

Methods for Monitoring and Controlling Freshwater Harmful Algal Blooms

by

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Abstract

Fresh water is critical for our survival, and its responsible use is necessary for the sustainability of our planet. Surface-water ecosystems are important sources of recreation and potable water are threatened by increasing urbanization, pollution, environmental degradation, and eutrophication. One major consequence of nutrient pollution is the development of harmful algal blooms (HABs). Such events have been documented to cause illness in humans and death in domestic animals and livestock. Autotrophic prokaryotes, called cyanobacteria, are the primary phytoplankton group responsible for HABs in freshwater ecosystems. Because of these threats water resource managers are interested in monitoring and controlling cyanobacterial blooms. Chapter 1 of my thesis aimed to develop a method using fluoremetry of the pigment phycocyanin to quickly and reliably estimate cyanobacterial abundance from whole water samples. Chapter 2 of my thesis aimed to determine the effectiveness of using grass carp as a biological control for the cyanobacterium *Lyngbya wollei*.

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FLUOROMETRY OF PHYCOCYANIN AS A RAPID APPROACH FOR ESTIMATING CYANOBACTERIAL ABUNDANCE

ABSTRACT

Cultural eutrophication continues to threaten freshwater resources through, in part, the promotion of harmful algal blooms that are usually dominated by autotrophic prokaryotic cyanobacteria. Strains of several cyanobacterial taxa, including *Anabaena*, *Microcystis*, and *Oscillatoria*, are capable of producing intracellular toxins or off-flavor compounds that can contaminate drinking water supplies, negatively affect associated organisms, and harm local economies. Traditionally, microscopy has been used to identify and quantify cyanobacteria, but this approach can be time consuming and challenging depending on the composition and abundance of phytoplankton taxa. Molecular technologies can provide fast turnaround times, however they can be costly and require extensive training and expertise for data collection and interpretation and their results are rarely confirmed with other techniques. Fluorometry, especially *in situ* probes, is gaining in popularity but few studies have developed strong correlations between cyanobacterial pigment concentrations and biovolume. Because of the need for a rapid and reliable way to quantify cyanobacterial biovolume from whole water samples, we developed a benchtop fluorometric method based on the analysis of the cyanobacterial pigment, phycocyanin, to estimate cyanobacterial abundance. Based on multiple laboratory experiments, a four-hour extraction at 4°C was chosen to be the most practical protocol for maximizing phycocyanin yields from our filtered water samples. In general, phycocyanin was significantly correlated with cyanobacterial biovolume across sixteen ponds that varied in productivity and phytoplankton species composition. Given that accurate estimates of cyanobacteria can be

efficiently achieved on the same day of sample collection, this tool should be considered by water resource managers interested in understanding complex spatiotemporal dynamics of toxigenic cyanobacterial blooms.

INTRODUCTION

Protecting diminishing freshwater resources is one of the most pressing environmental issues and will likely become more challenging as climate change, environmental degradation, and cultural eutrophication further reduce water quality and quantity. Harmful algal blooms (HABs) are a common consequence of excess nutrient loading and can impair water quality in many freshwater and coastal marine ecosystems. (Paerl and Huisman 2009). Cyanobacteria (i.e., blue-green algae) are the primary phytoplankton taxa responsible for freshwater HABs and have been implicated in the poisoning of food webs, pets, and humans (Chorus and Bartram 1999, Carmichael 2001). In addition, several cyanobacterial genera produce off-flavor compounds, such as geosmin and methylisoborneol, which may contaminate municipal drinking water systems (Juttner and Watson 2007). Given the ecological, economic, and human health concerns associated with cyanobacterial blooms, water resource managers need a rapid and accurate tool for quantifying the presence and abundance of cyanobacteria.

Microscopy has long been used to quantify phytoplankton biomass and species composition (Hasle 1978, Komárek and Anagnostidis 1986). Despite its advantages for fine-scale identification, microscopic enumeration of phytoplankton is extremely time consuming, depending on the abundance and composition of plankton and other organic and inorganic material. Moreover, using microscopy to obtain accurate estimates of phytoplankton biovolume can be time-consuming and challenging, especially for cyanobacterial taxa with irregular

morphologies (e.g., *Microcystis*). There has been a growing demand by regulatory agency and academic scientists for the development of new methods to more rapidly assess cyanobacterial abundance in water samples. For example, molecular techniques, such as qPCR and gene detection, are often used to rapidly detect toxin-producing cyanobacteria (Rinta-Kanto et al. 2009, Al-Tebrineh et al. 2011). However, molecular-based approaches are expensive, require extensive expertise, and often provide only crude estimates of cell density based on gene copies that are rarely calibrated with phytoplankton cell counts.

Phytoplankton contain a variety of pigments that are used for capturing sunlight for photosynthesis. The algal pigment, chlorophyll *a*, is widely used to estimate the abundance of freshwater and marine phytoplankton given its presence in all autotrophs and relative ease of analysis. Consequently, several approaches are available for measuring chlorophyll *a*, including spectroscopy and fluorometry (Lorenzen 1966, Leavitt et al. 1989, Riemann et al. 1989, Otsuki et al. 1994, Randolph 2007, Zimba 2012). Cyanobacteria contain phycobilin pigments, including phycocyanin (freshwater taxa) and phycoerythrin (marine taxa), that have absorption maxima between 550-650 nm (Rowan 1989, Lee et al. 1995) and provide many cyanobacterial taxa their distinctive blue-green color. Given its high market value in the food industry as a blue colorant (≈\$10-50 million a year), the extraction and purification of phycocyanin is well-studied (Siegelman and Kycia 1978, Santiago-Santos et al. 2004, Zhu et al. 2007, Chaiklahan et al. 2012). There is a growing interest by water resource managers and scientists towards using cyanobacteria-specific pigments to quantify cyanobacterial abundance (Stewart and Farmer 1984, Viskari and Colyer 2003, Lawrenz et al. 2011, Zimba 2012). For example, recent studies have compared efficacies of multiple phycocyanin extraction protocols (Viskari and Colyer 2003, Zimba 2012) or used *in situ* fluorometers with attached phycocyanin probes to estimate

cyanobacterial abundance (Lee et al. 1995, Brient et al. 2008, Marion et al. 2012). Marion et al. (2012) directly related *in situ* phycocyanin measurement with microcystin concentrations; however Bastien et al. (2011) reported no relationship between *in vivo* fluorescence of phycocyanin and the concentration of cyanotoxins. While some studies have related phycocyanin or chlorophyll *a* measurements to cyanobacterial abundance, there is limited data indicating the ability of these measurements to be used to estimate the abundance of cyanotoxins.

The primary aim of this study was to develop a rapid, reliable protocol to estimate cyanobacterial abundance in diverse lentic, freshwater habitats using fluorometry to measure phycocyanin. Ultimately, our results show that this new method can be used to quickly (i.e., same day) estimate cyanobacterial abundance from natural samples from many freshwater habitats including lakes and ponds that contain diverse phytoplankton assemblages.

MATERIALS AND PROCEDURES

Study Sites and Sample Collection

Sixteen ponds at the E. W. Shell Fisheries Research Center, Auburn University, varying in productivity were used for this study (Table 1.1). Seven ponds (G13, G19, G20, G48, G52, S11, and S22) were sampled 22 May 2012 using a clear polyvinyl chloride tube sampler (5 cm diam) for studies to optimize of our extraction procedure. In addition to our pond samples, we included two unialgal cultures, including the non-phycocyanin producing chlorophyte, *Ankistrodesmus falcatus* (as a negative control since green algae do not produce phycocyanin) and the cyanobacterium *Microcystis aeruginosa* (UTEX 2667) (as a positive control since cyanobacteria do produce phycocyanin). 10 additional ponds (E32, E33, E35, F4, F9, F11, S3,

S8, S10, and S28), including ponds G19, G20, G48, G52, S11, and S22 were sampled 7 September 2012 to further develop our relationship between cyanobacterial and phycocyanin concentrations. Pond S11 was sampled twice due to a change in the phytoplankton community between the two sampling dates.

Water samples from each pond were stored in coolers prior to being processed in the laboratory. Each sample was mixed well prior to collecting samples on 47 mm Pall A/E filters for chlorophyll *a* (two replicates) and phycocyanin (four replicates) analyses or poured into 20 mL glass vials and preserved with 1% Lugols solution for phytoplankton enumeration (two replicates). Filters were stored in the dark at -9°C until processed.

Chlorophyll *a* Analysis

Chlorophyll *a* concentrations were measured by extracting phytoplankton from filters in 90% ethanol for 24 h in the dark at 4°C followed by measurement with a Turner Designs Trilogy fluorometer using a non-acidification module (#7200-046) (Sartory and Grobbelaar 1984).

Phycocyanin Analysis

Filter grinding

To quantify phycocyanin, filters were ground in 10 mL of 50 mM phosphate buffer (Ricca Chemical Company, #5807-16) under reduced light, using a smooth Teflon grinder in a centrifuge tube for 1 min. Another 10 mL aliquot of phosphate buffer was used to rinse the grinder into the centrifuge tube, bringing the extract total to 20 mL. The centrifuge tube was capped, mixed well by hand, and immediately stored in darkness. The grinder was rinsed with clean buffer and dried between samples to prevent contamination.

