OPTIMIZING THE PRODUCTIVITY AND SUSTAINABILITY OF ALGAL BIOFUEL SYSTEMS: INVESTIGATING THE BENEFITS OF ALGAL DIVERSITY AND UTILIZING BREWERY WASTEWATER FOR CULTIVATION

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Integrative Biology—Doctor of Philosophy
Ecology, Evolutionary Biology and Behavior—Dual Major

2016

ABSTRACT

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The era of inexpensive fossil fuels is coming to a close, while society is beginning to grapple with the byproducts of their combustion. Identifying alternative and more sustainable energy sources is of the utmost importance. One extremely promising option is biofuel derived from microalgae. Although algal biofuels have the capability to generate a substantial amount of biodiesel, there are a number of limitations that are hindering its commercialization. In this dissertation, I examine two main limitations concerning the economic and environmental sustainability of mass algal cultivation: (1) achieving and maintaining high algal productivity and (2) identifying an inexpensive water and nutrient source. Through the application of some core principles of ecological theory and employing a trait-based approach, I will illustrate how fostering algal diversity within these biofuel systems can lead to high productivity and stability. Knowledge of algal eco-physiological traits is essential for assembling optimal algal communities. Thus, I conducted a large thermal trait survey for 25 different algal species to better understand their biomass and fatty acid production across a range of temperatures. Finally, I will present two experiments I conducted investigating the feasibility of coupling algal cultivation with wastewater remediation generated at breweries. This work has illustrated that wastewater is a highly suitable source of nutrients for algal cultivation and microalgae have high remediation potential as well, ultimately advancing the sustainability of both breweries and mass algal production.

With much love and thanks, this thesis is dedicated to the many amazing and inspirational people in my life. Marissa, this is for your undying love and support. Without it, this would not have been possible. Thank you for your relentless encouragement as I stumbled my way through. Ofelia, this is for you and your future. And to the algae, I pushed you to your limits, and you pushed me to mine. I'm glad we got through this together.

ACKNOWLEDGEMENTS

I would like to give my deepest thanks to my advisor Elena Litchman and my esteemed committee members: Christoph Benning, Christopher Klausmeier and Gary Mittelbach. Especially to Christoph Benning for the use of his laboratory and equipment, none of the fatty acid work would have been possible without your kindness. I would also like to thank all of the wonderful lab mates that have advanced and challenged my views on science and the world: Colin Kremer, Beth Miller, Danny O'Donnell, Ravi Ranjan, Simon Stump, Mridul Thomas and Paul Wilburn. Special thanks to Maria Stockenreiter for the endless support and advice as I navigated my graduate years, I am eternally grateful. I also wish to thank all of my undergraduates for the many summers of fun and hard work: Farhana Haque, Katie McCullen, Jacob Pino and Scott Schultz. Finally, I owe an unpayable debt of thanks to Pam Woodruff and Ally Hutchens for the endless support throughout experiments and sage advice as I fumbled through my years of graduate school.

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INTRODUCTION

Algae derived biofuels are emerging as an extremely promising alternative liquid fuel source as fossil fuel reserves begin to dwindle. Algae can generate copious amounts of biomass on limited, non-arable land with significantly reduced, if not neutral, carbon emissions all while being cultivated on waste streams from industry and municipalities. Yet, although promising, the commercial reality of algae-derived biofuel is still hampered by inconsistent yields and expensive infrastructure inputs (bioreactors, pond construction, water and fertilizers). In this dissertation, I investigate how algal systems can be made more productive and resilient under environmental fluctuations and pressures (invasion, predation, infection) through fostering algal diversity. I also present some research on how wastewater from the rapidly growing brewery industry might be a highly suitable and inexpensive source of water and nutrients for mass algal cultivation.

Using algae as an energy source is not a novel concept. The fossil fuels we are currently consuming are derived mostly from ancient aquatic plant life. In 1978, the Department of Energy funded the Aquatic Species Program aimed at researching algae as an energy source. Yet obtaining high, consistent yields within these systems still remains a challenge. In Chapter 1, I lay out an overview of how core concepts of community ecology research can be applied to algal biofuel systems. Through piecing together complementary algal communities that are tailored to local environmental conditions, these systems can be optimized to achieve consistently high yields and resist undesirable invasion, predation and infection. Although these concepts are well established in the field of ecology, their application to algal biofuel systems remains novel.

Mass cultivation of algae will most likely take place in large, open outdoor ponds due to low infrastructure costs. These outdoor systems will experience environmental fluctuations on multiple temporal scales (daily, seasonal). In Chapter 2, I investigate the implications of

fluctuating light on growth and fatty acid production of algal monocultures and polycultures. Increasing algal diversity led to higher biomass stability through time, not significantly impacted by the fluctuating light. The polyculture biomass achieved overyielding, out performing the averaged monocultures, which was attributable to niche complementarity. Fatty acid production though showed no difference between monocultures and polycultures. Light levels also had no significant impact on the species-specific fatty acid profiles.

In order to tailor species assemblages to local conditions, the eco-physiological responses to temperature will be important. In Chapter 3, I present a thermal trait survey of 25 algal species collecting growth rates, fatty acid production and fatty acid profiles across a range of temperatures (9-32°C). Thermal growth curves were constructed for the surveyed species, which we used to determine max growth rate, thermal growth optima, minimum and maximum, and niche width. We also derived the functional group level growth curves, illustrating that there are functional group level differences in temperature optima and maximum growth rates. There are also functional group specific fatty acid profiles, highlighting that some groups are more suitable for biodiesel production than others.

Chapter 4 is the first of two chapters investigating the feasibility of cultivating microalgae in brewery wastewater (BWW). This chapter establishes proof of concept that microalgae can be cultivated in brewery wastewater. Through an initial survey of 16 species from 3 functional groups, we identified 7 candidate species that achieved positive growth in minimally diluted BWW. *C. vulgaris*, *Ourococcus* sp., and a seven-species polyculture all achieved high biomass and fatty acid accumulation across 12 days. The polyculture also had the highest removal rates of total nitrogen and phosphorus, removing 76% and 83% respectively.

Finally, in Chapter 5 I extend the research of coupling algal cultivation and brewery wastewater remediation. In an effort to achieve even higher rates of nitrogen and phosphorus removal, we attempted a two-stage approach to remediation. The primary cleaning step utilized the candidate species from the previous experiment, cultured in monoculture and a collective polyculture. After 9 days, the filtered water was moved to a secondary cleaning stage where cyanobacteria were cultivated. Some cyanobacteria can fix atmospheric nitrogen, which would allow them to thrive in nitrogen deficient environments while still removing phosphorus. Supporting the results from the previous study, we found that the polyculture and *C. vulgaris* achieved high biomass and fatty acid production. By day 10, 94% of the total nitrogen and 88% of the total phosphorus had been removed. By day 15, more than 99% of the total nitrogen and 93% of the total phosphorus was removed, bringing levels to below the detection limit and 4 mg L^{-1}, respectively.

This research aims to advance the small, but growing body of work investigating the benefits of promoting algal diversity in algal biofuel systems. Also, this research shows the potential of brewery wastewater as a viable nutrient and water source for mass cultivation in an effort to advance the economic and environmental sustainability of the algae and brewing industry.

CHAPTER 1

Community ecology of algal biofuels: complementarity and trait-based approaches

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Published in *Industrial Biotechnology* (2014) Issue 10, Pages 191–201

ABSTRACT

Although open outdoor pond systems are the most economically viable option for mass cultivation of algae as a biofuel source, they face a number of limitations. Open-ponds will experience environmental fluctuations (i.e., light levels, nutrient ratios, and temperature), invasion pressure by undesired algal species, pathogen infections, and herbivory by invading zooplankton, which may negatively influence the system's overall productivity. Using ecological principles to address the limitations of the open-pond cultivation is a promising direction in algal biofuel research. This review surveys the growing body of work on these topics and offers a mechanistic framework for optimizing algal biofuel production while minimizing the negative effects of invasion, infection and herbivory. High levels of productivity (in terms of biomass and fatty acids) are crucial for viable biofuel production and can be achieved by increasing algal diversity and assembling communities based on species eco-physiological traits. Herbivory can be significantly reduced by choosing algal species resistant to grazing or by introducing biotic controls on herbivores. Diverse assemblages of algal species can be constructed to fill in the available ecological niche space leading not only to high productivity but reduced invasibility by undesired strains and potentially reduced susceptibility to algal diseases. Optimization of the mass cultivation of algae requires an interdisciplinary approach that includes using ecological principles for designing productive, resistant and resilient algal communities.

INTRODUCTION

Alternative renewable fuel sources have become an active area of research, as global fossil fuel reserves are estimated to be depleted within the next 60 years and unprecedented

anthropogenic combustion of hydrocarbons has led to increased atmospheric CO_2 levels resulting in climatic changes (Chapin *et al.* 2000; Stephens *et al.* 2010; IPCC 2013). One key renewable source is living biomass, or biofuels. But, for biofuel production to be economically viable and environmentally sustainable, the feedstock source must be highly and consistently productive, scalable to industrial levels, irrespective of food production networks, require little energy and nutrient inputs, and have a low carbon footprint (Schenk *et al.* 2008; Mata, Martins & Caetano 2010). Microalgae are considered among the most promising biomass-based renewable fuels.

Microalgae play a number of important ecosystem roles, from contributing over half of the global primary production, sequestering large amounts of CO_2 and supplying nutrition for primary consumers (Müller-Navarra *et al.* 2004; Guschina & Harwood 2006; Falkowski & Raven 2007; Ratha & Prasanna 2012). They also gained attention as a potential source of biofuel, first in the mid-1980's through the Department of Energy Aquatic Species Program (ASP) (Sheehan *et al.* 1998). Fifteen years after the end of the ASP, an interest in algal-based biofuel technology has become reinvigorated. Microalgae are an excellent biofuel crop due to their potentially high levels of productivity, limited land requirements for production and small carbon footprint from growth to combustion (Chisti 2007).

Although highly promising, the full potential of algal biofuels has not been realized in a commercial way. A number of limitations have emerged that must be dealt with, e.g., suitable strains, harvesting and extraction methods. Many studies have developed screening programs, looking to identify algal species that have high biomass fatty acid content (Rodolfi *et al.* 2009; Pereira *et al.* 2011; Ratha & Prasanna 2012).

Some of these "bioprospecting" studies found that algal cells can be stimulated to synthesize fatty acids when exposed to stressful conditions. The most common stressor is

nitrogen limitation that can increase fatty acid synthesis, resulting in higher fatty acid content (Borowitzka & Borowitzka 1988; Yamaberi, Takagi & Yoshida 1998; Griffiths & Harrison 2009). Some species, such as *Botryococcus braunii*, have been identified as having high internal fatty acid stores (>80% dry weight), making them ideal candidates for biofuel production (Ratha & Prasanna 2012). However, there is a trade-off between fatty acid production and growth, with high fatty acid content occurring in slow growing cells (Metting 1996). This trade-off underscores the need for an alternative approach where highly productive or fast-growing algae could still have high fatty acid content. Consequently, studies have focused on genetically manipulating species, attempting to hyper-activate the metabolic pathway responsible for fatty acid synthesis but still allowing high growth rates (Dunahay *et al.* 1996; Gimpel *et al.* 2013). Alternatively, there are also ecological options to optimize algal production without creating genetically modified organisms (GMO) that may pose a risk for natural communities. Through a review of ecological literature, we will present how algal biofuel production can be optimized through the promotion of functionally diverse algal assemblages that could achieve increased productivity, reduced invasibility by undesired algae, overall less susceptibility to pathogen infections while maintaining stable yields through highly variable outdoor environmental conditions.

We will draw upon the growing body of literature that has focused on this topic, but we aim to advance the ideas even further through presenting some of our own data analysis, discuss more in-depth trait based approaches, advance thoughts on reducing pathogen impacts, focus on a key mechanism for niche complementarity (light), and finally extend this ecological applications to aquaculture. Previous work has focused on reaching ecologically minded audiences, whereas here we outline our ideas in a concise and more broadly tangible dialogue to

foster more insight and investigation from an ecological level within the algal biofuel industry sector for a broader readership.

CULTIVATION SYSTEMS FOR MICROALGAL BIOMASS PRODUCTION

Two main cultivation approaches have been identified: one, more "industrial", requiring a large amount of infrastructure and technical upkeep and the other one, a more "agricultural" approach requiring less infrastructure and investment. Both have distinct benefits and limitations (Georgianna & Mayfield 2012). Photobioreactors (PBRs) are closed systems of clear plastic or glass tubes allowing solar radiation to penetrate the circulating algal culture ("industrial"). These systems are costly and often plagued by heat production and O_2 buildup. On the other hand, open-ponds, or raceway systems require limited amounts of construction and infrastructure, making them the most economical option available for scaling to industrial levels ("agricultural"). However, these open systems are exposed to the natural environment and, therefore, experience environmental fluctuations (e.g. temperature, light), invasions by unwanted algal species, herbivores and pathogens (e.g. chytrid fungi). Consequently, for these open-pond systems to be a viable mass-cultivation option we need to identify monocultures or polycultures of algal species that can persist throughout these fluctuations and invasion pressures while yielding high biomass and fatty acid content. As elucidated below, we suggest that through utilizing the local environmental conditions and algal trait data to inform the construction of a multi-species algal community that is adapted to the local environmental conditions, much like piecing together a puzzle (as shown in Figure 1). These ponds will not only realize increased energy output but also limit successful invasion events by undesired local algal species.

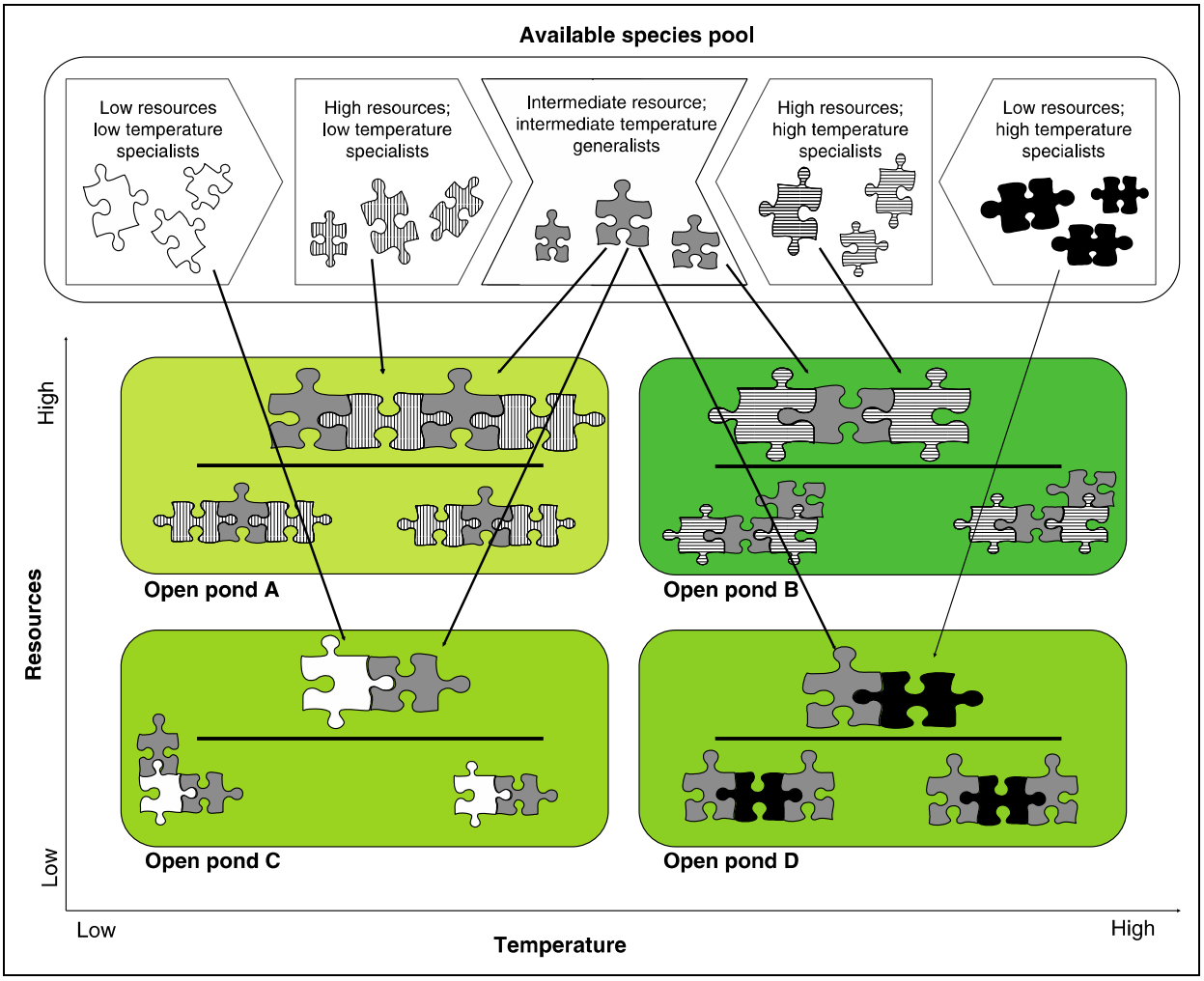

Figure 1. Open-ponds A-D represent hypothetical outdoor ponds experiencing different ranges of temperature and resource (light, nutrients) conditions. The available species pool represents a large number of species with known traits, allowing them to be grouped in to categories of specific temperature and resource ranges. Some species are more specialized in their temperature range tolerances and nutrient demands (i.e. low temperature and low resource adapted). There are also intermediate species, or "generalists," that thrive around the environmental averages, an essential attribute as these environments experience fluctuating conditions. When species compositions are "pieced-together" to optimize trait complementarity for the specific range of local environmental conditions (from both specialists and generalists) these systems can realize

overyielding in productivity even as conditions fluctuate. For example, Pond A's local conditions are high to moderate resource levels (light and nutrients) experiencing low to moderate temperatures. Trait-informed assemblages would be constructed from local taxa that draw from both low thermally adapted, high nutrient demanding species AND intermediate (or "generalist") species.

DIVERSITY-PRODUCTIVITY RELATIONSHIPS IN MICROALGAL COMMUNITIES

As described above, open-pond systems will be continuously invaded by other organisms with high dispersal rates, such as undesired algae and zooplankton grazers resulting in a diverse community (Tilman, Wedin & Knops 1996). However, a diverse community is not necessarily a disadvantage for the open-pond cultivation systems. Higher species diversity often leads to higher productivity (in terms of biomass and fatty acid content), as was first observed in grasslands (Loreau, Mouquet & Gonzalez 2003). At least two non-exclusive mechanisms have been proposed for the observed positive diversity-productivity relationship: first, a "selection effect" (see Table 1), whereby a highly productive species has a higher likelihood of being present in a more diverse community, thus increasing the overall biomass (Hooper *et al.* 2005). Secondly, a "complementarity effect", ", in which an assortment of species interact with collectively harmonious characteristics to better fill the available niche space (see Table 1), ultimately achieving highly efficient use of available nutrients, leading to an overall increase in productivity (Hooper *et al.* 2005; Balvanera *et al.* 2006). However, some studies in natural and artificial communities also found a negative diversity-productivity relationship, where

monocultures of high-yielding species outperformed communities of low-yielding species (Cardinale *et al.* 2007; Schmidtke *et al.* 2010).

Table 1. Definitions of Relevant Ecological Terms and Concepts Mentioned in the Text

COMPLEMENTARITY EFFECT	A mechanism of species co-existence through minimizing competition with similar species through differentiating resource requirements and resource acquisition traits. This differentiation results in a diverse array of species co-existing in an environment where individually there is little competition and collectively the community efficiently utilizes most, if not all, of the available resources leading to higher productivity.
FUNCTIONAL GROUP	For algae, a functional group describes a group of algae that all share specific prominent characteristics (biochemical, physiological, ecological). **Example: Diatoms are a functional group of algae that all contain a silicon based cell wall, or frustule.**
NET BIODIVERSTIY EFFECT (NBE)	Difference between observed yield in a mixture and its expected yield. The expected value is the weighted average (by the actual distribution of algae in the mixture) of the monoculture yields for each species in the mixture (see also Loreau and Hector 2001). **NBE = (Observed yield) – (Expected yield)**
NICHE	The range of all biotic and abiotic environmental factors where a given species can persist.
SAMPLING EFFECT	A highly productive species, within a set pool of species, has a higher probability of being included in a more diverse mixture, thereby increasing the overall productivity of diverse communities.
TROPHIC CASCADE	A predator impacts a particular ecosystem by suppressing the abundance of that prey item, subsequently releasing the predation pressure on the lower trophic level, leading to positive population growth. **Zooplankon (+) ✐ Algae (-)** **Add A Trophic Level: Fish (+) ✐ Zooplankon (-) ✐ Algae (+)**

These mechanisms can act in tandem (in most cases one is emphasized) and yield an overall net biodiversity effect or benefit. This net biodiversity effect is defined as the observed yield in the mixture minus the theoretical, or expected yield of the mixture estimated from the

mixture composition and the constituent species monoculture yields (Loreau *et al.* 2001). Recently, a positive diversity-productivity relationship has been described for microalgal communities where it is driven by complementarity, facilitation and resource use efficiency (Ptacnik *et al.* 2008; Striebel, Behl & Stibor 2009; Cardinale 2011; Stockenreiter *et al.* 2012).

A crucial component of investigating the diversity-productivity relationship is defining diversity, because diversity can have different meanings. The majority of studies define diversity simply as species richness or the number of species present, neglecting other crucial components of diversity . Other studies define diversity as functional diversity or functional composition (Díaz & Cabido 2001; Duru, Theau & Cruz 2012). A "functional group" represents species grouped based on their common biochemical and/or ecological functions. In microalgal communities these divisions often match taxonomic groups, e.g., Bacillariophyta, Chlorophyta, Cyanobacteria, etc. because major taxonomic groups differ in their physiologies and biogeochemical roles (Hood *et al.* 2006). Promoting species richness *per se* as a measure of diversity may result in a species pool with highly overlapping, homogeneous physiological characteristics (traits) (e.g., similar light-harvesting pigments, nutrient requirements), thus reducing the possibility for complementarity. Therefore, promoting diversity by increasing the number of functional groups or trait differentiation is often more efficient for resource use and, consequently, productivity.

DIVERSITY, LIGHT, AND FATTY ACID PRODUCTION

Most of the work on the diversity-productivity relationship focused primarily on biomass production. Recently, Smith *et al.* (2010) hypothesized that diverse microalgal communities in

open-pond bioreactors might store more solar energy as fatty acids, compared to single species cultures in closed photobioreactors . Shurin *et al.* (2013) also argue that certain species combinations might lead to more robust and productive biofuel systems, compared to monocultures. Using diversity as a tool to improve mass cultivation of algae might have important advantages.

Optimizing algal fatty acid production by applying ecological principles, such as the diversity-productivity theory, has been limited so far to only a few empirical studies (Stockenreiter *et al.* 2012, 2013). Fatty acid production in microalgal communities can be enhanced through two non-exclusive mechanisms. First, increased fatty acid content could derive from increased biomass production as a result of promoting diversity (either species richness or functional richness). Secondly, as Stockenreiter *et al.* (2012) showed, diversity may also influence the biomass-specific algal fatty acid content resulting in individual cells having higher than expected internal fatty acid content, further increasing the overall fatty acid production. However, the exact reason for this enhancement is unknown.

An important mechanism for a positive microalgal diversity-productivity relationship may be due to the algal species' ability to capture different wavelengths of the photosynthetically active radiation (PAR) spectrum (400 - 700 nm) (Striebel *et al.* 2009; Behl, Donval & Stibor 2011). About 90% of carbon is bound up in macromolecules, such as proteins, fatty acids and carbohydrates (Wilhelm & Jakob 2011). Photosynthesis is the key chemical process of carbon fixation to produce macromolecules, and therefore, fatty acids. Fatty acid synthesis has been shown to be affected by light. Moreover, light affects fatty acid metabolism: fatty acid quality can change with light fluctuations (Nalley *et al.*; Harwood & Jones 1989). Triacylglycerols (TAGs) are usually produced during light periods and polar fatty acid synthesis for membranes

occurs during dark periods (Thompson 1996). These findings, however, are restricted to single algal species grown in monoculture and the understanding of effects of interactions between light and diversity on fatty acid production of microalgae remains limited.

Light is traditionally considered to be a homogeneous resource, but light is a heterogeneous resource, made up of varying wavelengths of energy. Microalgae have an array of light harvesting structures (pigments), greatly outnumbering pigments of terrestrial plants (Gantt & Cunningham 2001). For each light harvesting pigment, there is a corresponding wavelength of light that can be captured, wavelengths that deviate from the pigments corresponding range cannot be harvested, representing an unused resource (Figure 2). All microalgae use chlorophyll *a* as their main light harvesting pigment, however, different algal groups are often characterized by their additional, secondary pigmentation (e.g. Cyanophyta contain phycoerythrine and/or phycocyanin, Bacillariophyta contain β carotene, etc). These accessory pigments capture only small portions of the PAR spectrum, but a functionally diverse assemblage of algae would contain a broad range of light harvesting structures resulting in a more efficient and overall higher total use of the available light resource compared to a single species (Figure 2B) (Striebel *et al.* 2009; Behl, Donval & Stibor 2011). This example highlights an important mechanism to explain the positive microalgal diversity-productivity relationship. Work on this topic is quite limited, but work by Stockenreiter *et al.* (2013) showed a possible link between the light harvesting efficiency and fatty acid production in algal communities. The majority of communities with species deriving from different functional groups (e.g. Chlorophyta, Bacillarophyta, Cyanophyta and Crysophyta together) showed higher fatty acid content compared to communities consisting only of one single functional group (e.g. Chlorophyta) (Smith *et al.* 2010). In functionally diverse communities, a higher variety of pigments present

resulted in greater overall light harvesting ability and higher light use efficiency. These findings show that certain complementary traits, such as pigmentation, when combined to optimize resource-harvesting capabilities (Figure 2B), may be a mechanistic driver of higher fatty acid production. Stockenreiter et al. 2013 showed a clear correlation between absorbance and fatty acid content. Additionally, trait variance in light acquisition might be even higher than in mineral resource acquisition traits (Ptacnik *et al.* 2008).

In open-pond cultivation systems, algae experience highly variable light regimes, from extremely low light levels in dense cultures due to self-shading to high, often photoinhibiting levels at the pond surface. Under well-mixed conditions (paddle wheels), algae are only exposed to high irradiance for a very short period of time, which may minimize photoinhibition and still allow high photosynthetic rates. Some algal groups appear to thrive (e.g. diatoms), while others decline (e.g. green algae) under fluctuating light, so choosing appropriate groups for different cultivation conditions may enhance yields (Litchman 2000).

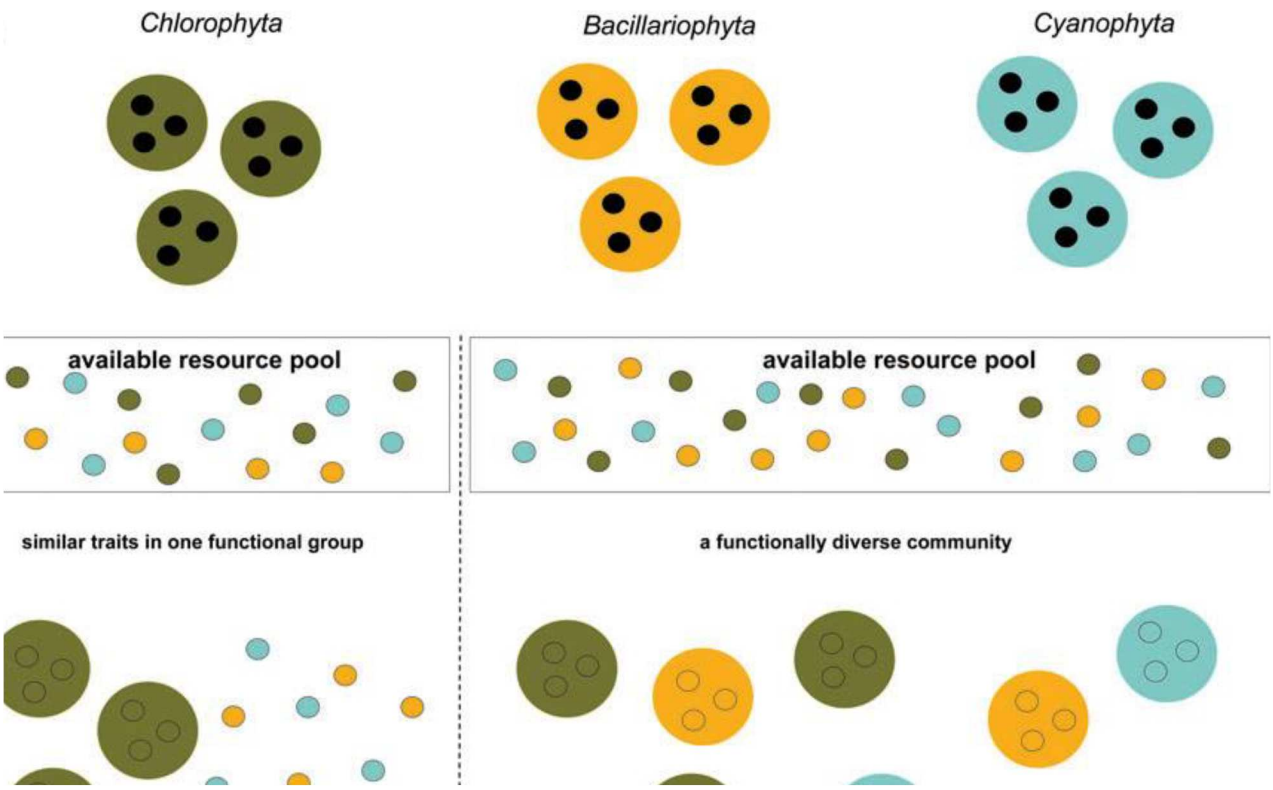

Figure 2. Biovolume estimates, growth rates and thermal growth response curves Investigating the role of trait complementarity: The top portion (A.) illustrates an algal community consisting of a single functional group (here Chlorophyta), which uses mainly Chlorophyll *a* and *b* to capture distinct wavelengths of the PAR spectrum, resulting in a largely unused portion of the light resource. The bottom illustration (B.) shows a diverse algal community consisting of multiple functional groups (Chlorophyta, Bacillariophyta, Crysophyta, Cyanophyta) that are utilizing a diverse collection of light capturing pigmentation, resulting in a larger portion of the PAR spectrum being captured. This light capture optimization leads to an efficient use of the entire light resource and can lead to increased algal productivity (i.e. biomass, fatty acids, energy content).

