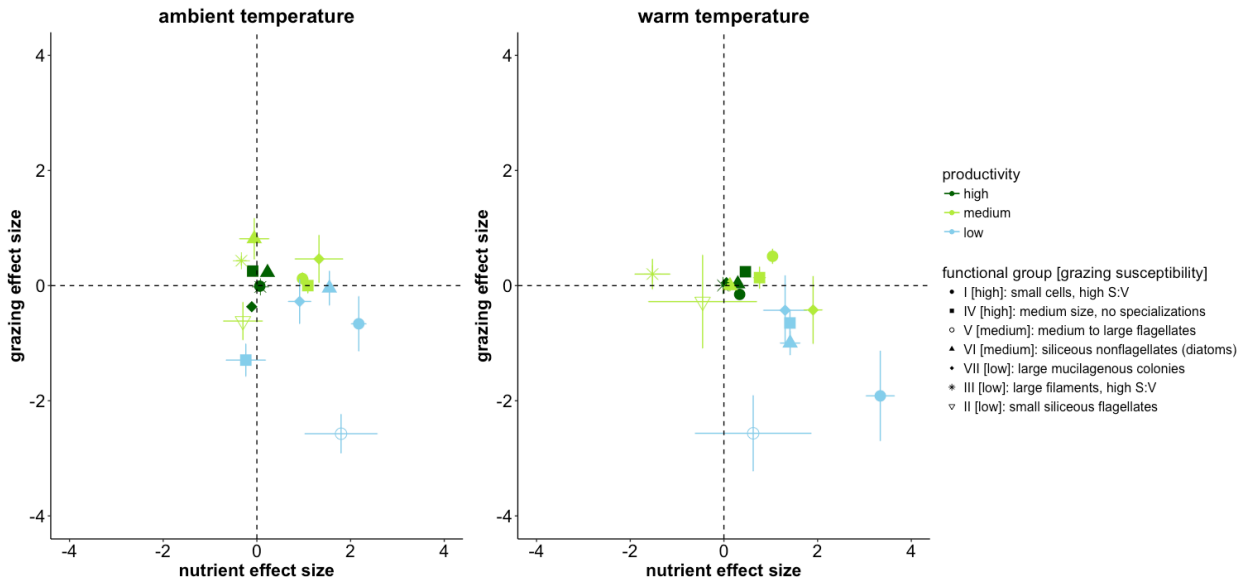

Figure 3.2: Effect sizes of grazing and nutrient addition on functional group biovolumes are shown at ambient and warmed temperatures. Colors indicate community productivity, while shapes indicate functional groups.

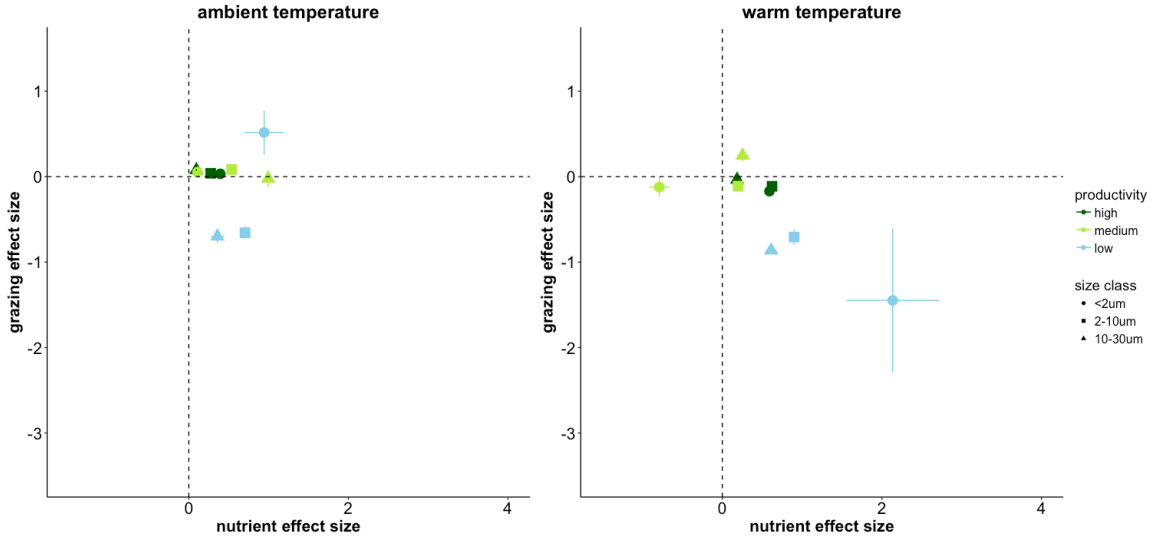

Figure 3.3: Effect sizes of grazing and nutrient addition on cell size abundances in the edible fraction (<2-30um) are shown at ambient and warmed temperatures. Colors indicate community productivity, while shapes indicate size class.