Extraction

Samples were stored in darkness at 4°C for 2 h and then placed in a dark room for another 2 h to warm to room temperature ($\approx 21^\circ\text{C}$) prior to analysis. Thus total extraction time was 4 h.

Filtering samples

Given the sensitivity of the fluorometer, extracts were required to be filter sterilized prior to analysis. Following the 4 h extraction, a 4 mL aliquot of the extract was purified through a series of three separate 25-mm inline filters (A/E; 0.45 μm SEP (VWR #28145-485); 0.20 μm SEP (VWR #28145-499)). All syringes, filters, and the cuvette were rinsed with fresh buffer between samples to prevent contamination.

Fluorometric analysis

Filtered extracts were analyzed for phycocyanin using a Turner Designs Trilogy fluorometer fitted with an orange module (#7200-044) that accommodated a four-sided clear glass square cuvette (10 mm width, Nova Biotech, G-550). The sides of the cuvette were wiped well prior to each analysis since smudges were shown to interfere with the readings. Raw fluorescence units (RFUs) were converted to phycocyanin concentrations using a standard curve (range: 0-500 $\mu\text{g L}^{-1}$) created with phycocyanin (Sigma-Aldrich #P2172-10MG) dissolved in phosphate buffer. Prior to creating our standard curve, the stock phycocyanin standard concentration was confirmed using this equation: $\text{C-Phycocyanin (Mg ml}^{-1}\text{)} = (\text{Abs } 615\text{nm} - (0.474 * \text{Abs } 652\text{nm}))/ 5.34$

Phytoplankton enumeration

Phytoplankton species abundance and composition were determined using compound microscopy after settling phytoplankton samples in Palmer-Maloney chambers. On average, twenty-five fields were counted at each of three magnifications (100, 200, and 400x) for each sample. All samples were enumerated in duplicate. Average algal cell volumes were calculated for each taxon for each sample using standard measurements of ≥ 10 individuals, when available. Biovolume for each species for each sample was calculated by multiplying cell density by cell volume. Dominate phytoplankton taxa ($> 10\%$ biovolume) were determined for each pond sample (Table 1.2).

Statistical Analyses

Analysis of variance (ANOVA) with Tukey's multiple comparison test was used to compare extraction time (4 and 24 hr) and temperature (4 and 21°C) on phycocyanin concentration for three ponds (G13, G20, and G52) or across extraction times (0, 2, 4, 6, 8, 10, or 12 hr) at 4°C for one composite sample from three ponds (G13, G19, and G20) as well as the ratio of algal pigments to biomass for up to six ponds (G19, G20, G48, G52, S11, and S22). A paired t-test determined if phycocyanin extractions generally differed between temperatures (4 and 21°C) for two extraction times (4 and 24 hr) across three ponds (G13, G20, and G52). Pearson's correlation coefficients were calculated to examine the relationship between phytoplankton biomass and chlorophyll *a* concentration, cyanobacterial biomass and phycocyanin concentration, or chlorophyll *a* and phycocyanin concentrations. Homogeneity of slopes was conducted to examine if the slopes between algal biovolume and pigment concentrations for ponds and algal cultures were different prior to analysis of covariance

(ANCOVA). Data were log-transformed as needed to conform to assumptions of parametric statistics given the wide range in algal biovolume and pigment concentrations across ponds. For all tests $\alpha = 0.05$.

ASSESSMENT

Optimization of extraction:

Extraction time and temperature test:

To determine if an optimal extraction time existed under one of two temperatures, four replicate samples were taken from a composite sample of water collected from three ponds (G13, G20, and G52) and extracted for 0, 4 or 24 h at 4°C (standard refrigerator) or 21°C (room temperature). In general, cold extractions (4 and 24 h across the three ponds) provided higher estimates of phycocyanin relative to warm extractions (Paired t-test $P = 0.04$, Fig. 1.1). This difference was most pronounced at the 24 h extraction point ($P < 0.05$ for all three ponds, Fig.1.1).

To determine if a more specific extraction time at 4°C further optimized phycocyanin extraction, four replicate composite samples of water pooled from three ponds (G13, G19, and G20) was extracted for 0, 2, 4, 6, 8, 10, or 12 h. Although longer extractions (10-12 hr) showed relatively reduced concentration compared to shorter extraction time (2 vs. 10 h Tukey $P = 0.03$, 2 vs. 12 h Tukey $P = 0.08$), extraction times ranging from 2-8 h produced similar concentrations (all comparisons for these extraction times, Tukey $P > 0.59$, Fig. 1.2).

Relationships between algal pigments and phytoplankton biovolume

We confirmed the strong relationships between phytoplankton abundance and chlorophyll *a* concentration or cyanobacterial abundance and phycocyanin concentration through analyzing a series of diluted samples (100% (not diluted), 80%, 60%, 40%, and 20%) (four replicates of each dilution) from six ponds and two algal cultures. To more broadly develop the associations between algal pigment and abundance, four replicate samples were collected from ten additional ponds and two algal cultures, and were analyzed as previously described for chlorophyll *a*, phycocyanin, and phytoplankton biovolume.

There were strong relationships between chlorophyll *a* concentration ($\mu\text{g L}^{-1}$) and phytoplankton biovolume ($\text{mm}^3 \text{L}^{-1}$) for all six pond samples and the two algal cultures (all relationships $P < 0.05$, Fig. 1.3, Table 1.3). Although the slopes varied between chlorophyll *a* and phytoplankton biovolume across all six ponds and two cultures (homogeneity of slopes, interaction term $P = 0.045$; Appendix Fig. 1.1A), no differences in slope existed when analyzing data for only the ponds (homogeneity of slopes, interaction term $P = 0.17$). Excluding the interaction term for the pond data, we found significant differences across the six ponds for the relationship between chlorophyll and phytoplankton biovolume (ANCOVA, Pond $P < 0.001$).

Strong relationships between phycocyanin concentration ($\mu\text{g L}^{-1}$) and cyanobacterial biovolume ($\text{mm}^3 \text{L}^{-1}$) also were observed for the four ponds with abundant cyanobacteria (G19, G20, G48, and G52) and the culture of *Microcystis aeruginosa* (all $P < 0.05$, Fig. 1.4, Table 1.4). Slopes for phycocyanin and cyanobacterial biovolume varied significantly across the four ponds and *Microcystis* culture (homogeneity of slopes, interaction term $P < 0.002$; Fig. 1.6B). However, slopes for the four ponds did not differ statistically (homogeneity of slopes, interaction term $P = 0.144$) and a significant effect of phytoplankton community was observed for the relationship between phycocyanin and cyanobacterial biovolume (ANCOVA, Pond $P < 0.001$).

These findings were not surprising given that different cyanobacterial taxa dominated the ponds, including *Anabaena*, *Cylindrospermopsis*, or *Planktolyngbya*. The pattern for pond S11 was intriguing given that the algal community was dominated by *Oscillatoria* (Table 1.2) yet phycocyanin concentrations were near detection limits. Although *Oscillatoria* was extracted efficiently for three other ponds (Fig. 1.5B, Table 1.4), such a finding suggests that some cyanobacterial taxa may require other procedures for effective extraction of pigment from the cells. The ratio of algal pigment to biomass varied considerably more for cyanobacteria (ratio range = 2.95-19.83, including only pond data with strong relationships between phycocyanin and cyanobacterial biovolume, Table 4) than other phytoplankton (ratio range = 2.86-7.43, including all ponds, Table 3). The ratio of chlorophyll *a* concentration to phytoplankton biovolume was significantly affected by pond (ANOVA, $F_{5,29}= 92.2$, $P < 0.001$). In addition, the ratio of phycocyanin to cyanobacterial biovolume also was significantly dependent on pond (ANOVA, $F_{3,19}= 35.29$, $P < 0.001$).

Across 16 ponds varying widely in productivity and algal diversity, and two strongly contrasting algal cultures (one chlorophyte and one cyanobacterium), we found strong relationships between chlorophyll *a* concentration ($\mu\text{g L}^{-1}$) and phytoplankton biovolume ($\text{mm}^3 \text{L}^{-1}$) ($P < 0.001$, $r^2 = 0.89$, Fig. 1.5A), and phycocyanin concentration ($\mu\text{g L}^{-1}$) and cyanobacterial biovolume ($\text{mm}^3 \text{L}^{-1}$) ($P < 0.001$, $r^2 = 0.63$, Fig. 1.5B). Such robust patterns were surprising given that the algal communities were dominated by a wide range of phytoplankton, including *Anabaena*, *Ceratium*, *Cylindrospermopsis*, *Microcystis*, *Oscillatoria*, *Planktolyngbya*, and *Pseudoanabaena*.