TRAIT-BASED APPROACHES AND POTENTIAL BENEFITS

Assembling a diverse algal community for biofuel production can have a multitude of positive effects discussed above. However, these effects will only be realized when species with the "right" traits are selected for the assemblage. The "right" traits can include a complementary use of resources such as light and nutrients, different thermal or pH preferences, etc., so that, in combination, the species outperform the best performing monocultures in a desired function (e.g., biomass or fatty acid production). Therefore, we propose that trait-based approach to assembling communities is more efficient than just manipulating species or functional group diversity and should be the next step in algal biofuel research (Figure 1).

Knowing the traits of species is crucial to using trait-based approaches (Litchman & Klausmeier 2008). Many key functional traits of microalgae have been measured, and there are several trait compilations that allow species to be characterized according to their maximum growth rates, cell sizes, and resource requirements (Litchman *et al.* 2007; Schwaderer *et al.* 2011; Thomas *et al.* 2012; Edwards, Litchman & Klausmeier 2013). The available information can help assemble communities tailored to local environmental conditions with species that have the desired trait values and combinations of traits. For example, species that are good competitors for nitrogen (N) can be placed together with the species that are good competitors for phosphorus (P). There is often a trade-off between N and P competitive abilities and such a trade-off can promote species coexistence and efficient use of resources (N and P) (Edwards, Klausmeier & Litchman 2011). Assemblages of species with complementary traits are expected to perform well under fluctuating conditions. Under light fluctuations, species with high and low light requirements can coexist and utilize varying light efficiently (Litchman & Klausmeier 2001; Litchman 2003). N:P ratios for optimal growth are species-specific and can vary between

20:1 and 50:1 (molar ratio) or more (Guildford & Hecky 2000; Geider & Roche 2002; Klausmeier *et al.* 2004). Microalgal biofuel production systems that include combinations of species varying in their stoichiometric carbon (C):N:P ratios could be used to maximize fatty acid production per unit of limiting nutrient.

A modular system based on complementary traits for the utilization of light and nutrients by microalgae may provide a promising method for optimizing microalgal cultivation for commercial uses under given environmental conditions. Using a trait-based approach, an assemblage of algal species could be put together that would be functionally diverse to increase light-harvesting capabilities, while the individual species within the community would also have different ranges of temperature tolerances, such that the overall system would maintain high growth under fluctuating temperatures. Finally, the species would also be selected to increase the difference in N and P requirements to maintain species coexistence, limit competition, and achieve highly efficient nutrient use. This process would be much like piecing together a multi-dimensional puzzle, such that species would complement other species to fill the available resource/environmental factor space defined by the local environmental conditions (Figure 1). As this suggests, a different algal assemblage could be constructed for each set of local environmental conditions. The selection of the best community should be aided by trait-based mathematical models that can achieve multi-objective optimization. Such models should include key aspects of algal physiology and ecological interactions and the information on relevant traits. For example, an algal-community model describing the growth dependence of each species on light, nutrients, and temperature can be forced by the specified environmental conditions, and different sets of species that is predicted to maximize a desired output (e.g. biomass, fatty acid yield) could be selected for experiments and cultivation.

Ecological and physiological trade-offs of microalgal species may also prove to be extremely beneficial in assembling highly productive algal biofuel systems. Algal growth rates inversely relate to cellular fatty acid content and also total energy content (Figure 3A) (Mata, Martins & Caetano 2010). There is also a trade-off between growth rate and cell size, where species with large cells have low growth rates (Figure 3B). These large-celled species though have higher grazer resistance, a positive attribute to avoid crop-loss from predation, as discussed later (Figure 3C). Thus, a logical extension of this knowledge of trade-offs leads us to posit that larger-celled and thus, more grazer-resistant species, may have higher levels of overall energy content (Figure 3D). It would be illuminating to test this hypothesis experimentally.

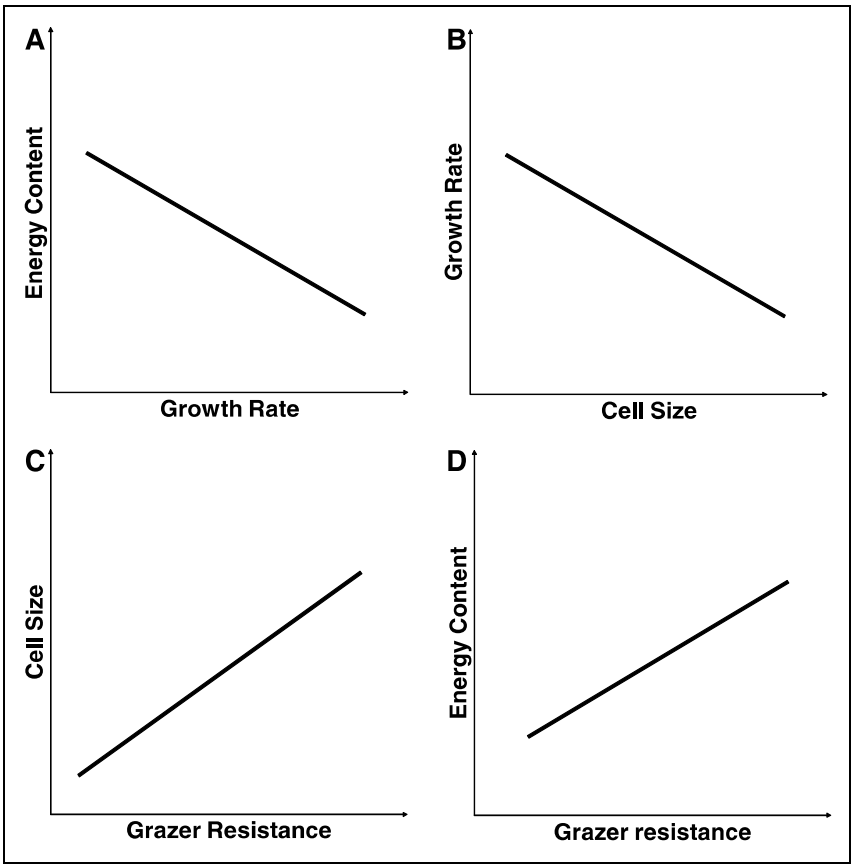

Figure 3. (A) The energy content of algae is species-specific and highly variable. However, total energy content tends to be inversely proportional to growth rate of algae (see also Metting et al. 1996). (B) Growth rates of smaller algae are observed to be higher than larger-sized algae. (C) Individual cell size is proportional to resistance to grazing pressures. Large cells have higher grazer resistance. Colonizing of small algae (increasing total size) and developing anti-predatory accouterments (spines, thick sheaths) effectively increase overall size and thus grazer resistance. (D.) Drawing from the logical progression of A-C, one could hypothetically assume that algae with high grazer resistance might have higher energy content.

It is important to identify the relevant traits and trade-offs in microalgae that also help to explain the mechanisms of species coexistence and diversity (Litchman *et al.* 2010). Different environmental conditions, such as varying light intensities and spectral characteristics, nutrient availability, and temperature have distinct impacts on microalgal cellular composition and community structure. Therefore, trying to identify species that grow best under given environmental conditions and then using those species to assemble microalgal communities suitable for different growth conditions is a promising strategy. Communities assembled by analyzing a priori how microalgal traits match growth conditions might provide a predictable system for a guaranteed supply of biomass and fatty acids (Figure 1). Trait-based approaches are being used in terrestrial plant ecology and phytoplankton ecology to explain community structure along major environmental gradients and to predict ecological community responses to global environmental change (Thomas *et al.* 2012; Edwards, Litchman & Klausmeier 2013). Trait-based approaches use trait information to help predict community assembly and ecological functioning (productivity, stability) (Lavorel & Garnier 2002; Westoby & Wright 2006). This framework has been applied to assembling native terrestrial plant communities that resist the establishment of exotic species. This same approach should also be developed and used in microalgal biomass and fatty acid production to achieve high ecological functioning and reduce invasibility.

MANAGING DIVERSE MICROALGAL COMMUNITIES IN OPEN-PONDS

Selecting the optimal combination of traits leading to a high fatty acid yield in diverse communities is an important step towards the mass cultivation of algal biomass for biofuel.

However, another critical requirement for the assembled communities is that they maintain stability and resilience (Kazamia, Aldridge & Smith 2012). Species with desired traits should be able to stably coexist and communities should not fluctuate widely in the face of disturbance (stability) or be able to rebound after it (resilience). Often, competition for resources can lead to a competitive exclusion of inferior competitors and a decreased diversity, the so-called competitive exclusion principle (Hardin 1960). Using species with complementary traits, e.g., good nitrogen and good light competitors may promote coexistence.

Several studies showed that diversity increases not only productivity in terms of biomass and/or fatty acids, but the stability of algal communities as well (Striebel, Behl & Stibor 2009; Cardinale 2011; Stockenreiter *et al.* 2012, 2013). A classical study by Tilman and Downing (1994) found that in grasslands higher levels of species diversity resulted in more stable biomass levels when communities experienced environmental perturbation (drought) and greater resilience, so that community biomass returned to the pre-disturbance level faster. The hypothesized mechanism for stability and resilience is that diverse communities had more drought-resistant species, thus underscoring the importance of certain trait combinations. A follow-up study by Tilman, Reich & Knops (2006) supported and extended their earlier results, showing that diversity helps hedge against temporal variation resulting in consistent biomass yields. Corcoran and Boeing (2012) showed that both species composition and species richness are important in driving patterns of stability. Additionally, a recent study by Cardinale *et al.* (2013) determined that biodiversity enhanced both productivity and stability, but these effects were independent of one another.

Cultivating algal communities in outdoor ponds poses a serious challenge to maintaining desired species compositions since these ponds experience fluctuating environmental conditions

(especially light and temperature) on multiple temporal scales (daily and seasonal). Fluctuations could have dramatic effects on the species assemblages. If they exceed the environmental tolerances of the desired species, the conditions could lead to culture crashes, invasion and establishment of undesired algal species, potentially resulting in lower productivity. Recent studies concluded that in a diverse community experiencing a single or multiple environmental stressors species composition and species-specific responses to stressors were crucial in predicting the overall community stability (Flöder & Hillebrand 2012; Schabhüttl *et al.* 2013). Assembling communities based on traits should help buffer against environmental fluctuations. For example, having species with different temperature optima should allow maintaining high growth under temperature fluctuations (Figure 2).

Fluctuating environmental conditions experienced by algae in outdoor ponds may also affect the overall fatty acid production and fatty acid composition. Nalley et. al. (unpublished, see chapter 2) found that light fluctuations caused changes in fatty acid composition in mixed- and single-species microalgae cultures and the changes were species-specific. Maintaining a consistent fatty acid feedstock is a crucial component of maintaining a commercially viable biodiesel product, so understanding the implications of these environmental fluctuations is essential (Knothe 2008).

INVASIONS

Outdoor algal ponds experience not only fluctuating environmental conditions but also face constant invasion from windborne or waterfowl-hitchhiking plankton. Several studies on terrestrial systems showed that another benefit of diversity is an increased resistance to invasion

pressures (Naeem *et al.* 2000; Kennedy *et al.* 2002; Fargione & Tilman 2005). The historical beginning of this diversity-invasion hypothesis comes from Elton (1958) where he states, "[T]he balance of relatively simple communities of plants and animals is more easily upset than that of richer ones; that is…more vulnerable to invasions." Elton's observations were supported by MacArthur's (1970) modeling work, concluding that higher levels of occupied niche space results in lower successful establishment rates by invaders. This phenomenon has become known as "resident biotic resistance." A number of empirical studies have supported this earlier work, finding an inverse relationship between resident species richness and invader biomass (Kennedy *et al.* 2002; Fargione & Tilman 2005). This inverse relationship was the result of diverse species composition within the plots, specifically high niche complementarity among the resident species (Fargione & Tilman 2005). However, some researchers find that promoting diversity may not enable communities to become completely resistant to invasion but would only constrain the abundance of already established invasive species (Levine, Adler & Yelenik 2004).

In the outdoor, open-pond systems algal assemblages will be constructed to optimize the overall productivity for biofuel generation, but these assemblages may be susceptible to invasions by undesired algae and could quickly change to unproductive systems. Developing an understanding of how these open-pond systems will respond to invasion pressure, specifically to invader establishment, is essential for maintaining consistent desired levels of productivity. Drawing from the resident biotic resistance theory, through promoting diverse assemblages of algal species with high levels of niche complementarity, should lead to a constraint on invading species abundance, reducing the impact of the inevitable undesired algal invasion on the overall productivity.

Another topic of concern is a potential "reverse invasion," or an event where algal species from the open-pond systems invade the surrounding natural water bodies. An advantage of tailoring a diverse algal community to the local environmental conditions is that it can be assembled of species that most likely are already present in the surrounding natural systems. GMOs however, can have a high potential for an accidental "reverse invasion" event. Strongly competitive GMO strains could drastically disrupt natural communities (Snow & Smith 2012).

BIOTIC CONTROLS

Top-Down Approaches

Ecological interactions in open-pond systems follow the same ecological principles of natural ecosystems. However, it is likely that artificial systems will have simpler food webs than most natural lakes and ponds, resulting in communities that are more sensitive to perturbations. Ecological interactions between microalgal communities and herbivorous zooplankton in artificially assembled food webs have to be studied in more detail in order to integrate them into commercial production systems to optimize biomass yields.

One such important process is a top down control of producer biomass by higher trophic levels. Herbivory by zooplankton can have tremendous impacts on the phytoplankton community. The direction and strength of phytoplankton responses to zooplankton grazing depends significantly on the size of phytoplankton species, as size influences the edibility of phytoplankton.

Herbivorous zooplankton can invade open cultivation ponds, creating a simple two level food web (primary producers and consumers). Trophic interactions can result in highly varying

microalgal biomass due to the shifting grazing pressures from the zooplankton, ultimately reducing biofuel feedstock (Smith *et al.* 2010). Drawing from the ecological principles of the top-down control and trophic cascades, these undesirable reductions in algal biomass can be minimized through the introduction of zooplanktivorous fish.

Zooplankton grazing can change the size distribution of phytoplankton communities, often towards the dominance by large, poorly edible algae (Shapiro & Wright 1984). *Daphnia*, in particular, can promote the growth of large, inedible and fast sinking algae that could be harvested more easily than small species. As most major microalgal groups include large, fast sinking species, the quality of fatty acid profiles can be influenced by selecting growth conditions (for example by resource supply ratios) benefitting large species within the desired microalgal groups (Figure 1 B, C). Grazing periodicity (e.g., diel vertical migration) and the initial algal composition can also mediate the responses to grazing (Haupt *et al.* 2012).

Pathogens

Another threat to algal ponds is pathogen infections, specifically by chytrid fungi. Chytrid fungi have been shown to alter the competitive abilities of algal species that could impact community composition and species succession (Ibelings *et al.* 2004). Chytrid infections can lead to pond wide epidemics, completely decimating phytoplankton species within days to weeks of observed infection, crashing the biomass in both natural and algal biofuel open-ponds (Ibelings *et al.* 2004; Shurin *et al.* 2013). One approach to control the chytrid infection is by applying a fungicide that can lead to a sharp reduction of the chytrid population (Shurin *et al.* 2013). Another approach could be by promoting a diverse assemblage of algal species, thus reducing the densities of potential hosts that would result in a lower infection susceptibility especially for the pathogens with high host specificity.

OTHER MICROALGAL APPLICATIONS - AQUACULTURE

The production of biofuel is only one of many possible applications of microalgal cultivation. Several other applications such as health food, animal feed, fertilizers and bioplastics are also important (Spolaroe *et al.* 2006; Natrah *et al.* 2007; Plaza, Cifuentes & Ibáñez 2008; Huerlimann, de Nys & Heimann 2010). Producing feedstock is one of the widely used algal applications: currently, 30% of the produced algal biomass goes into the feed market, with algae mainly used for fish feed in aquaculture The food quality of microalgae is crucial for transferring energy to higher trophic levels. It is determined by the cellular composition of carbohydrates, fatty acids, and proteins and the carbon to nutrient ratios. Low carbon to nutrient ratios (in most cases phosphorus and/or nitrogen) result in high quality food for herbivorous zooplankton (Urabe & Sterner 1996; Hill, Rinchard & Czesny 2011) . Another major aspect of food quality is the fatty acid composition in terms of essential ω3-polyunsaturated fatty acids (ω3-PUFAs) of primary producers, as animals are incapable of synthesizing certain ω3-PUFAs *de novo*.

Most modern aquaculture systems focus on particular microalgal strains that have the desired properties. However, in natural ecosystems, zooplankton are exposed to a variety of microalgal species, living in communities. The nutrient concentration (phosphorus) in the system positively correlates with the phytoplankton ω3-PUFA content (Müller-Navarra *et al.* 2004). Ambient nutrient levels also influence the diversity of primary producers, which in turn will affect their resource use efficiency and productivity. However, to what extend diversity has an impact on the food quality (in terms of fatty acids) of microalgae for zooplankton remains poorly known. Recently, Stockenreiter *et al.* (2013) tested how microalgal diversity influences fatty acid composition in algal communities. Diversity had a significant effect on primary producer communities and their fatty acid composition, especially on the essential ω3-PUFAs such as α-

linolenic acid (ALA). These results represent a first step towards investigating the effects of diversity on the properties of artificially constructed systems, like raceway ponds, beyond just the carbon-based primary productivity. The observed correlations between nutrients and the phytoplankton fatty acid composition could, therefore, be also mediated by diversity.

CONCLUSIONS

Open-pond algal systems are currently viewed as the most economically viable cultivation system for mass production of algae-derived biofuel. These systems will face a number of environmental pressures, from fluctuating conditions (light, temperature, nutrient ratios) to herbivory, infections and invasions by undesired algal species. One approach would be to artificially select or genetically modify algal strains that can persist under extremely harsh growth conditions, such as high salinity, so that no other organisms (competitors, pathogens or herbivores) can establish in such ponds. We argue that a more effective approach would be to use fundamental ecological principles to design algal assemblages with desired properties for outdoor open-ponds (Figure 2). Through promoting algal biodiversity within these systems and selecting species with complementary traits, biomass and fatty acid yields may exceed those of the highly productive monocultures. These artificial algal assemblages (in terms of functional diversity and complementary traits) can better weather environmental fluctuations and remain stable through invasion, herbivory, and pathogen infection events, all while yielding consistent biofuel feedstock. However, further work is needed to determine the optimal assemblages for local environmental conditions, as well as focusing on scaling-up to industrial-scale levels. We

believe that a number of benefits can be realized through this research, ultimately taking this developing technology a step further towards industrial-scale reality.

ACKNOWLEDGEMENTS

This work was supported by the NSF grant CBET-1134215 to (EL and C.A. Klausmeier). This is Kellogg Biological Station contribution No. 1716.

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CHAPTER 2

Complementarity and stability in algal biofuel communities under fluctuating light

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ABSTRACT

Outdoor open-pond cultivation of microalgae for biodiesel production has gained support due to its favorable economics and scale-up potential. Outdoor open systems experience varying environmental conditions (i.e., light levels, temperature, pH, etc.) at multiple temporal scales. Species diversity within these systems may help buffer environmental fluctuations but this has been largely unexplored. We investigated the effect of light fluctuations on the dynamics of biomass accumulation, growth rates, biomass stability, total fatty acid yield and fatty acid profiles in monocultures and three or six species polycultures drawn from three algal functional groups. We grew different diversity treatments under fluctuating light with a period of 4 days for 32 days. While the growth in monocultures was sensitive to light level fluctuations, growth in the six species polyculture was not significantly impacted by fluctuating light. The biomass within the six species polyculture was also significantly more stable throughout the fluctuating conditions compared to the averaged monocultures. We observed overyielding in mean biomass for both the three and six species polycultures, compared to the constituent monoculture yields. These benefits of biodiversity can be attributed to high levels of niche complementarity and/or facilitation. However, the overall fatty acid/mL values of polycultures were not significantly different from the averaged monoculture yields. The fatty acid profiles of both monocultures and polycultures showed little response to changing light levels, while different species combinations led to varying levels of desirable and undesirable biodiesel fatty acid composition. These results suggest that polycultures may be more stable under fluctuating environmental conditions leading to consistent biomass production through time, and with the correct combination of species, overall fatty acid yields can be optimized while maintaining ideal fatty acid composition.

INTRODUCTION

Biodiversity effects on various ecosystem functions, such as productivity, stability, resistance to invaders, increased resource use efficiency and others, have been well documented for many terrestrial and some aquatic ecosystems (Tilman & Downing 1994; Yachi & Loreau 1999; Naeem *et al.* 2000; Lehman & Tilman 2001; Kennedy *et al.* 2002; Fargione & Tilman 2005; Ptacnik *et al.* 2008; Stockenreiter *et al.* 2012; Cardinale *et al.* 2013). However, whether and how biodiversity affects the functioning of artificially created multispecies systems and, especially, engineered microbial assemblages such as algal biofuel communities remains underexplored. In particular, whether diversity increases stability of algal biofuel communities in the face of environmental fluctuations is poorly known. Here we investigate how increasing species diversity in algal biofuel communities affects biomass and fatty acid yields, and their stability under fluctuating light.

Algal biofuel communities may be a promising alternative to fossil fuels. At current rates of consumption, some estimates have placed the end of known fossil fuel reserves within the next 60 years (Stephens *et al.* 2010). In addition, the amount of CO_2 and other green house gases (GHG) coming from fossil fuel combustion has been exponentially increasing, leading to climatic changes. Therefore, developing alternative sources of energy to meet the increasing energy demand while reducing the amount of GHG emissions is a pressing societal need. A number of biologically derived fuel sources have been identified (i.e. corn, soy, rapeseed, jatropha), but there are limitations hampering large-scale production, such as significant land requirements, low energy yields and competition with food production (Mata, Martins & Caetano 2010). The use of microalgae for biodiesel production has gained considerable attention due to its potential for high-energy yields, low land requirements, separation from food

production, and numerous applications for bioremediation (Chisti 2007; Smith *et al.* 2010). Despite this promise, a report from the National Research Council (USA) concluded that the current methods of algal biofuel generation have a number of sustainability concerns, including the high water and nutrient demands, energy return on investment, and greenhouse gas emissions (Hunter-Cevera *et al.* 2012).

Two cultivation techniques have been developed for large-scale microalgae production: open outdoor cultivation in shallow or raceway ponds and closed photobioreactors. Closed systems allow for controlled conditions, reduced evaporation and contamination, and high surface-to-volume ratios, but often have high internal temperatures, light limitation due to biomass self-shading and high infrastructure costs (Pienkos, Laurens & Aden 2011). Open-ponds are more economically feasible and can easily be scaled to industrial levels, but are susceptible to undesired algal introduction, fungal and bacterial contamination, experience environmental fluctuations and high rates of predation (Pienkos, Laurens & Aden 2011; Shurin *et al.* 2013). Some suggest that although open-pond systems are artificially created, applying the knowledge obtained from natural systems can address some of these issues (Smith *et al.* 2010; Kazamia, Aldridge & Smith 2012; Shurin *et al.* 2013; Nalley, Stockenreiter & Litchman 2014).

There is also a growing body of work suggesting that biodiversity can offer a number of benefits for algal biofuel cultivation. Biodiversity can potentially enhance the "reliable, efficient, and sustainable supply" of biofuels in general (Tilman, Reich & Knops 2006), and the biomass accumulation and fatty acid yield in algal systems (Stockenreiter *et al.* 2012). Polycultures have also been shown to achieve higher rates of resource utilization than monocultures (Ptacnik *et al.* 2008; Striebel, Behl & Stibor 2009; Striebel *et al.* 2009; Cardinale 2011) and often display transgressive over-yielding, i.e., when polycultures perform better than the most productive

monoculture (Trenbath 1974; Weis, Madrigal & Cardinale 2008). Traditionally, this diversity-productivity relationship was shown for biomass, but a recent study investigated fatty acid production and concluded that more functionally diverse communities can utilize available light more efficiently through an increased number of light harvesting pigments, resulting in higher than expected fatty acid yields (Stockenreiter *et al.* 2013).

Perceived benefits of biodiversity (i.e. "overyielding") can be parsed into two main mechanisms: "the selection effect", when the community is dominated by a single, good performer , or "the complementarity effect", when there is a synergistic community, with many species contributing (Loreau & Hector 2001). The complementarity effect occurs when multiple species are able to use resources more efficiently than single species, or when interactions between the species facilitate their growth. Comparing observed to expected values estimated from monoculture yields and community composition is not sufficient to disentangle the two forces (Loreau & Hector 2001). These differences can be parsed using a modified Price equation (originally developed to describe evolutionary processes) and partitioning the differences between polycultures and monocultures into the effects of species and of diversity itself (Fox 2005). This is a powerful tool to test whether polycultures actually outperform monocultures in critical functions.

Outdoor ponds experience fluctuating environmental conditions (i.e. light levels, temperature, pH, etc.) on multiple temporal scales: from daily cycles to seasonal changes. Diversity is hypothesized to stabilize communities in the face of such variation, in part because different species have contrasting responses to fluctuations. Studies have shown that when plant communities experience an environmental perturbation, those with higher levels of biodiversity are more resilient, maintaining more stable levels of productivity (Tilman & Downing 1994;

Yachi & Loreau 1999; Tilman, Reich & Knops 2006). This is analogous to diverse stock holdings maintaining a more constant yield in a fluctuating market (Lehman & Tilman 2001). Within these large, outdoor algal ponds, light levels will be extremely variable dependent on the position within the water column, ranging from overexposure (photoinhibition) to limitation due to shading by biomass.

Although there is a large body of work addressing a number of the benefits of biodiversity, we are not aware of studies that have investigated the effects of environmental fluctuations on the performance of microalgal single and multi-species cultures for biodiesel production. Light fluctuations occur at different scales in natural environments and have been shown to have different effects on algal growth rates, depending on the species (Litchman 2000). Light fluctuations can also promote species coexistence and increase diversity (Litchman 1998, 2003; Litchman & Klausmeier 2001). Better understanding of how multispecies assemblages respond to a fluctuating environment, including light fluctuations, could help optimize open outdoor cultivation of microalgae. With an eye towards outdoor open-pond systems for algal biodiesel generation, we investigated how fluctuating light levels affect biomass accumulation and stability, total fatty acid production and the fatty acid profile of both polycultures at different levels of diversity and their constituent monocultures.

MATERIALS AND METHODS

Microalgal communities

To compare how cultures of single species versus mixed assemblages respond to light fluctuations, we established single-species and mixed microalgal cultures of two species diversity levels (hereafter termed monocultures and polycultures, respectively) and subjected them to step function low frequency fluctuations in light levels while all other conditions were held constant. These cultures consisted of six species of algae from three different functional groups (Table 2) obtained from the laboratory culture collection. To assess the impact of diversity, we established polycultures with a mixture of three functionally different species ("intermediate polyculture"), and then a collective mixture of all 6 species ("full polyculture"). Cultures were grown in the WC medium (Guillard & Lorenzen 1972).

Table 2. Experimental species combinations. List of all the species and different community compositions at the three- and six-species polycultures.

Monocultures
Chlorophyta: *Chlorella vulgaris*, *Scenedesmus obliquus*
Cyanophyta: *Anabaena* sp., *Microcystis* sp.
Bacillariophyta: *Cyclotella* sp., *Navicula pelliculosa*
Three Species Mixtures
Ch. vulgaris, Anabaena sp., *Cyclotella* sp.
Ch. vulagris, Microcystis sp., *Navicula pelliculosa*
S. obliquus, Anabaena sp., *Cyclotella* sp.
S. obliquus, Microcystis sp., *N. pelliculosa*
Six Species Mixture
Ch. vulgaris, S. obliquus, Anabaena sp., *Microcystis* sp., *Cyclotella* sp., *N. pelliculosa*

To compare the response of monocultures and polycultures to fluctuating light, we established three replicates of all individual species and of all respective polyculture combinations, resulting in a total of 33 cultures (Table 2). The starting cell density of all cultures was inoculated at a standardized biovolume of $7.5x10^6$ units biovolume mL^{-1}. Polycultures were inoculated with equal biovolumes of the constituent species that additively resulted in a total biovolume of $7.5x10^6$ units biovolume mL^{-1}.

