Species diversity of resident algae slows the establishment and proliferation of bloom causing *Microcystis*

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Abstract

As harmful algal blooms (HABs) have expanded in both size and scope, there have been increased efforts to design models that try to predict which ecosystems are at the greatest risk for a bloom. However, predicting blooms is a difficult process that has had limited success which we believe partly stems from the fact that efforts to date have considered a relatively small set of variables that influence algal growth. In particular, the focus on hydrology and abiotic conditions fails to consider biotic interactions that ultimately control the final composition of ecological communities.

In this study we examined how species richness of resident green algae influenced the growth and carrying capacity of *Microcystis* in both low and high nutrient environments. We found that when either nitrogen or phosphorous were reduced, competition with green algae had a significant negative impact on the growth rate and final biomass of *Microcystis*, but the species richness of the green algal assemblage had no discernible effect on outcomes. However, in a high nutrient environment, a species rich community of green algae had a greater negative impact on the invasion success of *Microcystis* than a species-poor community.

These results suggest that biotic interactions with phytoplankton can be important in limiting the establishment and proliferation of HAB species like *Microcystis*, and diverse communities can be more resistant to proliferation of HABs under nutrient conditions that favor blooms. In the future, the inclusion of biotic variables that influence bloom formation, in addition to the physical and abiotic requirements of a HAB species, could more accurately predict HAB events allowing for more effective management of our freshwater resources in the future.

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Introduction

Harmful algal blooms (HABs) are expanding in size and increasing in frequency around the globe, causing widespread problems with water quality (Hallegraeff 1993). Although HABs occur as natural events (Anderson et al. 2002), evidence suggests that one of the key factors promoting the recent increase in HABs has been the cultural eutrophication of freshwater systems (Heisler et al. 2008, Lopez 2008, Paerl and Otten 2013). The proliferation of blooms has caused widespread degradation to freshwater habitats as well as reduced the quality of goods and services that lakes and streams provide to humanity (Van Dolah et al. 2001, Dodds et al. 2009). For example, in the United States alone, it has been estimated that HABs have an annual cost of approximately 100 million dollars, with most of this from economic losses in the fishing, recreation and tourism industries (Davidson et al. 2014). As HABs have proliferated and their impacts increased, there has been more focus on developing predictive models that can accurately forecast the formation and spread of HABs so we might better manage them in the future.

Predicting when and where HABs will occur is a difficult process that, more often than not, has had limited success. We believe this limited success partly stems from the fact that efforts to date have considered a relatively small set of variables that influence algal colonization, establishment, and population growth. In most models of HAB formation, nuisance species are treated as passive dispersers where initiation of a bloom is primarily a function of the hydrologic conditions that deliver colonists to a site (Heisler et al. 2008). However, passive colonization is only one step in the formation of a bloom. More realistic models tend to complement the focus on hydrology with a consideration of abiotic conditions, such as water pH, temperature, and inorganic resources (nutrients and light) that influence the

ability of a nuisance species to survive and proliferate after they are passively delivered (Anderson et al. 2012). While hydrology and proper abiotic conditions are necessary precursors of bloom formation, biotic interactions were largely absent from the general discussion of HAB models {Anderson, 2012 #8;Heisler, 2008 #3}, despite ultimately controlling the final composition of ecological communities. The absence of biotic interactions in the study of HABs contrasts with numerous other fields of biology, like agricultural/forest pest management or invasive species biology. While not an exotic invader, we propose that there are parallels between HAB formation and the invasion of native communities by exotic pests. In particular, we suggest that the study of HABs could benefit from theory that attempts to predict the spread of nuisance species based on three common 'filters' that regulate the spread of all species (Webb et al. 2002, HilleRisLambers et al. 2012). Filter 1 is the dispersal filter which includes the barriers a species must overcome to be introduced to an environment where it was previously absent. Filter 2 is the environmental filter which includes the set of abiotic conditions that must be in place for a species to survive even if it successfully navigates through Filter 1 and is introduced to the new environment. Finally, filter 3 is the biotic filter which includes the suite of biological interactions with natural enemies and competitors that ultimately determine if the species will exhibit positive population growth even if the abiotic environment is conducive to growth. While the study of HABs has focused on Filter 1 and, to a lesser extent on Filter 2, we would argue that forecasting might be improved by more attention to Filter 3 – the biological interactions that regulate population growth.

One of the most important interactions that limit population growth of nuisance species is competition with species in the resident community (Shea and Chesson 2002, Levine et al. 2004). When a nuisance species invades an established community, competition with the resident

community can limit the spread of a nuisance species by restricting access to important resources. As competition between the resident community and the invader intensifies, theory suggests that the invader is increasingly less likely to successfully establish and become dominant in the community (Tilman et al. 1997b, Shea and Chesson 2002). While competition with the resident community is a common mechanism used to predict invasion success, the biological diversity of the community can also have a large influence on the strength of competition, and ultimately, the spread of a nuisance species (Levine and D'Antonio 1999, Levine 2000). There is solid empirical evidence that the diversity of a native community plays a key role in resisting the establishment and spread of a nuisance species (Tilman 1997, Naeem et al. 2000, Kennedy et al. 2002, Cardinale et al. 2012). For example, a meta-analysis of plant invasion experiments found that the resident species diversity consistently had a significant negative effect on both the establishment of the invader into the community as well as the performance of the invader after it had established (Levine et al. 2004). More intense competition for limited resources in diverse communities is one of the most commonly cited mechanisms by which diversity confers resistance to invasion. As the species richness of a community increases, inorganic resources presumably get used more completely (Knops et al. 1999), leaving fewer resources available for invading species to proliferate (Tilman et al. 1997a). When resources get used more completely, the intensity of competition experienced by an invading species increases, which in turn limits the ability of the invading species to establish and dominate the community (Knops et al. 1999, Levine et al. 2003, Catford et al. 2012). While heightened competition for resources within a diverse community is a common explanation for invader resistance in terrestrial plant communities, the role that species diversity and competition

play in limiting the proliferation of nuisance HAB species has not been studied to our knowledge.

While many species of algae are known to cause HABs, we chose to focus on species from the genus *Microcystis* because it is one of the most common and widespread cyanobacterial HAB species (Paerl and Otten 2013). This genus of cyanobacteria is particularly problematic due to the production of the toxin Microcystin , a hepatoxin that can impact both humans and other animals (Paerl et al. 2011). Microcystin has been especially problematic in Lake Erie where a bloom of *Microcystis* delivered enough of the hepatoxin into the water supply of Toledo, Ohio that the water was shut off for the entire city for 3 days (Frankel 2014). In order for *Microcystis* to bloom, the species must first be introduced into the water column, which generally occurs when hydrologic mixing re-suspends overwintering cysts from lake-bottom sediments (Verspagen et al. 2004, Michalak et al. 2013). Once re-suspended, abiotic conditions must be conducive to growth. For example, high temperatures (Johnk et al. 2008), a basic pH (McLachlan and Gorham 1962) and elevated nitrogen and phosphorus levels (Vézie et al. 2001) have all been associated with *Microcystis* blooms. However, in order to expand into a bloom, the growth of *Microcystis* must greatly exceed the growth of numerous other, often less harmful algal species, all of which potentially utilize the same pool of inorganic resources. To what extent does competition with resident algae for resources like nutrients limit the population growth and carrying capacity of *Microcystis*? And do higher levels of species diversity intensify these competitive interactions? In this paper, we attempt to answer these questions with the results of two laboratory experiments in which we looked at how the species diversity of resident green algae influenced the growth rate and carrying capacity of the nuisance cyanobacteria *Microcystis aeruginosa*.

We predicted that (1) competition with resident algae for nutrients [N, P] will significantly reduce the population growth rate and carrying capacity of *Microcystis*, but that these effects will be relatively weak at high nutrient concentrations, as often occurs at the beginning of bloom formation, and relatively strong at low nutrient concentrations. We anticipated this hypothesis would be true because we assume competition is comparatively weak when nutrients are abundant and that competition intensity will decrease with increasing resource supply rate (Huston and Deangelis 1994, Davis et al. 1998). We also predicted that (2) more diverse communities of resident algae serve to intensify competition by using nutrients more efficiently, thus reducing the population growth and carrying capacity of Microcystis. Again, we expected these effects to be relatively weak at high nutrient concentrations like those that precede the initiation of blooms, and relatively strong at low nutrient concentrations. To test these hypotheses, we performed two laboratory experiments. In the first, we quantified how the growth rate of *Microcystis* varied as a function of dissolved N or P concentrations to determine levels at which *Microcystis* maintained positive growth and maximized its growth rate. In the second experiment we examined how species richness of resident green algae influenced the growth and carrying capacity of *Microcystis* in low vs. high nutrient concentration environments. Our two experiments partially supported prediction 1, showing that competition with resident algae consistently reduced the growth rate and carrying capacity of *Microcystis* regardless of nutrient condition. Prediction 2 was also only partially supported. We found that species richness of green algae did reduce *Microcystis* growth and carrying capacity, but this only occurred in the high nutrient environments. Our work suggests that biotic interactions with phytoplankton can be important in limiting the establishment and proliferation of HAB species

like *Microcystis*, and that diverse communities can be more resistant to proliferation of HABs under certain nutrient conditions.