Regression equations associated with these relationships useful in estimating phytoplankton or cyanobacterial abundance from algal pigments.

$$\log \text{ phytoplankton biovolume (mm}^3 \text{ L}^{-1}) = (\log \text{ chlorophyll } a \text{ (}\mu\text{g L}^{-1}) * 1.041) - 0.672$$

$$\log \text{ cyanobacterial biovolume (mm}^3 \text{ L}^{-1}) = (\log \text{ phycocyanin (}\mu\text{g L}^{-1}) * 0.573) + 0.296$$

Chlorophyll *a* and phycocyanin concentration also were highly correlated ($P < 0.001$, $r^2 = 0.862$, Fig. 1.5C). The relationship of the two algal pigments also could be used to help estimate abundance of cyanobacteria in water samples.

$$\log \text{ phycocyanin (}\mu\text{g L}^{-1}) = (\log \text{ chlorophyll } a \text{ (}\mu\text{g L}^{-1}) * 1.385) - 0.819$$

DISCUSSION

Several expensive or technical methods exist for quantifying cyanobacterial biomass, however there is a need for a rapid, inexpensive, and reliable method for quantifying cyanobacterial biovolume from whole water samples. The aim of this study was to develop a protocol using bench-top fluorometry of the cyanobacterial pigment, phycocyanin, to accurately estimate cyanobacterial biovolume in freshwater habitats.

Using two laboratory experiments that manipulated temperature or extraction time, we identified conditions that most effectively extract phycocyanin from mixed phytoplankton communities. The first experiment measured the effects of extraction time (4 vs 24 h) and temperature (4 vs 21°C) on phycocyanin concentration. Unlike our chlorophyll *a* extraction protocol, we found that a 24 h extraction period significantly reduced phycocyanin concentrations, especially under the 21°C (Fig. 1.1). These results were not surprising given the sensitivity of phycocyanin pigment. For example, through our protocol development we learned that phycocyanin degrades more rapidly under light than chlorophyll *a*. Thus, all extractions and sample processing must be done under low laboratory light and absolutely no direct or indirect sunlight. These results are consistent with those of another study which examined the sensitivity

of phycobilin pigments to pH, temperature, and light (Moreth and Yentsch 1970). Our data also indicate that samples extracted at 4°C were less variable than the samples extracted at 21°C. Such findings are consistent with other studies that have documented variation in phycocyanin extraction related to uncontrollable fluctuations in room temperature (Sarada et al. 1999, Doke 2005).

A second experiment highlighted that extractions lasting between 2-8 h provide similar extraction efficiencies. In addition, although two h extractions yielded the highest absolute phycocyanin concentration, it is necessary to allow an additional two h for the extracts to slowly warm to room temperature prior to fluorometry. Thus, a total extraction period of four h was ultimately used for final protocol. Our methods and results differ from a study which used HPLC to measure phycocyanin (Otsuki et al. 1994), which showed the highest extractions at 12 h using 10 mM phosphate buffer at 3°C. One notable difference between Otsuki et al. (1994) and our study is that we ground our samples prior to extraction since we found that manually disrupting the cells helped shorten extractions times in prior studies (unpublished data).

Our results, demonstrated a significant relationship between phycocyanin concentration and cyanobacterial biovolume (Fig. 1.5C). Similar results have been reported for other studies (Brient et al. 2008, McQuaid et al. 2011), which promoted the use of phycocyanin to estimate cyanobacterial abundance. These studies also aimed to interpret their data World Health Organization (WHO) water quality guidelines (Ahn et al. 2007, Brient et al. 2008, McQuaid et al. 2011). For example, Brient et al. (2008) reported that a phycocyanin concentration of 30 $\mu\text{g L}^{-1}$ is equivalent to the WHO alert level 1 of 20,000 cyanobacterial cells mL^{-1} . For our data, there was a mildly significant relationship between chlorophyll *a* and phytoplankton cell density (Fig.

1.7A), and no significant relationship between chlorophyll *a* and cyanobacterial cell density (Fig. 1.7B) or between phycocyanin concentration and cyanobacterial cell density (Fig. 1.7C), therefore, we cannot assess the pigment concentrations relevant to WHO alert levels associated with cyanobacterial densities. Cell density does account for the morphology of cyanobacteria, and therefore is not a good metric for estimating pigment concentration. However, cyanobacterial biovolume was highly correlated with pigment concentration (Fig. 1.5B), thus we recommend that others consider using cell biovolume as opposed to cell density in water quality guidelines.

Several *in situ* fluorometers can measure phycocyanin across space and time, but there are many unresolved issues that may lead to inaccurate estimates of cyanobacterial biomass. For example, several studies reported problems with estimating cyanobacterial abundance accurately because of technical problems related to the *in situ* fluorometer (e.g. clogging of the probe and light saturation interfering with the readings), abiotic factors (e.g. turbidity and nutrient loading), and intrinsic factors relating to cyanobacteria (e.g. variation in phycocyanin production depending on cyanobacterial growth stage, morphology and size, and relative abundance to other phytoplankton taxa) (Gregor et al. 2007, McQuaid et al. 2011, Chang et al. 2012). In addition, other issues may compromise utility of *in situ* fluorometers regarding how they are calibrated. In some studies, manufacturer settings are used without additional calibrations (Bowling et al. 2012, Song et al. 2013) or standards are made available in solvents that are not commonly used for pigment extraction. In other cases, phycocyanin probes are not calibrated to convert the raw fluorescence units (RFUs) into pigment concentrations (Song et al. 2013). Since RFUs are specific to each instrument and protocol, it is impossible to accurately compare phycocyanin measurements across studies. In some cases where *in situ* probes are calibrated, the probes are

calibrated for one taxa, typically *Microcystis aeruginosa*, regardless of the dominant type in the waterbody (Bastien et al. 2011, McQuaid et al. 2011).

Despite the issues associated with *in situ* fluorometry, its use is spreading. However, some contend that laboratory-based methods need to confirm *in situ* measurements (Brient et al. 2008, Bowling et al. 2012). In our study, we showed strong relationships between algal pigments and biovolume. However, in one pond (S11) dominated by *Oscillatoria*, our method was unable to estimate cyanobacterial abundance accurately. It is possible that our inability to accurately estimate cyanobacterial abundance from this pond is because *Oscillatoria* does not produce a lot of phycocyanin (Moreth and yentsch 1970). Given the diversity of cyanobacterial morphologies and high cell densities under bloom conditions, estimating cyanobacterial biovolume can be challenging and may lead to an over-estimation of cyanobacterial abundance.

Overall, our study confirms that the use of bench-top fluorometry is an effective way to estimate cyanobacterial biovolume from diverse water samples. The short (4 hour) extraction time and relatively simple extraction method make it a simple alternative for measuring cyanobacterial abundance relative to complex molecular or chemical techniques that may take more time and be cost-prohibitive. This method may be useful for water resource managers interested in monitoring drinking water sources for the presence and abundance of cyanobacteria.

COMMENTS AND RECOMMENDATIONS

While other methods exist for extracting and measuring phycocyanin, such as qPCR or HPLC (Otsuki et al. 1994, Rinta-Kanto et al. 2009, Zimba 2012), our data show that bench-top fluorometry can rapidly (i.e., same day) and accurately estimate cyanobacterial biovolume from

diverse freshwater samples. Given the sensitivity of phycocyanin, care must be taken to keep extracts away from direct light or excessive temperatures for maximum phycocyanin measurements. Temperature should be kept constant because as temperature increases the sample will fluoresce less, and will lead to an underestimation of cyanobacterial abundance. While a temperature correction factor exists for the measuring chlorophyll *a* using the Turner designs fluorometer, no temperature correction factor exists for measuring phycocyanin. We also found that any smudges on the glass cuvette or non-sterile extracts caused significant measurement errors.

It is important to note that cyanobacteria are not the only taxa to produce phycocyanin, but that cryptophytes have been shown to also produce this pigment (Hoef-Emden 2008). So while phycocyanin is a more cyanobacteria-specific pigment compared to chlorophyll *a*, it is not exclusive to the taxa. Cryptophytes were not abundant in our samples, but it may be necessary to confirm phycocyanin measurements with microscopy to assure that cyanobacteria are the dominant taxa in samples. If not, then cryptophytes may cause overestimates of cyanobacterial abundance, and thus lead to inappropriate measures and associated management actions.

At the beginning of our method development, we created a standard curve with phycocyanin. Due to differences between fluorometers, modules, and calibration methods, it is necessary for others to calibrate their fluorometers before using any method, including ours. Therefore, the data presented here should be used a reference, but it may be necessary for others to develop their own calibration curves, and regressions to accurately measure phycocyanin concentration using their own equipment for their study systems. As more phycocyanin data are

published, we expect that a stronger link between phycocyanin concentration and water quality will be achieved as is currently available with chlorophyll *a*.

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Table 1.1. Characteristics of ponds at E.W. Shell Fisheries Research Center sampled for this study. Data source = Boyd and Shelton (1984).

