PRODUCTIVITY AND RESOURCE USE EFFICIENCY IN A MICROALGAL POLYCULTURE UNDER DIFFERENT LEVELS OF CO_2 AND PHOSPHORUS SUPPLY

By

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PRODUCTIVITY AND RESOURCE USE

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PHOSPHORUS SUPPLY

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Abstract: Although decades of research in the field of ecology have shown that productivity is a product of both species richness and resource supply, few studies have investigated the potential application of these principles to the production of biofuels. Recent studies provide evidence that species richness of microalgal communities is positively related to biomass production and resource use efficiency (RUE), but it is unclear how this relationship is affected by resource supply. Species differ in their ability to acquire resources and in how efficiently they are able to convert them into biomass. Therefore, changes in resource supply may alter the composition of algal communities, thus affecting productivity. I conducted an experiment in which I grew polycultures consisting of two algal species from different major taxa, Chlorophyceae and Cyanophyceae, at different levels of CO_2 and phosphorus (P) supply in order to investigate how resource supply affected species abundance. I compared the growth and elemental composition of mixed cultures to that of species grown in monoculture to evaluate the effects of increased species richness on productivity and RUE. The results show that species relative abundance was dependent on resource supply. There was no evidence for effects of increased richness on RUE, but the productivity of mixed cultures was greater than that of either monoculture when P supply was high. The increased productivity of mixed species cultures under high P supply appeared to be due to the weakening of negative competition effects by increased resource supply. These results suggest that growing multi-species cultures may be an effective way to enhance biomass yields and improve biofuel production.

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CHAPTER I

INTRODUCTION

As a consequence of emissions associated with increased fossil fuel usage, atmospheric CO_2 levels are expected to double by the end of the century–rising from current levels of approximately 390 parts per million (ppm) to between 700 and 1000 ppm (IPCC 2007). Concerns about the impact of increasing CO_2 on global temperatures and climate cycles have led to an increase in efforts to find alternative energy sources which could reduce anthropogenic CO_2 contributions. The production of fuels from renewable and sustainable sources, such as plant biomass, has the potential to reduce reliance on petroleum-based fuels and decrease net CO_2 emissions.

Currently, biofuels are being produced from food crops, creating competition with agriculture and potentially increasing food costs. Microalgae represent a promising alternative to the use of food crops as a feedstock for biofuel production, in part, because their potential photosynthetic efficiency is several times that of terrestrial crop plants, resulting in greater productivity per area of land (Chisti 2007, Rodolfi 2009). Additionally, many species are able to store assimilated carbon in the form of energy-dense neutral lipids or carbohydrates (U.S. DOE 2010), which are desirable feedstock for the synthesis of biofuels. One of the principle challenges to the production of algal-based

biofuels is establishing methods of cultivation that are both efficient and cost-effective on a large scale. At present, the two primary methods under consideration for large-scale cultivation are photobioreactors (PBRs) and open ponds. PBRs have the advantage of being closed systems, thereby allowing greater control over culture conditions. While PBRs can be highly productive, they are expensive to construct and maintain, making it unlikely that they will be practical on industrial scales (Mata et al. 2010, Smith et al. 2010). Open ponds are cheaper to construct but are susceptible to invasion by undesirable algal species, bacteria, fungi, and herbivorous grazers.

Regardless of cultivation method, it is desirable for cultures to be as productive as possible. To this end, a great deal of research has been targeted at identifying ideal algal species for biofuel production (e.g., Griffiths and Harrison 2009, Roldolfi et al. 2009, Tang et al. 2011). However, ecological theory and experimental data suggest that a positive relationship exists between species richness and biomass production (Tilman et al. 2001, Downing and Leibold 2002, Behl et al. 2011), implying that monocultures may not necessarily be more productive than cultures consisting of multiple algal species. Furthermore, there is evidence that resource use efficiency (RUE; the amount of carbon assimilated per unit of limiting nutrient) may increase with the number of algal species present in communities (Dickman et al. 2006, Ptacnik et al. 2008, Striebel et al. 2009).

The positive effects of increased diversity on ecosystem productivity are generally attributed to two nonexclusive mechanisms. The first, known as the "selection effect", occurs when a highly productive species dominates the productivity of the community. In more diverse communities, there is an increased probability that such a species will be present (Huston 1997, Tilman et al. 1997). The second mechanism is known as the

"complementarity effect" and may be observed when increased productivity is brought about through interactions which reduce the strength of interspecific competition relative to intraspecific competition (Hooper et al. 2005). In the context of biofuel production, complementarity effects are desirable because they may facilitate overyielding, in which the productivity of multi-species cultures exceeds that of monocultures (Vandermeer 1989). Overyielding may occur if facilitative interactions or resource partitioning allow species to make more complete use of available resources (Loreau and Hector 2001). Complementarity effects are expected to be strongest between species that differ greatly in their use of resources (Chesson et al. 2002). To a greater degree than terrestrial plants, algae are a large and diverse group of organisms, including both eukaryotes and prokaryotes. Algae from different major taxa may exhibit considerable differences in their resource requirements which may strengthen complementary effects and promote increased productivity even in cultures with relatively few species.

Theory predicts that the productivity of primary producers is a function of both species richness and resource availability (Schmid 2002). Moreover, the relationship between diversity and resource supply itself may be a dynamic one. Gross and Cardinale's (2007) model proposes that, while resource supply limits the biomass production and number of species that can coexist in a system, species richness directly impacts biomass production. Resource supply rates may also affect species interactions and may either enhance or nullify complementarity effects. Both high and low rates of resource supply can result in the dominance of superior competitors (Grover and Chrzanowski 2004, Harpole and Tilman 2007, Cardinal et al. 2009), which may not necessarily be the most productive species. Species differ in their ability to acquire

nutrients and the efficiency with which they convert them into biomass, suggesting that resource supply ratios could be an important control mechanism to optimize biomass production in algal cultures.