Methods

Experiment 1: Quantifying *Microcystis aeruginosa* growth rates at different nutrient supply rates.

Microcystis aeruginosa (strain LB 2061) was obtained from University of Texas Culture Collection. In the first experiment, we quantified the growth rate of *Microcystis* across a range of nitrogen (N) and phosphorus (P) concentrations. We then fit the growth rates to a Monod function to estimate the minimum concentration (R^*) of nitrogen and phosphorus required by *Microcystis* to maintain a positive growth rate. Using the R* value for both N and P, we calculated half saturation constants (K_s) that were used to choose N and P concentrations that were manipulated in the second experiment (described later).

Experimental Design

Experiment 1 was performed in 1-ml 48-well Falcon tissue culture plates (well plates) with low evaporation lids sealed with a parafilm wrap. The well plates were run as semi-continuous batch cultures with a 10% media exchange every other day. Well plates were kept in a 19^{o}C Percival chamber (model number AL41L3) on Thermo scientific MaxQ 2000 shakers at 100 rpm for 24 hours per day. Well plates received 100 µmol/m^{2}s light across the full visible spectrum and were exposed to a 16:8 light/dark cycle.

Nutrient dilutions

All wells were filled with 1-mL of modified COMBO growth media, a widely-used growth medium for freshwater phytoplankton (Kilham et al. 1998). Concentrations of K_2PO_4 and $NaNO_3$ were varied in the COMBO media to create gradients in N and P while holding the concentration of all other elements constant. The gradients of N and P were created by serial

dilution of stock solutions (stock $NaNO_3$ = 1000mM and stock K_2PO_4 = 50mM) to create eight concentrations for each nutrient ($NaNO_3$ = 0.0 µM, 0.2 µM, 2.0 µM, 20 µM, 100 µM, 200 µM, 500 µM, and 1000 µM, K_2PO_4 = 0.0 µM, 0.5 µM, 1.0 µM, 3.0 µM. 6.0 µM, 12 µM, 25 µM, and 50 µM). The concentrations of K_2PO_4 was maintained at 50mM in each of the nitrogen dilutions while the concentration of $NaNO_3$ was maintained at 1000mM in each of the phosphorus dilutions. The N and P portions of experiment 1 were conducted in separate well plates, with each plate receiving a different nutrient concentration in each column of the plate, and concentrations increasing from left to right. Though nutrient treatments were not randomly assigned to wells, we chose this design because it minimized the chance of contamination among wells with higher to lower nutrient concentrations. In total, each N/P concentration had six replicate wells for a total of 48 experimental units for each nutrient experiment.

Because of the difficulties of working with low concentrations of P, added precautions were taken during the P portion of experiment1 to limit potential contamination. All equipment that came into contact with either algae or media was soaked in a 10% HCL solution for 1 hour and then tripled rinsed with distilled water. Everything was then transferred to a distilled water bath where it was soaked for 4 hours followed by a second triple rinse with distilled water. *Microcystis* was also starved prior to use in the P portion of the experiment to remove all luxury stores of P from the cells. To accomplish starvation, *Microcystis* cells grown in COMBO media were spun down (4,000 rpm for 15 minutes) and the supernatant discarded. The cells were then rinsed three times with phosphate free COMBO media in three successive reconstitutions and spins. After the final rinse the cells were reconstituted with phosphate free COMBO media and allowed to grow for 4 days.

Experimental Procedure

Each well was inoculated with *Microcystis* at an initial cell density of 86,000 cells/ml. Inoculations were performed in a laminar flow hood using standard sterile techniques for algal culturing (Andersen 2005). The growth of *Microcystis* in the wells was subsequently tracked by measuring the florescence of Phycocyanin, which is a photo-pigment that is specific to cyanobacteria and absent in green algae (Izydorcyk 2005). Phycocyanin was measured on a BioTek Synergy HI hybrid plate reader after each media exchange (excited at 590 nm and emitted at 650 nm). The experiment ran for 15 days until all replicates were no longer growing exponentially, which we determined by tracking changes in Phycocyanin measurements every 2-days (described next).

Data Analysis

To estimate the growth rate of *Microcystis* in each of the nutrient concentrations, the Phycocyanin fluorescence measurements were plotted over time and fit to an exponential growth model in Excel (Microsoft 2013). *Microcystis* growth often exhibited a lag phase before the population entered exponential growth. To eliminate this lag phase and obtain more reliable estimates of exponential growth, data points at the beginning of the time-series were sequentially removed from day zero forward until fit to an exponential model (the R^2 values) was maximized. The growth rate was then estimated as the slope of the exponential equation (see inset, Figure 1A).

To determine the minimum concentration of nitrogen and phosphorus required by *Microcystis* to maintain positive growth, we took estimates of exponential growth rates and fit these to the Monod function.

$$\mu = \frac{\mu_{Max} * R}{R * \left(\frac{\mu_{Max}}{\alpha}\right)}$$

(1)

Where μ is the average growth rate of *Microcystis* at each nutrient concentration, μ_{Max} is the maximum growth rate that the species can achieve at unlimited nutrient concentrations, α is the initial rate of change showing how growth rates respond at low nutrient concentrations, and *R* is the concentration of the nutrient. From equation (1) we estimated the minimum requirement (*R**) for each nutrient where μ is equal to zero, which was then used to also calculate the substrate concentration where $\mu = 1/2\ \mu_{Max}$ as

$$K_s = \frac{½\mu_{Max}}{\alpha} + R^*$$

(2)

Ks is the nutrient concentration above which growth of *Microcystis* becomes less constrained by nutrient availability.

Experiment 2: Diversity Effects and Competition with Resident Algae

The goal of the second experiment was to explore how nutrient availability and competition with a resident community of algae influenced the growth and proliferation of *Microcystis* in green algae communities after being introduced at low densities. In particular, we were interested in how nutrient availability affected the importance of green algal diversity to the resistance of that community to invasion by a harmful cyanobacterium.

Focal Species Pool

All species used in experiment 2 were obtained from the University of Texas Culture Collection (UTEX) or the Culture Collection of Algae at Goettingen University (SAG). *Microcystis aeruginosa* (UTEX strain LB 2061) was chosen as the representative invading HAB species due to its history as a problematic HAB species in the Great Lakes region and, in particular, Lake Erie (Paerl and Otten 2013). Green algae were chosen to represent species that are common in Lake Erie, and therefore, that might reasonably be interacting with *Microcystis*. In a 1998 survey of Lake Erie performed by Barberio and Tuchman (2001), 22 green algae species were classified as dominant: defined as species comprising at least 5% of the sampled biomass. From the genera of dominant algae identified during the survey, we chose five species to represent the resident community: *Pediastrum tetras* (UTEX strain B 84), *Oocystis polymorpha* (UTEX strain 1645), *Monoraphidium minutum* (UTEX strain 2459), *Pediastrum duplex* (UTEX strain LB 1364), *and Scenedesmus acuminatus* (SAG strain 38.81). We focused on these five species because (1) they were morphologically distinguishable and could be separately identified in microscope counts, and (2) all five grow well in COMBO growth media, thus providing a common media for the competition experiment (Appendix A1).

Experimental Units

Experimental units used for this study were 125-ml glass flasks covered with 2x2 inch piece of aluminum foil to prevent contamination. Flasks were placed in randomized positions on Thermo scientific MaxQ 2000 shaker tables that were kept in a 19^{o}C Percival chamber (model number AL41L3). The shaker tables were rotated at 100 rpm 24 hours per day, and were placed under fluorescent lights that emitted 100 µmol/m^2s across the full visible spectrum on a 16:8 light/dark cycle.