Pond	Latitude	Longitude	Area (ha)	Average depth (m)
E32	32°38'57.75"N	85°29'4.92"W	0.04	0.91
E33	32°38'57.13"N	85°29'4.97"W	0.04	0.91
E35	32°38'56.25"N	85°29'5.01"W	0.04	0.91
F4	32°39'11.62"N	85°29'17.12"W	0.04	0.91
F9	32°39'11.04"N	85°29'16.42"W	0.08	1.37
F11	32°39'10.16"N	85°29'15.35"W	0.08	1.37
G13	32°39'43.07"N	85°29'46.82"W	0.04	0.91
G19	32°39'41.30"N	85°29'46.95"W	0.04	0.91
G20	32°39'41.33"N	85°29'46.06"W	0.04	0.91
G48	32°39'42.05"N	85°29'42.92"W	0.04	0.91
G52	32°39'40.74"N	85°29'43.13"W	0.04	0.91
S3	32°40'49.55"N	85°30'54.95"W	3.84	1.71
S8	32°40'20.30"N	85°30'30.35"W	4.49	1.49
S10	32°40'10.37"N	85°30'30.22"W	1.32	1.74
S11	32°40'23.71"N	85°30'25.45"W	1.15	1.37
S22	32°40'45.28"N	85°30'56.16"W	0.87	1.80
S28	32°40'12.30"N	85°30'19.99"W	1.92	1.65

Table 1.2. Dominant phytoplankton taxa (>10% total biovolume) present in sixteen ponds that were used to develop a relationship between phycocyanin concentration and cyanobacterial biovolume.

Pond	Dominant phytoplankton taxa (% of total biovolume)	
	Cyanobacteria	Other taxa
E32	<i>Oscillatoria</i> (94.6%)	none
E33	<i>Oscillatoria</i> (97.2%)	none
E35	<i>Oscillatoria</i> (99.2%)	none
F4	<i>Cylindrospermopsis</i> (24.6%)	<i>Gymnodinium</i> (32.9%) <i>Phacus</i> (21.6%)
F9	<i>Cylindrospermopsis</i> (71.5%) <i>Microcystis</i> (24.2%)	none
F11	<i>Cylindrospermopsis</i> (84.3%) <i>Anabaena</i> (13.7%)	none
G19	<i>Cylindrospermopsis</i> (98%)	none
G20	<i>Cylindrospermopsis</i> (68.4%) <i>Planktolyngbya</i> (15%)	none
G48	<i>Anabaena</i> (84.4%) <i>Cylindrospermopsis</i> (15.2%)	none
G52	<i>Planktolyngbya</i> (99.9%)	none
S3	<i>Cylindrospermopsis</i> (93.4%)	none
S8	<i>Cylindrospermopsis</i> (70.8%) <i>Microcystis</i> (27.6%)	none
S10	<i>Cylindrospermopsis</i> (92.1%)	none
S11 (5/22/12)	<i>Oscillatoria</i> (96.8%)	none
S11 (9/7/12)	<i>Cylindrospermopsis</i> (62.5%) <i>Microcystis</i> (32.3%)	none
S22	none	<i>Ceratium</i> (100%)
S28	<i>Pseudoanabaena</i> (47.2%) <i>Anabaena</i> (40.4%)	none

Table 1.3. Results for the relationship between chlorophyll *a* concentration ($\mu\text{g L}^{-1}$) and phytoplankton biovolume ($\text{mm}^3 \text{L}^{-1}$) of two cultures (the chlorophyte, *Ankistrodesmus*, and the cyanobacterium, *Microcystis aeruginosa* (UTEX 2667)) and six ponds at the Auburn University E. W. Shell Fisheries Research Center (ponds G19, G20, G48, G52, S11, and S22). Regression equation: phytoplankton biovolume = (chlorophyll *a* x slope) + y-intercept. Ratio = average chlorophyll *a* ($\mu\text{g L}^{-1}$) divided by phytoplankton biovolume ($\text{mm}^3 \text{L}^{-1}$) measured across the dilution gradient. S.E. = standard error. r^2 = proportion of variation. *p*-value = relationship between chlorophyll and phytoplankton biovolume. One chlorophyll sample for pond S11 was lost prior to sample analysis. Data are graphically shown in Figure 1.3.

Sample	Ratio	Slope (S.E.)	y-intercept (S.E)	r^2	<i>p</i>
<i>Ankistrodesmus</i> culture	6.28	0.205 (0.017)	-0.218 (0.215)	0.981	0.001
<i>Microcystis</i> culture	6.11	0.168 (0.020)	-0.095 (0.571)	0.961	0.003
G19 pond	6.56	0.162 (0.004)	-2.433 (1.484)	0.998	<0.001
G20 pond	7.43	0.143 (0.010)	-0.603 (1.764)	0.986	<0.001
G48 pond	3.23	0.372 (0.011)	-4.748 (1.439)	0.998	<0.001
G52 pond	4.61	0.226 (0.013)	-1.385 (4.239)	0.991	<0.001
S11 pond	6.10	0.134 (0.016)	2.774 (2.055)	0.973	0.013
S22 pond	2.86	0.342 (0.027)	0.357 (3.097)	0.982	0.001

Table 1.4. Results showing the relationship between phycocyanin concentration ($\mu\text{g L}^{-1}$) and cyanobacterial biovolume ($\text{mm}^3 \text{L}^{-1}$) of two cultures [the chlorophyte, *Ankistrodesmus*, and the cyanobacterium, *Microcystis aeruginosa* (UTEX 2667)] and six ponds at the Auburn University E. W. Shell Fisheries Research Center (ponds G19, G20, G48, G52, S11, and S22). Regression equation: cyanobacterial biovolume = (phycocyanin x slope) + y-intercept. Ratio = average of phycocyanin ($\mu\text{g L}^{-1}$) divided by cyanobacterial biovolume ($\text{mm}^3 \text{L}^{-1}$) measured across the dilution gradient. S.E. = standard error. r^2 = proportion of variation. p = relationship between phycocyanin and cyanobacterial biovolume. Data are graphically presented in Figure 1.4.

Sample	Ratio	Slope (S.E.)	y-intercept (S.E)	r^2	p
<i>Ankistrodesmus</i> culture	not applicable				
<i>Microcystis</i> culture	1.87	0.243 (0.029)	1.830 (0.384)	0.960	0.003
G19 pond	11.97	0.056 (0.005)	13.027 (4.041)	0.977	0.002
G20 pond	19.83	0.040 (0.007)	3.506 (3.132)	0.916	0.011
G48 pond	2.95	0.311 (0.030)	3.499 (4.076)	0.974	0.002
G52 pond	10.90	0.079 (0.009)	7.675 (7.232)	0.966	0.003
S11 pond	0.09	6.781 (4.445)	10.607 (10.343)	0.437	0.225
S22 pond	29.80	0.016 (0.017)	-0.015 (0.066)	0.238	0.404

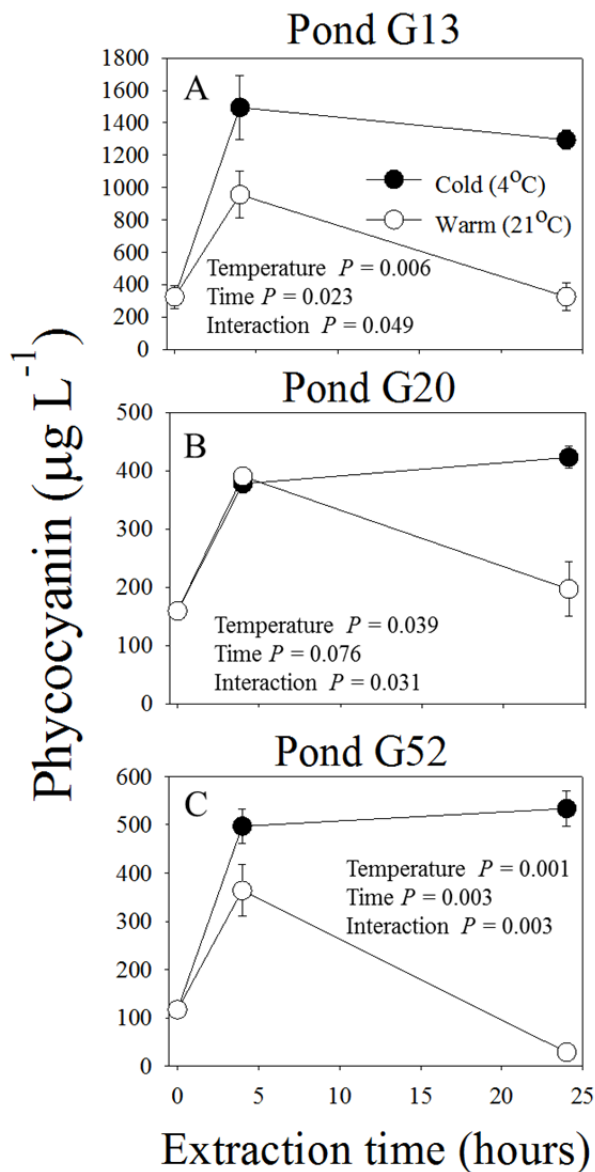


Figure 1.1. Phycocyanin concentration ($\mu\text{g L}^{-1}$) from three ponds at the Auburn University E. W. Shell Fisheries Research Center (ponds (A) G13, (B) G20, and (C) G52) analyzed over a range of extraction times (0, 4, and 24 h) and under two temperatures (cold = 4°C and warm = 21°C). Replicate subsample mean \pm standard error. All three pond samples showed significantly lower phycocyanin concentration after a 24 h extraction at 21°C relative to concentrations observed after a 24 h extraction at 4°C (G13= .012, G20= .046, G52 $p = 0.026$).