In addition to enhanced productivity, increased species richness may have other potential advantages for biofuel production including greater stability of algal cultures (Lehman and Tilman 2000), and reduced susceptibility to invasive species (Hooper et al. 2005). Incorporating multiple species into the cultivation of microalgae may be a cost-effective way to improve biofuel production.

CHAPTER II

PRODUCTIVITY AND RESOURCE USE EFFICIENCY IN A MICROALGAL POLYCULTURE UNDER DIFFERENT LEVELS OF CO_2 AND PHOSPHORUS SUPPLY

Introduction

Concerns regarding the effect of CO_2 on climate change have led to increased efforts to identify alternative energy sources. Replacing petroleum-based fuels with renewable biofuels has the potential to substantially reduce net CO_2 emissions. Microalgae are a promising feedstock for biofuels because their potential biomass productivity, growth rate, and photosynthetic efficiency is higher than that of any terrestrial crop currently cultivated for biofuel production (Chisti 2007, Rodolfi et al. 2009). Algae can also be grown on non-arable land using wastewater, minimizing competition with agriculture for fertile land and freshwater (U.S. DOE 2010). Despite these advantages, methods to efficiently cultivate algae for biofuel production are still in the early stages of development. A substantial amount of research has focused on the selection (or design) of species possessing desirable qualities (e.g., lipid content), with the expectation that monocultures of the selected algae could be cultivated under optimal conditions. However, there is increasing evidence which suggests that cultivating mixed assemblages containing multiple microalgal species may offer a number of advantages over monocultures for the production of biofuel feedstock.

Recent studies have shown that species richness is positively associated with increased algal biomass production (Ptacnik et al. 2008, Power and Cardinale 2009, Behl et al. 2011), lipid content (Stockenreiter et al. 2012, 2013), and cellular carbon quotas (Dickman et al. 2006). While the diversity-productivity relationship is generally positive, it is asymptotic, with the greatest gains in productivity resulting from relatively small increases in diversity (Turnbull et al. 2013). This suggests that even cultivating mixtures containing relatively few species may increase total biomass production. The positive effects of diversity on productivity have been attributed to niche differences, which allow species to use available resources more completely (Gross et al. 2007, Cardinale et al. 2011). Niche theory predicts that the number of species that may coexist in a resource-dependent community is determined by the number of limiting resources for which they compete (Tilman 1982). To achieve the high growth rates and biomass densities desirable for biofuel production, it will be necessary to supplement cultures with resources required for growth. High resource supply rates reduce the number of limiting resources which may result in dominance of the system by a small number of species best adapted to the reduced range of supply ratios (Grover and Chrzanowski 2004, Harpole and Tilman 2007). Differences in species' ability to acquire resources, and in their resource use efficiency (RUE; the amount of carbon assimilated per unit of limiting resource), may cause the presence of a superior competitor to outcompete more productive species.

The positive diversity-productivity relationship suggests that it may be possible to maximize microalgal biomass production by cultivating mixtures containing multiple species, but it is unclear if mixed cultures could be maintained or if they would

necessarily be more productive than monocultures. While methods of algal cultivation constitute artificial ecosystems, they are governed by the same ecological principles as natural systems, including competition for resources (Smith et al. 2010). The resource-ratio theory predicts that when the growth of multiple species is limited by two or more resources, the outcome of competition depends upon the ratio of resource availability (Tilman 1982). By manipulating the supply of resources to cultures, it may be possible to exploit differences in species' competitive ability to favor more productive species or to increase total productivity and RUE in mixed cultures.

Inorganic carbon (C_i) is a resource required for algal growth and can be limiting when photosynthetic (PS) activity is high (Spijkerman et al. 2011), therefore supplementing dense cultures with CO_2 may increase productivity. Although most microalgae possess carbon concentrating mechanisms (CCMs), which allow them to maintain CO_2 at PS-saturating levels by actively transporting CO_2 or HCO_3 into the cell, major algal taxa differ in their affinity for CO_2 and in CCM strategies (Beardall and Raven 2004, Riebesell 2004). Recent studies suggest that physiological differences in CO_2 utilization may cause shifts in the competitive balance between algal species grown under increased CO_2 (Low-Décarie et al. 2011, Verschoor et al. 2013). In addition to inherent species-specific differences, species' ability to acquire CO_2 may be affected by other factors, including P availability (Wu et al. 2012). The fact that algal CCMs require active transport means their operation is dependent upon ATP supply (Raven et al. 2011). This creates the possibility that, when P supply is low, algae may be simultaneously P- and C-limited (Beardall and Giordano 2002). As algal species differ in their ability to

acquire both CO_2 and P, the availability of these resources could potentially be manipulated to optimize biomass production.

Resource supply may affect RUE as well as productivity. Microalgae demonstrate a remarkable degree of flexibility in their stoichiometric composition in response to various abiotic factors, including nutrient supply (Hillebrand and Kahlert 2001). When growth is nutrient-limited, but C_i and light are in ample supply, rates of C fixation may exceed rates of nutrient uptake, resulting in an excess of fixed C and increased biomass C:nutrient ratios (Sterner et al. 1997). Thus, complementary resource use by species in mixed cultures may more fully consume available nutrients, creating nutrient-limited conditions and promoting increased RUE. As C is the principle component of energy storage molecules, an increase in per cell C quotas suggests that excess C is converted into energy storage molecules such as lipids or starch, desirable feedstocks for biofuel production.