Experimental Design

The experiment utilized a factorial design in which we grew seven assemblages of algae and/or cyanobacteria (treatment 1) in each of three nutrient concentrations (treatment 2). The seven assemblages included:

(1) *Microcystis* grown alone as a control so that we could quantify this species growth rate and carrying capacity in the absence of any interspecific interactions.

(2) Five monocultures, one per green algal species that were grown to steady-state before *Microcystis* was introduced at low densities so that we could measure the cyanobacteria's ability to proliferate in the presence of each individual species.

(3) A polyculture composed of all five green algal species, also grown to steady state before *Microcystis* was introduced at low density to measure the cyanobacteria's ability to proliferate in the presence of a more diverse community of species.

All seven assemblages were grown in COMBO media (Kilham et al. 1998) where the concentration of $NaNO_3$ and K_2PO_4 were manipulated to create three nutrient treatments: high nitrogen/high phosphorous (High N/P), low nitrogen/high phosphorous (Low N/High P), and

high nitrogen/low phosphorus (High N/Low P). Full strength COMBO was used to represent a High N/P environment, which had a $NaNO_3$ concentration of 1000 µM and K_2HPO_4 of 50 µM. To create the low nutrient environments, we used the estimated K_s values for N and P in experiment 1 to determine how much nitrogen and phosphorus should be reduced. For the High N/Low P treatment, the concentration of K_2HPO_4 was reduced to 3µM (equal to the K_s; Table 1) with an additional 94µM KCL added to maintain the concentration of potassium at 100µM, while the concentration of $NaNO_3$ was maintained at 1000µM. For the Low N/High P treatment, the $NaNO_3$ concentration was reduced to 240µM (3x the K_s; Table 1) while the K_2HPO_4 was maintained at 50µM. To maintain algal growth for the duration of the experiment, a 10% media exchange was performed every 2 days. All media exchanges and inoculations were done in a laminar flow hood using standard sterile techniques for algal culturing (Andersen 2005).

Construction of algal communities –All assemblages were inoculated at a biovolume of 5.0 x 10^6 μm^3/ml into experimental units containing 100-ml of the appropriate nutrient media. Thus, for polycultures, each species was inoculated at a biovolume of 1.0 x 10^6 μm^3/ml for a total biovolume of 5.0x10^6 μm^3/ml. Biovolumes for each species were determined by imaging ≥70 individual cells using a Fluid Imaging Benchtop FlowCam, and then using the area by diameter method to estimate cell volumes (ABD; Appendix B1).

Growth of Green Algae - After species of green algae were inoculated into the three nutrient treatments, we initiated the 10% media exchange every 2-days. Of the 10ml that was removed for each media exchange, 1-ml was transferred to a 48-well Falcon tissue culture plate with low evaporation lids to measure in-vivo fluorescence of chlorophyll-a (excited at 435 nm and emitted at 685 nm on a BioTek Synergy HI hybrid plate reader), which served as a measure for algal

growth. Maintenance of green algal communities proceeded until the communities reached a steady state: defined as the point at which populations had reached a growth rate equal to zero (Figure 1b). To quantify growth rates, we fit linear models (using R-studio version 3.1.2, 2008) to the last three time points of each replicate to estimate the slope/change in fluorescence. A t-test was used to determine if slopes within each diversity/nutrient treatment were different from zero (P-values > 0.1 were used to indicate steady state).

The High N/Low P treatment, while initially having reached a steady state, began to decline in fluorescence towards the end of our experiment even as other communities were still approaching steady state (Appendix C1). To ensure that all bottles still contained green algae which *Microcystis* could compete with after invasion, we counted all bottles in the High N/Low P treatment to estimate the community biovolume. Any bottle that had a biovolume below 5.0 x 10^6 μm^3/ml (the initial inoculation biovolume) was removed from the analysis (Appendix C2).

Re-establishment of Low N/ High P Treatment - For the Low N/High P treatment, we raised the concentration of $NaNO_3$ from 60μM to 240 μM forty days into the experiment and re-inoculated the green algae. This was done after the original concentration of nitrogen proved to be insufficient to maintain algae growth as intended and, as a result, communities were in decline (Appendix D1). After adding an additional 180 μM of N to all bottles in the Low N/High P treatment, each was re-inoculated at their original biovolumes. Before invading the cultures with *Microcystis*, all Low N/High P polycultures were counted to verify they contained multiple species of green algae and still represented diverse communities following re-establishment of the treatment (Appendix C2).

Invasion by Microcystis - On day 60 of the experiment all communities had reached a steady state and *Microcystis* was introduced at low densities to all experimental units (including controls). *Microcystis* was inoculated at an initial cell density of 60,000 cells/ml ($3.6x\ 10^6$ μm^3/ml) per flask. The subsequent growth rate of *Microcystis* was mesaured as the florescence of Phycocyanin (excited at 590 nm and emitted at 650 nm) using 1-ml of the media removed during media exchanges performed every 2 days. Communities were maintained through media exchanges and florescence was measured until *Microcystis* was determined to be at steady state (quantified by the same method as described previously for green algae). The majority of communities reached steady state after 18 days, but some treatments required up to 58 days of monitoring. Maintenance of the communities and measurement of Phycocyanin ceased after a community had reached steady state, but all flasks remained on the trays throughout the experiment to minimize changes in light availability for the remaining bottles.

Data Analysis

All statistical tests and model fitting were done in R-studio version 3.1.2 (2008). To quantify the success of *Microcystis* after invasion into all communities, fluorescence data for Phycocyanin was fit with either a logistic model to estimate growth rate (r) and carrying capacity (K), or an exponential model, to estimate r. We fit data to a logistic model whenever possible, but data for several experimental units could not be fit to the logistic because: (1) *Microcystis* failed to establish in the community, (2) there were less than three data points to estimate K, or (3) the logistic model was a poor fit due to erratic fluorescence time series (Appendix E). Additionally, there was a subset of communities where the logistic model gave a poor estimate of the carrying capacity due to overshoot. When the estimated carrying capacity deviated from the

last florescence measurement by more than 30%, the *K* estimate was dropped from analysis (Appendix E). To improve the fit of both exponential and logistic models, data points were removed from either end of the time series when an R^2 was below 0.75.

After estimating *r* and *K* for *Microcystis* in all communities, boxplots were used to look for outliers and any data point that was more than 1.5 times the 95% confidence intervals was removed. We used a one-way analysis of variance (ANOVA) to compare how the growth parameters of *Microcystis* (*r* and *K*) in the two different green algal diversity treatments differed from the growth of *Microcystis* when cultured alone in controls. A Welch's t-test, which is suitable for unequal sample size comparison, was then used to directly compare the growth parameters of *Microcystis* in the two different diversity treatments.

If it was found that *r* or *K* of *Microcystis* in polycultures differed from *r* or *K* in monocultures, the competition experienced by *Microcystis* in each green algae community for an entire nutrient treatment was calculated as followed.

$$S_{ir} = \left(\frac{r_{alone} - r_{invader}}{r_{alone}}\right) \times 100\%, \text{ or } S_{ik} = \left(\frac{K_{alone} - K_{invader}}{K_{alone}}\right) \times 100\% \quad (3)$$

S_{ir} or S_{ik} indicates the growth rate (*r*) or carrying capacity (*K*) reduction of *Microcystis* when being cultured with different green algae diversity treatments (5 monoculture and 1 polyculture). R_{alone} or K_{alone} is the estimated growth rate (*r*) or carrying capacity (*K*) of *Microcystis* when grown alone, and $r_{invader}$ or $K_{invader}$ is the estimated growth rate (*r*) or carrying capacity (*K*) of *Microcystis* after invasion into a green algae community. Within a nutrient treatment, every possible pairwise comparison between each replicate of the controls and each replicate of the diversity treatment was calculated. Sometimes the calculated S_{ir} or S_{ik} was greater than 100 or

below 0 because the growth rate of *Microcystis* after invasion was either higher than the growth of *Microcystis* in the control or below zero because *Microcystis* had a negative growth rate. When this occurred we recorded those above 100 as 100 and any that were below 0 were recorded as 0. We did this to restrict the S_i numbers between 0 and 100 because it represents the percent reduction in growth rate or carrying capacity.