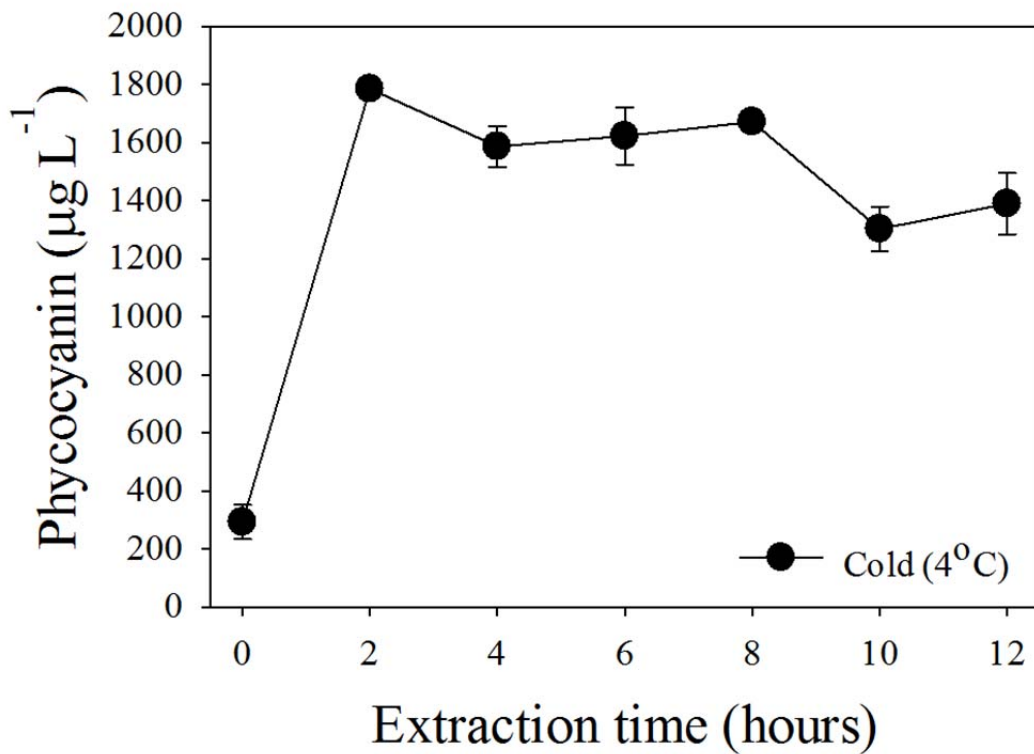


Figure 1.2. Phycocyanin concentration ($\mu\text{g L}^{-1}$) of a mixture of seston collected from several ponds at the Auburn University E. W. Shell Fisheries Research Center analyzed over a range of extraction times (0-12 hours) at 4°C . Replicate subsample mean \pm standard error.

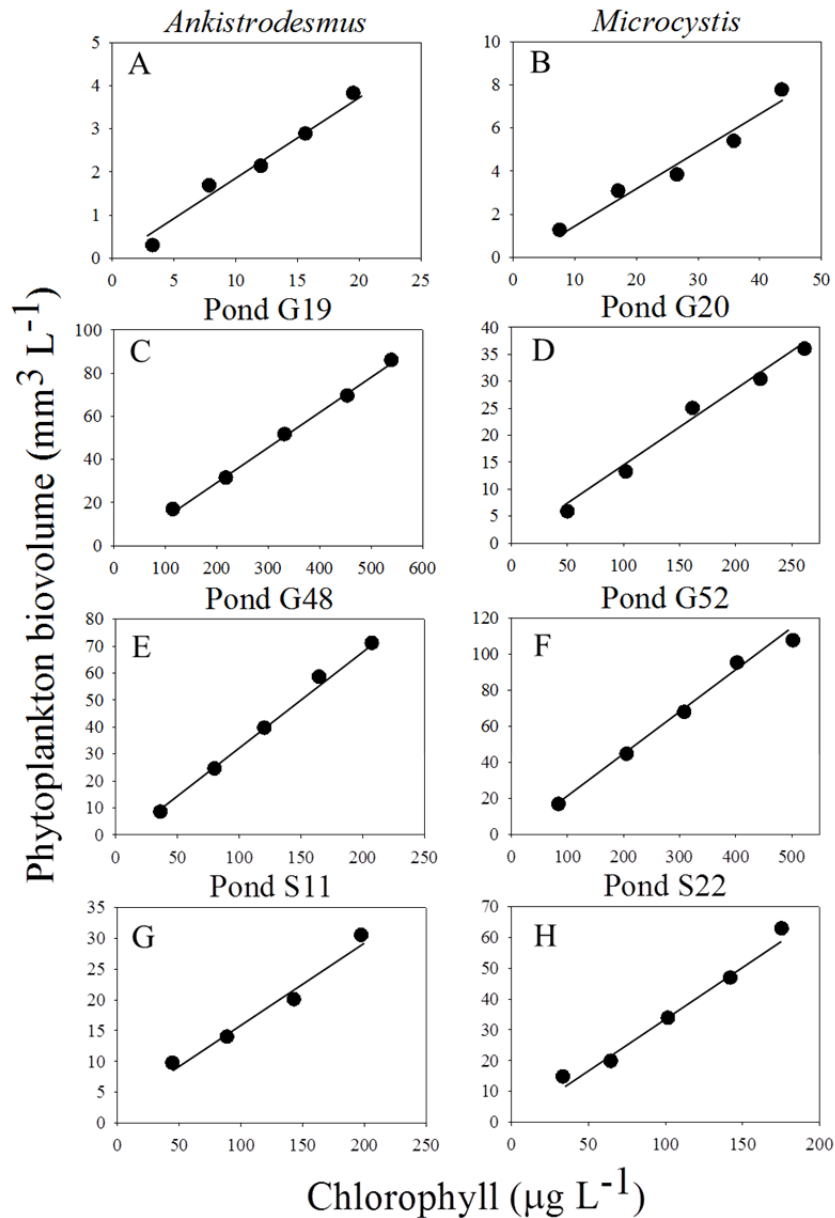


Figure 1.3. Relationship between chlorophyll *a* concentration ($\mu\text{g L}^{-1}$) and phytoplankton biovolume ($\mu\text{m}^3 \text{ml}^{-1}$) of two cultures ((A) the chlorophyte, *Ankistrodesmus*, and (B) the cyanobacterium, *Microcystis aeruginosa* (UTEX 2667)) and six ponds at the Auburn University E. W. Shell Fisheries Research Center (ponds (C) G19, (D) G20, (E) G48, (F) G52, (G) S11, and (H) S22). One chlorophyll *a* sample collected for the highest algal concentration for pond S11 was lost.

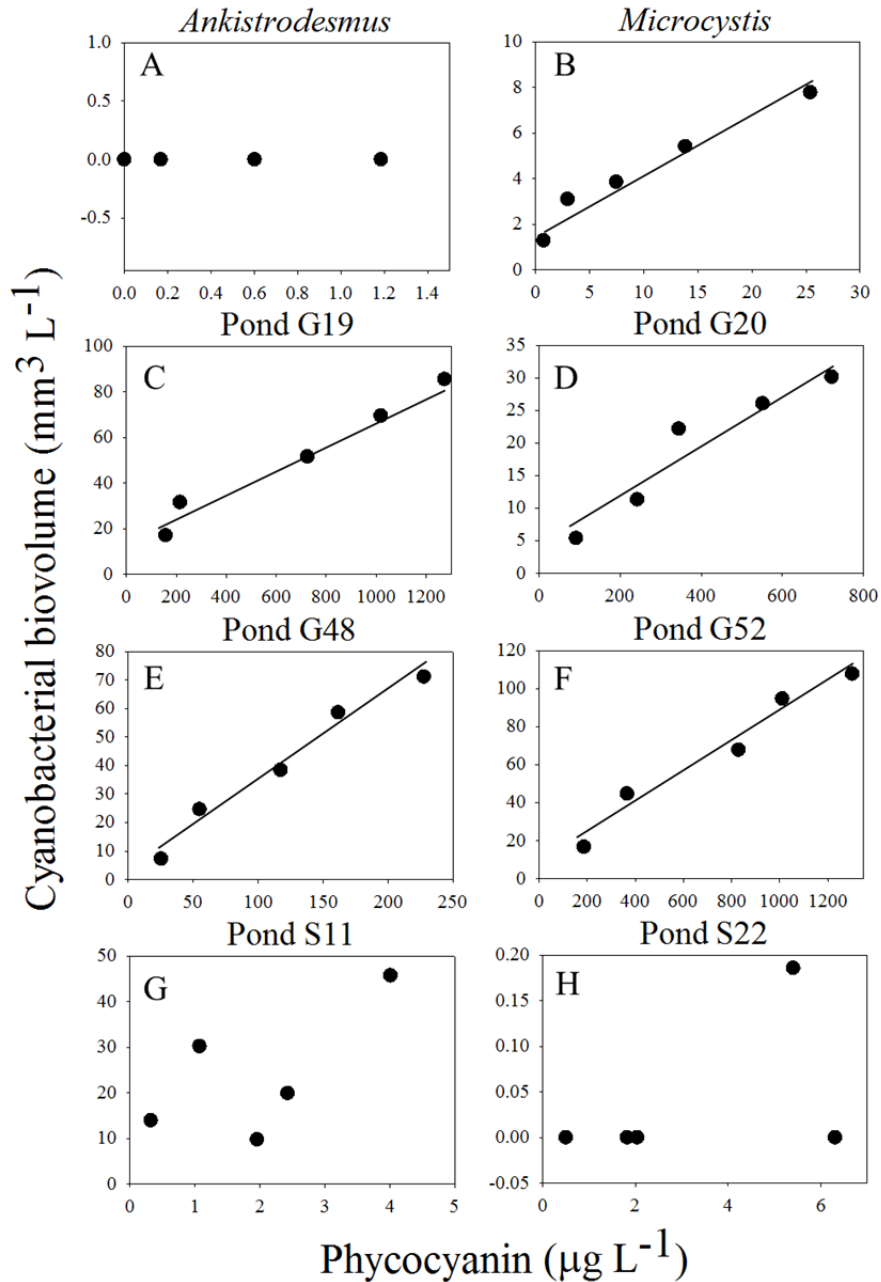


Figure 1.4. Relationship between phycocyanin concentration ($\mu\text{g L}^{-1}$) and cyanobacterial biovolume ($\mu\text{m}^3 \text{ml}^{-1}$) of two cultures (A) the chlorophyte, *Ankistrodesmus*, and (B) the cyanobacterium, *Microcystis aeruginosa* (UTEX 2667) and six ponds at the Auburn University E. W. Shell Fisheries Research Center (ponds (C) G19, (D) G20, (E) G48, (F) G52, (G) S11, and (H) S22).