The goals of this study were to determine: (i) the effects of species richness and resource supply ratios on biomass yields and RUE, and (ii) how CO_2 and P availability affect productivity and species abundance in mixed cultures of microalgae. I conducted an experiment in which I grew a cyanobacterium and a green alga at different levels of CO_2 and P. I chose to use algae from these two taxa because they are known to differ in their CCMs (Beardall and Raven 2004). Cyanobacteria have highly efficient CCMs and also possess a high affinity for P, making them superior competitors when C_i or P are limited (Caraco and Miller 1998, Posselt et al. 2009). However, many green algae are able to down-regulate CCM activity when CO_2 concentrations are high, while some CCMs in cyanobacteria are constitutive (Shapiro 1997). Thus, elevated CO_2 may benefit

green algae to a greater degree than cyanobacteria. I grew both organisms in monoculture and in mixed cultures to test the following hypotheses: (i) mixed cultures will have higher biomass production and RUE than monocultures under all nutrient regimes, (ii) low P treatments will favor cyanobacteria in competition with green algae, and (iii) increased CO_2 concentrations will increase the growth of green algae relative to cyanobacteria.

Materials and Methods

Species and culture conditions

The species used in this study were isolated from a pond in northeast Oklahoma and identified as *Synechocystis* sp. (Cyanophyta) and *Chlorella* sp. (Chlorophyta) based on morphological characteristics. Clonal cultures of both species were maintained in unbuffered AS-100 medium (Starr and Zeikus 1993), modified to reduce salinity (0 M NaCl, 0.1 mM $MgSO_4$, 0.08 mM KCl, 0.023 mM $CaCl_2$), for approximately six months under normal laboratory conditions. Stock cultures used as inocula for the experimental cultures were maintained under experimental CO_2 concentrations for two weeks prior to the experiment to allow species to acclimate.

To manipulate CO_2 levels, I conducted the experiment using two custom built incubation chambers constructed of clear acrylic. The chambers were illuminated by a 400 W metal halide lamp on a 14:10 light:dark cycle positioned directly above the chambers, providing an irradiance of 90 μmol m^{-2} s^{-1}. CO_2 was controlled by supplying chambers with either air or an air/CO_2 mix, resulting in CO_2 concentrations of 0.5% (+CO_2 treatment) and 0.039% (ambient CO_2; -CO_2 treatment). The chambers sat on shaker tables and cultures were continuously agitated to facilitate gas diffusion. To avoid overly acidifying cultures, supplemental CO_2 was only supplied during the photoperiod.

Temperature in the chambers varied between 20-22° C during the 16 day experimental period.

To initiate the experiment, algae from stock cultures in log phase growth were inoculated into 125 mL flasks containing 80 mL of AS-100 media modified to total P concentrations of either 367 μM (+P treatment) or 2.5 μM (-P treatment). All other nutrients were supplied well in excess of algal requirements. The N:P supply ratios in the two treatments were 33.2:1 and 211.8:1, indicating P limitation in low P cultures. Flasks were covered with Whatman No. 1 filter paper to allow gas diffusion and placed in incubation chambers. The factorial design of the study resulted in four combinations of CO_2 and P: high CO_2 and high P (+CO_2+P), low CO_2 and high P (-CO_2+P), high CO_2 and low P (+CO_2-P), and low CO_2 and low P (-CO_2-P). Both monocultures and mixed cultures were replicated five times for a total of 60 flasks. The target inoculum biovolume for monocultures and mixtures in all treatments was 0.01 μL mL^{-1}, with each species in mixed cultures contributing 0.005 μL mL^{-1}. Cultures were grown in batch mode for five days. Beginning on the fifth day, 40% of the culture volume was replaced with fresh medium daily to allow the effects of competition to be observed in actively growing cultures (0.4 d^{-1}). After five days of semicontinuous cultivation, dilutions were stopped and algae were allowed to grow in batch mode until biomass approached a maximum.

Growth measurements

Preliminary work showed a strong linear relationship between cell number and optical absorbance at 750 nm (R^2 = 0.99 and 0.97 for *Synechocystis* and *Chlorella*, respectively). Therefore, I monitored algal growth in monocultures by quantifying optical absorbance at 750 nm using a UV160U spectrophotometer (Shimadzu Scientific

Instruments, Columbia, MD, USA). In mixed cultures, species composition and biovolume was estimated microscopically from samples fixed using formaldehyde. Samples were photographed on a hemocytometer and cells were manually counted using ImageJ software (Abramoff et al. 2004). All growth measurements were made at photoperiod hour seven each day.

Due to differences in species' size, cell number is not necessarily an accurate estimate of which species dominates biomass production. In this study, I used species' biovolume as the measure of productivity. Biovolume was estimated by assuming species' shapes approximated a sphere (reasonable for these species) and was calculated from the average diameter of 30 cells from each species photographed and measured using ImageJ. Calculated mean biovolumes were 8.97 fL cell^{-1} for *Synechocystis* and 46.2 fL cell^{-1} for *Chlorella*. Total productivity for both monocultures and mixtures is expressed as the product of species cell number and biovolume.