Results

Effects of N and P supply on the growth of Microcystis

Microcystis had a high requirement for nitrogen, with a minimum concentration of 11.018 µM required to sustain a positive growth rate (Table 1). By comparison, the requirements for P were comparatively low, requiring just 0.72 µM of phosphorus to maintain a positive growth rate (Table 1). At low nutrient concentrations, *Microcystis* responded more slowly to increases in N ($\alpha = 0.005$) then increases in P ($\alpha = 0.076$) by nearly an order of magnitude.. The half-saturation constants, K_s, at which growth rates of *Microcystis* begin to decelerate were 60 µM for N and just 3 µM for P (Table 1).

Diversity impacts on the establishment and growth of Microcystis

For the High N/P treatment, there were significant differences between the growth rate (r) of *Microcystis* in controls (grown alone in monoculture) and growth rates when introduced into cultures containing resident species of green algae ($F = 9.40$, $p < 0.01$). Post hoc tests (Tukey HSD) showed that algal monocultures ($p < 0.01$) and polycultures ($p < 0.01$) both reduced r by 48.5% and 57.9% on average respectively. A Welch's t-test suggested that reductions in growth rates were equal between the two levels of resident algal diversity (Welch's $t=1.01$, $p=0.34$; Figure 2A). While the diversity of green algae did not impact r, it did have an impact on the carrying capacity K of *Microcystis*. Significant differences in K were noted between controls and cultures of green algae ($F = 4.42$, $p = 0.03$, Figure 2D). Post hoc tests showed that both the monocultures ($p = 0.05$) and polycultures ($p = 0.04$) significantly reduced K by 41.9% and 94.2% units of fluorescence, respectively. Furthermore, a Welch's t-test revealed that the polyculture

reduced the carrying capacity of *Microcystis* significantly more than the average monoculture ($t=2.59$, $p<0.05$).

We used equation (3) to calculate the strength of competition with green algae, estimated as the proportional reduction in *Microcystis* growth rates (*r*) and carrying capacity (*K*) in the presence of other species. In the High N/P treatments, competition with species monocultures as well as the full polyculture significantly reduced *r* ($F = 17.9$, $p < 0.01$). Estimated *r* reductions ranged from 3.79% for competition with *Pediastrum duplex* monoculture to a 100% reduction (e.g., no establishment or growth of *Microcystis*) in monocultures of *Monoraphidium minutum* (Figure 3B). Post hoc comparisons (Tukey HSD) showed that competition with *M. minutum* monocultures had the greatest impacts on *r* ($p < 0.01$) between all diversity levels. In addition, competition with polycultures was stronger than competition with monocultures of *Scenedesmus acuminatus* ($p < 0.01$), which is significant given that *S. acuminatus* dominated polycultures at the end of the experiment (98% of relative biovolumes, Figure 3A). Competition also reduced the carrying capacity (*K*) of *Microcystis* ($F = 86.01$, $p <0.01$). The same three species that were present at the end of the experiment in polyculture (*O. polymorpha, M. minutum, and S. acuminatus,* Figure 3A) all reduced *K* by 60 to 100% relative to controls where *Microcystis* was grown alone (Figure 3C). The two species of *Pediastrum* (*tetras* and *duplex*), which were absent from the final polycultures, did not impact the *K* of *Microcystis* (Figure 3C).

In the Low N/High P and High N/Low P treatments, competition with algae had negative impacts on the establishment of *Microcystis*, but there were no differences among levels of algal diversity in Microcystis ; ability to invade(Figure 2). In the Low N/High P environment, *Microcystis* exhibited an $r = 0.66$ and $K = 353.89$ when grown alone. Yet, *Microcystis* was

unable to invade and maintain positive growth in any assemblage of green algae regardless of the level of diversity (Figure 2B). This also meant final *K*'s in all cultures were zero (Figure 2E) and, given there was no establishment, there were no significant differences between algal mono- and polycultures ($p = 0.57$, Figure 2B). In the High N/Low P environment, interactions with green algae did not influence the *r* of *Microcystis* ($F = 1.01$, $p = 0.35$, Figure 2C); however, competition from green algal mono- or polycultures equally reduced *K* relative to controls ($F = 20.15$, $p < 0.01$; Fig 2F).

Discussion

We have reported results from two experiments that examined how competition between resident algae and *Microcystis* can limit the population growth and carrying capacity of this nuisance cyanobacterium. Our first prediction was that competition with resident algae would reduce the population growth rate and carrying capacity of *Microcystis*, and that the impacts of competition would be weakest when nutrient concentrations were high and strongest when nutrient concentrations were low. Consistent with this hypothesis, we found that competition with green algae substantially reduced the growth rate and carrying capacity of *Microcystis*, often up to a full 100%). In treatments where we manipulated N or P so they were scarce (the Low N/High P, and High N/Low P treatments), results were as anticipated. While *Microcystis* was able to establish and grow in the Low N/High P treatment when it was grown alone, *Microcystis* was unable to establish when grown in the presence of any species of green algae. The N* of *Microcystis* quantified in experiment 1 suggests that this cyanobacterium requires a particularly high concentration of N to maintain a positive growth rate; therefore, we suspect that the presence of any species of green algae reduced N to levels below requirements for *Microcystis* growth. Similarly, *Microcystis* was able to establish and grow in the High N/Low P treatment when grown by itself, but its growth rate and densities were greatly reduced when it was grown in the presence of any species of green algae. Where results diverged from our prediction was in the high N/P treatments where we had anticipated that competition would be weak because nutrients were abundant. In contrast, we documented substantial reductions in both the growth rate and carrying capacity of *Microcystis* when in the presence of green algal species in the high N/P treatments. While we are skeptical that nutrients became limiting in this treatment, it is possible that some other resource like light became scarce (Hautier et al. 2009).

Our second prediction was that competition would be more intense in diverse communities of algae, and that the effects of diversity would be most prevalent when nutrients were scarce and relatively weak when nutrients were abundant. Consistent with this hypothesis, we found that more diverse communities of resident algae were better at reducing the growth rate and carrying capacity of *Microcystis*, but in contrast, strong effects of diversity were only observed in the high nutrient environment. When either of the initial nutrient concentrations were low (Low N/High P or High N/Low P), it appeared that the presence of nearly any species of resident green algae was sufficient to reduce the growth rate and carrying capacity of *Microcystis*. Because all species exhibited competition with *Microcystis*, diverse polycultures were no more effective than a typical monoculture. However, when nutrients were abundant (High N/High P) there were large differences in the competitive abilities of the different green algal species. Only 3 green algal species proved to be strong competitors under these nutrients conditions, *S. acuminatus, O. polymorpha,* and *M. minutum*, and the rest of the species had comparably weak effects on *Microcystis*. These differences among species resulted in the monocultures having lower mean competition compared to the algal polyculture, which accounts for the stronger effects of the polyculture on *Microcystis* growth rates and carrying capacities.

The negative interactions between *Microcystis* and resident green algae were consistent with select empirical studies that have previously demonstrated how *Microcystis* was influenced by biotic interactions. For example, in experiments with the Chlorophyceaen green algae *Quadrigula chodatti*, the total biomass of *Microcystis* was reduced by up to 58% due to competition with the green algae (Zhang 2012). Our results were also consistent with patterns documented in observational studies, which had shown a negative correlation between species richness of phytoplankton and the probability of HAB formation (Roelke and Buyukates 2001,

Vazquez et al. 2005). While a negative correlation certainly does not indicate a causal relationship between diversity and HAB formation, the patterns were consistent with this study and others showing that competition can influence the formation of blooms, and that competition can be stronger in diverse communities.

Results of our study were also consistent with a large body of research in ecology that has demonstrated how competition with species in a resident community can limit the establishment and proliferation of nuisance species. For example, Levin et al. (2004) conducted a meta-analysis of 65 experiments comparing invader establishment and performance in intact communicates to treatments in which resident competitors were excluded. They showed that competition was a significant barrier to the establishment of invasive species, and that diverse communities were better at resisting invaders, presumably because they more completely utilized available resources. Studies of biological control in agricultural and forested habitats have also identified competition as a key interaction that limits the density of unwanted pests, and have shown that diverse systems are more efficient at biological control (Mack et al. 2000, Denoth et al. 2002, Shennan 2008, Letourneau et al. 2009). Given the widespread influence of competition in invader success, and the role of biodiversity in mediating the strength of competition, it would not be surprising if local interactions with resident algae, in addition to abiotic and physical parameters, were also important in mitigating the successful establishment and proliferation of *Microcystis* during the preliminary stages of bloom formation.