Discussion

We found no support for the hypothesis that interspecific tradeoffs would result in negative correlations between grazer and nutrient limitation across phytoplankton size or functional categories, either within or among lakes. Instead, we found that functional groups became less sensitive to *both* nutrient addition and grazing as productivity increased, such that almost all groups in the lowest productivity community were co-limited by both grazing and nutrient supply, and these effects diminished with increasing productivity among lakes (Fig. 2). Warming increased top-down control on some size classes in the low and high productivity communities, but had idiosyncratic effects on bottom-up control depending on the community. Our results indicate that (1) effects of both nutrient limitation and grazing decline across functional and size classes with increasing lake trophic status, (2) functional groups and size classes are more sensitive to both top-down and bottom-up control in the least productive lake, and (3) warming shifted both consumer and nutrient effects, but the effects varied by lake, size class and functional group. The experiments indicate that differences among factors limiting growth do not promote coexistence locally within lake phytoplankton, but rather contribute to regional turnover along gradients in productivity.

The decreasing effect of grazing on functional groups with increasing productivity is likely a result of community turnover along productivity gradients, with increasing abundance of inedible functional groups as productivity increases (Fig.1). Increasing abundances of large-celled, colonial or filamentous species with increasing phytoplankton productivity is consistent with expectations, as small-celled species are better nutrient competitors in low-nutrient environments while large-celled species thrive in high or fluctuating nutrient environments (Cloern 2018, Irwin et al. 2006, Kiørboe 1993, Chisholm 1992, Litchman et al. 2007; Litchman

et al. 2010; Edwards et al 2011). In general, *Daphnia* grazing impact on phytoplankton communities decreases as productivity increases, as *Daphnia* exhibit a Holling Type II functional response in which their ingestion rates increase linearly when prey density is low and saturate as prey availability increases (Colina et al. 2016, Holling 1959).

Additionally, the inedibility of filamentous cyanobacteria, which dominated the intermediate and high productivity communities in our experiments, has been established for *Daphnia*. These filamentous forms mechanically obstruct the *Daphnia* filtering apparatus especially for larger bodied species like *D. magna* used in our experiments (DeMott et al. 2001, Gliwicz & Lampert 1990, Gliwicz 1990). Additionally, filamentous cyanobacteria produce toxic and possibly allelopathic chemical compounds (Fulton 1988, Lampert 1981) and are of low nutritional quality (Brett & Müller- Navarra 1997). As a result, large-bodied *Daphnia* cannot maintain growth in the presence of high densities of toxic filamentous cyanobacteria, including in lake Loosdrecht (DeMott et al. 2001), the medium productivity community used in our experiment. Therefore, large *Daphnia* can be excluded from eutrophic lakes dominated by these forms (Hansson 2007). Inhibition of grazing and therefore growth of large *Daphnia* species by filamentous cyanobacteria has been shown to occur even when supplemented with edible algae (DeMott et al. 2001), indicating that a dominance by filamentous forms provides an associational defense for more edible phytoplankton species. This is consistent with our study, as the edible functional groups showed no significant losses to grazing in the two lakes dominated by filamentous cyanobacteria. Facilitation may thus allow grazing-resistant and -susceptible groups to coexist in highly productive environments.

The weakening effects of nutrient addition on functional groups as productivity increases is likely a result of decreasing nutrient limitation with increasing productivity, as productivity

scales with phosphorous availability (Elser et al. 2007, Watson et al. 1997, Smith 1979, Schindler 1978). In contrast to the expectation that functional groups within lakes would be differentially limited by nutrients, the effect sizes of nutrient addition varied more across lakes than within lakes. Functional groups in the lowest productivity lake showed strongest positive responses to nutrient addition, likely because low nutrient environments are dominated by strong nutrient competitors that acquire nutrients efficiently and can grow quickly (Hecky and Kilham 1988, Irwin et al. 2006, Litchman et al. 2007). Biomass of taxonomic groups in oligotrophic environments have been shown to increase sharply with TP (Watson et al. 1997). The weaker response to nutrient addition in the highest productivity lake for all functional groups may result from light limitation at high cell density in nutrient rich environments (Edwards et al. 2013). The dominance of cyanobacteria and especially filamentous forms in eutrophic lakes often occurs with increasing levels of TP (Watson et al. 1997) because they are strong light competitors (Scheffer 1997, Smith 1986). Filamentous cyanobacteria also increase turbidity relative to other algal species (Scheffer et al. 1997) and some species can both efficiently absorb photosynthetically active radiation (PAR) and grow well in low light environments, resulting in less total light penetration through the water column (Huisman 1999). Stronger light competitors have been shown to increase in biovolume as light becomes limiting, and therefore variation in community structure along productivity gradients may be driven by relative availabilities of light and nutrients (Edwards et al. 2013, Smith 1986).