I compared the productivity of species in mixed cultures to that of monocultures to identify effects of diversity. I quantified these effects by calculating the proportional deviation, D_i, of the total biovolume yield of species i in mixed cultures from their expected yield as:

$$D_i = \frac{O_i - M_i}{M_i}$$

where O_i is the total biovolume produced in the mixed culture and M_i is the expected biovolume that would be produced based on a species' biovolume yield in monoculture (Loreau 1998). In mixed cultures of two species, the M_i value for species i is one-half of the total biovolume produced by that species in monoculture. Positive D_i values indicate that a species' productivity in mixed culture is higher than would be expected based on

its yield in monoculture, and can be interpreted as evidence of complementary resource use or facilitation enhancing productivity. A negative D_i value indicate species' biovolume production is less than expected, and is evidence that productivity is adversely affected by competition.

Chl-a assays and elemental analysis

The high N content of chlorophyll and associated proteins may affect RUE by altering C:N ratios. Therefore, samples from three replicate cultures from each treatment were analyzed for changes in chlorophyll content three times during the experiment. Cultures were assayed twice during log phase growth and at the end of the experimental period. A known volume of culture was collected on GF/F filters and analyzed for chl-*a* content using spectrophotometric methods following extraction in dimethylformamide (see Porra et al. 1989 for equations).

On the final day of the experiment, algal biomass was collected from two replicate cultures from each treatment to assess changes in biomass stoichiometry. Biomass was harvested by centrifugation and rinsed with nutrient-free AS-100 medium. Cell pellets were freeze-dried and maintained at -20° C until analysis. A fraction of the pellet was weighed to the nearest 1.0 μg, wrapped in aluminum foil, and combusted in an Elementar Vario Micro Cube (Elementar GmbH, Hanau, Germany) to determine C and N content.

Data analyses

Final algal biovolume production, chlorophyll-*a* content, and stoichiometric data were analyzed for differences and treatment effects using three-way ANOVA with species composition (*Chlorella* monocultures, *Synechocystis* monocultures, or mixed

cultures), CO_2, and P as fixed factors. When significant main effects were identified, Tukey's HSD test was used to identify differences between groups. Student's t -tests were used to determine differences in biovolume production between species in mixed cultures and to test D_i values for significant differences from the expected value of zero. All analyses were performed using the SPSS statistical package (IBM Corp, New York, USA).

Results

Effects of CO_2 and P on growth and species composition

Increased CO_2 enhanced the productivity of both monocultures and mixtures, but only under P-replete conditions. There was no difference in total biovolume produced in monocultures of *Synechocystis* or *Chlorella* when grown under low P supply, regardless of CO_2 concentration (Fig. 1). Likewise, analysis showed no significant interaction effect between species and CO_2 level, but did indicate a significant three-way interaction between species composition, CO_2, and P concentration (Table 1). Average biovolume was higher in monocultures when supplemented with P and was further enhanced by elevated CO_2, increasing by 22% in *Synechocystis* and 60% in *Chlorella* grown at high CO_2. Differences in total productivity between species in monoculture were only observed in the $+CO_2$+P treatment, in which biovolume of *Chlorella* exceeded that of *Synechocystis* by approximately 33%.

In terms of total biovolume, mixed cultures followed the same trend as monocultures. Biovolume accumulation in low P cultures was unaffected by CO_2 supply, but increased with the addition of P and reached highest concentrations in the $+CO_2$+P treatment (Fig. 1). However, mixed cultures were significantly less productive than

monocultures at low P levels. The addition of P produced a roughly fourfold increase in the final biovolume of mixed cultures, resulting in significantly greater productivity than achieved in either monoculture. Mixed cultures in the $+CO_2$+P treatment demonstrated the highest productivity of any experimental culture, exceeding the highest monoculture biovolume concentration by an average of nearly 25%.

Species composition in mixed cultures was affected by CO_2 and P supply. D_i values indicate that *Synechocystis* performed worse than expected based on monoculture yields in mixed cultures under all conditions, but was most affected by the presence of *Chlorella* in the $+CO_2$-P treatment, in which it only reached 33% of expected biovolume (Table 2, Fig. 2C). *Chlorella* exceeded expected yields in P-replete conditions, comprising 78% and 75% of total biovolume in $+CO_2$+P and $-CO_2$+P cultures, respectively (Figs 2A and 2B). The presence of *Synechocystis* appeared to have no negative impact on the growth of *Chlorella* and its productivity was even greater than that attained in monoculture ($D_i > 1$). The higher total biovolume in $+CO_2$+P cultures was due solely to increased productivity of *Chlorella* in high CO_2 cultures as *Synechocystis* biovolume was not affected by CO_2 at high P. CO_2 had no effect on total productivity in either monoculture or mixtures at low P but strongly influenced species composition (Fig. 2C and 2D). At ambient CO_2 and low P, *Synechocystis* dominated cultures, accounting for approximately 75% of total biovolume. At elevated CO_2 and low P, 68% of biomass consisted of *Chlorella*.

Chl-a, carbon, and nitrogen content

Cellular chlorophyll-*a* quotas in monocultures were largely unchanged while cultures were grown semicontinuously, but generally increased in high P cultures as cell

densities increased after dilutions were stopped (Fig. 3A and 3B). In low P cultures, final chl-*a* quotas decreased or remained unchanged in *Synechocystis* while increasing in *Chlorella* under all treatments. In contrast to monocultures, chl-*a* increased in mixtures during semicontinuous culture in both low and high P cultures grown at elevated CO_2 (Fig 3C). Final chl-*a* quotas in mixed cultures were similar to those in monocultures, with the lowest values observed in low P cultures dominated by *Synechocystis*.