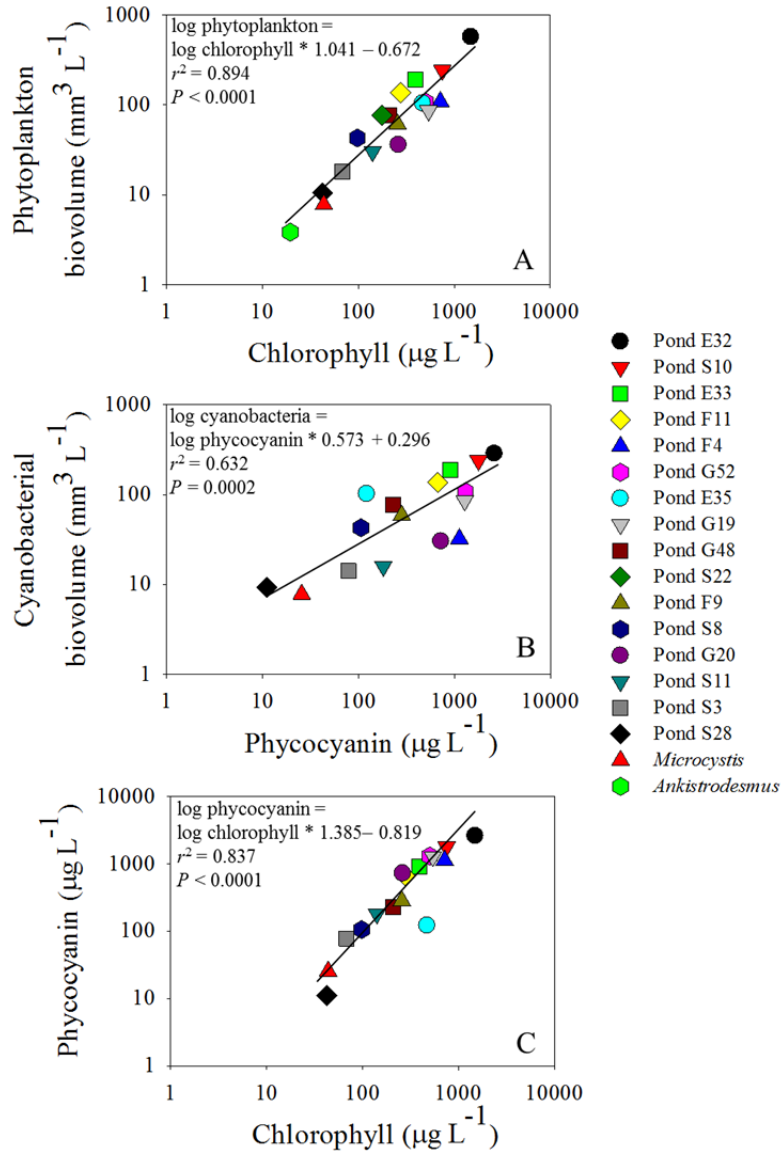


Figure 1.5. General patterns between (A) chlorophyll *a* concentration (μg L⁻¹) and phytoplankton biovolume (μm³ ml⁻¹), (B) phycocyanin concentration (μg L⁻¹) and cyanobacterial biovolume (μm³ ml⁻¹), or (C) chlorophyll *a* concentration (μg L⁻¹) and phycocyanin concentration (μg L⁻¹) of two cultures (the chlorophyte, *Ankistrodesmus*, and the cyanobacterium, *Microcystis aeruginosa* (UTEX 2667)) and 16 ponds at the Auburn University E. W. Shell Fisheries Research Center (ponds G19, G20, G48, G52, S11, S22, S28, S3, S8, F11, E33, F4, S10, E32, and E35).

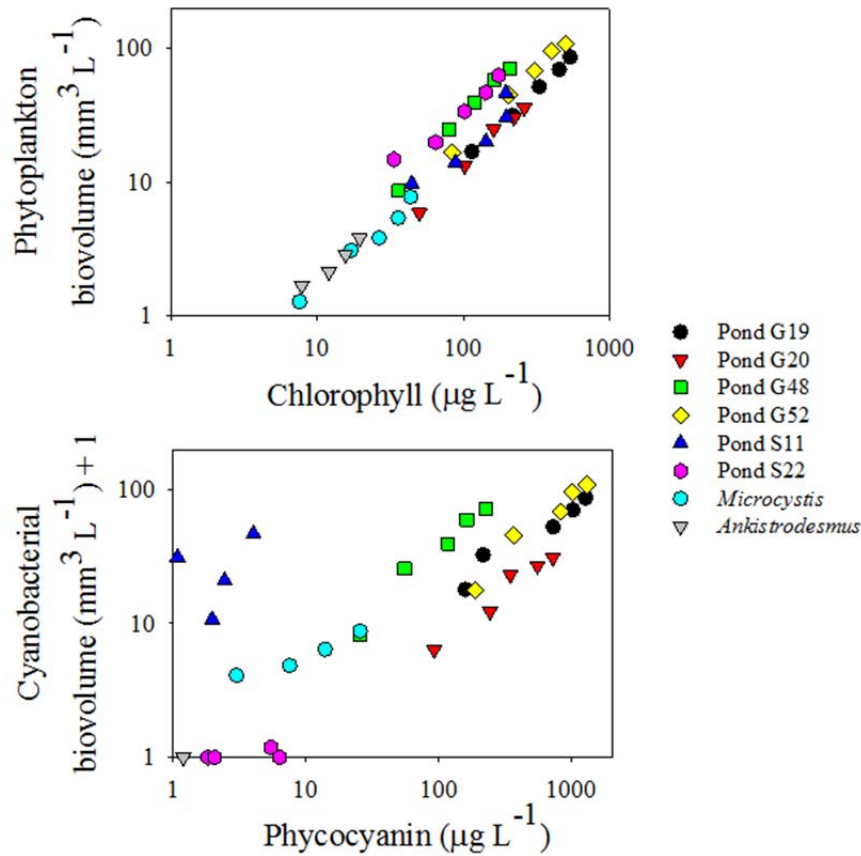


Figure 1.6. General patterns between (A) chlorophyll *a* concentration ($\mu\text{g L}^{-1}$) and phytoplankton biovolume ($\mu\text{m}^3 \text{mL}^{-1}$), and (B) phycocyanin concentration ($\mu\text{g L}^{-1}$) and cyanobacterial biovolume ($\mu\text{m}^3 \text{mL}^{-1}$), of two cultures (the chlorophyte, *Ankistrodesmus*, and the cyanobacterium, *Microcystis aeruginosa* (UTEX 2667)) and 6 ponds at the Auburn University E. W. Shell Fisheries Research Center (ponds G19, G20, G48, G52, S11, S22).