Experiments were run in Erlenmeyer flasks (150 mL) with a working volume of 100 mL for thirty-two days with light levels changing every fourth day at 20°C with 12:12 h light:dark cycles. Light levels varied between 30 and 130 $\mu E\ m^{-2}\ s^{-1}$, which are limiting and saturating,

respectively, for the growth of most phytoplankton species (Richardson, Beardall & Raven 1983; Litchman 2000). As the main goal of this study was to compare and contrast the responses of polycultures vs. monocultures to light fluctuations, there was no need to have a constant light treatment. The different light levels were achieved through manipulating the proximity of the cultures to the main light source and with the addition/subtraction of mesh screening. Light levels were verified at each light shift using the QSL light meter (Biospherical Inc., CA). The temperature within the flasks was checked throughout the experiment and no significant difference was observed under the different light conditions. Every fourth day, a "harvesting event" occurred where 40% of the homogenized culture volume was replaced with fresh WC medium. The decanted volume was used for analyses.

Cell counts, biovolume estimates, growth rates

Cell counts were performed using a haemocytometer with improved Neubauer ruling. For the polycultures, each individual species was counted and summed for overall cell count. To estimate biovolume of each species, 40 random cells were measured using Image Pro Plus version 4.5.1 software and the averaged values were then used to estimate cell volume (Hillebrand *et al.* 1999).

Growth rates were calculated for two distinct portions of algal growth: exponential (Day 0-19) and stationary (Day 20-32). Biovolume measurements collected at the beginning and end of each light step were used for calculations (ex. Day 8 and 12). These growth rates were then categorized by light level and averaged for across the appropriate timeframe.

Fatty acid analysis

To understand how fluctuating light influenced the total fatty acid accumulation and the composition of fatty acids, we performed a fatty acid extraction and fatty acid methyl ester analysis that followed the protocol established by Wang & Benning (2011) and modified for algae by Boyle et al. (2012). At each sampling event, approximately 35mL of culture were filtered onto GF/B 2.1cm diameter glass microfiber filters (volume dependent on biomass values), immediately frozen and then analyzed following the protocol established by Boyle et al. (2012). We deviated from the protocol slightly and did not perform thin-layer chromatography, thus we analyzed all extracted neutral fatty acids together, without separating the sample into specific fatty acid classes (i.e., triacylglycerols (TAGs), diacylglycerols (DAGs), etc.). In brief, algal samples and an internal standard were pre-treated with acid and heated to lyse the cells and begin the extraction. Through centrifugation fatty acids were forced into the hexane layer that was then extracted, dried, and resuspended in hexane. Fatty acid methyl esters were then identified using gas chromatography and flame ionization detection. We observed strong signal strength for all of our samples.

Biodiversity effects

To calculate the overall benefit arising from the biodiversity (ΔY), we utilized the Price equation established by Fox (Fox 2005) and previously used by Stockenreiter et al. (Stockenreiter *et al.* 2012):

$$\Delta Y = Y_{obs} - Y_{exp} = Z + \text{cov1} + \text{cov2} \quad (1)$$

where:

$$Z = S \times \bar{M} \times \overline{RY} \quad (1a)$$

$$cov1 = S \times Cov\left(M_{obs}, \frac{RY_O}{RYT} - RY_E\right) \quad (1b)$$

$$cov2 = S \times Cov\left(M_{obs}, RY_O - \frac{RY_O}{RYT}\right) \quad (1c)$$

The overall biodiversity benefit (ΔY) is given by the difference between observed (Y_{obs}) and expected (Y_{exp}) biomass (1). This can be further decomposed into the product of the number of species in the polyculture (S), the average of all monoculture biovolumes ($\overline{M}$), and the average yield of all the species in the polyculture ($\overline{\mathrm{RY}}$) plus two covariance terms (1). The term Z (1a) represents the synergistic effect of the diversity arising from niche complementarity and/or species facilitation ("trait-independent complementarity"). The term cov1(1b) gives the covariance between the observed monoculture biovolume (M_{obs}) and the difference between the observed (RY_O / RYT) and expected (RY_E) frequencies, where the quotient RY_O / RYT is the observed yield of each species over the total observed yield and RY_E is simply 1/S, illustrating the extent to which a single species disproportionately influenced the overall yield, akin to the "selection effect". The term cov2 (1c) gives the covariance between monoculture biovolume and the difference between observed yield and observed frequency, or what can be attributed to when high achieving monocultures achieve high relative yields in the polyculture without hampering the performance of other species ("trait-dependent complementarity").

We could not apply this analysis to fatty acid yields because we were unable to measure individual species' fatty acid yields in the polyculture. To assess the effect of biodiversity on measure of fatty acid yields, we used standard analysis of variance approach using R.

Biomass stability

To simulate the harvesting strategies of a fully functional pond, we assessed stability of the culture biomass through time once the culture achieved its carrying capacity (Day 20-32, "stationary"). The exponential phase of the experiment was eliminated from the stability assessment due to this periods "ramping up". We assessed stability by calculating the coefficient of variation of biomass yields. Comparisons of the biomass stability in monocultures and polyculture were conducted using the Dunnett-Tukey-Kramer Pairwise Multiple Comparison Test with the DTK package in R.

RESULTS

Biovolume dynamics and growth rates

Both the three- and six-species polycultures achieved higher mean biomass compared to the averaged monoculture biomass (Figure 4). The averaged three-species polyculture had significantly higher biomass at day 12, 16 and 28 ($p<0.05$), with no significant difference detected on other dates. Whereas with little power of three replicates from the six-species polyculture, we observe significantly higher biomass over the monocultures only at day 12 ($p<0.05$). Overall, biomass in all cultures shows a quick increase in total biomass (Day 0-19) until the biomass begins to saturate (Day 20-32).

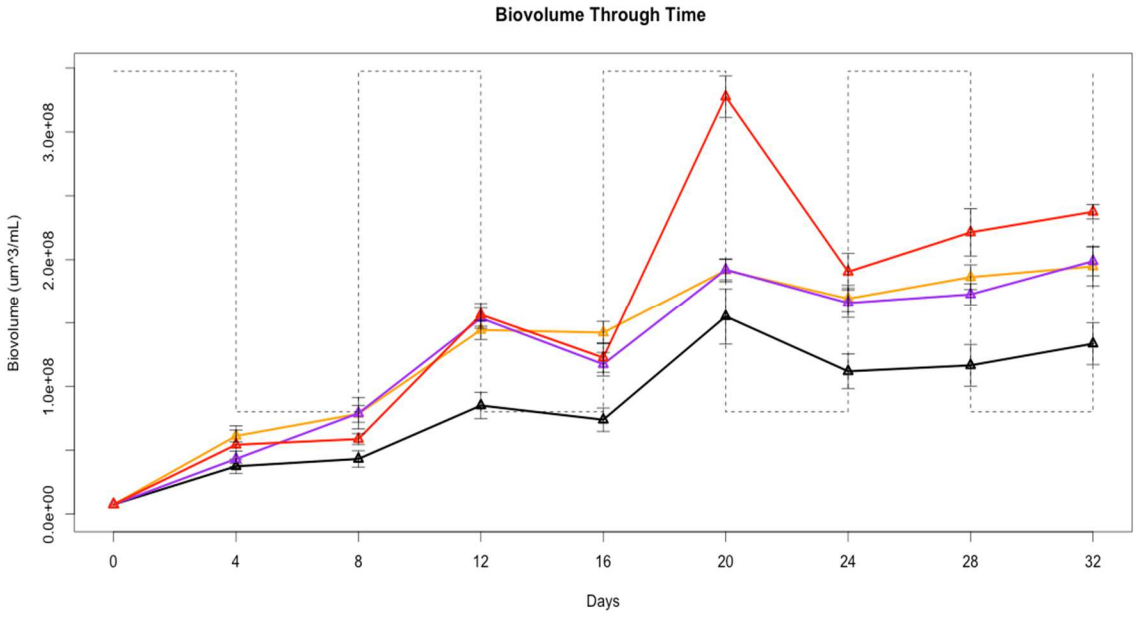

Figure 4. The dynamics of biomass (biovolume, $\mu m^3\ mL^{-1}$) in averaged monocultures (Black), the best performing monoculture (*C. vulgaris*, Red), three- (Orange) and six-species (Purple) polycultures. Each data point represents biovolume measurements prior to a 40% dilution, or a "harvesting" event. Dashed, thin line shows fluctuating irradiance (130 $\mu E\ m^{-2}\ s^{-1}$ and 30 $\mu E\ m^{-2}\ s^{-1}$). Error bars denote ± 1 standard error.

During the exponential phase of the experiment, light fluctuations significantly impacted the growth rates of all levels of diversity: monocultures, three- and six-species polycultures (Figure 5, $p<0.0001$, $p<0.0001$ and $p=0.008$, respectively). At the stationary phase though, the growth rate of the full polyculture was not significantly reduced under low light ($p=0.105$) while the growth rates of both the monocultures and the intermediate polycultures were significantly reduced ($p=0.0006$ and $p<0.0001$, respectively).

Individual monocultures and polycultures responded differently to light fluctuations. These species-specific responses to the different light levels can be explained through differences in growth rates between high and low light levels (Figure 5 and Supplemental Figure 1. These differences can also be visualized in their individual biomass through time plots (Supplemental Figure 2). During the exponential growth phase (Day 0-19), the biomass accumulation of *C. vulgaris*, the best performing monoculture, was dramatically impacted by the light levels it experienced, with a four-fold increase in growth under high light compared to low light ($p<.001$). A significant impact of light on *C. vulgaris* growth was also observed during the stationary phase. Whereas there was no significant difference in the growth rate of *S. obliquus* under the fluctuating light conditions at either period of the experiment (Supplemental Figure 1a).

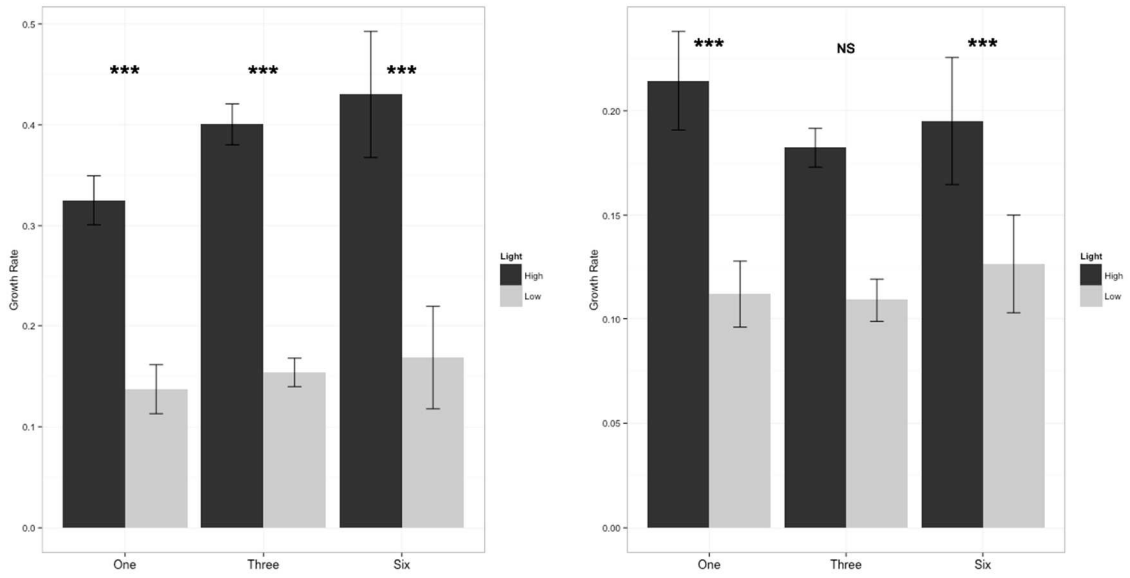

Figure 5. Growth rates under different light conditions: The averaged growth rates of the monocultures and polyculture under high (black) and low (grey) light conditions. A.) Exponential Phase (Day 0-19) B.) Stationary Phase (Day 20-32). Error bars denote ± 1 Standard Error. Significance denoted $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***) and not significant (NS).

To assess the overall effect of biodiversity on biomass, we used the tripartite approach developed by Fox (Fox 2005). Through this approach, we found a large net benefit from the increased diversity on the overall biomass at both levels of diversity. Within the six species polyculture, this benefit was primarily (84%) attributable to the species within the polyculture occupying separate niche dimensions and/or facilitating one another, the "complementarity effect" (Equation 1a), resulting in the constituent species all out-performing their individual monocultures. We also observed a slight "dominance effect" (18%) (Equation 1b) arising from *C. vulgaris* and *S. obliquus* contributing a large proportion to the overall biovolume (Figure 6). For the three species polyculture, we found very similar results, with the large benefit of biodiversity arising from niche complementarity and/or facilitation (90%) and a smaller portion attributable to a dominant species (10%). Overall, the positive net biodiversity effect on biomass is predominately due to niche differentiation and interspecific facilitation ("complementarity effect"), resulting in the observed increase in biomass. This indicates that the increase in productivity is due to biodiversity *per se*.

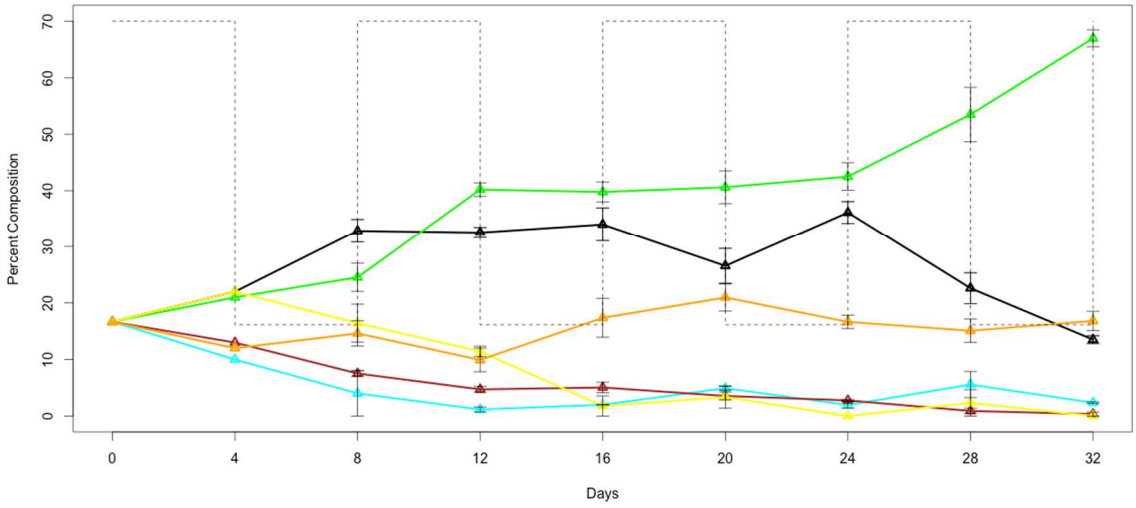

Figure 6. The compositional makeup of the six species polyculture. *Chlorella vulgaris* (black), *S. obliquus* (green), *Microcystis* sp. (orange), *Anabaena* sp. (ice blue), *Cyclotella* sp. (yellow) and *N. pelliculosa* (red). Dashed, thin line shows fluctuating irradiance (130 µE m^{-2} s^{-1} and 30 µE m^{-2} s^{-1}). Error bars denote ± 1 standard error.

Polyculture composition

Through employing the tripartite approach, we can mathematically illustrate that our polycultures maintained diversity throughout the experiment. But it is also helpful to visualize the community dynamics through time (Figure 6). The six-species polyculture maintained high diversity throughout the experiment. *S. obliquus* and *C. vulgaris* (*Chlorophyta*) made up roughly 70% of the overall biomass, with *Microcystis* sp. and *Anabaena* sp. (*Cyanophyta*) comprising roughly 20% and *Cyclotella* sp. and *N. pelliculosa* (*Bacillariophyceae*) contributing the final 5-10%. This maintenance of diversity breaks down at the three-species level. Our three-species cultures were had high proportions of Chlorophytes within their mixture. By the end of the

experiment, *C. vulgaris* and *S. obliquus* achieved a compositional average that generally exceeded 95%.

Biomass stability

Biomass stability, estimated using the coefficient of variation, was compared between the monocultures and two levels of polyculture over the stationary phase of the experiment (Day 20-32). The six species polyculture was significantly more stable than the averaged monocultures (Table 3). There was no significant difference between the three- and six-species polycultures, or the three-species polyculture and the monocultures.

Table 3. Biomass stability during the stationary phase. The coefficient of variation (CV) for the three levels of diversity during the "saturated growth" phase (Day 20-32). CVs with different letters are significantly different. Significance set at $p<0.05$.

Diversity Level (species)	Coefficient of Variation
One	24.35 ± 1.96 [a]
Three	17.38 ± 2.27 [a,b]
Six	12.07 ± 1.44 [b]

Fatty acid content of cultures

The averaged three-species polycultures achieved the highest mean total fatty acid productivity throughout the duration of the experiment, while the six-species polyculture produced the least total fatty acid yield (Figure 7). Yet, we only detected significant lower fatty

acid production in the six- compared to the three-species polycultures at day 8, 15, 20 and 24 ($p<0.05$), while the six-species polyculture only produced significantly lower fatty acid content than the averaged monocultures at day 20 ($p<0.05$). This spike in monoculture fatty acid yields and high variability at Day 20 can be attributed to the individual spikes in *C. vulgaris* and *Microcystsis* sp. (Supplemental Figure 3a).

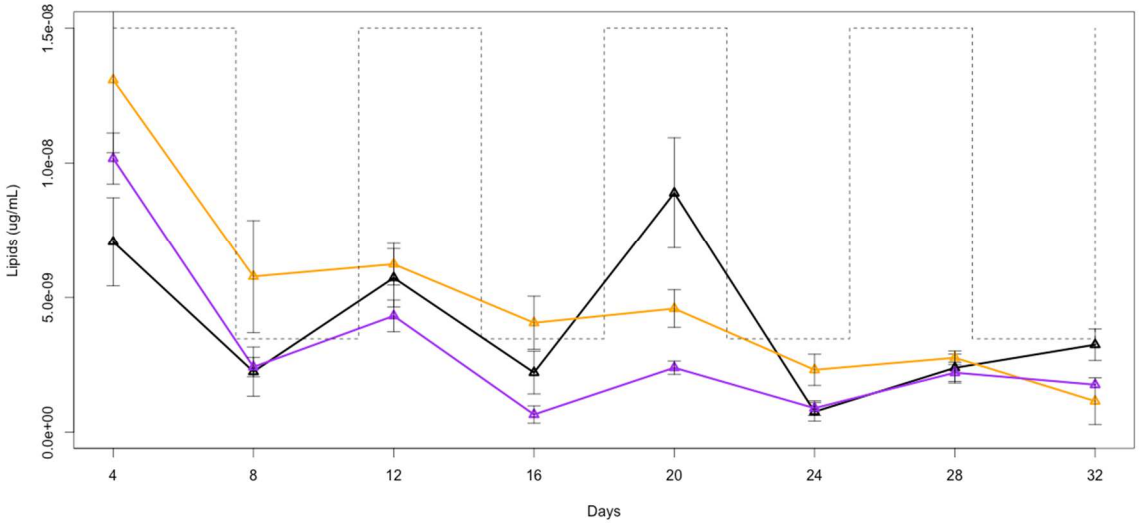

Figure 7. Overall fatty acid productivity (μg mL^{-1}) of averaged monocultures (red), three- (blue) and six-species (yellow) polyculture. Error bars denote ± 1 Standard Error. Thin dashed line shows light fluctuations (130 μE m^{-2} s^{-1} and 30 μE m^{-2} s^{-1}).

Apart from the trends at the level of diversity, we also detected differences in total fatty acid yields across individual monocultures and polycultures (Supplemental Figure 3). As with our findings with biomass, *C. vulgaris* is the highest fatty acid producing species of the experiment, at least doubling the total fatty acid production of other species (Supplemental Figure 3a). In three-species polycultures, species combinations have a large effect on the total

fatty acid production. When *C. vulgaris* can competitively exclude the other species in the mixture we see similar fatty acid yields as the *C. vulgaris* monocultures, yet when *Microcystis* sp. is present to compete with *C. vulgaris*, this total fatty acid yield is dampened (Supplemental Figure 3b).

Fatty acid profiles

The fatty acid profiles of monocultures and polycultures showed little differences under low and high light levels (Figure 8). With the six-species polyculture, the fatty acid profiles of all the cultures consisted predominantly (~55%) of palmitic acid (16:0) and palmitoleic acid (16:1), with approximately 20% linolenic acid (18:3).

Although there was not much impact of light on the fatty acid composition, there are distinct species-specific fatty acid profiles. Such as with *Anabaena* sp. which has a distinctly high percentage (38%) of linolenic acid (18:3), whereas its cyanobacterial counterpart, *Microcystis* sp., is made up of a majority palmitic acid (60%) with limited linolenic acid (~10%). The best performing monoculture for both total fatty acid production and biomass accumulation, *C. vulgaris*, has high levels of polyunsaturated fatty acids, with 33% linolenic acid and 20% hexadecatrienoic acid (16:3). The profile of *Cyclotella* sp. is made up almost exclusively of C_{16} hydrocarbons: palmitic (20%), palmitoleic (33%) and hexadecatrienoic (16%).

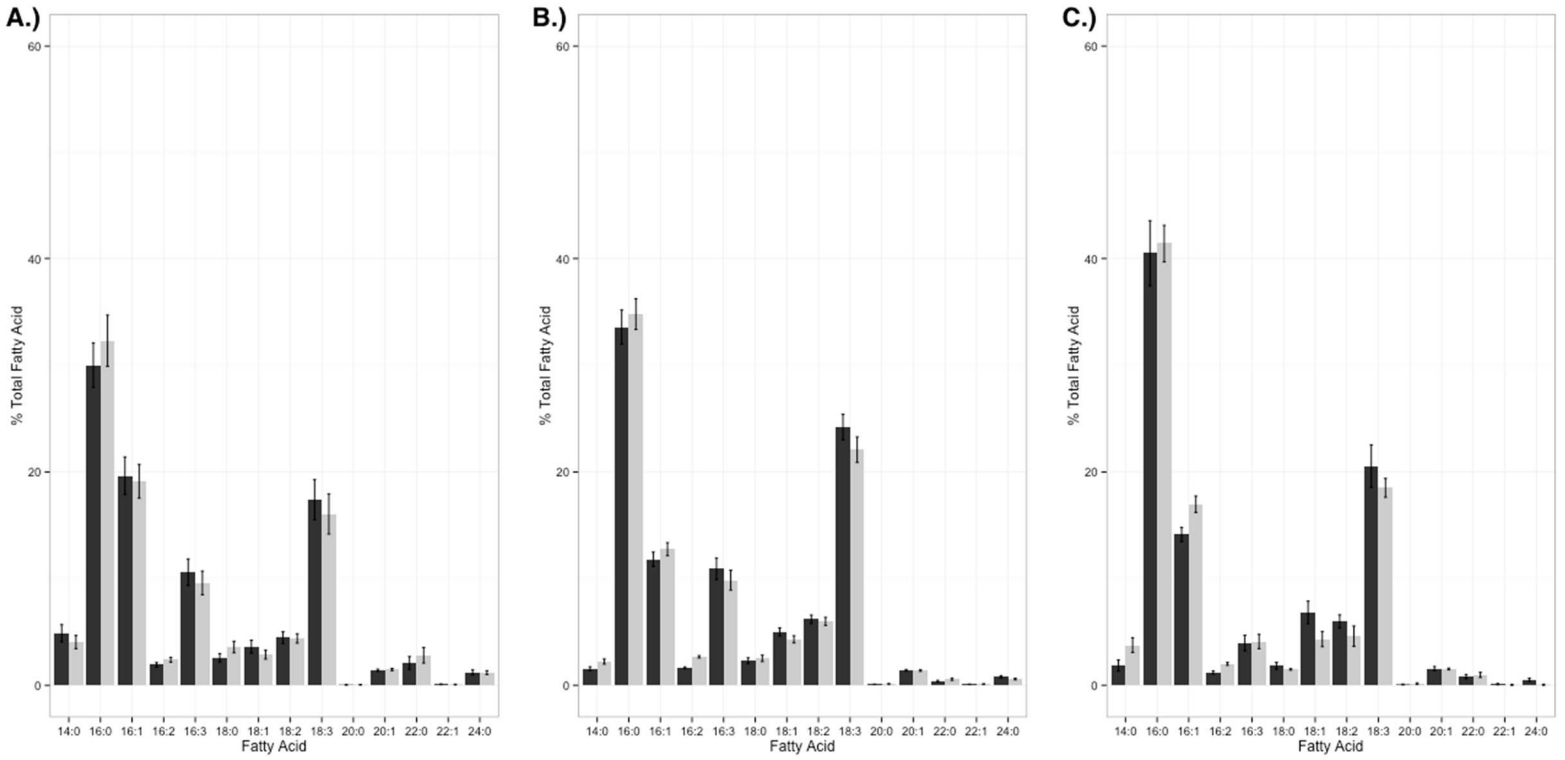

Figure 8. Fatty acid profiles in the (A) averaged monocultures and the (B) three-species and (C) six-species polycultures. The percent composition of each fatty acid under high (black) and low (grey) light conditions. Error bars denote ± 1 Standard Error.

These species-specific profiles carry over to fatty acid composition of the polycultures. For example, the three-species polyculture containing *N. pelliculosa, C. vulgaris* and *Anabaena* sp., a majority of the profile is highly unsaturated fatty acids (57%) with limited palmitic acid. Whereas, replacing *C. vulgaris* with *S. obliquus* results in a shift away from highly unsaturated fatty acids to more saturated palmitic and palmitoleic acids (40 and 19%, respectively). Within the six-species polyculture, the presence of all species leads to a fatty acid profile more similar to an averaged monoculture profile, where clear species-specific signals are present, with *Microcystsis* sp. and *S. obliquus* contributing to the high palmitic acid makeup, *N. peliculosa* and *Cyclotella* sp. providing palmitoleic acid, while *Anabaena* sp. and *C. vulgaris* are contributing to the linolenic acid percentages.

Fatty acid composition is extremely important in determining the overall quality of the biodiesel feedstock (see Discussion). To better identify algal species and polycultures with desirable fatty acid profiles, we computed the distance between the specific sample and the ideal biodiesel mixture. We established the "ideal biodiesel mixture" to a composition of 45% palmitic acid (16:0), 45% palmitoleic acid (16:1) and 10% polyunsaturated fatty acids (16:2, 18:3). We calculated the percentage of each of these components for each sample. This calculation places each sample and the ideal mixture at a location defined by its coordinates in three dimensions (% palmitic acid, % palmitoleic acid, and % polyunsaturated fatty acids). The distance between each sample and the ideal composition is the Euclidian distance between the location of a sample point and the ideal. We then plotted this distance from the ideal composition against the total fatty acid production of that culture to visualize their relationship (Figure 9). Within our study, there is a clear trade-off between overall fatty acid production and closeness to the ideal composition.

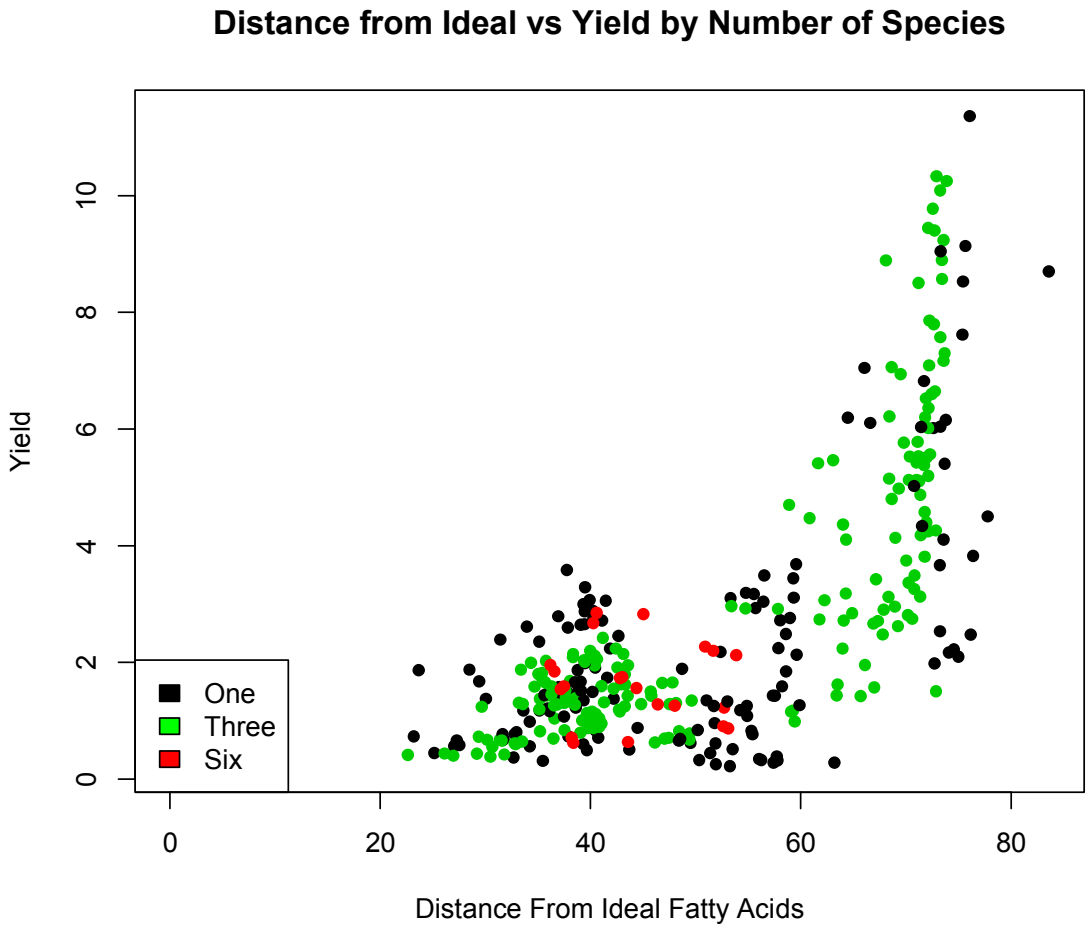

Figure 9. Trade-off between distance from ideal biodiesel feedstock FA composition and overall fatty acid production.