CO_2 addition did not affect algal C or N content in monocultures or mixtures in high P treatments and biomass C:N ratios were close to the Redfield ratio (Figs 4A-4C), consistent with N-replete conditions. *Synechocystis* monocultures exhibited a marginal increase in C:N ratios in low P treatments, due to reduced N content, but C content appeared unaffected by CO_2 or P concentration. C:N ratios were significantly higher in mixed cultures in low P treatments, relative to high P treatments (Tukey's HSD, $p < 0.05$). In *Chlorella*, C:N ratios at low P were dependent upon CO_2 supply, showing no change in the +CO_2-P treatment but increased to 16.7 ± 0.31 under ambient CO_2. Mixed culture C:N ratios with low P suppy ranged from 11.4-13.7 and were not affected by CO_2 concentrations in low P treatments.

Discussion

Growth and species composition

There is substantial evidence that species richness is positively associated with biomass production in primary producers (Tilman et al. 2001, Hooper et al. 2005, Cardinale et al. 2007). Increased productivity is often attributed to species interactions, such as complementary resource use, which weaken interspecific competition. It should be noted that increased productivity, in itself, is not sufficient evidence for positive

effects of species interactions. This is because total yields are an aggregate measure of many possible species interactions occurring simultaneously (Loreau 1998). Positive effects, including complementary resource use and facilitation, are two possible interactions; negative effects, such as interference or exploitation competition, may also affect yields. Based on prior knowledge of differences between green algae and cyanobacteria in CO_2 uptake mechanisms and P affinity, I hypothesized that niche differences would facilitate complementary resource use, resulting in greater biovolume yields in mixed cultures than monocultures. Furthermore, I predicted that *Synechocystis* would be dominant when P supply was low, and that *Chlorella* would be a superior competitor at elevated CO_2.

I found that the productivity and species composition of mixed cultures was dependent upon resource supply. Under low P conditions, monoculture productivity exceeded that of mixed cultures, indicating that negative effects of competition were stronger than the sum of any potential positive species interactions when growth was P-limited. Moreover, the results indicate that the outcome of this competition was affected by CO_2 supply. As expected, *Synechocystis* was the superior competitor for P at ambient CO_2. This is consistent with resource competition theory, which predicts that when the growth of multiple species is limited by a single nutrient, the superior competitor will reduce concentrations to a level at which other species are unable to exploit it (Tilman 1982). However, species' ability to exploit a resource may be affected by a number of factors, including changes in the availability of other resources (Dybzinski and Tilman 2007). In this study, elevated CO_2 increased the growth of *Chlorella* relative to *Synechocystis* even when P supply was low. This is in agreement

with the results of a recent study by Low-Décarie et al. (2011), in which they found that higher CO_2 concentrations enhanced the competitive ability of chlorophytes to a greater degree than cyanobacteria.

Biovolume increased in both monocultures and mixed cultures in P-replete treatments, relative to low P, but was highest in mixtures. The higher productivity in P-replete mixtures was due to the dominance of *Chlorella,* which accounted for 75% and 78% of total biovolume in ambient CO_2 and high CO_2, respectively. In the same mixtures, *Synechocystis* achieved nearly 90% of its expected volume, based on monoculture yields, suggesting that increased resource supply lessened competitive effects. There was no noticeable effect of CO_2 on competition between the two species, but the increase in productivity of *Chlorella*, in response to elevated CO_2, was greater than that of *Synechocystis* in both monoculture and mixed cultures.

These results agree, in part, with the results of other studies which have found that biomass production increased with species richness (Griffin et al. 2008, Power and Cardinale 2009, Vanelslander et al. 2009). However, I found that monocultures were more productive than mixtures when P-limited, suggesting that, at least at low levels of species richness, diversity effects are dependent on resource supply. In a study employing the same low P concentration used in this study, Schmidtke et al. (2010) manipulated diversity levels using eight algal species and reported no evidence of overyielding. They attributed this to high resource consumption by species with low productivity. This is an unlikely explanation for underyielding of low P mixtures in this study because, although the dominant species differed with CO_2 level, total biovolume was unchanged. It is

therefore more likely that, in this study, reduced productivity was caused by an increase in the strength of competition effects induced by low P supply.

Increased productivity of *Chlorella* in response to elevated CO_2 may be explained by reduced CCM activity. Many algae have the ability to down-regulate CCMs activity when CO_2 concentrations are above a threshold level (Raven et al. 2011). Xia and Gao (2005) reported that CCM activity in *C. pyrenoidosa* decreased at CO_2 concentrations of approximately 0.1%. The elevated CO_2 level used in this study was substantially higher than this, suggesting that energy demanding CCM functions were likely reduced and that *Chlorella* was able to rely on passive diffusion of CO_2 to meet requirements. This may enhance the competitive ability of *Chlorella* when P supply is low because it could free P that would otherwise be used to acquire CO_2 for other purposes.

Elemental composition

RUE did not increase in mixed cultures. Instead, the highest C:N ratios were observed in P-limited monocultures of *Chlorella* in ambient CO_2. There was no effect of CO_2 or P supply on *Synechocystis* monocultures, indicating that increased C:N ratios observed in low P mixed cultures were due to the presence of *Chlorella* instead of complementary resource use by the two species. Furthermore, lower N-content coincided with reduced cellular chl-*a* quotas, suggesting that shifts in pigment content may explain, in part, increased C:N ratios. These results contrast with previous studies in which RUE increased with algal species richness (Dickman et al. 2006, Ptacknik et al. 2008, Striebel et al. 2009, Behl et al. 2011). The failure to identify effects of diversity on RUE may be explained by the low level of species richness tested in this study. Striebel et al. (2009) found that, under low P conditions, increased RUE only became apparent at high levels

of diversity. Conversely, nutrient replete conditions may also negate diversity effects by allowing rates of nutrient uptake to be coupled with the rate of carbon fixation (Sterner and Elser 2002). Nonetheless, recent studies have shown that microalgal lipid production is higher in more species rich communities (Stockenreiter et al. 2012, 2013), suggesting that mixed cultures may be a promising approach to algal biofuel production.