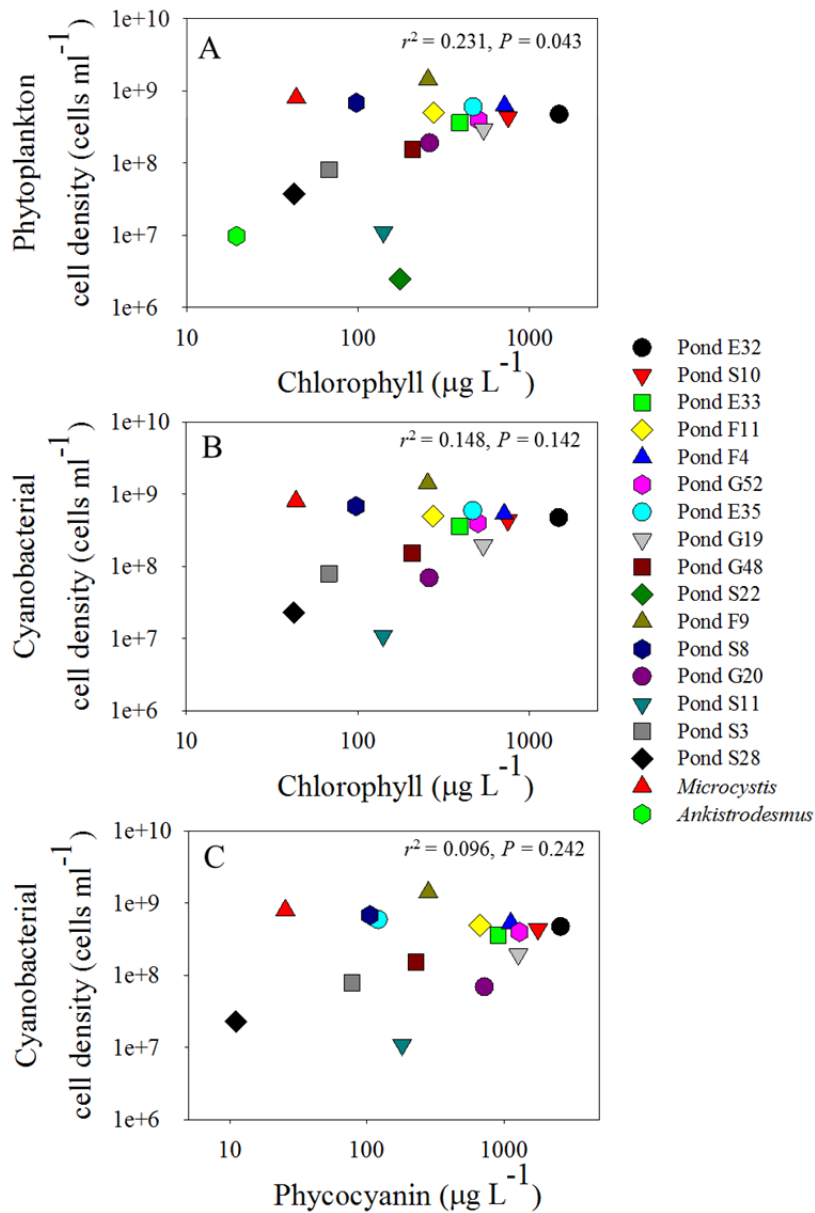


Figure 1.7. General patterns between (A) chlorophyll *a* concentration ($\mu\text{g L}^{-1}$) and cyanobacterial cell density (cells mL^{-1}), and (B) phycocyanin concentration ($\mu\text{g L}^{-1}$) and cyanobacterial cell density (cells mL^{-1}), of two cultures (the chlorophyte, *Ankistrodesmus*, and the cyanobacterium, *Microcystis aeruginosa* (UTEX 2667)) and 16 ponds at the Auburn University E. W. Shell Fisheries Research Center (ponds G19, G20, G48, G52, S11, S22, S28, S3, S8, F11, E33, F4, S10, E32, and E35).

GRASS CARP DO NOT CONSUME THE INVASIVE BENTHIC CYANOBACTERIUM,
LYNGBYA WOLLEI

ABSTRACT

Grass carp have been well studied for their ability to control a variety of aquatic weeds, including submersed and floating macrophytes. Despite limited data, grass carp are promoted as a form of control for filamentous algae, including cyanobacteria. One important nuisance cyanobacterium, *Lyngbya wollei*, is an invasive species in North America that forms benthic and surface mats and can produce multiple intracellular toxins. Current management of *Lyngbya* calls for approaches similar to those for other nuisance aquatic plants, including chemical control using herbicides and biological control using grass carp (*Ctenopharyngodon idella*). While pond managers recommend stocking grass carp to control filamentous algae, no conclusive experimental data exist that show that grass carp consume *Lyngbya*. We conducted mesocosm experiments where different densities of grass carp of varying sizes were fed diets either containing a palatable macrophyte, *Myriophyllum*, and/or *Lyngbya*. In general, grass carp did not consume *Lyngbya* when offered by itself or with *Myriophyllum*. These results were consistent when fish densities or sizes were varied. Additional feeding experiments suggest that the secondary chemistry, but not the physical structure, of *Lyngbya* prevents grass carp consumption. Thus, if grass carp are an effective biological control for *Lyngbya* in ponds, then consumption is not the mechanism of control.

INTRODUCTION

Since their introduction into the United States in 1963 for aquatic plant management (Courtenay et al. 1984), grass carp (*Ctenopharyngodon idella*) have been well studied for their feeding preference and ability to control aquatic weeds (Pine et al. 1989, Hanlon et al. 2000, Dibble and Kovalenko 2009). Grass carp, especially small individuals ($\approx 10\text{-}13$ cm), can consume two to three times their body weight of plant material per day (Wright and Reeves 2004) and have been shown to effectively control submerged and floating macrophytes under certain conditions (Stoot and Orr 1970, Osborne and Sassic 1981, Hanlon et al. 2000, Piplova 2006). However, despite limited data and disagreement in the literature, grass carp are routinely promoted for controlling filamentous algae, including benthic cyanobacteria (Dyck 1994, Madsen et al. 2012, Zolcynski and Smith 1980).

Lyngbya wollei (hereafter *Lyngbya*) is an invasive, benthic, filamentous cyanobacterium that is aesthetically displeasing and potentially harmful to other organisms because of the ability of some strains to produce intracellular dermatotoxins, hepatotoxins, and neurotoxins (Carmichael et al. 1997, Burns 2004). It also produces musty off-flavor compounds, including methylisoborneol and geosmin, which can taint drinking water and aquaculture-raised fishes (Schrader and Blevins 1993). *Lyngbya* is tolerant of reduced light (Speziale et al. 1988, Speziale et al. 1991) and can form dense mats on lake bottoms in nutrient enriched habitats. Mats may become displaced and float to the water surface where they effectively shade other autotrophs (Paerl and Huisman 2009). In addition, *Lyngbya* is a perennial species that can grow throughout the year, allowing it to create massive standing crops that exceed those of other nuisance filamentous algae (Speziale et al. 1991).

Due to the nuisance and potential negative economic impacts caused by this species, recreational fisheries and water resource managers are interested in controlling *Lyngbya*. Grass carp have often been promoted as an effective biological control of *Lyngbya* (Zolczynski and Smith 1980; Wright and Reeves 2004), yet, limited data indicate that grass carp may control filamentous algae, including *Lyngbya* (Zolczynski and Smith 1980). One study reported that grass carp may be ineffective at controlling *Lyngbya* because it is not a preferred diet item (Dyck 1994); however, we are unaware of any replicated studies documenting that grass carp consume *Lyngbya*. High grass carp stocking rates (62-125 grass carp per ha) are often suggested for controlling filamentous algal blooms, including *Lyngbya* blooms (Wright and Reeves 2004). Data are needed that clearly document the trophic interaction between grass carp and *Lyngbya*.

The primary objectives of our study were to determine the effect of grass carp size and density, as well as food choice, on consumption of *Lyngbya*. In general, we found that grass carp do not consume *Lyngbya* with or without alternative diets. Secondly we were interested in examine if morphological defenses of *Lyngbya* deterred grass carp consumption. Plant defenses against consumers are diverse and can include structures (thorns, sheaths), size, and secondary metabolites (Ehrlich and Raven 1964, Gomez and Zamora 2002). Feeding assays using pellets created from ground, dried *Lyngbya*, lettuce and fish feed suggested that secondary metabolites, but not morphological properties, of *Lyngbya* deter grass carp consumption.

METHODS

Study Site

All experiments were conducted in the wet lab facility at the E. W. Shell Fisheries Station, Auburn University. A recirculating tank system without a biofilter, was used for all experiments. Eighteen aerated 150 L tanks were partially filled (95-112 L) with dechlorinated water and

covered with window screen prior to holding grass carp. The ground plant material experiment was conducted in aerated 25 L mesocosms partially filled (13 L) with dechlorinated water and covered with window screen.

Study Organisms

Prior to each experiment, grass carp were seined from one of three holding ponds, including pond M1 (small fish, 7-12 cm TL), pond M6 (medium fish, 14-19 cm TL), and pond M4 (large fish, 21-24 cm TL), and housed in a sump attached to the recirculating tank system or directly in experimental tanks. Fish were fasted for 2-3 d prior to the start of each experiment to increase feeding rate. Mortality replacement fish were housed in the sump.

Lyngbya was collected from pond S1 at the E. W. Shell Fisheries Station and returned to the laboratory to remove large debris, and alternative food for grass carp, including, dragonfly larvae, snails, leaches, worms, sticks, and leaves. Given the structural complexity of *Lyngbya* in the field, smaller items, including other minor components of the algal community, were not removed. Thus, *Lyngbya* used for our experiments was not a pure culture but instead a “*Lyngbya* complex” consistent with material found in nature. *Lyngbya* accounted for $\approx 95\%$ of the biomass fed to grass carp.

Myriophyllum heterophyllum (variable leaf water milfoil; hereafter *Myriophyllum*) is a native aquatic plant that grass carp are known to consume and served as a control diet in the experiments. *Myriophyllum* was collected from Okhussee Thloko, a public fishing pond in Tuskegee, Alabama, and returned to the laboratory for cleaning prior to being used in feeding experiments.