Individual species have vastly different responses. Both the diatoms (*Cyclotella* sp. and *N. pelliculosa*) exhibit close to ideal fatty acid composition while achieving the lowest levels of fatty acid production, whereas *C. vulgaris* achieves the highest fatty acid production while being compositionally furthest from ideal, thus creating a trade-off. The six-species polyculture falls in the middle of this trade-off, exhibiting both intermediate overall production and distance from the ideal composition. The signal of the three-species polycultures is dependent on whether *C.*

vulgaris or *S. obliquus* is present. Cultures containing *C. vulgaris* have high yields while being compositionally undesirable, whereas cultures containing *S. obliquus* fall at the intermediate level.

DISCUSSION

Growth and biomass accumulation

The observed biovolume in both the three- and six-species polycultures achieved higher mean values than the averaged monocultures (Figure 4). Through employing the revised Price equation, we were able to determine that this higher biomass could be achieved by promoting species diversity for both the three- and six-species polycultures. Our analysis identified that this benefit was predominantly attributable to "complementarity" that could be due to niche differentiation (high vs. low light preference) among species creating more efficient use of the overall niche space or interspecific facilitation where one species creates a better environment for another. Within the six-species polyculture, we also observed similar growth rates under the fluctuating light levels, whereas the intermediate polycultures and monocultures showed significant reduction in growth under low light (Figure 5). For outdoor mass cultivation, maintaining consistent growth of algal crop is integral for the overall productivity and economic feasibility. These results suggest that some large monoculture algal ponds could be highly sensitive to natural light fluctuations, leading to reductions in overall productivity, while polycultures may be more resilient.

Biomass stability

We observed that the six-species polyculture had significantly higher biomass stability compared to the monocultures. A higher polyculture stability is due to its lower sensitivity to fluctuations (less growth rate fluctuations and species complementarity). Therefore, promoting species diversity can lead to more consistent and predicable yields under fluctuating light and, perhaps, under other environmental fluctuations.

Fatty acid profiles and biodiesel composition

For biodiesel to be marketable in the United States and the European Union, it must meet standards outlined in the American Society for Testing and Materials (ASTM) D6751 and European Standard EN 14214 respectively. These standards place specifications on cetane number (combustion quality), viscosity, oxidative stability, and cold-flow properties (Knothe 2008, 2011; Stansell, Gray & Sym 2011). Long hydrocarbons with limited unsaturation have high cetane numbers, but also have high melting points potentially causing crystallization at cold temperatures. Higher levels of unsaturation have much lower melting points, making them ideal for cold weather combustion. Balancing the mixture of hydrocarbon length and unsaturation is essential in producing a biodiesel that meets ASTM standards. The European Standard also sets limits on the percentage blend of linolenic acid (18:3) at <12% (Chisti 2007).

Fatty acid yield and composition trade-off

The three-species polycultures achieved the highest mean fatty acid production (Figure 7). When we compared the individual fatty acid productivity of the monocultures and the three-species polycultures, the highest levels of productivity were found in the cultures containing *C. vulgaris* (Supplemental Figure 3). But, as discussed below, the composition of the high fatty

acid yields was highly unfavorable for biodiesel production in terms of fatty acid composition, thus suggesting a trade-off between fatty acid yield and composition.

Our results also show that there is no significant increase in overall fatty acid production at the six-species level compared to the constituent monocultures. This similar fatty acid yield contrasts with the previous study by Stockenreiter et al. (Stockenreiter *et al.* 2013), where an increase in functional diversity led to a significant increase in total fatty acid yields.

Generally, we observed little effect of light levels on the overall fatty acid composition. But, there were distinct species-specific responses that produced varying levels of suitable and unsuitable biodiesel feedstock. *C. vulgaris* receives considerable attention as a biodiesel candidate due to its high fatty acid productivity and its amenable cultivation potential (Rodolfi *et al.* 2009; Mitra, van Leeuwen & Lamsal 2012; Farooq *et al.* 2013). We observed this high level of productivity as well, but on closer inspection, its fatty acid profile is highly unsaturated, with roughly 35% of the overall fatty acid coming from linolenic acid. Levitan et al. (Levitan *et al.* 2014) highlight that diatoms have ideal fatty acid profiles. Our results from the diatoms we cultured, *Cyclotella* sp. and *N. pelliculosa*, support their conclusions, with both species having high percentages of palmitoleic acid and very low levels of polyunsaturated fatty acids, specifically linolenic acid. But, these species are not overall very productive compared to other algal species from different functional groups (Figure 9).

Our results show that a promising possibility to simultaneously achieve high productivity and a close to ideal fatty acid profile for biodiesel generation, despite the reported trade-off, may be a synergistic multispecies community of microalgae (Nalley, Stockenreiter & Litchman 2014). This idea was supported when we investigated the profile of the six-species polyculture.

Through having a diverse polyculture, we were able to achieve moderate levels of productivity while maintaining high stability and a more suitable overall fatty acid profile (Figure 9).

From the observed fatty acid profiles, we can conclude that certain species have a more "ideal" fatty acid composition than other species. A better understanding of a species' fatty acid profile and its overall productivity is integral to understanding their broader implications for mass production of biofuel. The ability to distill and visualize this relationship between composition and productivity is an effective way to identify the overall potential of a given species or a polyculture for biodiesel production. This procedure can be expanded to include more dimensions if other components of the fatty acid profile are of interest. Of the species tested under fluctuating light, *Cyclotella* sp., *N. pelliculosa*, *S. obliquus* and *Microcystis* sp. all had profiles that would generate an acceptable biodiesel mixture according to the American biodiesel standards, due to high levels of saturation and monounsaturation, and relatively low levels of polyunsaturated fatty acids. As discussed in Levitan et al. (Levitan *et al.* 2014), it is unlikely for a single species to produce an ideal fatty acid profile, but identifying species with high levels of monounsaturated fatty acids could produce close to optimal blends (i.e., diatoms). Additionally, assembling communities with contrasting responses to fluctuating light in terms of fatty acid content could also help optimize fatty acid composition under dynamic conditions.

CONCLUSIONS

In open-pond outdoor systems, microalgae will experience fluctuating environmental conditions. To design systems that generate continuous and consistent yields, we must

understand the influence of these fluctuations. In this study we tested how changing light levels could influence the dynamics of single-species and mixed microalgal cultures. Previous work has reported a number of benefits associated with biodiversity, such as higher biomass and fatty acid yields, as well as reduced susceptibility to establishment of undesired species. Here we found a significant positive effect of biodiversity on biomass yield and stability under environmental fluctuations and fatty acid composition, but little effect on fatty acid yields.

Although this work only addressed fluctuations of a single resource, a positive effect of biodiversity on biomass was observed, where differences in individual species' responses to light levels within the community lead to higher and more stable biomass. Fluctuating light had significant effects on algal growth and fatty acid production, but little effect on fatty acid profiles. The six-species polyculture growth rates showed no strong response to light fluctuations during the stationary phase and there was a large benefit of biodiversity within the polycultures that can be attributable to complementarity. Polyculture biomass was also significantly more stable than the monocultures. The species-specific fatty acid profile shifts are extremely relevant for maintaining the ideal biodiesel quality. Ultimately, under fluctuating light conditions, a more diverse community of microalgae can lead to more consistent and predictable biomass and fatty acid yields, all while maintaining a fatty acid composition that is more ideal for biodiesel production.

In summary, our results suggest that diverse algal systems could be beneficial for biofuel generation under fluctuating conditions. It is possible that more species-rich communities composed of functionally diverse species could yield greater benefits of biodiversity under fluctuating conditions. Fluctuations in other resources or environmental factors, such as temperature and pH, must also be considered and ultimately combined to simulate natural

conditions. The design of optimized algal biofuel polycultures must take into account multiple trade-offs and constraints, not only the biomass or fatty acid yields, so that multiple goals of sustainable algal biofuels are met.

ACKNOWLEDGEMENTS

This work was in part supported by the National Science Foundation grant CBET 1134215 to EL and C.A. Klausmeier. JON was supported by NSF GK-12 graduate fellowship. We thank P. Woodruff, A. Hutchens, E. Poliner, and B. Liu for their technical support throughout the study. A special thank you to C. Benning and J. Stevenson for the use of their lab facilities. This is W.K. Kellogg Biological Station contribution #1844.

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CHAPTER 3

Temperature effects on growth rates and fatty acid content in freshwater algae and cyanobacteria

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ABSTRACT

Mass cultivation of algae for biofuel and other bioproducts will have considerable economic advantage if these systems are grown in outdoor, open raceway ponds. But, these systems would be subjected to environmental fluctuations. Temperature fluctuations can have dramatic effects on the growth of algal species, which could impact the overall productivity of targeted algal crops. This study sought to identify the influence temperature has on algal growth rates, biomass accumulation, fatty acid production and fatty acid composition. We surveyed 25 different algal species from 5 different functional groups, growing them in 6 different temperatures from 9-32°C. For the species surveyed, we collected eco-physiological trait data determining maximum growth rate, temperature optima, niche width, and temperature maxima and minima for growth. We also calculated the trait data at the functional group level. Thermal responses were species specific, but, generally, at a functional group level we identified that cyanobacteria have the highest thermal optima (30.6 ± 2.3°C), followed by chlorophytes (25.7 ± 0.1°C) and diatoms (24.0 ± 0.4°C). Species also had temperature-specific fatty acid production, mostly driven by growth rate differences, while some was attributable to changes in the cellular fatty acid content. We also determined that temperature directly influences the composition of the fatty acid profiles, but universal trends were not evident. Overall, this study showed that temperature can significantly impact the overall production of algal biofuel systems, specifically by influencing growth rates, and thus biomass and fatty acid production. Finally, fatty acid composition is essential for producing consistent biofuel and/or bioproducts, therefore, cultivation temperature must be taken into account for commercialization.

INTRODUCTION

The era of inexpensive, fossil fuel-derived energy is rapidly approaching its end, while demand for energy is ever increasing. Within the next century, alternative fuel sources will have to be developed, implemented and optimized to meet this demand (Stephens *et al.* 2010). Although a number of alternative energy sources have been explored, all options have impediments limiting their commercial viability. Algal-derived biofuels have gained considerable attention as a viable renewable energy source, attributable to its high productivity, limited land use, potential carbon neutrality and many synergistic applications for bioremediation (Olguın 2003; Spolaroe *et al.* 2006; Chisti 2007; Smith *et al.* 2010). Microalgae have also been shown to produce high value bio-products that are of use to the pharmaceutical, agricultural and food industries (Schwartz *et al.* 1990; Borowitzka 1999; Hemaiswarya *et al.* 2011).

To achieve the mass production of these high value products, industrial-scale cultivation of microalgae will be necessary. Two strategies for mass cultivation have been explored: outdoor, open cultivation in shallow, raceway ponds and closed photobioreactors. Closed photobioreactors have more controlled conditions with little risk for undesired infections and invasions, but can suffer from extreme internal temperatures and dense cultures leading to self-shading (Smith *et al.* 2010; Kazamia, Aldridge & Smith 2012). Outdoor ponds may be the most economical option for mass production, but these pond systems are highly susceptible to invasion by undesired local phytoplankton, fungal infection, strong herbivory, and fluctuating local environmental conditions (Pienkos, Laurens & Aden 2011; Shurin *et al.* 2013). One main drawback for both of these systems is the effects of varying temperature on the growth and fatty acid production of crop algal species. Although a small number of studies have investigated the influence of temperature on fatty acid production and growth of different microalgae, the

differences among these studies limit cross comparison and general conclusions of how temperature affects algal eco-physiology, specifically in terms of biofuel production. In this study, we use the same growth conditions and uniform experiments to test the effects of different temperatures on a diverse set of freshwater algae and cyanobacteria.

Algal species differ greatly in thermal ranges, the temperatures where positive growth can be maintained (Thomas *et al.* 2012; Thomas & Litchman 2016). Falling outside of this range leads to negative growth, whereas within the thermal range there is an optimal growth point. This thermal range can generally be categorized as a left skewed curve, illustrating that positive growth is possible at a wider range of temperatures below the optimum but growth rates precipitously decline at temperatures that exceed this optimum (Figure 10). This trend can be explained through the Arrhenius function, which illustrates that an increase in temperature towards the thermal optimum exponentially increases photochemical reactions that ultimately lead to an increase in algal growth (Talling 2012; Falkowski & Owens 2016). Whereas temperatures surpassing the thermal optimum lead to heat stress, resulting in decreased enzymatic functionality, modifying photosynthetic-related proteins, and even triggering the synthesis of stress proteins (Raven & Geider 1988; Salvucci & Crafts-Brandner 2004; Bajguz 2009). These responses to high temperatures limit the downstream production of necessary cellular products and ultimately lead to the observed precipitous decline in growth.

Temperature directly affects the fatty acid amount and fatty acid composition of organisms as well, specifically within the cellular membrane, but the magnitude of this change is variable across species and not well characterized (Harwood & Jones 1989; Guschina & Harwood 2006). At lower temperatures, cells will modify their cellular membrane to incorporate more unsaturated fatty acids, while under higher temperatures the cellular membrane becomes

more saturated (Stumpf & Bradbeer 1959; Hilditch & Williams 1964). The cellular response of modifying fatty acid composition has been widely viewed as a mechanism to maintain normal levels of cellular function (Thompson 1996). For example, through increasing the trienoic fatty acid (16:3 and 18:3) content in tobacco plants, through the introduction of an omega-3 fatty acid desaturase gene (*fad7*), the cold tolerance of these plants increased. Also, through the suppression of this same gene and subsequent decrease in trienoic fatty acids, tobacco plants were significantly better at acclimating to high temperatures (Murakami *et al.* 2000). In non-transgenic plants, it has been observed that low temperatures activate fatty acid desaturases leading to overall fatty acid unsaturation, while at high temperatures the post-translational stability of fatty acid desaturases becomes unstable, resulting in the decrease in trienoic fatty acids (Somerville 1995; Thompson 1996; Matsuda *et al.* 2005). Similar trends (high levels of unsaturation at low temperatures and high levels of saturation at high temperatures) have been documented in various functional groups of microalgae (Sato & Murata 1980; Lynch & Thompson 1982; Renaud *et al.* 2002). It is important to note that there is also evidence that suggests this trend is species-specific and not universal in both algae and higher order plants (Canvin 1965; Patterson 1970; Guschina & Harwood 2006).

The knowledge of these eco-physiological responses to temperature (temperature traits) will not only inform our basic understanding of algae, but can also be utilized to assemble more productive algal communities that experience a changing thermal environment, e.g., in outdoor ponds. Temperature traits are among the key functional traits that help characterize the performance of microalgae under different conditions. Trait-based approaches can help develop a framework that utilizes known characteristics of target organisms in order to maximize a desired outcome, whether that be prairie restoration, limiting pathogen infection, or optimizing biofuel

production. Collecting the desired traits is paramount for these trait-based approaches (Litchman & Klausmeier 2008; Litchman *et al.* 2010). Work characterizing microalgae by other key traits has already been conducted, from growth rates, resource requirements and cell size (Litchman *et al.* 2007; Schwaderer *et al.* 2011; Thomas *et al.* 2012; Edwards, Litchman & Klausmeier 2013). Research on thermal traits however, specifically those concerning overall fatty acid and fatty acid production, is quite limited and, within these studies, there are few methodological commonalities.

This study aims to fill in the knowledge gaps by surveying the temperature traits of 26 freshwater algal species from six different functional/taxonomic groups. We measured growth rates, fatty acid production and fatty acid composition at six different temperatures over a range of 23°C (9-32°C). The conditions within the experiment have been standardized, so that both general, functional group trends and species-specific differences can be identified. Also, the collection of these eco-physiological traits will inform future work on using algal traits to explore the relationship between temperature and algal performance.

MATERIALS AND METHODS

Microalgal and cyanobacterial cultures

To survey algal thermal responses, we established monocultures of 26 microalgae, including cyanobacterial species from the lab grown cultures (Table 4 –species list, UTEX and University of Göttingen). The selected species belong to six different taxonomic groups: Bacillariophyceae, Chlorophyta, Chrysophyceae, Cryptophyta, Cyanobacteria and

Dinoflagellata. While more focus in biofuel research has been on eukaryotic microalgae, we included cyanobacteria in our study because they were also shown to have fatty acids suitable for biodiesel production, along with high growth rates and wide environmental tolerance limits (Wahlen, Willis & Seefeldt 2011; Karatay & Dönmez 2011; Lynch *et al.* 2015).

Table 4. Species surveyed and their temperature-dependent growth and fatty acid production parameters. Taxonomic/functional group level parameters are in bold. All estimates are ±95% CI. Estimates for which 95% CI were unobtainable are designated "± N/A".

Functional group, *Species*	**Niche Width**	**Max Growth Rate**	**Growth Optimum**	**CT_{min}**	**CT_{max}**
Cryptophyceae	**20.70 ± 0.80**	**0.35 ± 0.001**	**20.27 ± 0.024**	**9.69 ± 0.078**	**30.40 ± 0.015**
Cryptomonas erosa	18.44 ± 0.014	0.42 ± 0.001	20.67 ± 0.016	12.53 ± 0.012	30.97 ± 0.006
Cryptomonas ovata	18.68 ± 0.016	0.36 ± 0.001	18.53 ± 0.015	9.80 ± 0.013	28.48 ± 0.010
Rhodomonas sp.	25.39 ± 0.20	0.36 ± 0.001	16.77 ± 0.056	5.43 ± 0.18	30.83 ± 0.088
Cyanobacteria	**36.73 ± 4.90**	**0.76 ± N/A**	**30.62 ± 2.28**	**-0.99 ± 3.26**	**35.74 ± 2.25**
Anabaena cylindrica	27.47 ± 0.31*	1.29 ± N/A*	31.71 ± 1.01*	8.89 ± 0.28	36.36 ± 0.12*
Anabaena flos-aquae	38.38 ± 0.11*	1.03 ± 0.003*	33.71 ± 0.066*	7.77 ± 0.029	46.15 ± 0.088*
Merismopedia sp.	19.33 ± 0.17	0.74 ± 0.003	28.47 ± 0.035	13.02 ± 0.16	32.35 ± 0.051
Microcystis wesenbergii	30.63 ± 0.23	0.57 ± 0.008	28.67 ± 0.27	2.77 ± 0.28	33.40 ± 0.19
Bacillariophyceae	**35.57 ± 0.87**	**0.49 ± 0.001**	**24.02 ± 0.35**	**0.65 ± 0.87**	**33.22 ± 0.014**
Asterionella formosa	31.06 ± 0.36	0.42 ± 0.001	25.99 ± 0.042	3.49 ± 0.34	34.54 ± 0.15
Cyclotella sp.	35.53 ± 0.049	0.57 ± 0.001	25.61 ± 0.012	-2.60 ± 0.046	32.93 ± 0.010
Fragilaria crotonensis	27.66 ± 0.015	0.70 ± 0.001	23.19 ± 0.011	5.66 ± 0.010	33.32 ± 0.007
Navicula pelliculosa	29.84 ± 0.050	0.36 ± 0.0003	17.66 ± 0.016	1.47 ± 0.048	31.30 ± 0.008
Dinophyceae	--	--	--		--
Peridinium sp.	24.36 ± 0.33	0.31 ± 0.082	17.12 ± 0.10	6.77 ± 0.30	31.13 ± 0.17
Chrysophyceae	--	--	--		--
Synura sp.	23.39 ± 0.078	0.54 ± 0.001	23.22 ± 0.023	8.09 ± 0.076	31.48 ± 0.012
Chlorophyceae	**34.36 ± 0.43**	**0.68 ± 0.001**	**25.66 ± 0.057**	**-0.16 ± 0.43**	**34.20 ± 0.016**
Ankistrodesmus falcatus	32.93 ± 0.15	0.86 ± 0.002	27.33 ± 0.018	0.70 ± 0.15	33.63 ± 0.045
Botryococcus braunii	27.68 ± 0.063	0.55 ± 0.001	22.87 ± 0.020	9.94 ± 0.018	37.62 ± 0.059
Chlamydomonas reinhardtii	30.90 ± 0.028	0.93 ± 0.001	27.45 ± 0.008	3.00 ± 0.017	33.90 ± 0.013
Chlorella vulgaris	32.73 ± 0.087*	0.63 ± 0.001	30.09 ± 0.045*	8.80 ± 0.028	41.54 ± 0.071
Crucigenia tetrapedia	35.16 ± 0.13	0.89 ± 0.002	27.87 ± 0.021	-0.53 ± 0.11	34.63 ± 0.046
Desmodesmus sp.	24.22 ± 0.026	0.74 ± 0.001	27.62 ± 0.008	10.30 ± 0.016	34.52 ± 0.013
Monoraphidium sp.	33.21 ± 0.25	0.46 ± N/A	23.96 ± N/A	2.66 ± 0.29	35.88 ± 0.21
Oocystis sp.	26.78 ± 0.010	0.53 ± 0.001	27.24 ± 0.006	6.27 ± 0.006	33.05 ± 0.005
Ourococcus sp.	36.86 ± 0.32	0.69 ± 0.002	26.90 ± 0.087	1.64 ± 0.25	38.07 ± 0.22
Pediastrum simplex	19.57 ± 0.11	0.70 ± 0.003	28.78 ± 0.034	14.75 ± 0.074	33.71 ± 0.065
Scenedesmus obliquus	29.78 ± 0.035	0.89 ± 0.001	25.59 ± 0.012	7.08 ± 0.021	36.86 ± 0.024
Scenedesmus quadricuada	32.38 ± 0.046	1.08 ± 0.001	26.84 ± 0.010	3.81 ± 0.035	35.37 ± 0.021
Selenastrum sp.	20.22 ± 0.24	0.56 ± 0.002	27.93 ± 0.033	12.30 ± 0.23	32.52 ± 0.065

* T_{opt}, TNW and CT_{max} estimates from fits in which the estimated T_{opt} was higher than the maximum assay temperature are unreliable.

To assess the impact of temperature on microalgae, all 26 species were cultured at six different temperatures (9, 15, 20, 25, 30 and 32°C). This temperature range was selected such that the algal strains would likely experience high and low temperature extremes in order to accurately characterize their thermal ranges and optima. Batch cultures of each species were established at 20°C to obtain high biomass that were then divided into 6 smaller batch cultures. These batch cultures were then acclimated for seven days at each of the six experimental temperatures. Triplicate monocultures were then established from these acclimated stocks and standardized to have starting biovolumes (measure of biomass) of about $2.5x10^8$ μm^3 ($3.333x10^6$ μm^3 mL^{-1}). Cultures were grown in 125mL Erlenmeyer flasks with a working volume of 75mL WC medium (Guillard & Lorenzen 1972) and light levels of 100 μE m^{-2} s^{-1} with 12:12 h light:dark cycles. Monocultures were then grown under these different temperatures for a total of 96 hours with sampling and culture resuspension taking place every 24 hours.

Biovolume estimates, growth rates and thermal growth response curves

To investigate the impact temperature on algal growth, we calculated growth rates from the change in biovolume through time. Biovolumes were collected at all three sampling events using the CASY Cell Counter and Analyzer System Model TT (Roche Innovatis AG). Growth rates were calculated as the slope of a linear regression fit to the Ln(biovolume) readings through time (Fogg & Thake 1987).

Each species' growth rates across six temperatures were fit statistically to the Norberg (2004) equation using maximum likelihood estimation (Nelder-Mead method) (Figure 1; Thomas et al., 2012). From these thermal reaction norms, we extracted species' thermal niche width (TNW), thermal optimum for population growth (T_{opt}), maximum growth rate at T_{opt}, (μ_{opt}), and the lower and upper critical temperatures ($CT_{min,}$ CT_{max}). Confidence bands in Figure 1A and

Figures S1-S27, as well as uncertainties associated with all Norberg parameter estimates were estimated using the parametric bootstrapping approach described in the supplemental materials of Thomas et al. (2012). Thermal reaction norms and bootstrapped parameter distributions for whole functional groups were obtained as above by fitting the Norberg equation to growth rate data pooled by functional group. Mean trait values can be compared statistically at both the species and functional group levels by using 95% confidence intervals of bootstrap distributions.

Fatty acid production and fatty acid composition

To understand how temperature influenced the total neutral fatty acid accumulation and the composition of fatty acids, we performed a fatty acid extraction and fatty acid methyl ester analysis according to the protocol by Wang & Benning (2011) and subsequently modified for algae by Boyle *et al.* (2012). At the final sampling event (t = 96 hr), 30-75mL of culture were filtered onto GF/B 2.1cm glass microfiber filters, immediately frozen and then analyzed following the protocol established by Boyle et al. (2012). Filtered sample volume depended on the culture density, and all fatty acid results were standardized to the filtered volume. We did not perform thin-layer chromatography, thus, we analyzed all extracted neutral fatty acids together, without separating the sample into specific fatty acid classes (i.e., triacylglycerols (TAGs), diacylglycerols (DAGs), etc.). In brief, algal samples and an internal standard were pre-treated with acid and heated to lyse the cells and begin the extraction. Through centrifugation fatty acids were forced into the hexane (top) layer that was then extracted, dried via compressed nitrogen gas, and resuspended in hexane. The sample was then identified using gas chromatography and flame ionization detection. Although sample sizes differed between groups, adequate signal strength was recorded for all samples included in the analysis.

From this process, we were able to sum all the fatty acids for a total fatty acid contribution that was then divided by the total milliliters sampled to yield fatty acid per milliliter, a representative measure for overall culture productivity. The fatty acid per milliliter of medium was then divided by the culture density in units of biovolume per milliliter to yield fatty acid per unit biovolume, representing the biomass-specific fatty acid content. From here on, we will refer fatty acid per milliliter as "total fatty acid production" and fatty acid per unit biovolume as "biomass-specific fatty acid content." By characterizing fatty acid content in these two ways, we can distinguish high-biomass producing but fatty acid-poor species from low biomass producers but fatty acid-dense species and how temperature affects these measures of biomass and fatty acid production.

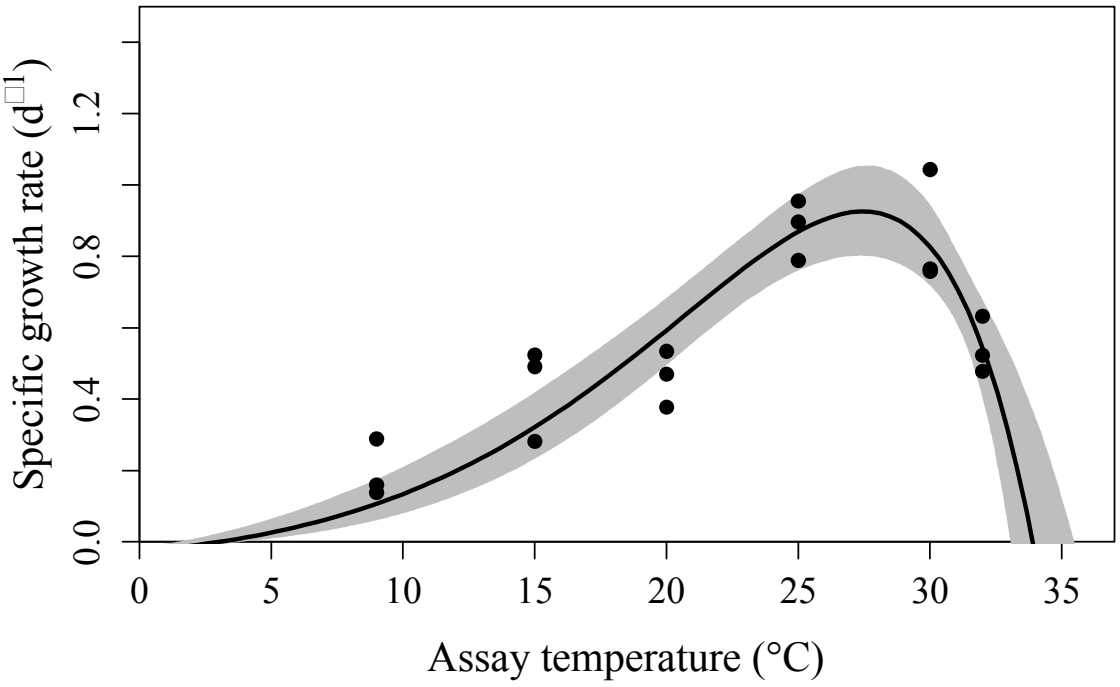
RESULTS

Thermal growth curves, thermal optima and niche widths

We found that major taxonomic/functional groups differed in their temperature optima, average maximum growth rates and niche widths (Figure 10B). The surveyed dinoflagellate had the lowest optimal growth temperature (17.12 ± 0.1°C), followed by the cryptomonads (20.3 ± 0.1), chrysophyte (23.2 ± 0.1), diatoms (24.0 ± 0.4) and chlorophytes (25.67 ± 0.1). Cyanobacteria achieved the highest optimal growth temperature that was estimated to be 30.6 ± 2.3. At the optimal temperatures, average growth rates for the functional groups differed as well, with cyanobacteria exhibiting the highest growth rates (0.76 d^{-1}), followed by green algae (0.68 ± 0.001 d^{-1}) and diatoms (0.49 ± 0.001 d^{-1}). Cryptomonads had the lowest growth rates (0.35 ±

0.001 d^{-1}). Functional groups also had different temperature niche widths. Cryptomonads had the narrowest niche width, spanning 20.7 ± 0.8°C on average, with an upper critical temperature of 30.40 ± 0.015°C. We found much higher upper critical temperatures for green algae (34.2 ± 0.1°C) and diatoms (33.2 ± 0.1°C), as well as broader thermal niche widths, 34.4 ± 0.4°C and 35.6 ± 0.9°C respectively. Cyanobacteria had the highest upper critical temperature (35.7 ± 2.3) with the broadest niche width (36.7 ± 4.9).