Conclusions

In this study I have shown that species composition and resource supply can influence the quality and quantity of algal biomass, which has implications for algae cultivated for biofuel production. Specifically, the results indicate that (i) growing mixtures containing even relatively few algal species has the potential to significantly increase total biomass production and that (ii) differences in resource supply can alter species composition in mixed cultures. Ecological theory predicts that the productivity of primary producers is a function of both resource supply and species richness (Schmid 2002), and this is clearly illustrated here. However, the cultures used in this experiment were both small in scale and were maintained for a relatively short duration, allowing conditions to be highly controlled. In real-world applications, microalgal cultures would be exposed to multiple biotic and abiotic factors that can be expected to affect species' productivity (e.g., temperature, light, invasive species). Therefore, future studies should investigate if the productivity of multi-species cultures can be maintained for longer periods in variable and suboptimal conditions.

Research in the field of algal biofuels has reached an unprecedented level. Much of this research is focused on the engineering of cultivation systems and genetic manipulation of organisms. In contrast, relatively few studies have considered the

application of fundamental ecological principles to biofuel production; Smith et al. (2010) and Stockenreiter et al. (2012, 2013) are rare exceptions. The results of this study provide evidence that further research regarding the productivity of diverse algal communities will assist in optimizing methods to improve the production of microalgal biofuels.

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I thank Jared Goos for assistance with elemental analysis.

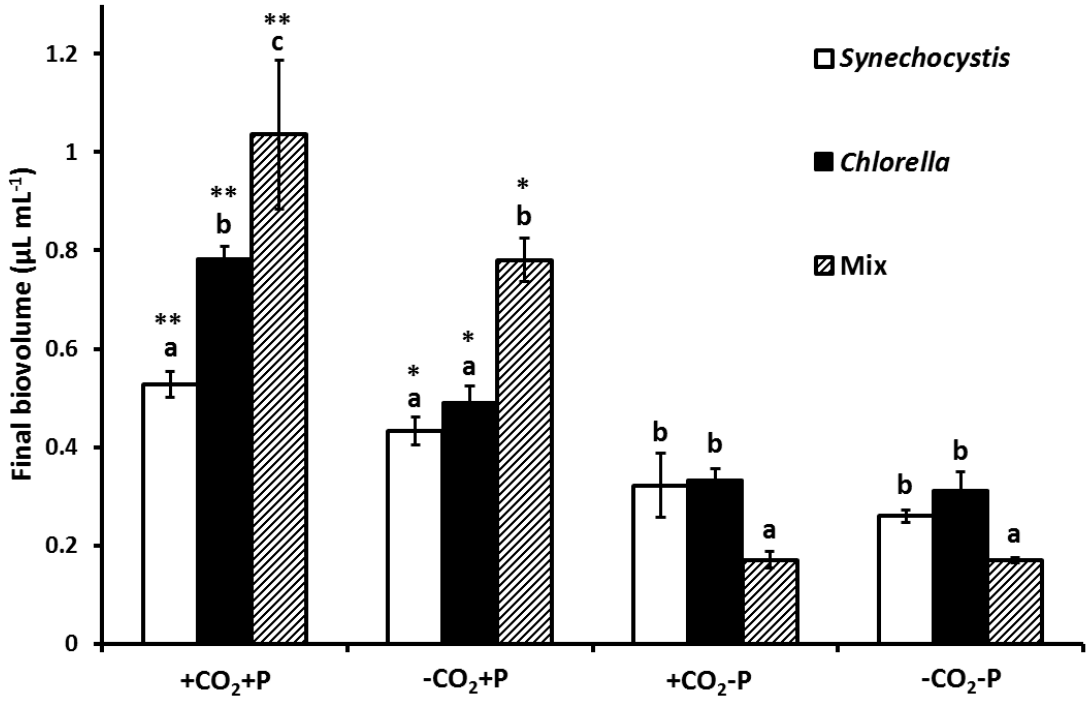

Fig. 1. Final biovolume produced by *Synechocystis* and *Chlorella* monocultures and mixed cultures under different levels of CO_2 and P availability. $+CO_2$+P, high CO_2 and high P; $-CO_2$+P, ambient CO_2 and high P; $+CO_2$-P, high CO_2 and low P; $-CO_2$-P, ambient CO_2 and low P. Values are means ± 1 SD of five replicate cultures. Letters indicate significant differences ($p < 0.05$) between biovolume production by monocultures and mixed cultures *within* a given treatment. Asterisks indicate differences in biovolume production by monocultures or mixed culture compared to its production across all treatments.