Though our study suggests that biotic interactions may be important for limiting the proliferation of HAB species like *Microcystis*, there are some obvious caveats to our work. Most notably, while the simplified laboratory environment used for our experiment allows for high

replication and experimental control, it was in no way meant to mimic the complexity of real lakes. Real lakes are characterized by greater levels of diversity and more complex food-webs than those considered here. Perhaps most importantly, our study only focuses on competition within the producer trophic level, and did not consider trophic interactions like herbivory that are known to influence the fate of producers (Cyr and Pace 1993), and which can influence the dominance of nuisance species like *Microcystis* (Vanderploeg et al. 2001). Real lakes also exhibit coupling of pelagic and benthic habitats where hydrologic mixing can control not only the delivery of propagules (Davis et al. 2014), but also the supply of nutrients (Chen et al. 2002). Also, real lakes exhibit fluctuations of abiotic conditions (temperature, sunlight, etc.) that we did not consider, but which can regulate the successful growth of algae. Therefore, studies like ours that are performed in simplified laboratory environments need to be complemented with more realistic field experiments (e.g., in situ bottle studies that vary resident diversity through dilution-series) as well as observational studies that use high resolution time-series to quantify the role of species interactions during bloom formation (Ives et al. 2003).

Despite the limited realism, our study does suggest that biotic interactions with resident phytoplankton may potentially limit the establishment and proliferation of HAB species like *Microcystis*. Assuming these results hold in future work, this knowledge could help managers design strategies that not only mitigate the negative consequences of HABs, but also reduce the probability of bloom formation in the future by more accurately describing the conditions that promote the growth of this nuisance species. For example, our work suggests that either a reduction in N or P or an increase in the species richness of the phytoplankton community could increase the competition experienced by *Microcystis* and help reduce the probability of a HAB. Currently, biotic interactions are largely absent from HAB research and predictive models, but

we would argue that these are necessary for a complete understanding of these events and as such should be more widely incorporated into the HAB field for a more complete understanding of bloom dynamics and behavior.

Table 1. The parameters for the Monod model for the response of *Microcystis* to nitrate and phosphate concentrations

Nutrient	***α***	***μMax***	***R****	***K_s***
Nitrate	0.005057	0.4871	11.0181	60 umol/L
Phosphate	0.07583	0.5753	0.72217	3 umol/L

The growth response parameters for Microcystis for changing concentrations of either Nitrogen or Phosphorus where α *is the initial rate of change showing how growth rates respond at low nutrient concentrations,* μ_{Max} *is the maximum growth rate that the species can achieve at unlimited nutrient concentrations,* R *is the concentration of the nutrient required by Microcystis to maintain a positive growth rate, and* Ks *is the nutrient concentration above which growth of* Microcystis *becomes less and less constrained by nutrient availability*

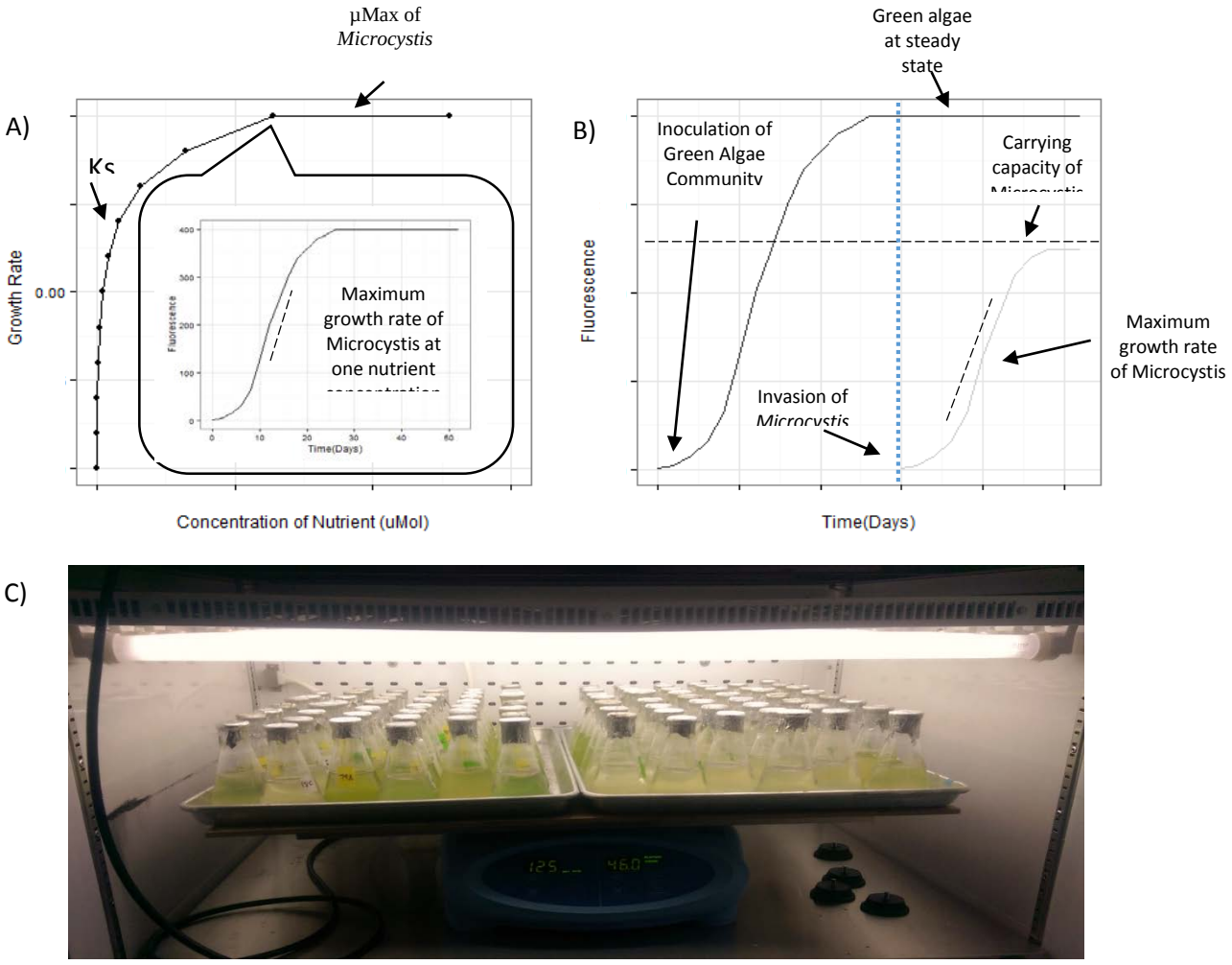


Figure 1: (A) A schematic of the experimental method used to determine the concentration of nitrate and phosphate needed by *Microcystis* to maintain a positive growth rate (R*).At a particular nutrient concentration a community of *Microcystis* was monitored over time to determine the maximum growth rate at that nutrient concentration. This was repeated at different nutrient concentrations and the maximum growth rates were used to fit a Monod model to determine the concentration at which the growth rate is zero. (B) A schematic of how the harmful algal bloom (HAB) experiment was designed. The communities of green algae were inoculated and allowed to grow until they reached a steady state. At that point, *Microcystis* was invaded a low densities and its growth was then tracked until it population had reached a steady state. From the invasion growth curve, the maximum growth rate and the carrying capacity of *Microcystis* was determined by fitting a logistic growth model to data. (C) A picture of the experimental set up with two trays on the shaker table positioned directly under the lights in the temperature controlled chamber.