A.)



B.)

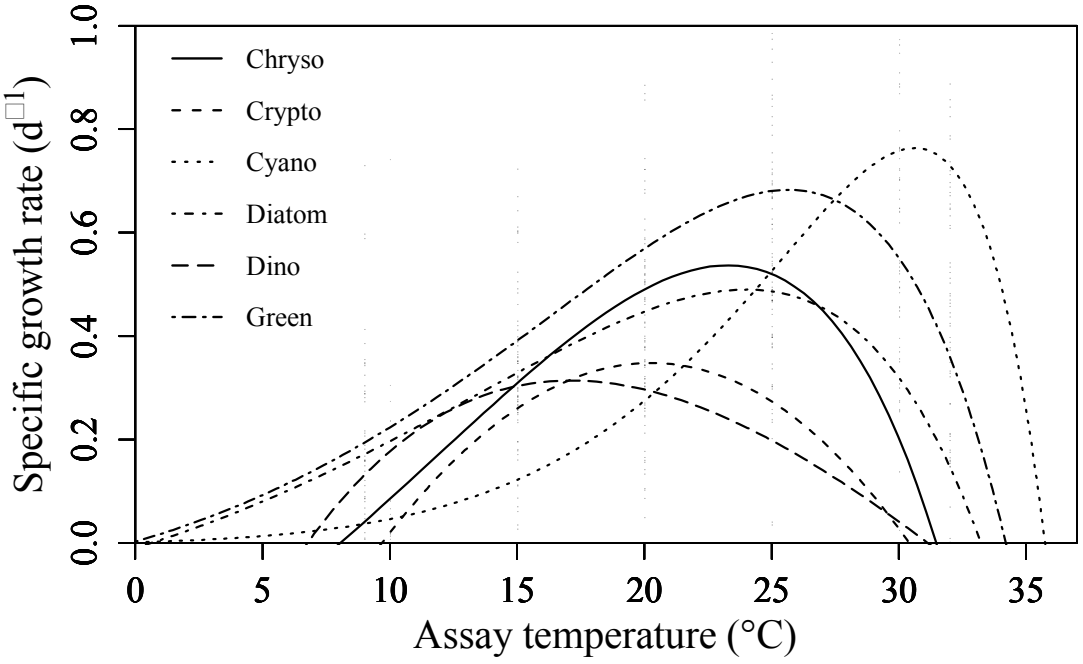


Figure 10. (A.) Growth curve for *Chlamydomonas reinhardtii*. (B.) Comparison of temperature curves for different algal functional groups: Chrysophyta (solid), Cryptophyta (dashed), Cyanophyta (dotted), Bacillariophyta (dashed and dotted) and Dinophyta (dashed).

At the species level, maximum growth rates and niche widths differed across and within the functional group level, while thermal optima remained relatively similar for within functional group comparisons (See Supplemental Figure 1). As an example, chlorophytes displayed the largest variation within the captured functional traits. Growth rates ranged from 0.46 d^{-1} (*Monoraphidium* sp.) to 1.1 ± 0.001 d^{-1} (*S. quadricauda*), with thermal niche widths ranging from 19.6 ± 0.11°C (*P. simplex*) to 36.9 ± 0.32°C (*Ourococcus* sp.). Despite this variation, almost all chlorophytes have thermal optima at 25.7 ± 0.057°C. The dinoflagellate *Peridinium* sp. and the cryptomonad *Rhodomonas* sp. had the lowest growth rates (0.31 ± 0.1 and 0.36 ± 0.001 d^{-1}) at their (lowest among the tested species) thermal optima (17.1 ± 0.1 and 16.8 ± 0.1°C). The cyanobacterium *A. cylindrica* achieved the highest measured growth rate of 1.29 d^{-1} at its thermal optimum of 31.7 ± 1.0°C.

Fatty acid production

We used two different ways to characterize the fatty acid production for the surveyed algal species, calculating total fatty acid production (fatty acid mL^{-1}) and biomass-specific fatty acid content (fatty acid (unit biovolume)$^{-1}$). Both measures provide unique insights into what mechanism is driving the overall productivity (see Discussion).

Focusing first on total fatty acid production, we determined that it strongly depended on temperature. Regardless of the functional group or genus, the highest total fatty acid production was at or close to the thermal optimum for growth, with productivities mirroring species thermal growth curves. For example, *S. obliquus* has a thermal optima of 25.6 ± 0.1°C, and we find that fatty acid mL^{-1} reaches its highest levels at 25 and 30°C, driven by the high growth rate at this temperature, with a significant decrease in fatty acid production at 32°C in part due to low growth rate (Figure 11). The same trend is observed across almost all species surveyed, as can be

visualized for a small sample of species belonging to different functional groups in Figure 11 (also, see Supplemental Figure 2). The growth optimum of *Anabaena* sp. was outside the experimental temperature range, and, similar to the growth curve, we find that the fatty acid production also continues to increase with temperature, presumably until the thermal optimum is reached, after which a decline would be predicted. The cryptomonad *C. ovata* had one of the lowest thermal growth optima, and we find a corresponding low thermal optimum for fatty acid production, where the highest values were observed between 15-20°C.

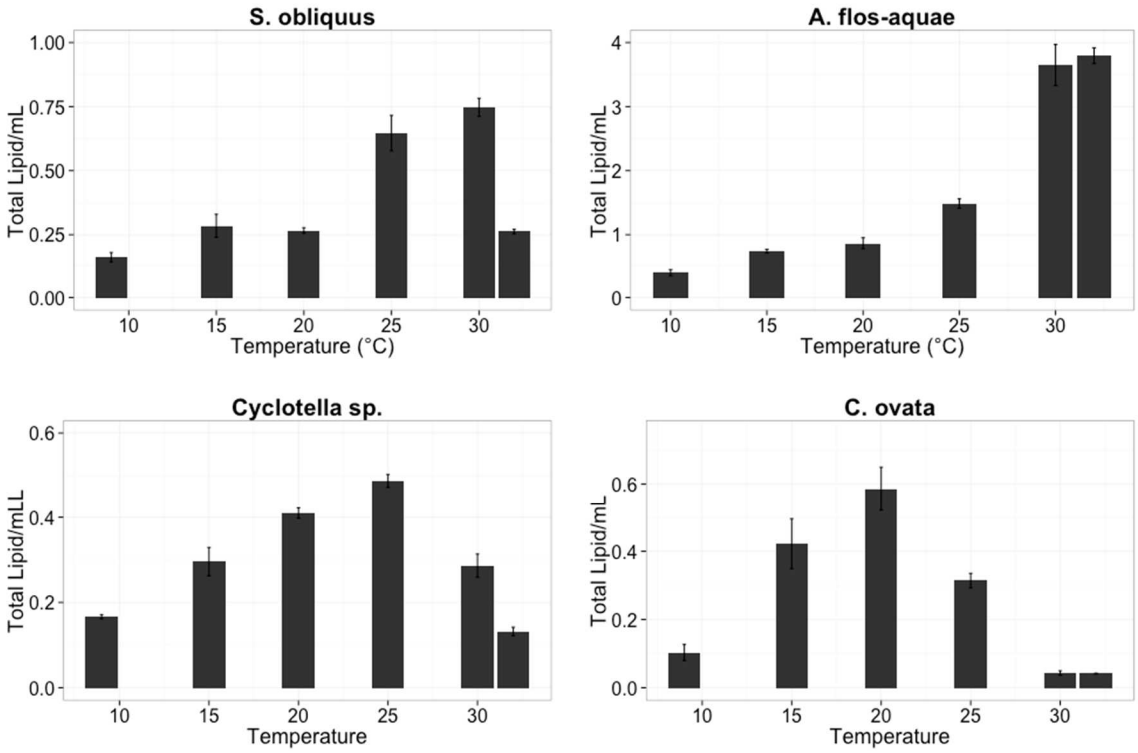


Figure 11. Temperature dependence of total fatty acid mL^{-1} production. Intra- and inter-species level differences in total fatty acid mL^{-1} production across a range of temperatures.

There is a large variation in the magnitude of total fatty acid production across functional groups and species, following differences in temperature optima for growth (Figure 11, Supplemental Figure 2). As Figure 11 illustrates, comparing species from different functional groups, such as *C. ovata* and *Anabaena* sp., we can see a difference in the thermal fatty acid production optima and a significant difference between the magnitude of the total fatty acids produced at these optima. The cyanobacterium *Anabaena* sp. has over a 6-fold higher total fatty acid production compared to *C. ovata* (3.7 ± 0.2 and 0.6 ± 0.04 μg mL^{-1}, respectively). Although differences are not always as drastic, we were able to identify both high and low fatty acid producing species in our survey. Several top producing species spanned functional groups, with the green alga *C. vulgaris* being the highest producing species in our experiments (12.6 ± 1.1 μg mL^{-1}), followed by the diatom *N. pelliculosa* (6.04 ± 0.06 μg mL^{-1}), cyanobacterium *Anabaena* sp. (3.7 ± 0.2 μg mL^{-1}), and the green alga *B. braunii* (1.9 ± 0.1 μg mL^{-1}). A vast majority of other species surveyed had fatty acid yields under the 1μg mL^{-1} range.

Another measure of fatty acid production was the biomass-specific fatty acid content. Generally, we did not find a significant temperature effect on this measure of fatty acid production (Supplemental Figure 3 – Fatty acid per biovolume plots). This holds for almost all chlorophytes, cyanobacteria, crytomonads, the dinoflagellate and chrysophyte we surveyed. But, we did find that at higher temperatures the biomass-specific fatty acid content of diatoms decreased (except in *N. pelliculosa*).

Fatty acid composition

We also determined fatty acid composition for the surveyed species. Functional groups have distinct fatty acid profiles, with varying relative contributions of specific fatty acids (Figure

12). We found that temperature can significantly influence the fatty acid composition of certain species, while having little to no effect on other species.

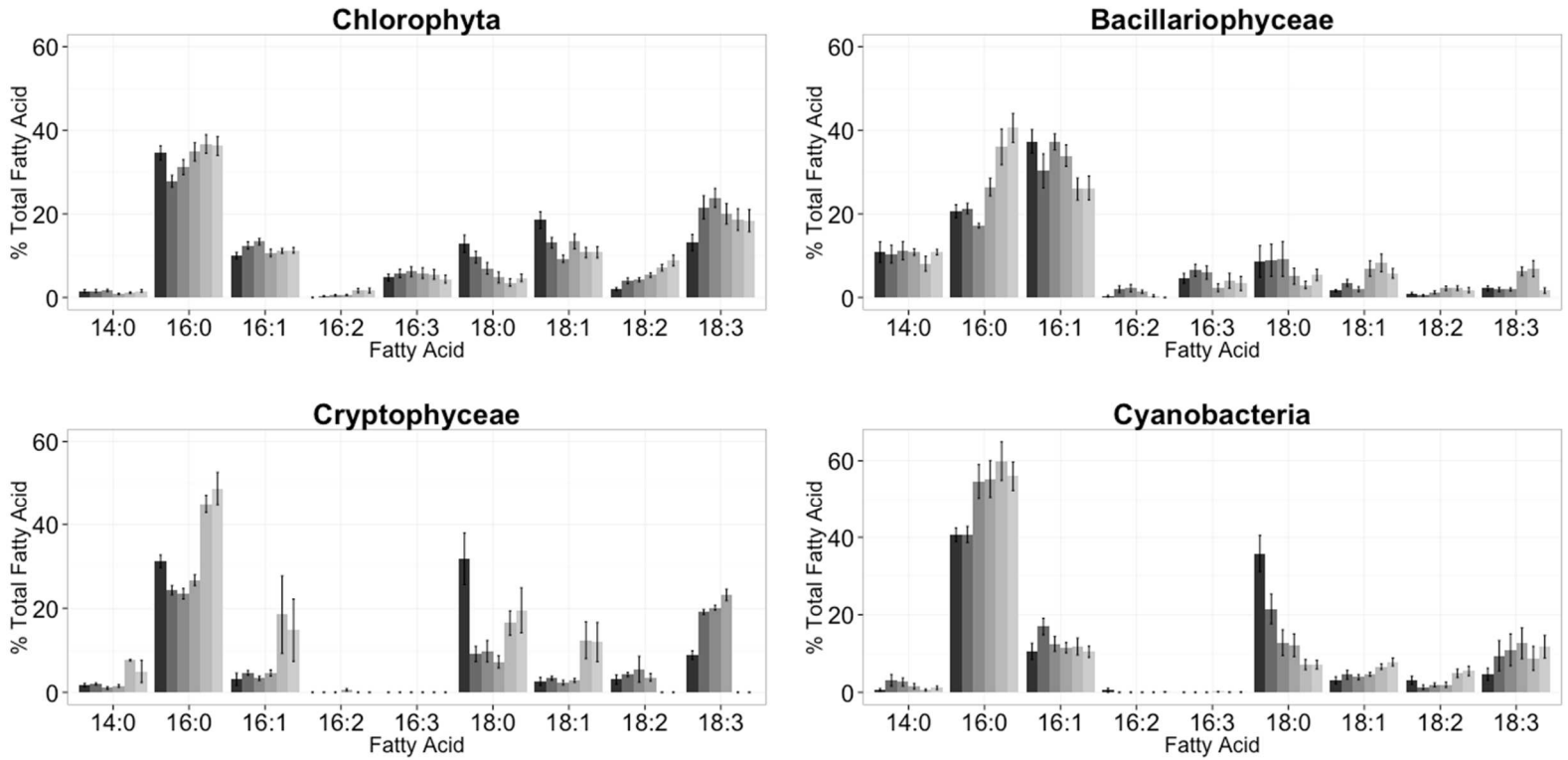


Figure 12. Temperature dependence of fatty acid profiles for different taxonomic groups. From left to right: 9, 15, 20, 25, 30, 32°C (black to light grey).

The fatty acid profile of chlorophytes is dominated by palmitic (16:0, ~35%) and linolenic acids (18:3, ~25%). At higher temperatures, we find a significant decrease in stearic acid (18:0), while linoleic acid (18:2) increases significantly. Diatoms' FA profiles are markedly different. The fatty acid composition of diatoms is a majority palmitic and palmitoleic acid (16:0 and 16:1, respectively), with minor contributions coming from the longer chain and more unsaturated fatty acids (such as 18:1 and 18:3). Temperature did not significantly influence the fatty acid profile of diatoms, other than an increase in palmitic acid under warmer conditions. Cyanobacteria have a high relative content of palmitic acid, at times reaching greater than 50%, which significantly increases at higher temperatures (Figure 12). In cyanobacteria, we also found relatively high levels of stearic acid at 9°C with significantly lower percentages at higher temperatures. Finally, cryptomonads had higher percentages of saturated fatty acids (16:0 and 18:0) and monounsaturated fatty acids (16:1 and 18:1) at higher temperatures, whereas polyunsaturated fatty acid composition was the highest at lower temperatures. Cryptomonads also had small percentages of long chain fatty acids (LCFA).

We also identified species-specific fatty acid profiles with their contrasting responses to temperature. For example, when comparing the two algae from the same functional group (Chlorophyta), with similar growth parameters, *C. vulgaris* and *S. obliquus*, these profile differences are quite apparent (Figure 13). The fatty acid profile of *C. vulgaris* is predominantly longer chain fatty acids (18-C's), with a majority of those derived from polyunsaturated fatty acids (PUFAs), specifically linolenic acid. *C. vulgaris'* profile also changes with temperature, containing more palmitic, hexadecadienoic (16:2) and linoleic acids at higher temperatures, while decreasing stearic and linolenic acids. Palmitic and palmitoleic acids make up less than 20% of the overall profile. Whereas, *S. obliquus* is compositionally over 60% palmitic and

palmitoleic acid with roughly 10% attributable to linolenic acid. *S. obliquus* shows very little change in fatty acid profile across the temperature range, with the only shift coming from a strong decrease in stearic acid as temperatures surpass 9°C. The profile of the diatom *N. pelliculosa* is highly stable across its thermal range, being heavily composed of palmitic, palmitoleic and stearic acids (>85%). The cyanobacterium *Merismopedia* sp. fatty acid content is composed of roughly 75% palmitic acid, with an observable increase in stearic and oleic acids at 32°C. For additional fatty acid profiles of the species not discussed directly in the text, please see Supplemental Figure 4.

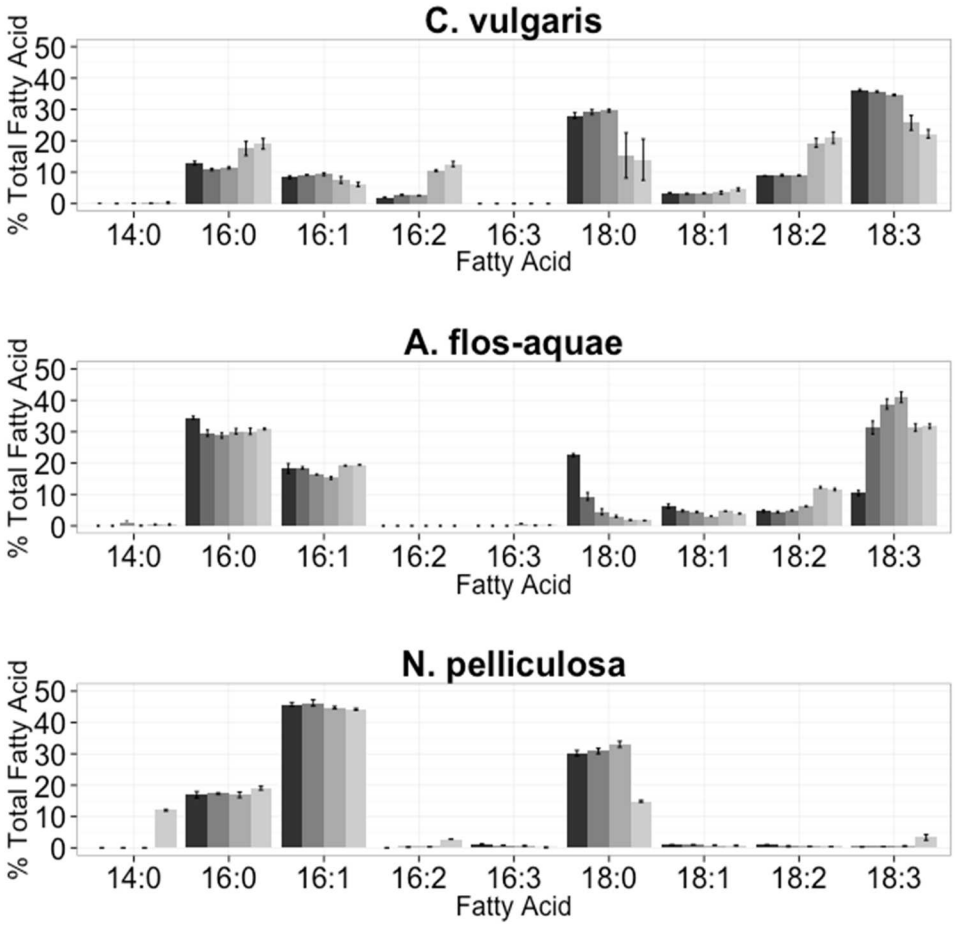


Figure 13. Temperature dependence of the species level fatty acid profiles. (A.) green alga *Chlorella vulgaris* (15-32°C), (B.) cyanobacterium *Anabaena flos-aquae* (9-32°C) and (C.) diatom *Navicula pelliculosa* (9-25°C).

DISCUSSION

The commercial cultivation of algae for feed, fuel, food and/or nutraceuticals, whether in contained or open systems, will experience variable and, at times, extreme temperatures. We conducted the present study to better understand and assess the performance of algal species that could be used for biofuel. Through collecting relevant performance measures (growth rates and

fatty acid content), or traits, across a wide range of temperatures, this study can provide a better understanding of biomass and fatty acid yields under specific temperature regimes or can serve as a method for selecting candidate algal species tailored to specific thermal ranges.

Through collecting thermal growth curves, we can gain a considerable amount of information about algal species, from both fundamental and applied perspectives. Our results on growth performance of diverse species at different temperatures support the general patterns of thermal preferences of different groups, such as the association of cyanobacteria with high temperatures and cryptomonads with lower temperatures, as is often observed in nature (Reynolds 1984). These temperature differences likely contribute to the observed patterns of seasonal succession in freshwater temperate lakes. In addition to the general thermal performance information, our study adds novel data on fatty acid yield and composition under different temperatures, making it particularly useful for algal cultivation challenges. Identifying the range of temperatures at which species can achieve positive growth and their growth optima allows for a simple method for including or excluding species for cultivation. At the functional level, cyanobacteria are better suited for high temperature environments, with the highest upper critical temperatures and highest optimal growth temperature compared to all other groups surveyed. At the same time, cyanobacteria have significantly reduced growth at temperatures falling below 25°C, resulting from a relatively high lower critical temperature (lowest temperature for growth). From our surveyed species, we also identified that chlorophytes have wide thermal niches, falling within an intermediate range of temperatures, suggesting that green algae might be best suited for growing in environments that have seasonality, or large temperature fluctuations. Although diatoms and cryptomonads had lower growth optima, we

found that generally green algae still had higher growth rates at those temperatures, suggesting that green algae would be also well suited for biomass generation at lower temperatures.

But for biofuel production, these growth parameters take on more significance when discussing fatty acid production. We assessed two different aspects of fatty acid production, one a raw overall measure of fatty acid production and the other focused on the specific fatty acid contribution on a cellular level. This comparison yielded two interesting observations: (1) highest total fatty acid production aligns with species-specific growth optima, as fatty acid yield is proportional to growth and (2) biomass-specific fatty acid production generally does not vary much across temperatures. These observations suggest that growth rates are the main driver in the overall productivity, since internal fatty acid content is not increasing at the optima, so that increased levels of biomass is the main driver. Diatoms might be an exception to this trend, as we see that, with increased temperature, the biomass-specific fatty acid content significantly decreases.

Our survey also identified a number of highly productive species that are consistent with previous studies documenting them as biofuel candidate species, while we also identified a few additional under-researched biofuel candidate species (Chisti 2007; Rodolfi *et al.* 2009). *C. vulgaris* had the highest observed fatty acid production. *Chlorella* species have been widely researched for biofuel applications, due to their high growth rates and high overall productivity. We also found that cyanobacteria produce high quantities of fatty acids as well, highlighting an area of future research into cyanobacteria, as this group are not traditionally identified as biofuel candidates. Recently, however, several studies have discussed the significant potential of cyanobacteria for biofuel (Wahlen, Willis & Seefeldt 2011; Karatay & Dönmez 2011; Lynch *et al.* 2015). The high productivity of cyanobacteria, coupled with their high thermal growth optima

and tolerance of eutrophic conditions suggests that these species would be ideal for aligning their cultivation with a high nutrient, anaerobically digested effluent (with high temperatures) for bioremediation. In addition, cyanobacteria can produce toxins that deter grazers and may inhibit other algae, including wild strains invading open cultivation ponds.

In agreement with previous studies, we also determined that the diatom *N. pelliculosa* achieves high fatty acid production at much lower temperatures (15-20°C) than the other species tested and may be a good candidate for cultivation under mild temperatures.

In addition to total fatty acid production, the composition of the fatty acids making up biodiesel feedstock is extremely important for biofuel applications. The composition of the fatty acid feedstock must meet quality standards as outlined in the American Society for Testing and Materials (ASMT) D6751 or the European Standard EN 14214, depending on location. This standard ensures that the biodiesel produced maintains adequate combustion quality, viscosity, oxidative stability and cold-flow-properties (Knothe 2008, 2011). These properties are highly influenced by the percentage of polyunsaturated fatty acids and the hydrocarbon length. The European Standard requires that linolenic acid (18:3) be below 12% of the total fatty acid blend.

As in previous studies, we found that fatty acid profiles of specific algal species can significantly change at different temperatures. With the biodiesel fuel standards in mind, temperature-induced shifts in fatty acid profiles could have significant effects on algal biofuel production, where a simple fluctuation in cultivation temperature can shift a viable crop to a less desirable feedstock. We found that certain algal species transitioned to undesirable fatty acid profiles as temperature increased (e.g., *Desmodesmus*, *C. erosa* and *C. ovata*) or decreased (e.g., *C. vulgaris*). With significant shifts in fatty acid profiles according to cultivation temperature, our results would suggest that in a highly variable thermal environment, identifying species with

highly stable fatty acid profiles across a range of temperatures would be extremely beneficial (e.g., *N. pelliculosa*).

Our work also highlighted that strictly selecting candidate strains based on total fatty acid production can be misguided. As stated earlier, *C. vulgaris* is researched quite heavily for its biofuel applications, but its fatty acid composition would suggest that it is a poor candidate due to high levels of linolenic acids (>30%). We also identified that *Anabaena* sp. was also a high fatty acid producer, but, again, high levels of linolenic acid may limit its biofuel candidacy. *Merismopedia* sp. however, achieved high growth, driving high fatty acid production, and its fatty acid profile is heavily enriched with palmitic acid, suggesting that this may be a strong biofuel candidate. Our study also agrees with previous suggestions that diatoms have a superior fatty acid composition for biodiesel production (Levitan *et al.* 2014). Furthermore, based on our results showing relatively little impact of temperature on the overall fatty acid composition, diatoms remain viable candidates, although growth rates of the species/strains we tested are lower than desired.

Previous work has also shown that as temperature increases, fatty acid composition shifts towards longer and more saturated fatty acids, while at low temperatures, the fatty acids are more unsaturated and shorter. Where this theory generally addresses membrane dynamics, our study extracted total fatty acid content, not yielding the precision to address the membrane dynamics directly. Based on our indiscriminant fatty acid extraction, we did not find higher relative contributions of longer, more saturated fatty acids at higher temperatures or shorter, more unsaturated fatty acids at lower temperatures. The only generalizable trend we identified was a shift towards more palmitic acid at higher temperatures. All other relationships were more species-specific and varied considerably within and among functional groups.

Overall, temperature can have dramatic effects on the overall productivity of algal species. Whether the goal of cultivating algae is for food, feed, fertilizer, high value nutraceuticals, or biodiesel, the impact of variable temperature must be understood for desirable outputs. This survey sought to help characterize the role that temperature plays in the growth, fatty acid production and fatty acid composition of freshwater algae and cyanobacteria. Through collecting these eco-physiological traits, we hope this study can serve as a tool to inform researchers and practitioners to use temperature-related traits to generate predictable and productive outcomes with respect to biomass and fatty acid yields in variable environments.

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CHAPTER 4

Coupling algal biofuel generation with brewery wastewater remediation

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ABSTRACT

The commercialization of mass algal cultivation suffers from several technical and economical bottlenecks at every step from production to product. For cultivation, these systems require large volumes of inexpensive water and nutrients to achieve the necessary economies of scale. Wastestreams, from industrial to municipal, present a unique source of inexpensive, if not free, nutrient-rich water source that is available in high quantities. We investigated the potential of coupling algal cultivation with brewery wastewater remediation through monitoring algal growth, total fatty acid production, fatty acid profiles, cell size and total nitrogen and phosphorus removal. Through bioprospecting, we identified 7 candidate species of algae that achieved positive growth in anaerobically digested brewery wastewater. Those candidate species were cultivated in unfiltered, unsterilized 80:20 brewery wastewater:water medium for 12 days, with sampling every 4th day. From these candidate species, we also assembled and grew a seven-species polyculture. The polyculture, and the *C. vulgaris* and *Ourococcus* sp. cultures all achieved high levels of productivity, in terms of biomass and fatty acid accumulation. We also found that *S. obliquus* and *quadricauda* performed well, while *A. falcatus* and *C. reinhardtii* had minimal production. In 12 days, the polyculture achieved the highest level of total nitrogen and phosphorus removal, 76% and 83% respectively. Our results show that brewery wastewater can be used as an inexpensive source of water and nutrients, and through coupling these two systems there is a unique opportunity to advance the economic and environmental sustainability of both mass algal production and the brewing industry.

INTRODUCTION

Microalgae continue to emerge as an extremely rich, and still underutilized, source for an array of bioproducts. Algal derived biofuels are amassing an increasing amount of interest as the demand for renewable fuels continues to grow. They are extremely promising due to their high productivity, small overall footprint, the ability to be cultivated on marginal lands, and the coupling of their cultivation to wastewater sources for fertilizer (Chisti 2007; Wijffels & Barbosa 2010; Smith *et al.* 2010, 2015). Yet, although promising, the commercial viability of algal biofuel generation is hampered by the systems' overall sustainability, both economically and environmentally (NRC 2012; FAO 2013). Two main challenges to realizing this economic and environmental sustainability are (1) achieving the high productivity potential within these systems and (2) identifying an inexpensive water and nutrient source at an industrial scale (NRC 2012; FAO 2013; Smith *et al.* 2015).