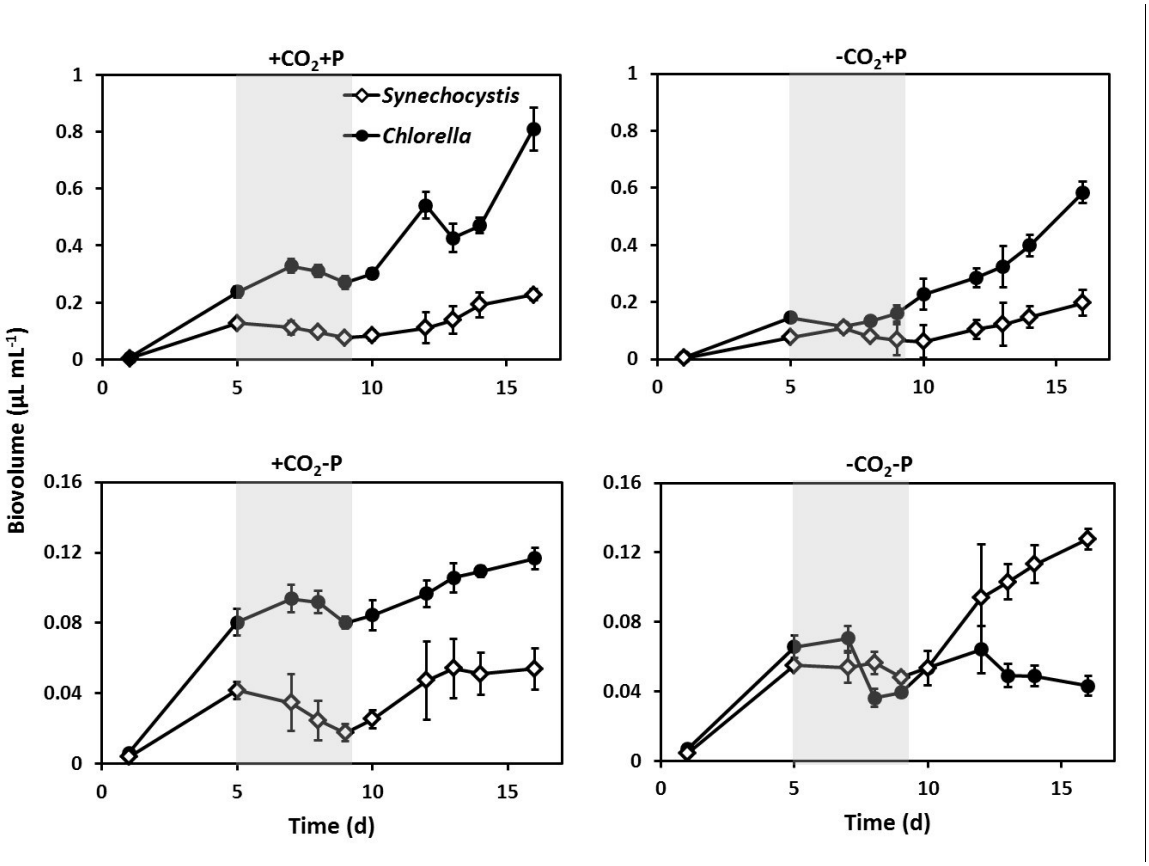

Fig. 2. Biovolume concentration of *Synechocystis* and *Chlorella* in mixed cultures over the course of the 16 day experiment. Shaded area indicates period of semicontinuous culture during which cultures were diluted by 40% daily. CO_2 and P treatments are as described in Fig. 1. (A) Biovolume in the +CO+P treatment, (B) biovolume in the -CO_2+P treatment, (C) biovolume in the +CO_2-P treatment, and (D) biovolume in the -CO_2-P treatment. Values are means ± 1 SD of five replicate cultures. Note that y-axis scales differ across panels.

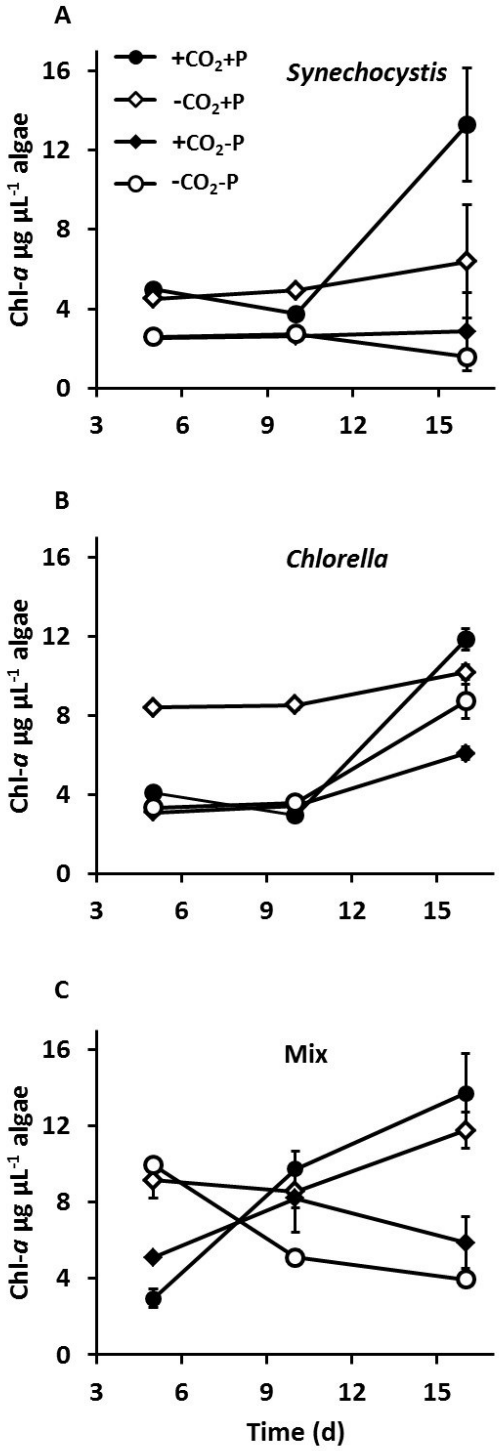

Fig. 3. Change in cellular chlorophyll-*a* content in (A) *Synechocystis* monocultures, (B) *Chlorella* monocultures and (C) mixed cultures during the experiment. Note that cultures were diluted by 40% daily for days 5-9. CO_2 and P treatments indicated are as described in Fig. 1. Values are means ± 1 SD of three replicates.