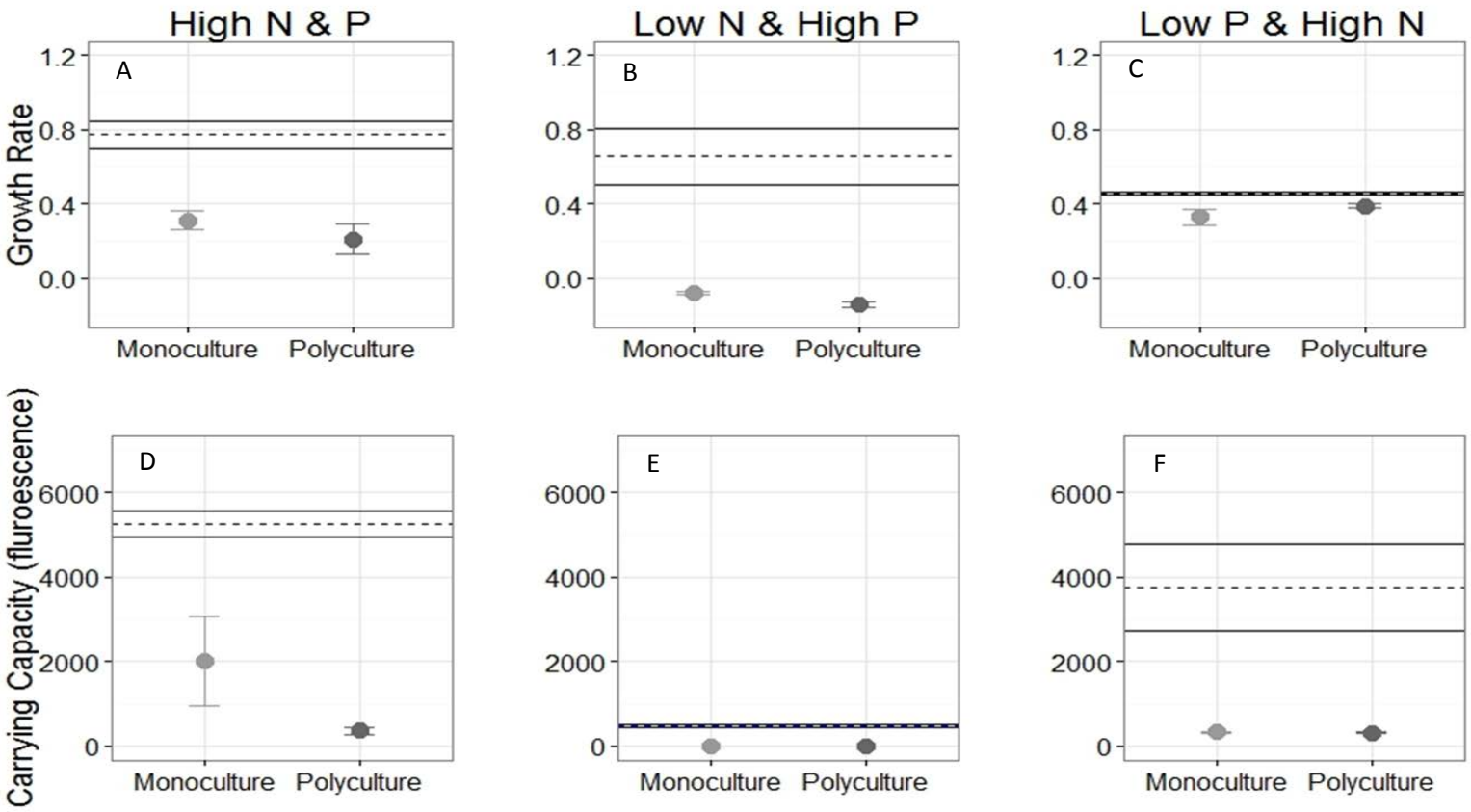


Figure 2: The growth rate and carrying capacity of *Microcystis* after invasion into the average monoculture and the polyculture of green algae. The doted line and two solid black lines represent the average growth rate or carrying capcity of *Microcystis* when grown alone and the standard error. All error bars represent one standard error. A star represents a significant difference.

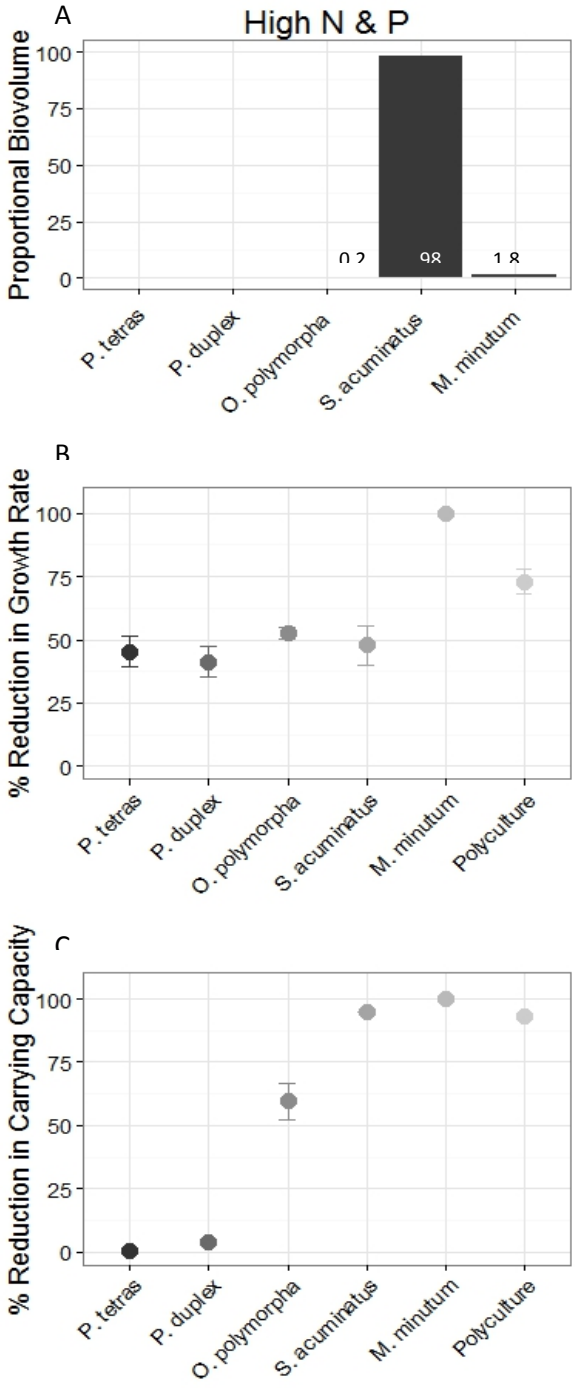
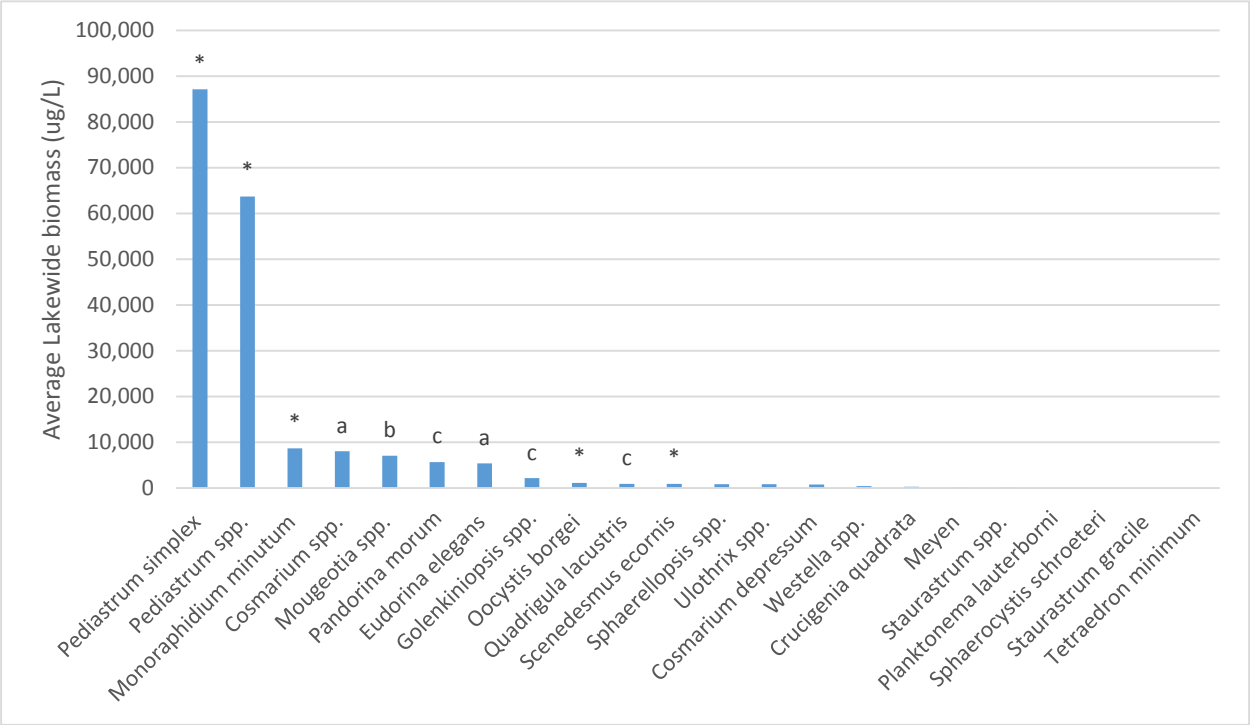


Figure 3 In the high nutrient treatment (High N&P) the relative proportion of each species of green algae represented in the polyculture (A), and the percent reduction of the growth rate (B) and the carrying capacity (C) of Microcystis after invasion into each community of green algae. All competitive effects have been kept between 0 and 100, and all error bars represent standard error

Appendix A: The green algae species present in Lake Erie during a lake wide survey in the summer of 1999.