To address these main bottlenecks to commercialization, a substantial amount of work has investigated coupling algal cultivation with wastewater remediation, i.e. phycoremediation. Initially this work focused on simply using algae for its remediation potential (Abeliovich 1986; de la Noue & Proulx 1988; Hashimoto & Furukawa 1989; Tyagi & Vembu 1990; de la Noüe, Laliberté & Proulx 1992; Travieso, Benítez & Dupeyrón 1992; Olguın 2003; Oswald *et al.* 2010), yet recently the research places an emphasis on the synergy of remediation while generating bioproducts (Chinnasamy *et al.* 2010; Raposo *et al.* 2010a; Park, Craggs & Shilton 2011; Mata *et al.* 2012; Mehrabadi, Craggs & Farid 2015; Shin *et al.* 2015; Stockenreiter *et al.* 2016; Wuang *et al.* 2016). Wastewater sources offer an extremely inexpensive fertilization source that can generate high levels of algal productivity. Algae have been used to successfully remediate a number of industrial and municipal wastewater sources: dairy, swine, carpet milling,

paper pulp, agro-industrial, aquaculture, distillery and brewery (Tarlan, Dilek & Yetis 2002; Mulbry, Kondrad & Buyer 2008; Travieso *et al.* 2008; Chinnasamy *et al.* 2010; Wang *et al.* 2010; Raposo *et al.* 2010a; Rawat *et al.* 2011; Mitra, van Leeuwen & Lamsal 2012; Stockenreiter *et al.* 2016; Wuang *et al.* 2016).

Identifying suitable wastewater streams for algal cultivation is challenging. Although studies have shown that algae can be cultivated on various wastewater sources, the waste streams need to be highly diluted (50-90%), filtered, sterilized, and/or require additional nutrient additions to ensure hospitable growth conditions (Kong *et al.* 2012; Mitra, van Leeuwen & Lamsal 2012; Shin *et al.* 2015; Stockenreiter *et al.* 2016; Wuang *et al.* 2016). But brewery wastewater, with its high levels of essential nutrients and low levels of toxic substances, antibiotics, and pathogenic bacteria, has emerged as a promising wastewater source for mass cultivation (Olajire 2012). Brewery wastewater also contains high levels of organic material that can be utilized by mixotrophic algal species, combining autotrophic and heterotrophic modes of nutrition. Yet even with these promising characteristics, the coupling of algal biomass generation with brewery wastewater remediation remains relatively unexplored.

In the US alone, the beer market exceeds $100 billion dollars, a five-fold increase over the past decade. In 2015, the craft beer industry made up 22% of the overall US market, growing almost 13% in a year, including a 16% increase in total exports. In the same year, the US beer industry produced over 196 million fluid barrels of beer, or ~6.2 billion gallons('The New Brewer Magazine' 2016). The sustainability of the brewing industry has generated a lot of attention and innovation. The brewing process is energy intensive and generates a large volume of wastewater (Olajire 2012). Even with an increased amount of innovation focusing on water consumption, a brewery generates up to ten liters of wastewater for every liter of beer produced,

with extremely efficient breweries generating 4-7 liters (European Commision 2006; Kanagachandran & Jayaratne 2006; Association 2013). It is important to note that this is only a small fraction of the overall water consumption if the cultivation process of the cereal grains and hops were included.

Wastewater is produced at every stage: from sequestered water in the grains during mashing and the dense settled trub after fermentation, to the large volumes of cleaning water used for sterilizing equipment, cans and bottles. The composition of the wastewater is beer specific, greatly changing between beer styles (i.e. India pale ale versus stout) due to the grain bill and yeast strain used for beer construction. The nitrogen and phosphorus concentrations in the effluent both range between 30-100 mg L^{-1}, with nitrogen coming directly from the cereal grains while phosphorus is derived from mostly the cleaning and sterilization process (Brewers of Europe 2012; Olajire 2012).

Brewery's wastewater remediation protocols depend on the size of the brewery. Small-scale breweries, i.e., microbreweries, can simply send their wastewater directly to the local municipal wastewater treatment facility, incurring remediation costs according to the organic content (BOD) of the effluent. But, as breweries continue to expand, local wastewater treatment facilities can no longer handle the increased brewery wastewater, forcing these municipalities to either expand operations at an increased cost to the brewery or set effluent limits, ultimately limiting brewery operations. In the case of Lagunitas Brewing Company, instead of paying the City of Oakland $1 million in annual treatment costs, they invested in an $8 million wastewater treatment facility on the brewery campus (Gribbins 2013).

Brewery wastewater poses serious sustainability issues, both economic and environmental, and as the brewing sector continues to grow it is imperative to develop

innovative, efficient and inexpensive methods of remediation. High rate algal ponds (shallow, open raceway ponds), have been used to treat municipal and industrial wastewater for half a century (Oswald & Golueke 1960). High rate algal ponds have been shown to achieve high nutrient removal while operating at low economic costs (Park & Craggs 2010; Park, Craggs & Shilton 2011). Optimizing these systems for algal production and nutrient removal poses some unique challenges because these systems are open ponds, susceptible to environmental fluctuations, invasions, infections and predation. But, an emerging line of research has illustrated that through growing diverse algal communities within these outdoor systems, algal productivity and nutrient removal can be increased, while maintaining system stability under environmental pressures (Ptacnik *et al.* 2008; Smith *et al.* 2010; Cardinale 2011; Stockenreiter *et al.* 2012, 2013; Kazamia, Aldridge & Smith 2012; Shurin *et al.* 2013; Nalley, Stockenreiter & Litchman 2014).

Although promising, growing algal biofuel polycultures with a simultaneous brewery wastewater remediation has not been explored. This study sought to first establish proof of concept that algae can be cultivated in true, unfiltered, anaerobically digested brewery wastewater. We also wanted to investigate the benefits of cultivating diverse algal communities vs. monospecific cultures for brewery wastewater remediation.

METHODS

Experimental design

Wastewater was collected post-anaerobic digestion at a craft brewery in Galesburg, Michigan. Brewery effluent was first sent to an equalizing tank on-site with a retention time of 3

days (170,000 gallon capacity). Since the chemical composition of the brewery effluent can vary considerably depending on the beer that is being produced, the equalizing tank homogenizes the wastewater across multiple days to ensure consistent loading. After equalizing, wastewater was diverted to one of three anaerobic digesters (40,000 gallons capacity), then recombined in a final anaerobic digester and subsequently collected for experimentation. Table 5 provides the chemical composition of the brewery wastewater over a six-month period. Post-collection, the wastewater was centrifuged at 800 rpm for 2 minutes to simulate gravitational settling of solids. The centrifuged wastewater still contained suspended solids and remained opaque. We also detected microbial activity in the wastewater post centrifuging, likely coming from the anaerobic digesters. Wastewater was then blended with reverse osmosis water resulting in an 80:20 BWW:H_2O ratio. This minor dilution was aimed to decrease the opacity of the BWW.

Table 5. Brewery wastewater chemical composition after anaerobic digestion.

Chemical Oxygen Demand (COD)	2268.4 ± 60.4 mg L^{-1}
Total nitrogen	143.3 ± 9.4 mg L^{-1}
Total phosphorus	57.1 ± 5.1 mg L^{-1}

We used algal cultures from our laboratory collection. At first, we screened 16 species of algae from 3 functional groups to assess their viability in brewery wastewater (Table 6). From these 16 species, we identified 7 candidate species, all chlorophytes, that were able to grow in the BWW and selected them for subsequent experiments. Candidate strains were acclimated for 4 weeks in 500 mL of WC-medium (Guillard & Lorenzen 1972) at 30°C under 12:12 light:dark cycle at 130 μE m^{-2} s^{-1}. After acclimation, monocultures of each species were established in

triplicate, as well as a polyculture containing all seven species, also in triplicate. Cultures were grown in 200 mL of BWW, placed in a growth chamber at 30°C with a 12:12 light:dark cycle at 130 μE m^{-2} s^{-1}. Cultures were inoculated with staring biovolumes of $1.5x10^{10}$ μm^3 ($7.5x10^7$ μm^3 mL^{-1}), with the polyculture consisting of equal proportions of the seven constituent species ($1.714x10^7$ μm^3 mL^{-1}). We used biovolumes (total volumes of the species) as proxies for biomass, which is a common approach in algal research. Conversion to biomass is done assuming that algal cells have density of water, 1 mm^3 L^{-1}=1 mg L^{-1} (Suthers and Rissik 2009). Manual mixing was performed daily, and sampling took place every 96 hours.

Table 6. Species surveyed to assess viability of cultivation in brewery wastewater. Check marks indicate species that had positive growth in our pilot experiment.

Species	Taxonomic Group	Candidate Species?
Cyclotella sp.	Bacillariophyceae	X
Fragilaria crotonensis	Bacillariophyceae	X
Navicula pelliculosa	Bacillariophyceae	**X**
Ankistrodesmus falcatus	Chlorophyta	**X**
Botryococcus braunii	Chlorophyta	**X**
Chlamydomonas reinhardtii	Chlorophyta	☐
Chlorella vulgaris	Chlorophyta	☐
Desmodesmus sp.	Chlorophyta	**X**
Oocystis sp.	Chlorophyta	☐
Ourococcus sp.	Chlorophyta	☐
Scenedesmus obliquus	Chlorophyta	☐
Scenedesmus quadricauda	Chlorophyta	☐
Anabaena flos-aquae	Cyanophyta	**X**
Anabaena cylindrica	Cyanophyta	**X**
Merismopedia sp.	Cyanophyta	**X**
Microcystis wessenbergii	Cyanophyta	**X**

Measurements

To understand biomass dynamics within the mono- and polycultures, we collected samples every fourth day of the experiment, without any medium recharge. Initial estimates of biovolume used for inoculating experimental cultures were collected using the CASY Cell

Counter and Analyzer System Model TT (Roche Innovatis AG). Due to high particulate matter, samples in the brewery wastewater could not be counted on the CASY Cell Counter, so 5mL of well mixed sample was preserved with a Lugol's iodine solution. Cell morphology and biovolume was determined through measuring forty randomly selected cells, averaged values were used to determine average cell volume for each species using the approximating geometric shapes (Hillebrand *et al.* 1999). Preserved samples were subsequently identified, counted and biovolumes (biomass proxy) were measured by taking the product of number of cells mL^{-1} and average cell volume mL^{-1}.

Fatty acid productivity and fatty acid profiles of our cultures were also measured every fourth day. We performed total neutral fatty acid extraction, followed by fatty acid methyl ester (FAME) analysis following the protocol first established by Wang & Benning (2011) and later tailored to algae by Boyle *et al.* (2012). In brief, samples were filtered onto GF/B 2.1cm glass microfiber filters, frozen, and then neutral fatty acids were extracted, transesterified to constituent fatty acids, subsequently identified using a gas chromatograph (Boyle *et al.* 2012). The filtered volumes were adjusted based on cell density (smaller volumes for denser cultures), and all fatty acid samples were standardized by filtered volume (generally between 10-25mL). All FAME samples achieved satisfactory signal strength. We did not perform thin layer chromatography, meaning all fatty acids were analyzed together, rather than being separated into specific fatty acid classes prior to FAME analysis.

To determine the nutrient removal/remediation capabilities of the different cultures, we determined the total nitrogen and phosphorus in the medium (after removing the algae) every four days. After filtering algal biomass for fatty acid analysis, 25mL of filtrate was immediately frozen at -4°C for later chemical analysis. For total nitrogen (TN) concentration, filtrate was

digested with persulfate at 121°C for 30 minutes, converting all inorganic nitrogen content to nitrate that could then be quantified via 2nd degree spectroscopy on a Shimadzu Spectrophotometer (Crumpton, Isenhart & Mitchell 1992). TN digestion was performed at a 10x dilution, and values were quantified using a standardized curve (R^2=0.989). Total phosphorus (TP) concentration was determined separately following the standard molybdate method using a spectrophotometer. The digestion converts available phosphates to ortho-phosphate, which then can be quantified colorimetrically. TP digestion was performed at a 100x dilution, and phosphorus content was quantified using a standardized curve (R^2=0.974).

RESULTS

Biomass dynamics, polyculture composition and cell size

We observed positive growth across all of the cultures selected for the experiments, except for *Oocystis* sp., but growth rates differed substantially across species (Figure 14). Both *A. falcatus* and *C. reinhardtii* performed poorly, achieving significantly lower biomass than all other cultures ($p<0.001$) due to low growth rates, 0.105 ± 0.004 and -0.024 ± 0.018 d^{-1} respectively (± 1 standard error reported). *C. vulgaris* and *Ourococcus* sp. thrived in the BWW, and by Day 12 both species achieved significantly higher biovolumes than all other cultures ($p<0.001$), with growth rates of 0.242 ± 0.021 and 0.215 ± 0.006 d^{-1} respectively. The seven-species polyculture underperformed compared to *C. vulgaris* and *Ourococcus* sp., ultimately achieving modest biomass accumulation similar to both *S. obliquus* and *S. quadricauda*. The growth rates for the polyculture, *S. obliquus* and *S. quadricauda* were 0.204 ± 0.018, 0.191 ± 0.016 and 0.241 ± 0.019 d^{-1} respectively. Although our pilot study identified *Oocystis* sp. as a

candidate species, the experimental culture of *Oocystis* sp. did not grow, thus is not reported or shown in Figure 14. Interestingly, *Oocystis* sp. did grow in the seven species polyculture.

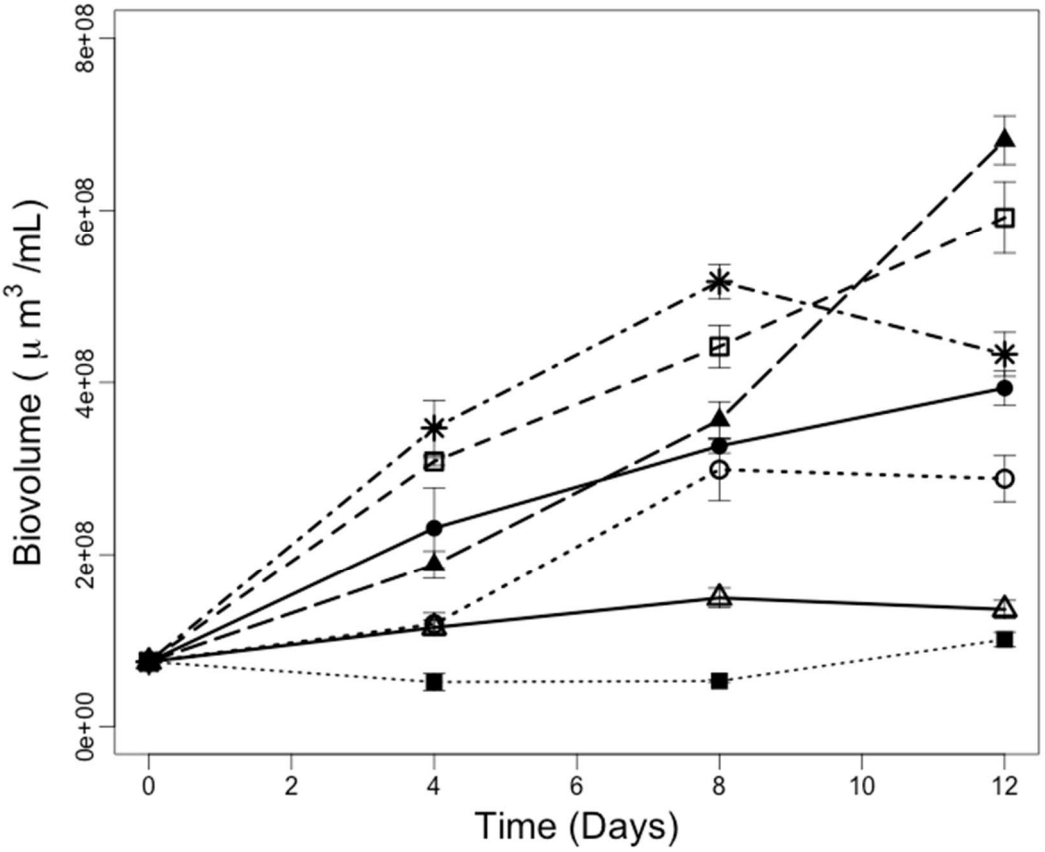

Figure 14. Biovolume dynamics through time of *A. falcatus* (open triangles, solid line), *C. reinhardtii* (filled squares, dotted line), *C. vulgaris* (open squares, dashed line), *Ourococcus* sp. (filled triangles, long dashed line), *S. obliquus* (open circles, short dashed line), *S. quadricauda* (asterisk, dot dashed line), and the polyculture (filled circles, solid line).

The seven species polyculture was inoculated with equal biovolumes of each species. While all species remained in the polyculture, *C. vulgaris* quickly dominated the overall culture,

while *S. obliquus* and *S. quadricauda* (collectively) maintained approximately 20-30% of the overall biovolume (Supplemental Figure 1). *Oocystis* sp. comprised roughly 10% of the overall biovolume, while *A. falcatus*, *C. reinhardtii and Ourococcus* sp. quickly became rare, composing less than 5% of the overall culture by Day 4.

Through visual counting of algal communities, we discovered that the algal cells of some cultures seemed enlarged compared to cultures grown in regular WC medium. Cell size was measured and used to estimate cellular biovolumes. We found that for 5 of 7 species, the cells grown in brewery wastewater were significantly larger than cells cultured in laboratory standard WC medium under the same light and temperature (Figure 15). *A. falcatus*, *C. reinhardtii*, *Oocystis* sp., *S. obliquus* and *S. quadricauda* all had significantly larger cell size in the brewery wastewater ($p=0.0006$, 0.03, 0.002, 0.03, 0.04, respectively), while *C. vulgaris* and *Ourococcus* sp. were not significantly larger ($p=0.518$ and 0.278, respectively). Figure 15 does not show *A. falcatus* on the plot due to its cellular biovolume being an order of magnitude higher than the other species. It is important to note that some *A. falcatus* cells formed nodules, or "beer bellies," on the exterior of the cells. It is unclear what caused these malformations.

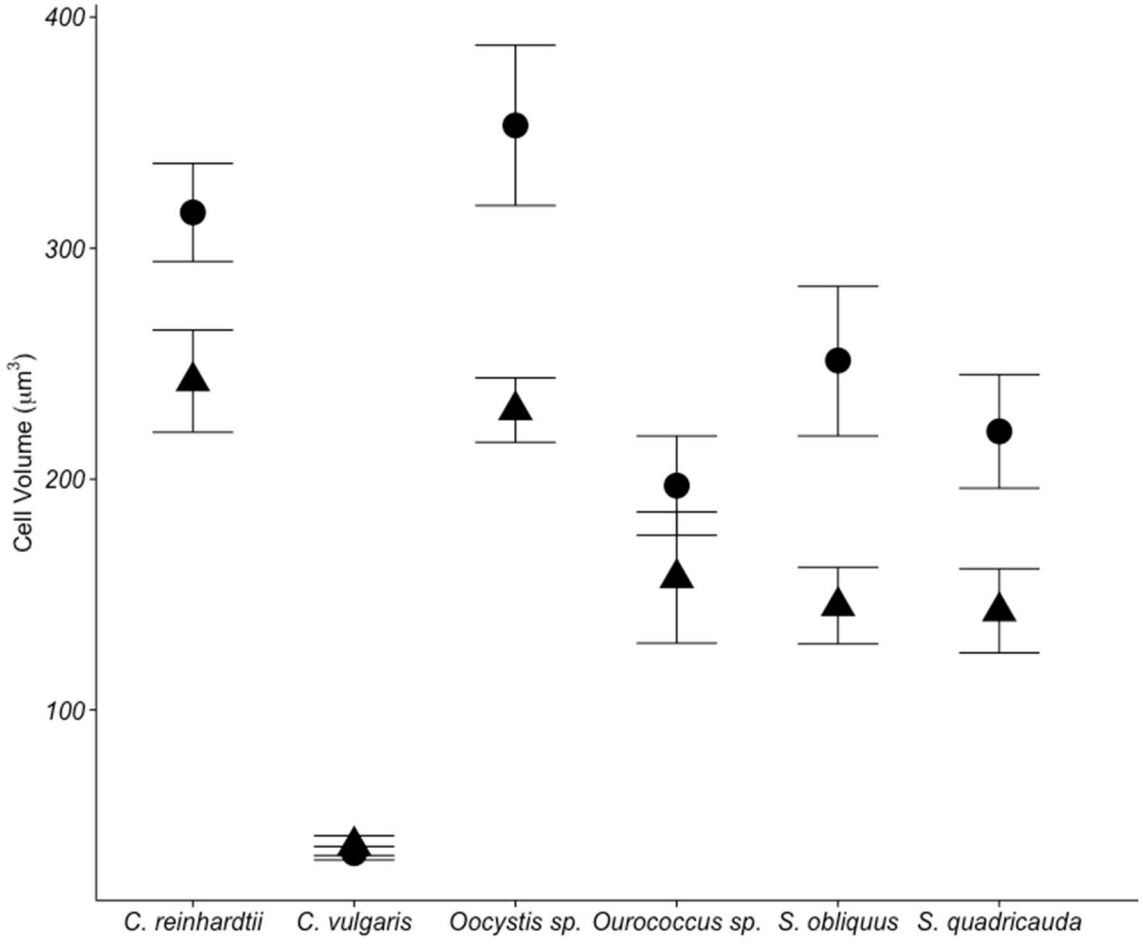

Figure 15. Comparing cell size in brewery wastewater (filled circles) and WC medium (same temperature and light conditions, filled triangles).

Fatty acid production and fatty acid profiles

Total neutral fatty acid mL^{-1} represents the overall productivity of the culture, taking into account both the cellular fatty acid content and the total biomass within the culture. The productivity dynamics of our experimental cultures showed a steady, general increase in overall fatty acid content through time, driven primarily by the increasing amount of biomass accruing in culture through time. *C. vulgaris* and the polyculture showed similar fatty acid production through day 8, but by day 12 *C. vulgaris* fell while the polyculture's fatty acid content kept

increasing (Figure 16). As observed for biomass accumulation, both *A. falcatus* and *C. reinhardtii* achieve the lowest total fatty acid production.

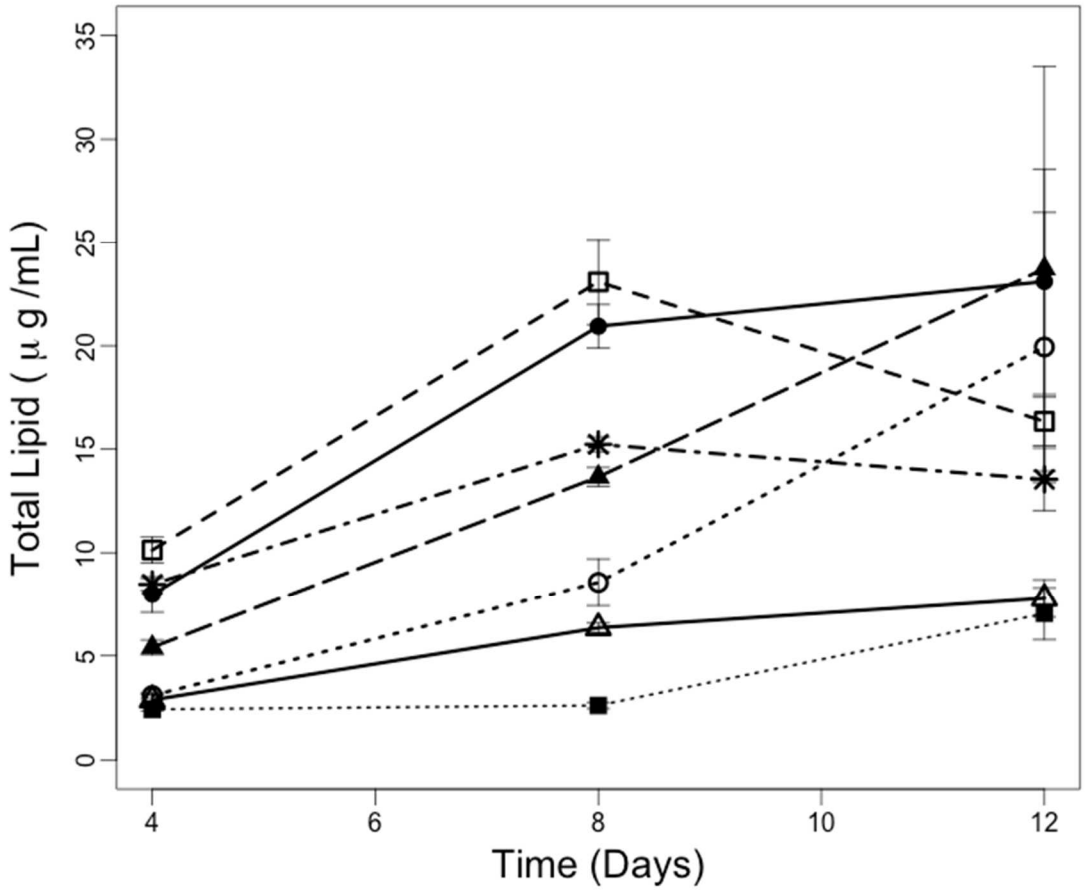

Figure 16. Total fatty acid production (µg mL^{-1}) through time of *A. falcatus* (empty triangles, solid line), *C. reinhardtii* (filled squares, dotted line), *C. vulgaris* (empty squares, dashed line), *Ourococcus* sp. (filled triangles, long dashed line), *S. obliquus* (open circles, short dashed line), *S. quadricauda* (asterisk, dot dashed line), and the polyculture (filled circles, solid line).

As expected, the fatty acid profiles of individual species and the collective polyculture had distinct compositions. Highlighted in Figure 17 are four truncated fatty acid profiles (see

Supplemental Figure 2 for full fatty acid profiles). *A. falcatus* is composed of a majority palmitic acid (16:0), with approximately 20% monounsaturated fatty acids (16:1 and 18:1). In comparison, *C. vulgaris* is composed of over 50% polyunsaturated fatty acids (>2 double bonds), with limited percent makeup of palmitic and stearic acids (16:0 and 18:0, respectively). The polyculture contains a higher percentage of palmitic acid, with limited hexadecatrienoic acid (16:3). The fatty acid profile of the polyculture appears to be very similar to the profile of *S. quadricauda*.

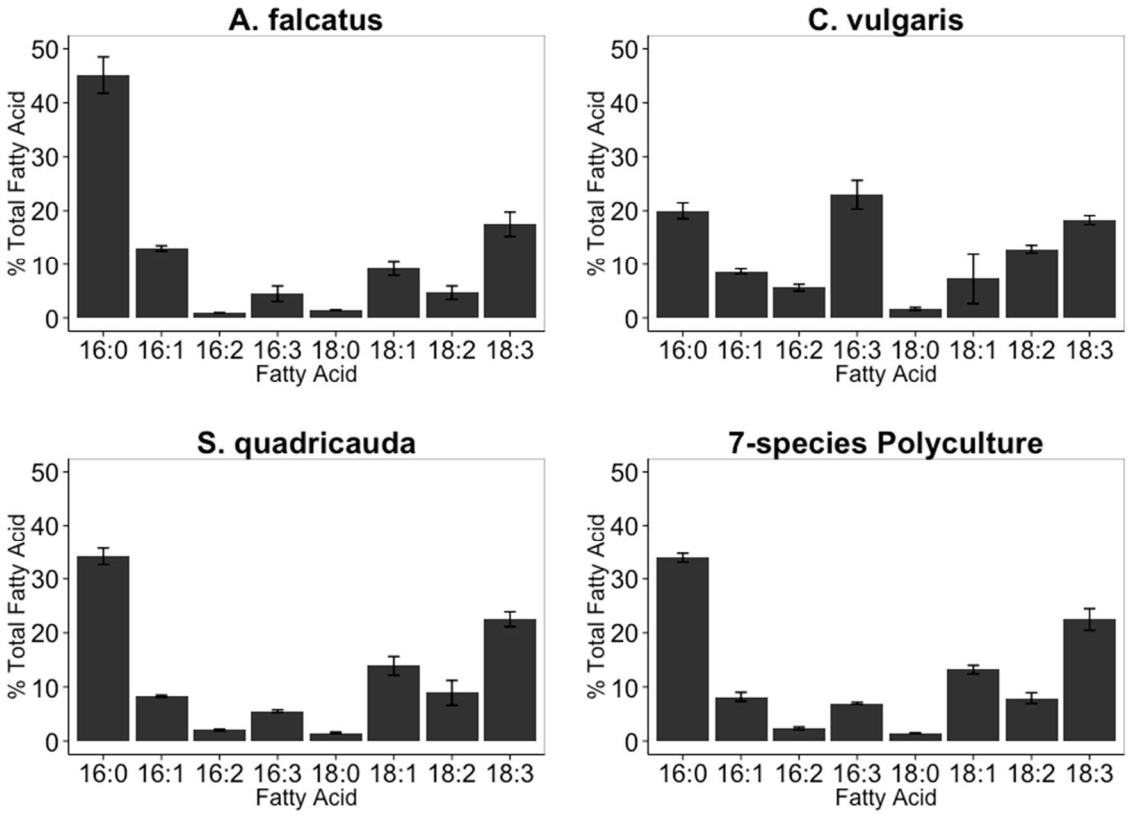

Figure 17. Percent fatty acid composition of a select three monocultures and the polyculture. Profiles have been truncated to only include fatty acids between 16 and 18 carbons. See Supplemental Figure 2 for all the complete fatty acid profiles.