The variable effects of warming and its interactions with nutrient addition on functional group and cell size abundance in each community is consistent with a body of literature indicating that the effect of warming depends on trophic state and species composition of phytoplankton in lakes. Warming effects on phytoplankton productivity can range from positive

to negative depending on P availability, with constrained or negative effects under P scarcity and positive effects under eutrophic conditions (Tadonléké 2010). Similarly, model simulations have shown that the effects of warming on phytoplankton blooms vary among species but are often more pronounced at higher nutrient levels (Elliott et al. 2006; Huber et al. 2008). Our experiments support these conclusions as warming and its interaction with nutrient addition had positive or additive effects on functional group and size classes in the most productive community, and the least effect on the least productive community. Although the lowest productivity community also experienced the lowest magnitude of warming (see methods), its response is consistent with the expectation that temperature should have a greater effect on phytoplankton in more productive lakes (Tadonléké 2010, Elliott et al. 2006; Huber et al. 2008, Rigosi et al. 2014). However, in the medium productivity community, warming reduced filamentous cyanobacteria abundance and had an antagonistic effect with nutrient addition on all edible size classes (Figs. 2 & 3). This unexpected negative interaction between nutrient addition and warming on this community might have been a result of photosynthetic inhibition at elevated temperature (Tadonléké 2010) or nutrient competition between heterotrophic bacteria and phytoplankton at elevated temperatures (Chrzanowski and Grover 2001). Additionally, warming beyond optimal temperatures for growth has been postulated to have an antagonistic effect with nutrient addition (Rigosi et al. 2014), which may have occurred for this community in our experiment because it had the highest temperature in the warmed treatment (20°C, see methods), although the mean temperature optima for cyanobacteria tends to be higher (29.2°C; Lürling et al. 2013).

The increased effect size of grazing on small size class abundances with warming is also consistent with predictions that warming magnifies top-down control, especially when resources

are abundant. Metabolic theory posits that warming increases metabolic rates of heterotrophic consumers faster than photosynthesis by autotrophs, resulting in increased energetic demands at higher temperatures (Allen et al. 2005, Brown et al. 2004), and this has been demonstrated in experimental studies (Shurin et al. 2012, Kratina et al. 2012, Velthuis et al. 2017). However, this effect depends on nutrient availability, as higher resource levels can support an increased heterotroph to autotroph biomass ratio with warming (O'Connor 2009). Similarly, increased effect sizes of grazing on small size class abundances in the low and high productivity communities occurred when warming was combined with nutrient addition. Positive effects of grazing that occurred for functional groups in the medium and high productivity lakes might have resulted from nutrient recycling by grazers (Elser and Urabe 1999).

Tradeoffs have the potential to promote diversity and coexistence when competing taxa experience limitation by different factors. Our experiments indicate that the competition-defense tradeoff does not occur at the local scale of communities within lakes, or communities among lakes across a productivity gradient. However, there was a robust pattern in which both top-down and bottom-up control decreased among lakes along a productivity gradient due to turnover in functional groups among lakes, with increasing abundances of inedible filamentous cyanobacteria in more productive lakes. These findings support the idea that spatial turnover in communities occur at the regional scale along productivity gradients (Ptacnik et al. 2010, Kneitel & Chase 2004). Additionally, we found that warming had idiosyncratic interactions with grazing and nutrient addition that depended on the trophic state and functional group composition of each community, suggesting that lake ecosystem responses to a warmer climate depend on a complex interplay between top-down and bottom-up forces.

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CONCLUSIONS AND FUTURE DIRECTIONS

Warming has been found to alter the strength of top-down and bottom-up forces (Shurin et al. 2012, O'Connor et al. 2011), but how temperature interacts with resource supply to shape autotrophic and heterotrophic aquatic microbial communities is poorly understood. My dissertation seeks to understand how warming and resource supply independently and interactively affect aspects of bottom-up control including community composition, stoichiometry, and functional tradeoffs in microbial communities across resource supply gradients.