Figure A1– The dominant Chlorophyta (>5% of the biomass at any one sampling site) that were present in Lake Erie during a lake wide survey by Baraberio and Tuckman (2001) in the summer (summarize from information in Table 2). Green algae species were chosen based on the dominance hierarchy above, the algae species available and the biology of the species. When possible the species level classification was met, but in many cases a different species was used to represent the genus. In the above figure, a star (*) above a species represents the four genus' that were represented by the five species in this experiment. We were unable to choose the top 5 species from this list due to a number of logistical reason denoted by either an (a), (b), or (c) above the species name in the figure. When there is an (a) denoted above the species, the algae either would not grow in media for the experiment (E. elegans) or the batch culture did not reach sufficient densities to inoculate the experiment (Cosmarium). A (b) signifies that the algae is a known water quality problem itself and a (c) denotes genus' that were not represented in the available algae library.



Appendix B: Green algal growth curves and biovolume estimates for the Low Phosphorus/High Nitrogen treatment before invasion by *Microcystis* on Day 60

Figure B1: growth curves for the green algae communities in the low phosphorus/high nitrogen treatment. Each graph represents one bottle: (1-5) are polycultures while the remaining 25 bottles are different monocultures: (6-10) Pediastrum tetras, (11-15) Pediastrum duplex, (16-20) Oocystis polymorpha monocultures, (21 - 25) Scenedesmus acuminatus monocultures, and (26-30) Monophoridium minutum.

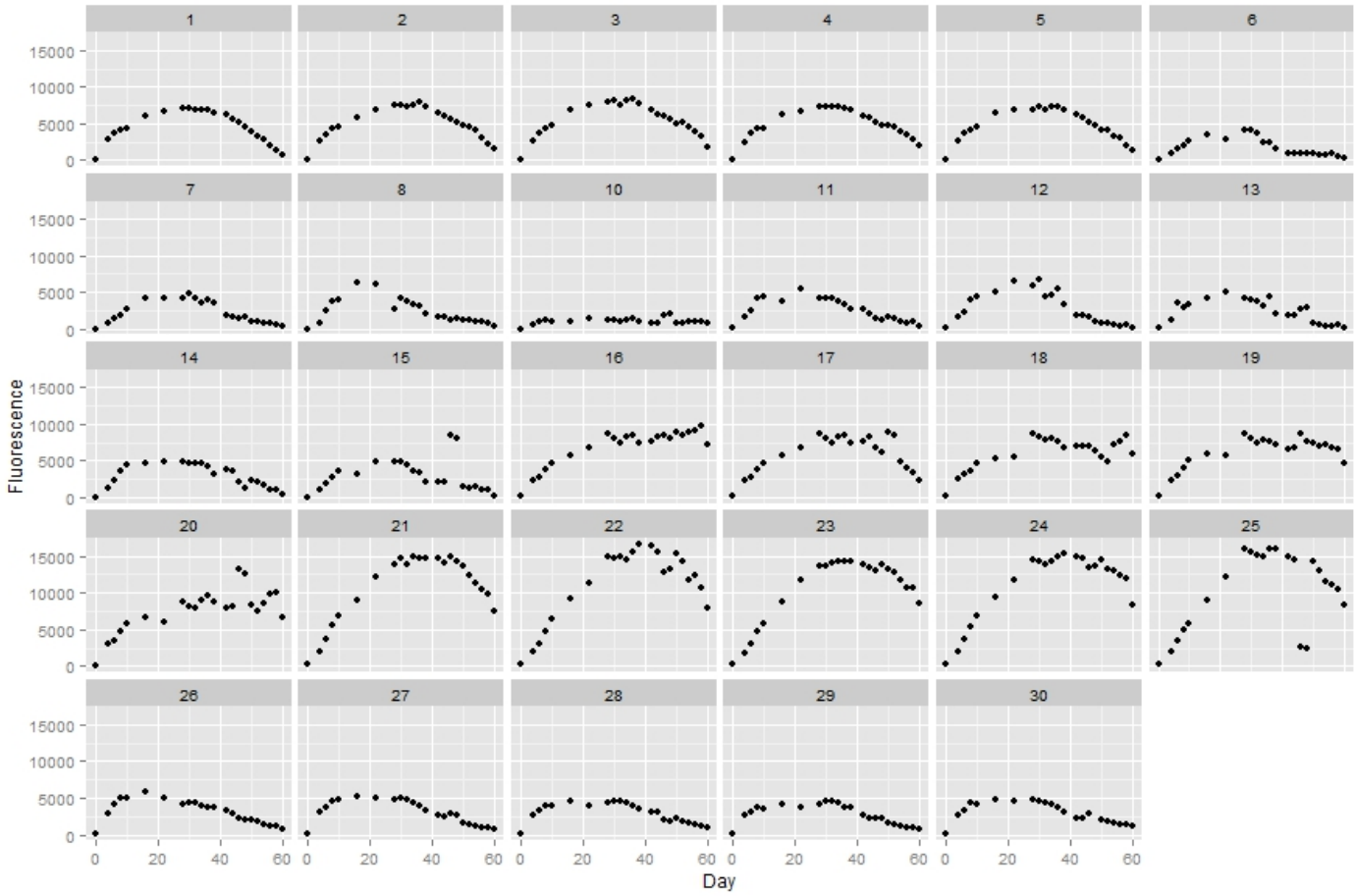


Table B2: Biovolume counts for all bottles in the Low Phosphorus/High Nitrogen (High N/Low P) treatment on the day of Microcystis invasion. For each of these communities, the bio-volume for each community was determined and those that were at a lower density then initially inoculated are noted with a * and were removed from calculations.

Experimental Unit	Green Algae Species	Biovolume on Day 40
1	Polyculture	$1.87x10^7$ µm^3/ml
2	Polyculture	$1.36x10^7$ µm^3/ml
3	Polyculture	1.01x107 µm^3/ml
4	Polyculture	$7.7x10^6$ µm^3/ml
5*	Polyculture	$2.8x10^6$ µm^3/ml
6	*Pediastrum tetras*	$2.09x10^7$ µm^3/ml
7	*Pediastrum tetras*	$1.86x10^7$ µm^3/ml
8	*Pediastrum tetras*	$7.34x10^7$ µm^3/ml
10	*Pediastrum tetras*	$5.25x10^7$ µm^3/ml
11	*Pediastrum duplex*	$7.69x10^6$ µm^3/ml
12	*Pediastrum duplex*	$5.17x10^6$ µm^3/ml
13	*Pediastrum duplex*	$5.3x10^6$ µm^3/ml
14	*Pediastrum duplex*	$5.8x10^6$ µm^3/ml
15	*Pediastrum duplex*	$5.42x10^6$ µm^3/ml
16	*Oocystis polymorpha*	$3.93x10^7$ µm^3/ml
17	*Oocystis polymorpha*	$1.35x10^7$ µm^3/ml
18	*Oocystis polymorpha*	$3.82x10^7$ µm^3/ml
19	*Oocystis polymorpha*	$8.33x10^6$ µm^3/ml
20	*Oocystis polymorpha*	$9.74x10^6$ µm^3/ml
21	*Scenedesmus acuminatus*	$1.08x10^7$ µm^3/ml
22	*Scenedesmus acuminatus*	$1.75x10^7$ µm^3/ml
23	*Scenedesmus acuminatus*	$1.69x10^7$ µm^3/ml
24	*Scenedesmus acuminatus*	$2.01x10^7$ µm^3/ml
25	*Scenedesmus acuminatus*	$2.15x10^7$ µm^3/ml
26	*Monophoridium minutum*	$1.56x10^7$ µm^3/ml
27	*Monophoridium minutum*	$1.43x10^7$ µm^3/ml
28	*Monophoridium minutum*	$1.52x10^7$ µm^3/ml
29	*Monophoridium minutum*	$1.85x10^7$ µm^3/ml
30	*Monophoridium minutum*	$1.72x10^2$ µm^3/ml

Appendix C: Biovolume estimates for each of the green algae species and for the polycultures on the day of *Microcystis* invasion

Table C1: the estimated bio-volume for the average single cell of each species of green algae and Microcystis

Species	Average Bio-volume (μM^3) (single cell)
Pediastrum tetras	3049.29
Pediastrum duplex	680.85
Oocystis polymorpha	345.98
Scenedesmus acuminatus	121.98
Monoraphidium minutum	62.41
Microcystis aeruginosa	60

Table C2: the calculated bio-volume of each species present in the polycultures. Experimental unit (1-5) low phosphorus/high nitrogen treatment, experimental units (36-40) low nitrogen/high phosphorus, and experimental units (71-75) high nitrogen and phosphorus treatment.