Nutrient removal rates

The diluted brewery wastewater (80% BWW:20% water) initially contained a total nitrogen concentration of 89 mg L^{-1}. Within four days, the nitrogen levels in the medium (with algae filtered out) were reduced by 30% in all of the cultures (Figure 18). By day 8, *A. falcatus* and *C. reinhardtii* mostly stopped taking up N, with TN remaining at ~70% of the initial TN concentration, while *C. vulgaris*, *Ourococcus* sp., *S. obliquus*, *S. quadricauda* and the polyculture have taken up approximately 50% of the total nitrogen. Finally, by day 12, *A. falcatus* and *C. reinhardtii* maintained a 30% removal (60.11 and 56.36 mg L^{-1}, respectively), *Ourococcus* sp., *S. obliquus* and *S. quadricauda* had nitrogen removal of 50% (39.99, 44.04, 42.68 mg L^{-1}, respectively), while *C. vulgaris* and the polyculture reduced total nitrogen by 66% and 76% (29.28 and 21.01 mg L^{-1}). By day 12, most of the cultures, except *A. falcatus* and *C. reinhardtii*, brought nitrogen levels at or below the EPA MCL standard for nitrates (44.3 mg L^{-1}), with *C. vulgaris* and the polyculture exceeding this threshold. Moreover, the polyculture treatment reduced TN to significantly lower level than any of the monocultures.

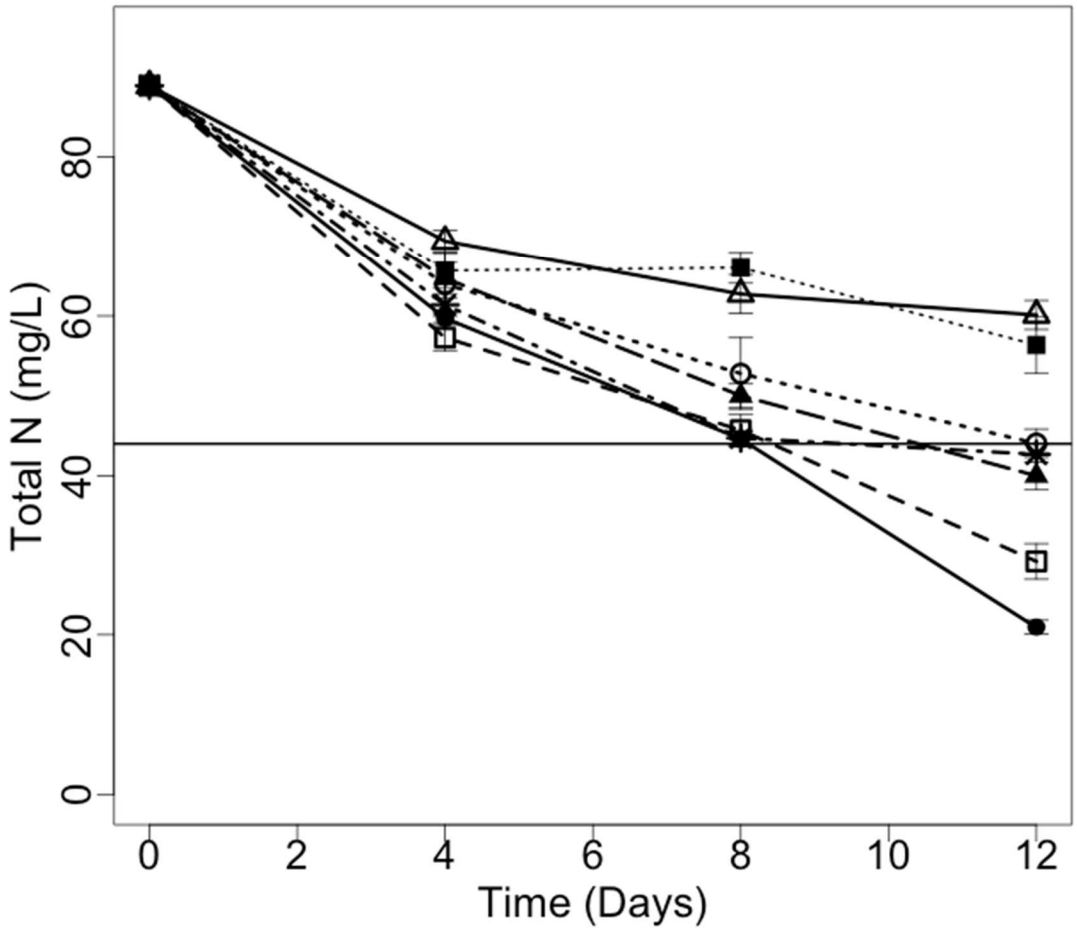

Figure 18. Total nitrogen concentration through time for *A. falcatus* (empty triangles, solid line), *C. reinhardtii* (filled squares, dotted line), *C. vulgaris* (empty squares, dashed line), *Ourococcus* sp. (filled triangles, long dashed line), *S. obliquus* (open circles, short dashed line), *S. quadricauda* (asterisk, dot dashed line), and the polyculture (filled circles, solid line). Solids horizontal line is set at the EPA MCL total nitrogen level of 44.3 mg L^{-1}.

The brewery wastewater initially had total phosphorus concentrations of 34 mg L^{-1}. *A. falcatus* and *C. reinhardtii* cultures achieved roughly 60% of TP removal by day 12 (Figure 19).

Within the first four days, *C. vulgaris*, *Ourococcus* sp., *S. obliquus*, *S. quadricauda* and the polyculture has assimilated about 50% of the total P. These cultures continued to draw TP levels down, and by day 12 the polyculture has reduced phosphorus by 83% (5.86 mg L^{-1}), the lowest level observed in our experiment, while *C. vulgaris*, *Ourococcus sp.*, *S. obliquus* and *S. quadricauda* have brought levels down by roughly 75% (7.40, 8.26, 7.63 and 11.33 mg L^{-1}, respectively). Although there is not a national discharge standard for phosphate, generally 1 mg L^{-1} is used. After 12 days, none of the cultures were able to bring TP levels down to that threshold.

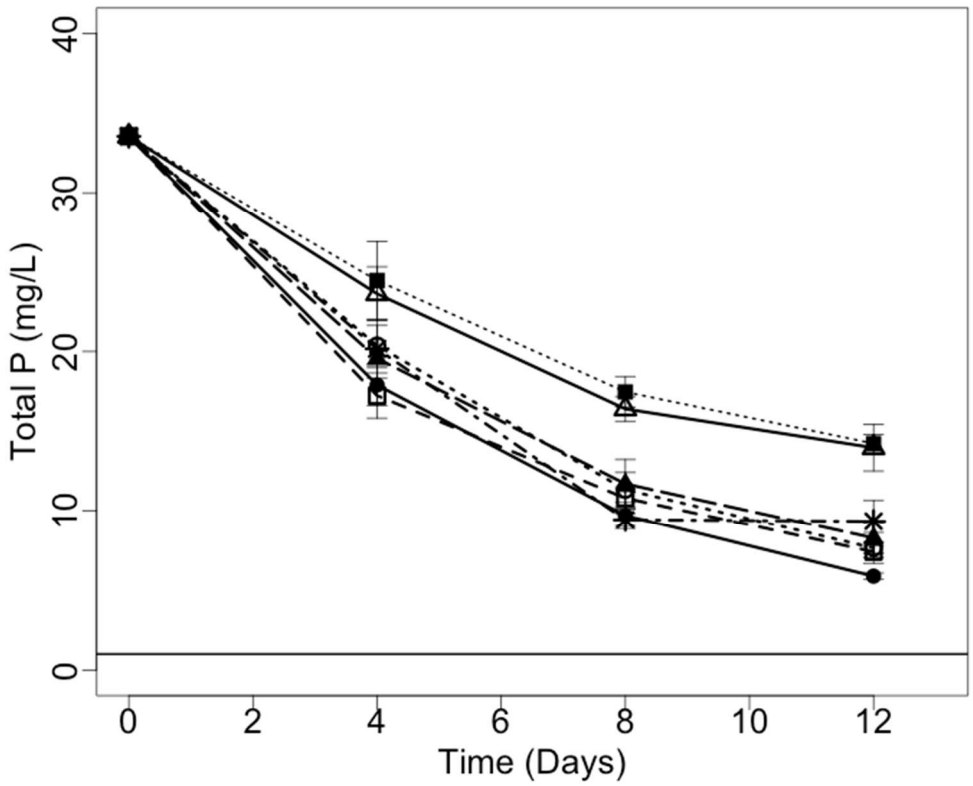

Figure 19. Total phosphorus concentration through time for *A. falcatus* (empty triangles, solid line), *C. reinhardtii* (filled squares, dotted line), *C. vulgaris* (empty squares, dashed line), *Ourococcus* sp. (filled triangles, long dashed line), *S. obliquus* (open circles, short dashed line), *S. quadricauda* (asterisk, dot dashed line), and the polyculture (filled circles, solid line). Solids horizontal line is set at the Michigan Department of Environmental Quality discharge regulation of 1 mg L^{-1} for total phosphate.

DISCUSSION

Our experiments showed that it is possible to couple algal cultivation for biofuel and other bioproducts with brewery wastewater remediation, thus increasing the sustainability of two

important industrial processes, biofuel generation and beer production. Our ability to grow microalgae in minimally diluted, unfiltered, anaerobically digested brewery wastewater suggests that brewery wastewater might be a highly suitable nutrient source for mass cultivation of algae, requiring minimal to no pre-treatment prior to its usage. While algae grew in the 80% BWW, we were unable to cultivate any species of algae in BWW before it was anaerobically digested, suggesting that this remediation approach may be limited to larger scale breweries that anaerobically digest their wastewater. The inability to cultivate any species other than chlorophytes in minimally diluted brewery wastewater may limit the potential bioproducts, such as some specialized nutraceuticals, while maintaining the ability to generate biofuels, animal feed, food and fertilizer. However, higher dilution ratios may allow growth of other taxonomic groups.

We were able to achieve the high density of algae in BWW in a short amount of time, which makes coupling of algal growth with BWW remediation a promising avenue of research. In terms of fatty acid production, we found that the polyculture achieved the highest total fatty acid value by day 12. Previous studies have discussed the benefit of fostering algal diversity as a method to achieve higher than expected yields (Smith *et al.* 2010; Stockenreiter *et al.* 2012, 2013, 2016; Nalley, Stockenreiter & Litchman 2014). Although the polyculture was composed predominately of *C. vulgaris*, the polyculture still achieved higher fatty acid production than the *C. vulgaris* monoculture, suggesting that the other algae contributing ~30% of biovolume significantly increased the overall fatty acid yield. Previous studies have also shown that not only can diversity lead to higher levels of productivity, but diversity can also lead to increased culture stability and reduced invasion, infection and predation rates (Nalley *et al.*; Smith *et al.* 2010; Kazamia, Aldridge & Smith 2012; Nalley, Stockenreiter & Litchman 2014). *Ourococcus* sp. also

showed marked fatty acid production in comparison to other monocultures. We observed a noticeable decline in *C. vulgaris* fatty acid production at day 12 and although we are confident in the finding, we are unsure of its cause.

The fatty acid composition of the biomass is extremely important in the production of biodiesel, deviating from the optimal mixture can lead to a host of issues from inadequate combustion, increased risk of fuel oxidation to undesirable fuel viscosity at extreme temperatures. Ideal fatty acid mixtures have been discussed to be blend of palmitoleic (16:1), oleic (18:1) and myristoleic (14:1) acids at a 5:4:1 ratio (Schenk *et al.* 2008). Others have suggested that although there is no "perfect" species to adhere to this optimal blending ratio, targeting species with high levels of monounsaturated fatty acids (16:1, 18:1), and limited saturated and polyunsaturated fatty acids might be closest to an optimal mixture (Levitan *et al.* 2014). Our results illustrate that chlorophytes cultivated in BWW are composed largely of saturated and polyunsaturated fatty acids, with limited monounsaturated fatty acids. Some of our earlier studies have shown that in polycultures, a more ideal biodiesel fatty acid profile can be achieved through diversifying species-specific fatty acid contributions, but here we find that the polyculture still contains high percentages of palmitic (16:0) and linolenic (18:3) fatty acids.

In addition to the potential for producing a valuable bioproduct from an inexpensive nutrient source, we also wanted to assess the feasibility of using algae as a remediation agent, specifically for brewery wastewater. Our results illustrate that algae can rapidly take up high both nitrogen and phosphorus, thus effectively remediating the brewery effluent. Moreover, we found that the polyculture achieved the highest removal rate of total nitrogen and phosphorus compared to monocultures; in line with previous findings that increasing algal diversity leads to higher resource use efficiency (Ptacnik *et al.* 2008; Stockenreiter *et al.* 2016). While nitrogen

levels were reduced below the EPA MCL standard of 44.3 mg L^{-1} within twelve days in most cultures, none of the cultures decreased phosphorus levels below the 1 mg L^{-1} threshold. Future research should be done to investigate how phosphorus levels can be brought below the 1 mg L^{-1} level, possibly by selecting different species combinations and increasing the duration of the cultivation.

Although all of the species selected for the experiment were able to achieve positive growth, *A. falcatus* and *C. reinhardtii* performed poorly in terms of biomass accumulation, and may not be the best species for further experimentation. As expected based on previous studies, *C. vulgaris* maintained high growth and accumulation throughout the duration of the experiment (Raposo *et al.* 2010b; Farooq *et al.* 2013). We also found that *Ourococcus* sp. achieved similar biomass levels as *C. vulgaris*, which to our knowledge this study is the first to highlight *Ourococcus* sp. as a candidate species for wastewater remediation. It is important to note that although most of the species achieved desirable levels of biomass, growth rates were less than those achieved in regular WC medium for all species surveyed (Nalley *et al.*). For example, with all other conditions standardized, *C. vulgaris* grown in BWW achieved an average growth rate of 0.24 ± 0.02 d^{-1}, while its average growth rate in WC medium was 0.68 ± 0.01 d^{-1}. This reduction in growth may be indicative that BWW is not entirely hospitable for algal cultivation, even after a 20% dilution. Also supporting this claim is the significant increase in cell size that we observed. Although we do not have a direct explanation for the increase in cell size, it may be due to the very high concentrations of available nutrients.

In summary, we found that brewery wastewater can be used as a nutrient-rich medium for successful algae cultivation and fatty acid production, with a simultaneous reduction of nutrient concentration in the brewery wastewater. Moreover, using multispecies algal assemblages

instead of monocultures may increase the effectiveness of fatty acid production and nutrient remediation.

With the US brewing industry continuing to expand, specifically at the craft brewery level, and water becoming more and more scarce, the priority to decrease the water footprint in breweries will become increasingly important. Coupling brewery wastewater remediation with microalgae cultivation seems to be a promising way to simultaneously increase the overall sustainability of brewing while generating a value-added product, turning an expense into a potential revenue stream. Future work must continue to expand on identifying an optimal mixture of algal species tailored to generate an ideal feedstock for the bioproduct of choice, while also reducing nutrient levels to state and/or federal discharge concentrations.

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CHAPTER 5

Optimizing the phycoremediation of brewery wastewater: a two-stage approach

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ABSTRACT

Coupling algal production with brewery wastewater remediation addresses some of the most pressing economic and environmental sustainability concerns of both the brewery and algae industry. Cultivating algae in brewery wastewater alleviates the economic burden of treatment on breweries while providing a nutrient-rich water source that is abundant and inexpensive. In this study, we build on our previous work to optimize algal productivity and remediation potential by employing a two-stage cultivation system. To assess the performance of the system, we monitored algal biomass and total fatty acid production, fatty acid profiles and total nitrogen and phosphorus concentrations in the medium. Algal cultures were inoculated in 100% brewery wastewater and monitored for eighteen days, with sampling every third day. During stage one, we cultivated four chlorophyte species in monoculture and in a collected polyculture for nine days. After fully harvesting all biomass from stage one, a four-species polyculture of cyanobacteria was cultivated for an addition nine days (stage two). We identified that during stage one, the polycultures and *Chlorella vulgaris* achieved higher biomass and fatty acid production compared to our previous work, while *Scenedesmus obliquus* and *Scenedesmus quadricauda* did not perform as well. During stage two, the cyanobacteria polyculture achieved limited biomass and fatty acid production. Within twelve days, most cultures reduced total nitrogen levels below the EPA MCL standard of 10 mg L^{-1}, and achieved >99% total nitrogen removal by day eighteen. Phosphate content was reduced by ~87% within the eighteen days, but was still not under the desired 1 mg L^{-1} discharge standard. We can conclude that brewery wastewater is a good source of inexpensive nutrients for mass algal cultivation, ultimately advancing the sustainability of both industries, but large area for innovation and optimization remain.

INTRODUCTION

As I have discussed throughout this dissertation, microalgae have the potential to be a highly productive, inexpensive biofuel source, with a minimal land and carbon footprint and has the potential to be cultivated in various wastewater sources (Chisti 2007; Wijffels & Barbosa 2010; Smith *et al.* 2010; Pienkos, Laurens & Aden 2011). But its commercial success depends on optimizing the overall system to achieve consistently high productivity while utilizing an inexpensive, and abundant source of water and nutrients (NRC 2012; FAO 2013; Smith *et al.* 2015).

As highlighted in Chapter 4, wastewater utilization from various waste streams has emerged as a promising solution for identifying an inexpensive, nutrient-rich water source. Algae have been used to remediate wastewater for quite some time (phycoremediation), but now there is renewed interest as the effort to generate algal biofuels and bioproducts intensifies (Abeliovich 1986; de la Noue & Proulx 1988; Hashimoto & Furukawa 1989; Tyagi & Vembu 1990; Travieso, Benítez & Dupeyrón 1992; de la Noüe, Laliberté & Proulx 1992; Olguın 2003; Chinnasamy *et al.* 2010; Oswald *et al.* 2010; Park & Craggs 2010; Raposo *et al.* 2010; Park, Craggs & Shilton 2011; Mata *et al.* 2012; Shin *et al.* 2015; Stockenreiter *et al.* 2016). With such high nutrient content, these waste streams can support extremely high densities of algal biomass and are no longer the growth limiting factor. Previous studies have shown that algae can be cultivated in an array of wastewater sources, from industrial, to agricultural to municipal (Travieso, Benítez & Dupeyrón 1992; Tarlan, Dilek & Yetis 2002; Mulbry, Kondrad & Buyer 2008; Chinnasamy *et al.* 2010; Wang *et al.* 2010; Raposo *et al.* 2010; Rawat *et al.* 2011; Mitra, van Leeuwen & Lamsal 2012; Stockenreiter *et al.* 2016; Wuang *et al.* 2016).

As we showed in our previous study (see Chapter 4, Nalley *et al.* 2016b), brewery wastewater might be a nearly ideal source for algal cultivation with its high levels of biologically available nitrogen and phosphorus sources and limited load of toxic substances, antibiotics and pathogenic bacteria (Olajire 2012). The organic content of the wastewater also presents a unique opportunity such that heterotrophic, or mixotrophic, algae could be cultivated in addition to autotrophic organisms.

The brewing industry is quite substantial, generating over $100 billion dollars annually. Mass-producers and imports dominate the market, with craft breweries on a significant rise, making up 22% of the overall market. In 2014, in the US alone, over 6.2 billion gallons of beer were produced ('The New Brewer Magazine' 2016). Yet the rapid increase in product consumption has highlighted some of the main sustainability challenges the brewing industry faces, specifically its water consumption (Olajire 2012). On average, for a single gallon of beer produced, there is between three to ten gallons of wastewater produced depending on system efficiency (European Commision 2006; Kanagachandran & Jayaratne 2006; Brewers Association 2013).

The process of brewing is extremely water intensive, and wastewater is generated at each step in the process, from the mash tun to the bottling floor. The wastewater chemical composition beer and brewery dependent, since the grain bill, hop additions and cleaning agents all carry different chemical compositions. Generally, the nitrogen is coming from the organic materials in the brewing process (i.e. cereal grains, hops and yeast), while the phosphorus content is mainly from the cleaning/sterilizing agents used (Brewers of Europe 2012; Olajire 2012).

For breweries, wastewater remediation can be a large capital expense. Some small breweries simply pay local municipal rates for remediation, while larger breweries have determined that the economics support construction of wastewater facilities on-site (Gribbins 2013). Economic analysis has shown that if a brewery is exceeding $250,000 annually for wastewater treatment, they should construct a wastewater treatment facility of their own (Shah-Gania 2011).

It is clear that breweries face a large sustainability challenge as water becomes more scare and expensive. High rate algal ponds (HRAPs), outdoor, open-pond systems, just might be the answer, with their low infrastructure costs and high nutrient removal potential (Oswald *et al.* 2010; Park & Craggs 2010; Park, Craggs & Shilton 2011). But because these systems are outdoors and open to the environment, there are a number of challenges with maintaining the desired algal species or community within the system as the pond experiences environmental fluctuations (i.e. temperature and light levels) and invasion from unwanted, locally adapted algae, grazers and pathogens. A growing line of research aims to address these concerns by promoting algal diversity within these ponds, ultimately leading to higher productivity, resilience and stability in the face of these environmental perturbations (Smith *et al.* 2010; Cardinale 2011; Stockenreiter *et al.* 2012, 2013; Kazamia, Aldridge & Smith 2012; Shurin *et al.* 2013; Nalley, Stockenreiter & Litchman 2014).

Our initial work on coupling algal cultivation and brewery wastewater remediation illustrated proof of concept that algae can be cultivated on brewery wastewater, achieving high biomass and fatty acid production, but we were unable to reach our target goals for nutrient remediation (Nalley *et al.* 2016b). This study sought to improve upon our previous work to achieve higher nutrient removal rates with the goal of falling below the nutrient regulations for

direct discharge post-treatment. Removal of phosphorus was our main challenge, so we decided to implement a two stage cleaning approach. First, we would cultivate the species we had identified earlier that thrived in the brewery wastewater for an initial quick removal of nutrients, specifically total nitrogen. This would be followed by a second stage, or polishing stage, focusing on removal of phosphorus. This would be achieved through promoting cyanobacteria that either had low N:P requirements or had the ability to fix atmospheric nitrogen (Mitsui *et al.* 1986; Havens *et al.* 2003).

METHODS

Experimental design

For cultivation medium, we collected wastewater from an anaerobic digester post-digestion at a craft brewery in Galesburg, MI on June 24, 2016, identical to the sampling procedure outlined in Nalley *et al.* (2016b). In summary, wastewater from the brewery is first sent to an equalizing tank, with a 3-day retention time, where it is homogenized to ensure consistent chemical profiles. Next, the wastewater is split into three anaerobic digesters and then recombined for a secondary round of digestion. Our experimental wastewater was collected after this secondary digestion. The chemical composition of the effluent post-digestion can be seen in Table 6 of Chapter 4. Immediately following collection, the wastewater was centrifuged for 1000 rpm for 90 seconds to settle larger suspended solids, although suspended solids and microbial activity still remained in the samples. We then used 100% brewery wastewater as our cultivation media.

In an effort to increase the overall remediation potential of our algal systems, we chose to treat the BWW with a two-stage approach, first with chlorophytes followed by cyanobacteria. Highly productive candidate species that were identified in Nalley *et al.* were used for the first stage. Experimental cultures were initially established as monocultures (one of four species), a 4-species polyculture of chlorophytes, or an 8-species polyculture consisting of both chlorophytes and cyanobacteria (Table 8). At the end of day nine, all biomass was filtered out of the cultures and the remaining filtrate was inoculated with a four-species cyanobacteria polyculture. Stock cultures for all experimentally used species were initially acclimated for 14 days in 500 mL WC-medium (Guillard & Lorenzen 1972) at 30°C under 12:12 light:dark cycle at 130 μE m^{-2} s^{-1}. Following the acclimation, triplicate cultures for each stage one experimental combination were established in 200 mL of 100% BWW with a starting biovolume density of 1 $x10^{8}$ μm^{3} mL^{-1} (2 $x10^{10}$ μm^{3} total). Similarly, after filtering out all biomass at day nine, the filtrate from all cultures was inoculated with a community of cyanobacteria at a starting biovolume of 1 $x10^{8}$ μm^{3} mL^{-1}. Biovolumes were used as proxies for biomass, a common approach in algal research, with the assumption that algal cells the same density of water, 1 mm^{3} L^{-1}=1 mg L^{-1}. For all multi-species cultures, each individual species contributed equal additions of biovolumes, totaling to the overall biovolume of 1 $x10^{8}$ μm^{3} mL^{-1}. Cultures were swirled daily, and sampling took place every 72 hours.

Table 7. Species combination for experimental monoculture and polycultures.

Stage One (Day 0-9)	**Stage Two (Day 10-18)**
Monocultures *Chlorella vulgaris*, *Scenedesmus obliquus*, *Scenedesmus quadricauda*, *Ourococcus* sp., *Anabaena flos-aquae*, *Anabaena cylindrica*, *Microcystis* sp., *Synechococcus* sp. **Four Species Mixture** *Chlorella vulgaris*, *Scenedesmus obliquus*, *Scenedesmus quadricauda*, *Ourococcus* sp. *Anabaena flos-aquae*, *Anabaena cylindrica*, *Microcystis* sp., *Synechococcus* sp. **Eight Species Mixture** *Chlorella vulgaris*, *Scenedesmus obliquus*, *Scenedesmus quadricauda*, *Ourococcus* sp., *Anabaena flos-aquae*, *Anabaena cylindrica*, *Microcystis* sp., *Synechococcus* sp.	**Four Species Mixture** *Anabaena flos-aquae*, *Anabaena cylindrica*, *Microcystis* sp., *Synechococcus* sp.

Measurements

All measurements were taken every third day of the experiment with a daily total volume sampled of 20mL, without any media/BWW. Acclimated stock cultures were used for the initial inoculations of the experimental cultures, and inoculation biovolumes were estimated using the CASY Cell Counter and Analyzer System Model TT (Roche Innovatis AG). Subsequent biovolume estimates could not be made via flow cytometry, due to high particulate load in the brewery wastewater, so a 5mL sample of homogenized culture was fixed in Lugol's idodine solution for later visual counting. To determine cell volumes for each species, forty randomly

selected cells were measured using the Image Pro Plus version 4.5.1 system, and then biovolumes were estimated using the approximating geometric shapes (Hillebrand *et al.* 1999). Species density and community composition was determined via performing optical cell counts with a haemocytometer.

The total fatty acid production ($\mu g\ mL^{-1}$) and fatty acid profiles were also collected every third day. After we extracted the total neutral fatty acid from the sample, we performed fatty acid methyl ester (FAME) analysis as established by Wang & Benning (2011) and later adapted to algae by Boyle *et al.* (2012). In summary, 15 mL of algal samples was filtered onto a Whatman GF/B 2.1cm glass microfiber filter, frozen, and later neutral fatty acid extraction was performed. Extracted fatty acids were then transesterified and these constituent fatty acids were then identified and qualified using gas chromatography (Boyle *et al.* 2012). At this density of biomass, we achieved satisfactory signal strength on all samples. Since we were focused on attaining total fatty acid content, we did not perform the thin layer chromatography portion of the protocol.

To quantify the nutrient removal rates of the different experimental combinations, we also analyzed the filtrate from the filtered fatty acid samples to determine total nitrogen and phosphorus left in the BWW medium. The filtrate collected every third day was immediately frozen at -4°C for later analysis. To determine total nitrogen (TN) content, we performed a persulfate digestion at 121°C for 30 minutes converting all inorganic forms of nitrogen to nitrate which could be quantified using 2nd degree spectroscopy on a Shimadzu Spectrophotometer (Crumpton, Isenhart & Mitchell 1992). Total phosphorus (TP) content was quantified using the molybdate method via spectrophotometry. The persulfate digestion, again at 121°C for 30 minutes, converts all forms of phosphates to ortho-phosphates which were then quantified

colorimetrically. TN and TP digestion were performed at a 10x and 100x dilution, respectively. Both methods relied on standardized curves for quantification ($R^2 > 0.97$).

RESULTS

Biomass and community dynamics

During the first stage of treatment, we observed positive growth for all mono- and polycultures throughout the first nine days, except for a sharp decline in *S. quadricauda* biomass between days six and nine (Figure 20). The top performers in terms of biomass were the polycultures (8-species and 4-species), and the *C. vulgaris* culture, also achieving the highest averaged growth rates across the first nine days, while *S. quadricauda* only had positive growth (0.257 ± 0.065 d^{-1}) for the first six days followed by negative growth (-0.233 ± 0.085 d^{-1}) to day nine. Although our previous work had identified *Ourococcus* sp. as a candidate species to be cultivated in BWW, we were unable to cultivate *Ourococcus* sp. in either monocultures or the collective polycultures so no data was collected or reported. Also, no monocultures of the cyanobacteria or the four-species cyanobacteria polyculture successfully established during stage one of the experiment, so no monoculture or four-species cyanobacteria polyculture data is reported. However, *Microcystis* sp. did remain in the eight species polyculture, as discussed later.

After the harvesting event at the end of day nine and the subsequent inoculation of those experimental cultures with a cyanobacterial community, we observed insignificant growth from day ten to eighteen. The averaged growth rates for almost all the cultures were not significantly different from zero. The cyanobacterial community in the *S. quadricauda* filtrate did increase its

biomass from day fifteen to eighteen, resulting in an average growth rate being significantly different from zero.

Table 8. Mono- and polyculture growth rates for both Stage One and Stage Two of the experiment. Note, all Stage 2 growth rates reported are for the 4-species cyanobacteria polyculture. Growth rates (d^{-1}) are reported with ± 1 Standard Error.