In Chapter 1, I showed that inorganic and organic resources structure prokaryotic and eukaryotic microbial communities along natural climatic gradients in mountain lakes. However, there was no indication of synergies or interference with temperature. Climate and bottom-up factors may therefore operate independently in structuring some aspects of microbial assemblages, indicating that temperature may play a secondary role to resource supply in structuring these lake microbial communities. We also found that relative abundances and membership in the two communities were strongly correlated, suggesting that biotic interactions between certain taxonomic groups may place constraints on community membership and abundance, as has been found in marine ecosystems (Lima-Mendez et al. 2015). Finally, the reduction of OTU richness for both prokaryotes and eukaryotes as N increases is consistent with other findings in plant and soil microbial communities (Harpole & Tilman 2007, reviewed in Cleland & Harpole 2010, Ramirez et al. 2010) and may indicate a universal response to N deposition of decreasing richness and increasing dominance of taxa across a diverse range of ecosystems. Disentangling the interactions among the diverse members of microbial communities and understanding their effects on ecosystems remains a major challenge, and these

interactions and functional roles of microbes warrant further study, using -omics techniques such as transcriptomics, metabolomics, and metagenomics. Pairing these techniques with fine scale environmental time series data would enable a better understanding of how these taxonomic groups and their interactions respond to environmental fluctuations through time.

In addition to affecting microbial community composition, changes in temperature and nutrient supply can affect biogeochemical cycling and stoichiometric ratios in phytoplankton. In Chapter 2, I found that the effects of climate warming, eutrophication, and grazing elicited distinct responses in phytoplankton communities depending on the trophic state, community composition and size structure. I show that across a gradient of increasing productivity, the fraction of small cells in communities decrease, resulting in strongest impacts of grazing and consumer-driven nutrient recycling on growth, biomass buildup, C:P and N:P in the lowest productivity lake with the highest fraction of small cells. Additionally, C:P responses differ by size fraction for all three communities examined, indicating that traits associated with cell size will mediate community stoichiometry in response to various stressors. The variable effects of warming and its interactions with nutrient addition in each community across our productivity gradient is consistent with numerous studies indicating that the effect of warming on phytoplankton communities in lakes depends on nutrient supply and species composition, with stronger effects in eutrophic and hypereutrophic systems (Tadonléké 2010, Rigosi et al. 2014, Elliott et al. 2006, Huber et al. 2008). This work raised questions about how temperature and nutrients can interact to have either synergistic or antagonistic effects on phytoplankton communities, and the mechanisms for these responses warrant further study. These mechanisms can be studied by measuring which traits and species are associated with these two divergent

responses, and conducting further experimentation to understand which abiotic or biotic conditions drive synergistic or antagonistic responses between nutrients and warming.

In Chapter 3, I show that the competition-defense tradeoff does not occur at the local scale within lakes, or among lakes across a productivity gradient. However, there was a robust pattern in which both top-down and bottom-up control decreased among lakes along a productivity gradient due to turnover in functional groups and increasing abundances of inedible filamentous cyanobacteria. These findings support the idea that beta diversity and turnover in communities along productivity gradients is due to regional, not local processes (Ptacnik et al. 2010, Kneitel & Chase 2004). Additionally, we found that warming had idiosyncratic interactions with grazing and nutrient addition that depended on the trophic state and functional group composition of each community, suggesting that lake ecosystem responses to a warmer climate depend on a complex interplay between top-down and bottom-up forces. Future work could examine whether a growth-defense tradeoff, rather than a competition-defense tradeoff, is driving the response we see, as small, grazer-limited taxa also have fast growth rates which may be driving their positive response to nutrient pulses, as compared to slower-growing, grazer-defended taxa. It would be illuminating to measure growth rates associated with each functional group present in these communities, and understand whether their nutrient responses are indeed tied to *r* vs. *K* growth strategies.

Predicting the effects of simultaneous environmental changes on aquatic microbial communities, food webs and ecosystems remains a challenge but is important for managing biodiversity and ecosystem services. My work shows that resources are strong structuring forces in lake bacterioplankton and phytoplankton communities, and that temperature may play a secondary role in driving community structure (Chapters 1, 2, 3). However, temperature is likely

to interact with resource supply in determining phytoplankton species composition, biomass, and stoichiometry; and these interactions are likely to depend on the trophic state and traits present in communities (Chapters 2 and 3). As traits mediate community responses to environmental stressors via interactions with predators and uptake of nutrients, predicting the effects of warming and other stressors on lake phytoplankton communities using a trait-based approach (Meunier et al. 2017, Litchman et al. 2012, Litchman & Klausmeier 2008) may offer the most accurate predictive framework for assessing how specific phytoplankton communities will respond to global environmental change. Microorganisms are central to biogeochemical cycling, primary production, decomposition, and energy flow in food webs, thus understanding microbial community responses to warming and nutrient pollution is critical for maintaining ecosystem functioning and important ecosystem services such as recreation, fishing, and water quality.

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