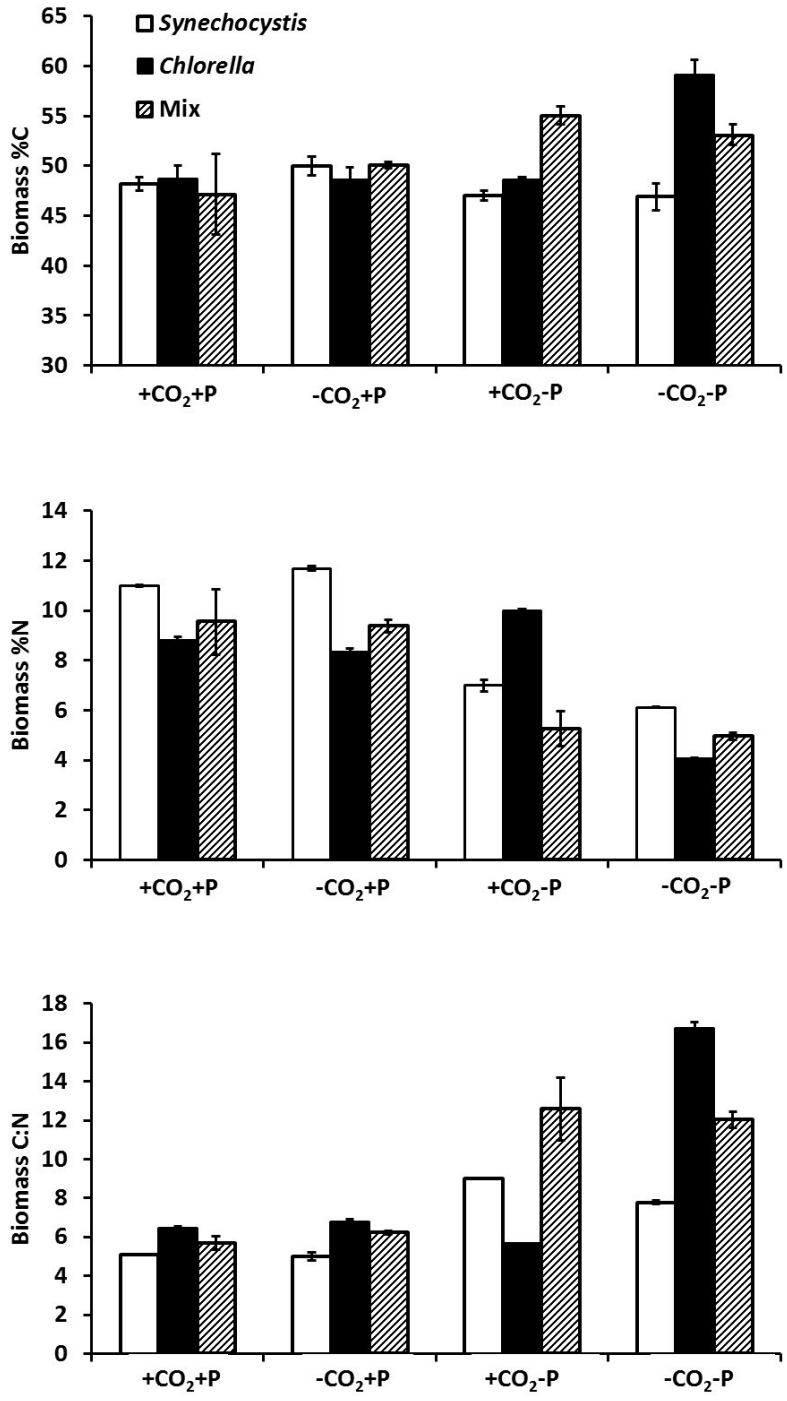

Fig. 4. Effects of treatment conditions on biomass C and N content. (A) C content as a percentage of algal biomass. (B) N content as a percentage of algal biomass. (C) Biomass atomic C:N ratio. X-axis labels are as described in Fig. 1. Values are means of two replicates. Error bars indicate range.

Table 1. P-values of three-way ANOVAs on factors affecting algal growth and C and N stoichiometry in monocultures and mixed cultures. Species refers to both *Chlorella* and *Synechocystis* monocultures and mixed cultures.

Trait	Species	CO_2	P	Species x CO_2	Species x P	CO_2 x P	Species x CO_2 x P
Biovolume	**< 0.0001**	**< 0.0001**	**< 0.0001**	0.076	**< 0.0001**	**< 0.0001**	**0.001**
Chl-*a*	**< 0.0001**	**0.011**	**< 0.0001**	**0.001**	**< 0.0001**	**0.001**	0.662
C content	**< 0.0001**	**0.001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**0.019**	**< 0.0001**
N content	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**
C:N ratio	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**

Table 2. Proportional deviation (D_i) of species final biovolume yield in mixed cultures from that expected based on monoculture yields. Positive values indicate species were more productive than expected; negative values indicate species were less productive than expected. Bold values differ significantly from zero ($p < 0.05$).

Species	+CO_2+P	-CO_2+P	+CO_2-P	-CO_2-P
Synechocystis	**-0.138**	**-0.091**	**-0.664**	-0.018
Chlorella	**1.065**	**1.380**	**-0.299**	**-0.725**

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