Species	Growth Rates (d^{-1})	
	Stage 1 (Days 0-9)	**Stage 2 (Days 10-18)**
Chlorella vulgaris	0.233 ± 0.025	0.146 ± 0.104
Scenedesmus obliquus	0.182 ± 0.058	0.082 ± 0.090
Scenedesmus quadricauda	-0.583 ± 0.085	0.097 ± 0.079
Four-species polyculture	0.199 ± 0.064	0.043 ± 0.043
Eight-species polyculture	0.234 ± 0.020	0.052 ± 0.057

Within the eight-species polyculture, four of the eight species persisted throughout the first nine days: *C. vulgaris*, *S. obliquus*, *S. quadricauda*, and *Microcystis* sp. Initially contributing 12.5% of the overall biovolume, *C. vulgaris* and *S. obliquus* quickly became dominant, contributing 30 and 50% of the overall biovolume by day 3, respectively. By day nine, that percentage had increased further, with *C. vulgaris* composing roughly 35% and *S. obliquus* roughly 60%. By the end, *Microcystis* sp. made up 5% of the overall culture, *S. quadricauda* mirrored its monoculture production maintaining ~12.5% for the first three days and then declining to below 1% by day nine. The four-species polyculture consisting of chlorophytes had different community dynamics. Within the first three days, the composition was similar to the eight-species polyculture with *C. vulgaris* at ~30% and *S. obliquus* at ~60%. But by day six, *C. vulgaris* made up a majority of the community (~75%), and by day nine it dominated the culture

making up ~85% of the overall biovolume. After establishing the cyanobacterial community for Stage Two of the experiment, the community quickly became dominated by *Synechococcus* sp. (~90%) and *Microcystis* sp. (~10%). This composition was observed across all different medium sources (from different cultures of stage One) for Stage Two.

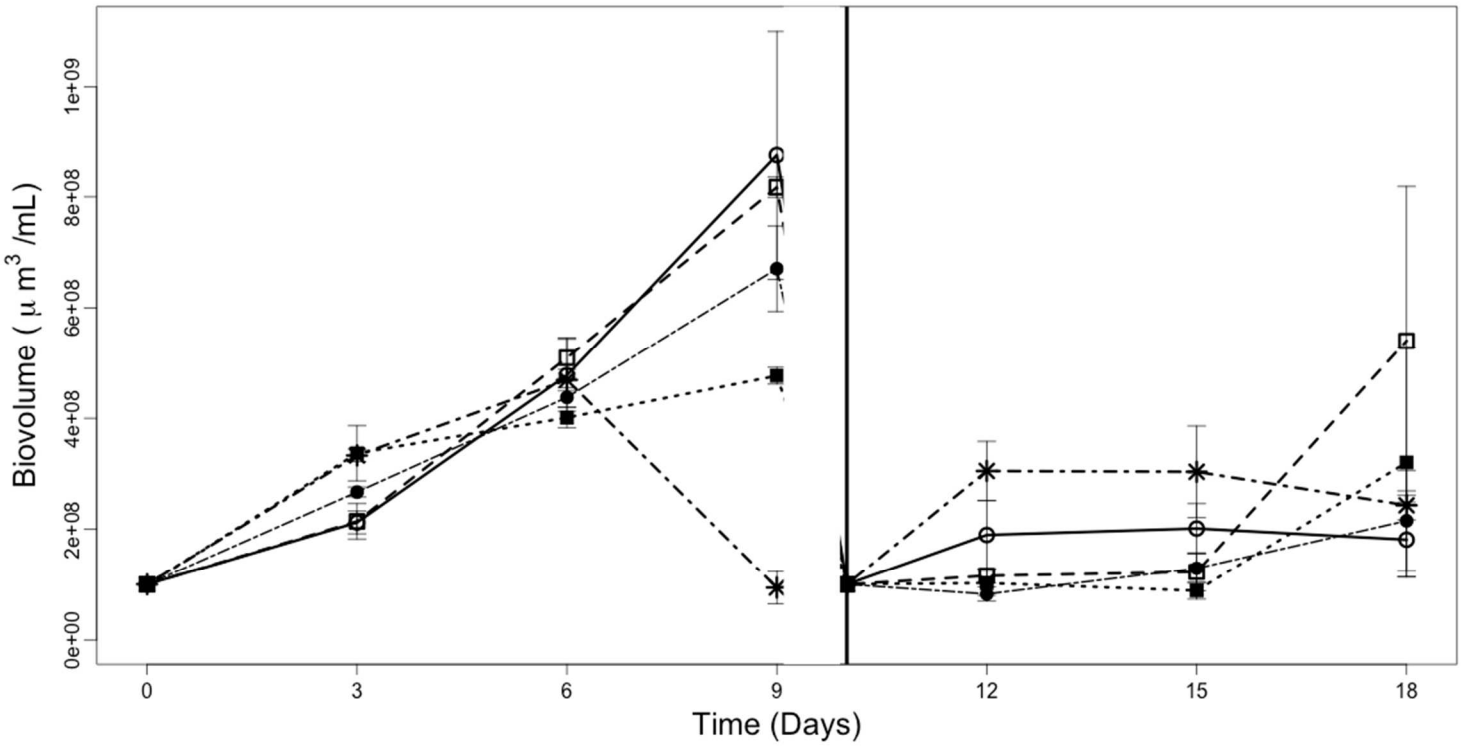

Figure 20. Biomass dynamics through time of *C. vulgaris* (open boxes, dashed line), *S. obliquus* (closed boxes, dotted line), *S. quadricauda* (asterisks, dotted dashed line), four-species chlorophyte polyculture (closed circles, dashed, dotted line), and eight-species polyculture (open circles, solid line). Vertical line at day 10 represents the harvesting event, where all biomass was removed prior to inoculating cultures with four-species polyculture of cyanobacteria.

Fatty acid production and fatty acid profiles

During the first nine days, we observed an increase in total fatty acid production in all cultures except *S. quadricauda* (Figure 21). The polycultures and *C. vulgaris* roughly doubled total fatty acid content every three days, leading to high totals by day nine. Both *S. obliquus* and *S. quadricauda* yielded significantly less total fatty acid content than the polycultures and *C. vulgaris*, and *S. obliquus* significantly out produced *S. quadricauda*. After the harvesting event at the end of day nine, the cyanobacterial polyculture produced significantly lower amounts of fatty acids through time, roughly an 80% reduction in fatty acid production compared to the peak of fatty acid production at day nine.

The fatty acid profiles in different cultures were species- and community-specific (Figure 22). We see that the *C. vulgaris* monocultures and the eight-species polycultures have almost identical fatty acid profiles, while the four-species profile is quite similar although it has a higher percentage of hexadecatrienoic acid (16:3). The fatty acid composition of *S. obliquus* is predominately saturated fatty acids (>70%), from both palmitic (16:0) and stearic acid (18:0). *S. quadricauda* has a lower percentage of overall saturated fatty acids, but has a high percentage (>40%) of palmitic acid and higher percentages of unsaturated fatty acids. The Stage 2 polyculture's fatty acid composition was quite similar across all the different experimental cultures, dominated by palmitic (~40%) and palmitoleic (~40%, 16:1) acids.

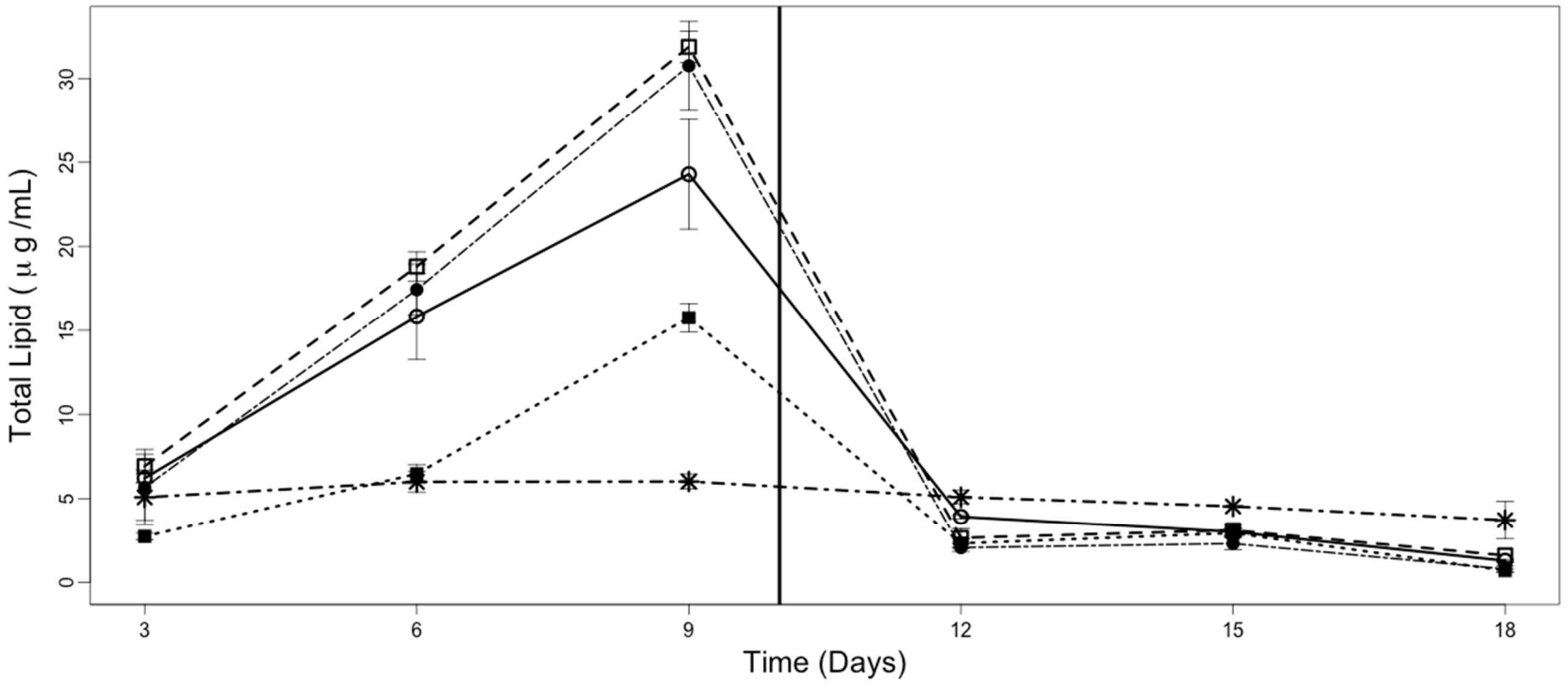

Figure 21. Total neutral fatty acid production (µg mL^{-1}) through time for *C. vulgaris* (open boxes, dashed line), *S. obliquus* (closed boxes, dotted line), *S. quadricauda* (asterisks, dotted dashed line), four-species chlorophyte polyculture (closed circles, dashed, dotted line), and eight-species polyculture (open circles, solid line).

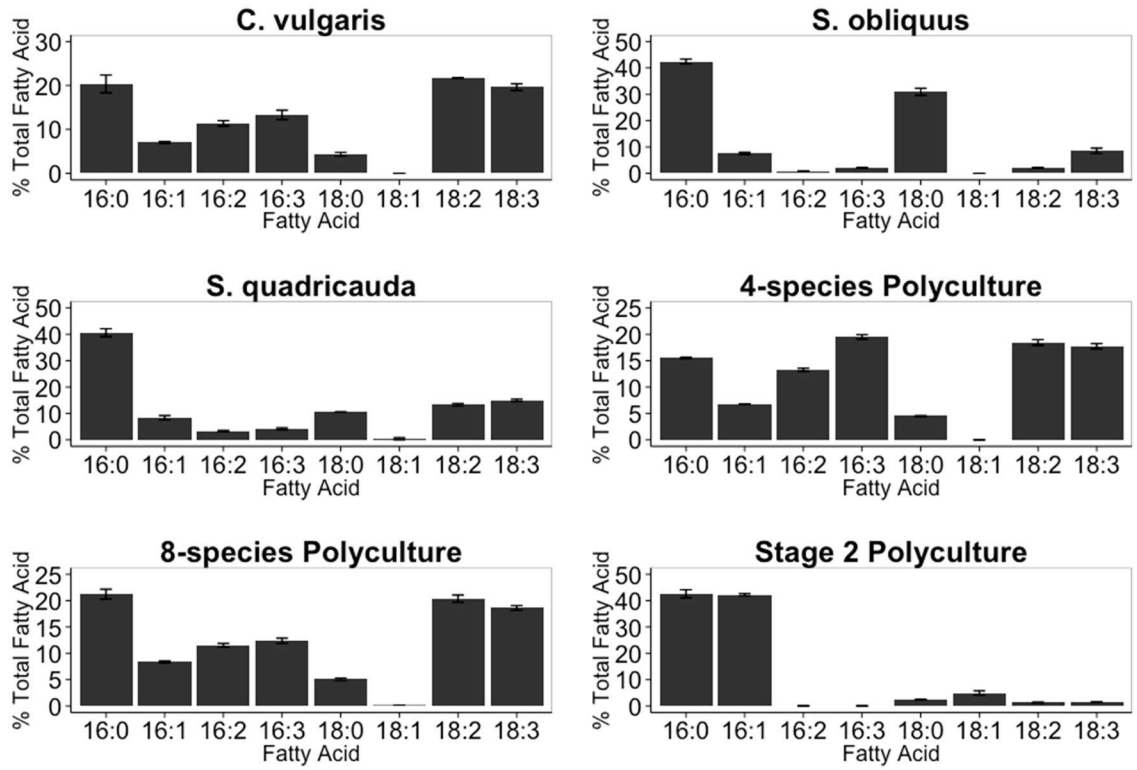

Figure 22. Percent fatty acid compositions for the Stage One monocultures and polycultures, and the Stage Two polyculture. These profiles are truncated to the most relevant fatty acids, but full profiles can be in the Supplemental Materials.

Nutrient remediation

At the time of collection, the brewery wastewater contained a total nitrogen (TN) concentration of 102.3 mg L^{-1}. We observed that within the first three days of treatment, levels of TN had been reduced by 30-35% (Figure 23). By day six, ~65% of the total nitrogen had been removed by all of the experimental cultures. At day nine, TN within the *S. quadricauda* showed a slight uptick, potentially arising from cell death and nutrient release. All other cultures continued removing TN, but at a slower pace by day nine. After inoculating the filtrates of cultures from stage one, the cyanobacterial polyculture continued to remove TN, bringing level

below the 10 mg L^{-1} mark by approximately day 12. Ultimately, by day 18 TN levels had been reduced below our detection levels (<1 mg L^{-1}). –

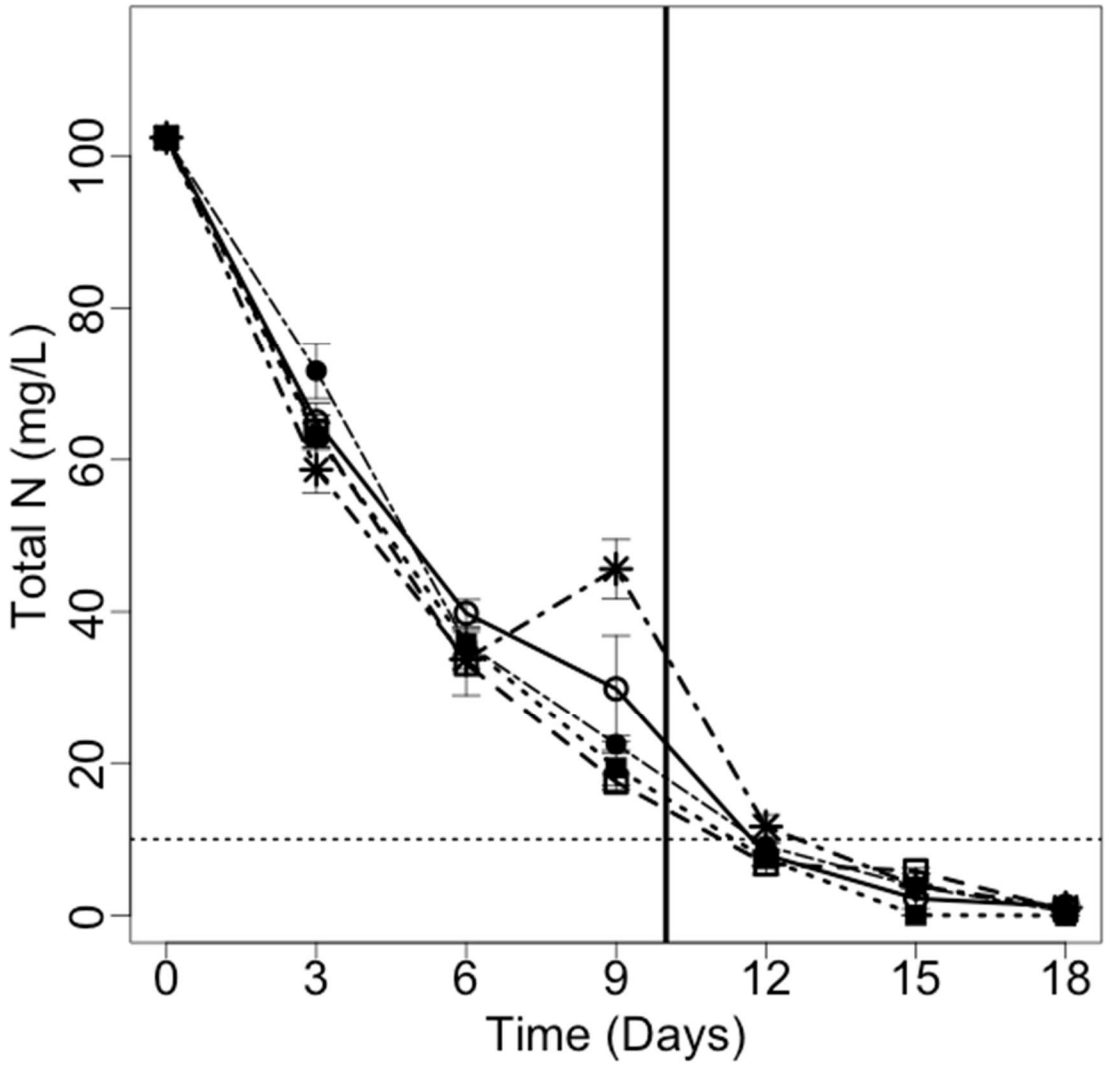

Figure 23. Total nitrogen concentrations through time for *C. vulgaris* (open boxes, dashed line), *S. obliquus* (closed boxes, dotted line), *S. quadricauda* (asterisks, dotted dashed line), four-species chlorophyte polyculture (closed circles, dashed, dotted line), and eight-species polyculture (open circles, solid line). Dotted horizontal line represents the 10 mg L^{-1} EPA MCL standard for clean drinking water.

The total phosphorus (TP) concentration at the start of the experiment was 77.9 mg P L^{-1}. Removal rates were very similar to the TN, with approximately 35% of the TP removed within the first three days (Figure 24). By day six, TP had been reduced by roughly 60% in all the experimental cultures. As with TN, we observed an increase in TP at day nine for *S. quadricauda*, while the other cultures continued to remove TP just at a slower rate. But in contrast to the trends we observed for TN removal, post-harvest (second stage) communities achieved limited, to no phosphate removal. After day ten, phosphate levels remained within the 10 mg L^{-1} range throughout the duration of the experiment. Although there is not a national discharge level for phosphates, the Department of Environmental Quality in Michigan stipulates phosphate to be at or below 1 mg L^{-1}. None of our experimental cultures remediated phosphate concentrations below this threshold.

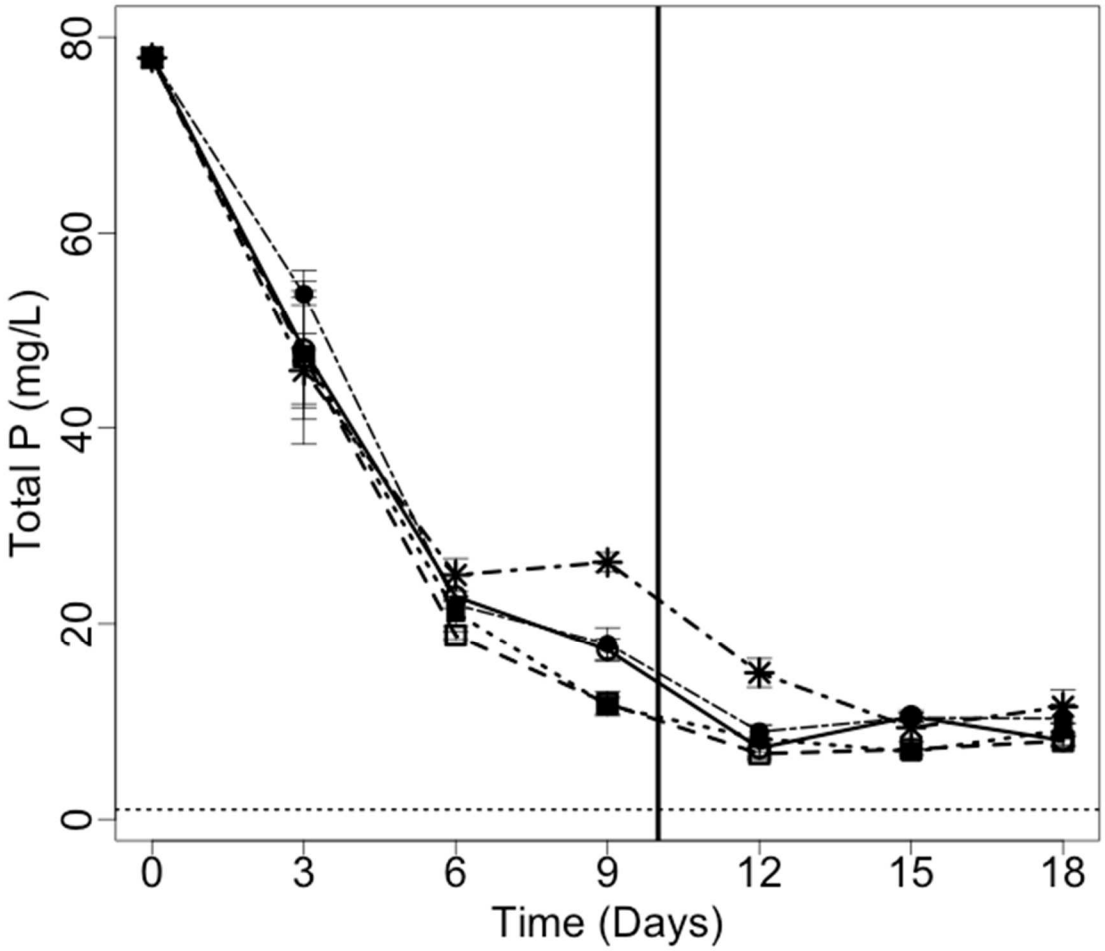

Figure 24. Total phosphate concentrations through time for *C. vulgaris* (open boxes, dashed line), *S. obliquus* (closed boxes, dotted line), *S. quadricauda* (asterisks, dotted dashed line), four-species chlorophyte polyculture (closed circles, dashed, dotted line), and eight-species polyculture (open circles, solid line). Dotted horizontal line represents the 1 mg L^{-1} MI DEQ standard for legal dischargeable levels.

DISCUSSION

We have previously shown that cultivating algae on brewery wastewater was possible, but highlighted some limitations to the study (Nalley *et al.* 2016b). Specifically, the inability to remediate nutrient levels below regulation limits was a main obstacle to concluding that the experiment was successful. Building from that study, we were able to cultivate a select number of microalgae in 100% unfiltered (compared to the 80% in the previous study), anaerobically digested BWW and achieve higher nutrient removal. This study advances the conclusions that utilizing BWW for mass algal cultivation could be an extremely promising, inexpensive nutrient source. The use of anaerobically digested wastewater makes the potential application of this technology relevant only to breweries that are large enough to have an anaerobic digester on-site, treating only wastewater generated at the brewery. Also, as in the previous study, we were only able to cultivate a small number of microalgae in the BWW, suggesting that using BWW as a cultivation medium may limit the bioproduct potential of the algal system. But, BWW dilution could increase cultivation potential.

For the selected species, we observed strong growth throughout the first stage of the experiment, except for the day six to nine growth of *S. quadricauda*. The starting biomass was 25% higher than in our previous study. This increase in biomass ultimately led to the overall higher biomass achievement by day nine, contributing to higher total fatty acid production and increased TN and TP removal. Generally, we saw that biomass roughly doubled every third day for the polycultures and *C. vulgaris*, where as *S. obliquus* and *S. quadricauda* exhibited lower total biomass values, and lower growth rates through time. We also determined that the growth rates for the algal mono- and polycultures were quite low, similar to the rates we observed in our

previous work and significantly lower than growth rates we identified under ideal laboratory conditions (Nalley *et al.* 2016a; b).

After the harvest event and the inoculation of the stage two community of cyanobacteria, we observed very limited growth in all of the cultures. This limited growth may be linked to the inability of cyanobacteria to be cultivated in 100% BWW, as illustrated in the first stage of the experiment. But, it is important to note that cyanobacteria were able to survive, just not thrive, in the BWW post-stage one remediation, suggesting that the presence of the chlorophytes modified the environment in such a way that made it more suitable for the growth of cyanobacteria, most likely from nutrient and turbidity reduction and/or uptake of other chemicals or organic material that we did not monitor.

Total fatty acid production mirrored biomass trends, suggesting that fatty acid production is driven by biomass accumulation rather than internally storing more fatty acid through time, similar to conclusions we have made earlier (Nalley *et al. 2016a*). During stage one, the polycultures and the *C. vulgaris* cultures achieved higher total fatty acid production in a shorter amount of time compared to our previous work too (Nalley *et al.* 2016b). Cyanobacteria generally do not have high fatty acid yields, and this held true in stage two. This is also a result of a very low growth rate during this time as well.

For biofuel or bioproduct production, the fatty acid profile is extremely important. For biofuels, the optimal mixture of fatty acids for biodiesel feedstock has been identified to be a mixture of palmitoleic (16:1), oleic (18:1) and myristoleic (14:1) acids at a 5:4:1 ratio, while others simply suggest to target species with high monounsaturated fatty acid with limited saturated and polyunsaturated fatty acids (Schenk *et al.* 2008; Levitan *et al.* 2014). Our results show that monounsaturated fatty acids are limited within our experimental algal culture. The

polycultures and the *C. vulgaris* cultures were composed of high levels of polyunsaturated fatty acids (16:2, 16:3, 18:2 and 18:3), with roughly 20% of the overall composition being palmitic acid. This trend clearly illustrates that the main fatty acid contributor within the polycultures is *C. vulgaris*. The *Scenedesmus* species are distinctly rich in saturated fatty acids, both palmitic and stearic acids (~70%). The cyanobacteria polyculture in stage two had very similar fatty acid profiles throughout all cultures and time. The cyanobacteria FA profile is dominated by palmitic and palmitoleic acids, comprising over 80% of the overall FA content. We conclude that none of the species or their combinations generate an ideal biodiesel mixture, but the biomass could be suitable as animal feed or fertilizers, either terrestrial or aquaculture (Benemann 1992; Posten 2012; Yaakob *et al.* 2014).

Previous studies have discussed the benefit of promoting algal diversity within algal biofuel systems in order to achieve higher than expected yields, both biomass and fatty acid content (Smith *et al.* 2010; Stockenreiter *et al.* 2012, 2013, 2016; Kazamia, Aldridge & Smith 2012; Shurin *et al.* 2013; Nalley, Stockenreiter & Litchman 2014; Nalley *et al.* 2016c). In this study, the polycultures did achieve higher biomass and fatty acid content compared to the averaged monoculture yields (overyielding), but we did not see any significant differences between the polycultures and the best performing monoculture, *C. vulgaris*. As we have elucidated previously, there are a number of other benefits that can arise from promoting species diversity, such as community stability, decreased invasibility, and decreased susceptibility to pathogens and/or predators (Nalley, Stockenreiter & Litchman 2014; Nalley *et al.* 2016c). But these parameters were not investigated in this study.

Another benefit of diversity is an increased nutrient removal rate (Ptacnik *et al.* 2008). In our study, we did not identify a significant difference between removal rates in monocultures and

polycultures. This may be due to a lack of functional diversity within the system, as our polycultures consisted only of green algae or cyanobacteria, or their mixtures. We did find that the candidate species achieved a high rate of removal for both TN and TP. Nitrogen levels were reduced below the EPA MCL level of 10 mg L^{-1} by day twelve. This rate of removal is substantially higher than the rates we observed in our previous experiment. However, we did not achieve as much removal of TP as we had desired. No cultures came below the 1 mg L^{-1} mark, within eighteen days TP remained at roughly 10 mg L^{-1}. This level of TP is still extremely high, and discharge would not be allowed. At the time of harvest, both TN and TP were still at high concentrations, suggesting that had the chlorophytes remained, biomass would have continued to rise while nutrient levels would have dropped.

The use of a two-stage cleaning system with these specific communities of algae does not appear to be the most efficient way of remediation and biomass generation. Identifying algae that have low N:P ratios would be extremely helpful so that a community could be assembled having high TN and TP removal. Importantly, these species should be able to achieve high growth in BWW. If the cultures were maintained at high densities, supplemented with CO_2 and light sources were optimized for algal photon capture (i.e. blue and red LEDs), we believe the timeline for remediation could be drastically reduced.

Ultimately, the coupling of algae cultivation and brewery wastewater remediation is a promising concept that merits further development. With the brewery industry continuing to expand, and increasing the self-awareness of their sustainability shortcomings, this technology just might be poised to answer a number of the brewery industry's concerns, while generating a valuable biofuel and/or bioproduct.

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