Experimental Unit	**Bio-volume of green algae species in polyculture ((μm^3)/ml)**					**Community Bio-volume**
	Pediastrum tetras	Pediastrum duplex	Oocystis polymorpha	Scenedesmus acuminatus	Monoraphidium minutum	
1	18069867	0	2626885	587311	115574	21399637
2	10728983	0	1409548	1219800	34672	13393004
3	3952783	0	1281407	1513456	46230	6793876
4	5646833	0	640704	880967	0	7168504
5	0	0	384422	564722	104017	1053161
36	0	0	8841711	3117267	72464944	84423922
37	10164300	0	4228644	14773133	80647589	109813667
38	0	0	2306533	10707133	94031067	107044733
39	0	0	1153267	13553333	107622578	122329178
40	0	0	384422	9351800	105680933	115417156
71	0	0	384422	134178000	3120500	137682922
72	0	0	1153267	209399000	5339522	215891789
73	0	0	0	111815000	3813944	115628944
74	0	0	1153267	176328867	8390678	185872811
75	0	0	1153267	157896333	6032967	165082567

Appendix D: Green algal growth curves for the Low Nitrogen/ High Phosphorus treatment before invasion by *Microcystis* on Day 60

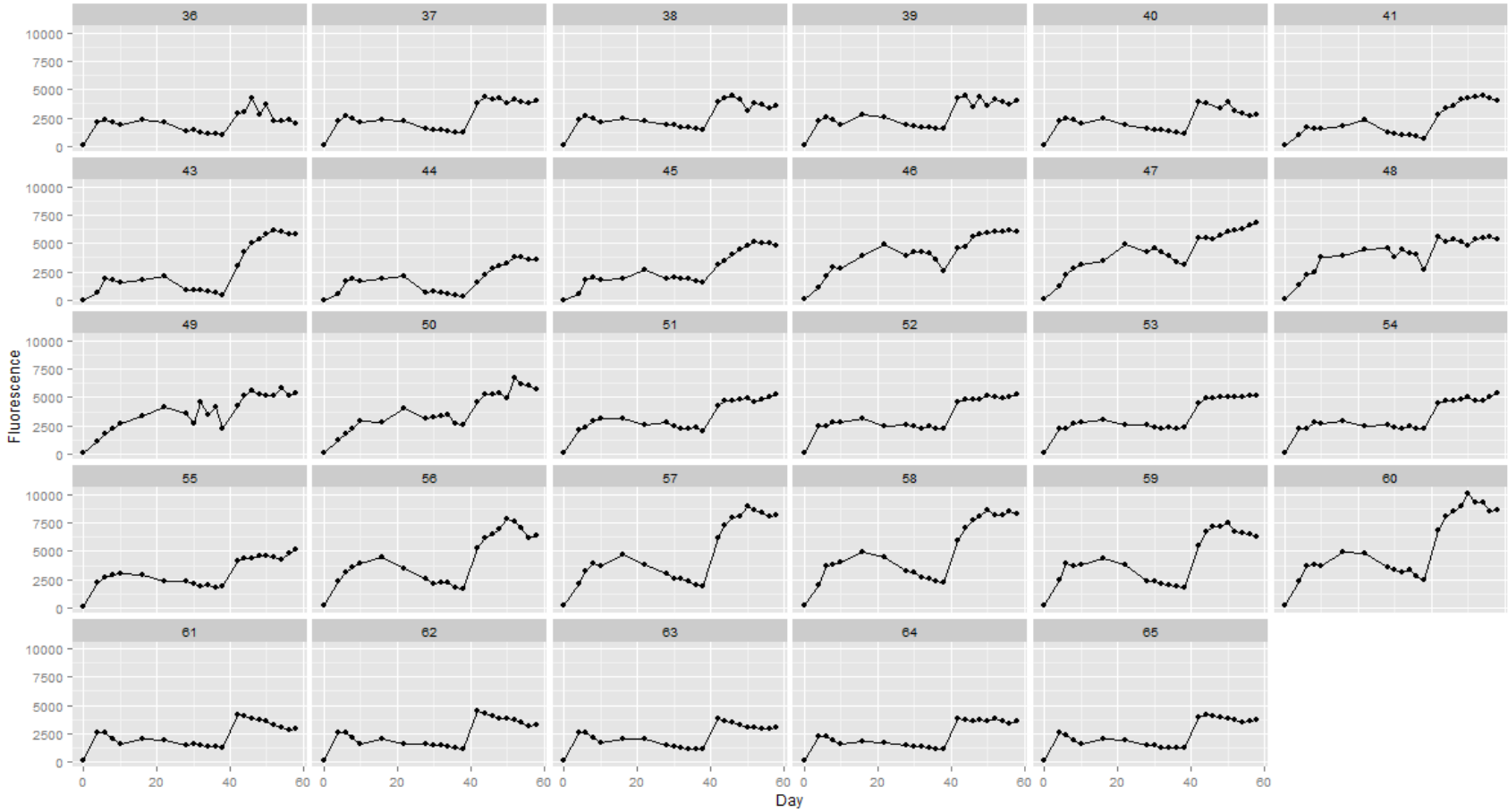


Figure D1: the green algae growth curves in the Low Nitrogen/High Phosphorus treatment. Each bottle was amended on day 40 of the experimental after systematic and consistent decreases in green algal growth was seen in all bottles. *Each graph represents one bottle: (36-40) polycultures, (41-45) Pediastrum tetras monocultures, (46-50) Pediastrum duplex monocultures, (51-55) Oocystis polymorpha monocultures, (56-60) Scenedesmus acuminatus monocultures, and (61-65) Monophoridium minutum monocultures.* After the amendment, all green algae communities grew exponentially and reached a steady state prior to inoculation with *Microcystis*.

Appendix E: A summary of the bottles removed from analysis and types of models fit to each Experimental Unit

Table E1: the number of replicates within each type of green algae community that were fit with a logistic model. The replicates that could not be fit with a logistic model are noted with one of three explanations for removal: (1) growth rate estimated with an exponential model due because logistic model could not be fit, (2) the carrying capacity estimate was greater than 30% of the measured fluorescence value but estimated growth rate from logistic model was still used, or (3) the bottle was removed from the experiment due.

Species ID	**Number of replicates fit with logistic model**	Bottle(s) removed or fit with exponential model	**Reason**
Low Phosphorus/High Nitrogen			
Polyculture	4	5	(3)
Pediastrum tetras	4	9	(3)
Pediastrum duplex	2	11,12,13	11,12– (1) 13- (2)
Oocystis polymorpha	2	16,18,20	(1)
Scenedesmus acuminatus	1	22-25	22,24,25 – (1) 23-(3)
Monophoridium minutum	3	26&27	26 – (1) 27 – (2)
Microcystis aeruginosa	4	34	(1)
Low Nitrogen/High Phosphorus			
Polyculture	0	36-40	(1)
Pediastrum tetras	0	41-45	41, 43-45 – (1) 42 – (3)
Pediastrum duplex	0	46-50	(1)
Oocystis polymorpha	0	51-55	(1)
Scenedesmus acuminatus	0	56-60	(1)
Monophoridium minutum	0	61-65	(1)
Microcystis aeruginosa	3	66 & 69	66 – (2) 69 – (1)
High Nitrogen and Phosphorus			
Polyculture	3	71 & 73	(1)
Pediastrum tetras	2	76, 79, 80	76, 80 - (2) 79 – (3)
Pediastrum duplex	2	81,82, 85	81 – (3) 82 – (2) 85 – (1)
Oocystis polymorpha	4	88	(1)
Scenedesmus acuminatus	4	91	(1)
Monophoridium minutum	0	96-100	96,97,99,100 - (1) 98 – (3)
Microcystis aeruginosa	5		

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