Grass carp feeding assays

Lyngbya vs. *Myriophyllum* choice experiment

Five small (length range = 7-12 cm TL) grass carp (hereafter fish) were added to each of nine tanks where they were fasted for two d before being simultaneously offered two different diets, including *Myriophyllum* (10 g wet weight) and *Lyngbya* (5 g wet weight), that were attached to 7.6 cm by 7.6 cm plastic mats secured to the bottom of each tank. Both plants were also added to nine other control tanks that lacked fish to measure autogenic changes in plant mass during the experiment. Dataloggers were used to measure temperature from all enclosures during the feeding trial (mean water temperature = 27°C). Four additional samples of each diet were collected at the start of the experiment to estimate plant wet-to-dry weight conversion factors. Plant dry weights were estimated by drying samples for 24 h at 50°C. Remaining plant materials was collected from each tank after 24 h and dried. Percent change in plant dry weight was estimated for each plant species in each tank with the following equation: $100 * [(T_f / (T_i * \text{correction factor})) - 1]$ where T_i and T_f were the initial and final weights for the treatment diet (modified from Stachowicz and Hay 1996). The correction factor was calculated as the average ratio of the control diet final to initial weights.

Lyngbya or Myriophyllum no-choice experiment

Five small (length range = 7-12 cm TL) fish were added to each of ten tanks where they were fasted for two d before being separately offered one of two different diets, including *Myriophyllum* (21.4 g wet weight, ~1.5 g dry weight) or *Lyngbya* (5 g wet weight, ~1.5 g dry weight), which were attached to plastic mats secured to the bottom of each tank. Dataloggers collected temperature data from all enclosures during the feeding trial (mean water temperature = 23°C). Each plant also was added separately to four control tanks lacking fish to measure autogenic changes in plant mass during the experiment. Four additional samples of each diet

were collected at the start of the experiment to calculate plant wet-to-dry weight conversion factors. Plant material in the tanks was collected after 24 h and dried as previously described to estimate change in dry weight of plant material in the experimental tanks.

In order to confirm that grass carp response was not a function of the fish being satiated, after the *Lyngbya* was removed from tanks with fish in the second no-choice experiment, *Myriophyllum* (21.4g wet weight) was added to each tank. After 24 h any remaining plant material was removed, dried, and percent change estimated.

Grass carp density no-choice experiment

A third experiment was conducted to determine if fish density influenced preference for *Lyngbya* or *Myriophyllum*. In response to a disease outbreak prior to the start of this experiment, experimental fish were quarantined in a separate tank system and treated for one wk prior to use with formalin (100 ppm every day and 200 ppm every third day) to remove any external parasites. These formalin treatments helped to reduce fish mortality during our experiments. Small grass carp (length range = 7-12 cm TL) were added to each of 12 tanks (six tanks with 5 fish, and six tanks with 10 fish) where they were fasted for three d before being separately offered either including *Myriophyllum* (21.4 g wet weight) or *Lyngbya* (5 g wet weight), attached to plastic mats secured to the bottom of each tank. Thus, a total of three replicates per density treatment containing fish was used. Each plant species was also added to three control tanks to measure autogenic changes in plant mass during the experiment. Dataloggers collected temperature data from all enclosures during the feeding trial (mean water temperature = 22°C). After 24 h, two fish were found to have escaped their tanks and were replaced with similar sized fish. After an additional 24 h (48h total), all plant material was collected and processed as

described above for earlier experiments to calculate wet and dry weights. Initial dry weights were estimated from a wet-to-dry weight regression created for *Myriophyllum* or *Lyngbya* from preliminary experiments.

Grass carp size no-choice experiment

A fourth experiment was conducted to determine if fish size influenced their preference for *Lyngbya* or *Myriophyllum*. Eight small (length range = 7-12 cm TL), four medium (length range = 14-19 cm TL), or two large (length range = 21-24 cm TL) fish were added to one of 12 tanks (four tanks per fish size treatment) where fish were fasted for two d before being offered *Lyngbya* (5 g wet weight) attached to a plastic mat secured to the bottom of each tank. Dataloggers collected temperature data from all enclosures during the feeding trial (mean water temperature = 25°C). Four additional samples of *Lyngbya* were collected to estimate a wet-to-dry weight conversion factor for the start of the experiment. Plant material in tanks was collected after 24 h and dried as above estimate change in dry weight of plant material in tanks.

After the *Lyngbya* was removed from treatment tanks, *Myriophyllum* (20g wet weight) was added for 24 h and any remaining plant material was removed and dried to determine if grass carp avoidance of *Lyngbya* could be attributed to fish satiation. Prior to replacing *Lyngbya* with *Myriophyllum*, four dead fish in separate tanks were replaced with appropriately sized mortality replacement fish. Relative change in dry weight in plant material was estimated as above.

Ground plant material experiment

To determine if the structure of *Lyngbya* deters grass carp consumption, *Lyngbya* was freeze-dried and ground into a fine powder that was then incorporated into agar-based pellets (15%

sodium alginate for every pellet). To promote grass carp consumption of pellets, feed used to maintain the fish was crushed and added to all agar pellets (accounted for 28% of each pellet by dry mass). Thus, all pellets contained 28% fish feed and 15% sodium alginate. To include a palatable control plant to the agar pellets as a replacement for *Lyngbya* biomass, iceberg lettuce was lyophilized and ground prior to being incorporated into agar pellets. Four pellet diets were included in this experiment: (1) 57% lettuce, 0% *Lyngbya* (control), (2) 40% lettuce, 17% *Lyngbya*, (3) 19% lettuce, 38% *Lyngbya* and (4) 0% lettuce, 57% *Lyngbya*. All percentages are of the total dry mass of the pellets. Each pellet type (total wet weight fed to fish = 0.3 g) was offered separately to three small (length range = 7-12 cm TL) grass carp that were fasted for seven d to maximize potential consumption. There were four replicates per treatment with fish and two replicates of each treatment in mesocosms without fish to account for changes in pellet mass not due to fish. After 30 min, pellets were removed and final wet weights were recorded. Percent change in pellet weight was calculated, as described before for percent change in plant mass.

Statistical analysis

Analysis of variance (ANOVA) with Tukey's pairwise comparisons was used to assess differences among treatment means. One-way student T-tests determined if treatment means were equal to 0. For two experiments (*Lyngbya* or *Myriophyllum* no-choice experiment and Grass carp size no-choice experiment) where *Myriophyllum* was offered to the same fish previously exposed to *Lyngbya*, a paired T-test was used to compare treatment means for plant weight change of control (*Myriophyllum*) and experimental (*Lyngbya*) diets. All values were considered significant at $\alpha < 0.05$.

RESULTS

When given a choice between *Myriophyllum* and *Lyngbya*, grass carp consumed significantly more *Myriophyllum* than *Lyngbya* ($F_{1,16} = 676, P < 0.001$). In fact, grass carp reduced *Myriophyllum* biomass by >80% whereas had no effect on *Lyngbya* biomass (t-test mean = 0, $T_8 = 0.47, P = 0.65$) (Fig. 2.1). In the no-choice experiment, grass carp consumed >75% *Myriophyllum* biomass when offered alone. Similar to the diet choice experiment, grass carp did not consume *Lyngbya* when it was offered separately (t-test mean = 0, $T_8 = 1.46, P = 0.22$) (Fig. 2.2). Again, there was a strong difference in the amount consumed by grass carp when comparing consumption of both diets ($F_{1,8} = 232, P < 0.001$). To determine if fish not eating *Lyngbya* were satiated, we replaced *Lyngbya* with *Myriophyllum* and found that fish previously avoiding *Lyngbya* consumed a similar amount of *Myriophyllum* compared to fish initially offered this edible plant ($F_{1,9} = 0.18, P = 0.69$) (Fig. 2.2). The same fish that were initially offered and avoided *Lyngbya* ($T_4 = 14, P < 0.01$) and then fed *Myriophyllum* consumed >75% of the control diet (t-test mean = 0, $T_4 = 12, P < 0.001$) (Fig. 2.2), indicating that these fish were hungry but actively avoided *Lyngbya*. Three fish died during this experiment; however, we did not observe any effects of these deaths on the feeding behavior of other fish in the affected tanks.

Fish density (5 or 10 fish) showed to have effects on plant consumption (ANOVA, food type ($F_{1,8} = 107, P < 0.001$), fish density ($F_{1,8} = 7, P = 0.03$), food type x fish density ($F_{1,8} = 13, P = 0.01$)). For example, fish had a large negative effect on *Myriophyllum* but no effect on *Lyngbya* (Fig. 2.3). And, as expected, the high fish density treatment resulted in higher consumption of *Myriophyllum* ($F_{1,5} = 14, P = 0.02$), whereas fish at both densities avoided *Lyngbya* (t-test mean = 0; five fish treatment; $T_5 = 0.70, P = 0.56$; ten fish treatment; $T_5 = -0.40, P = 0.73$).

Fish size also had no significant impact on amount of *Lyngbya* consumed (ANOVA, $F_{2,9} = 0.74$, $P = 0.50$; t-test mean = 0; small fish: $T_3 = 0.04$, $P = 0.97$; medium fish: $T_3 = -1.55$, $P = 0.22$; large fish: $T_3 = 0.63$, $P = 0.57$) (Fig. 2.4). As in the *Lyngbya* or *Myriophyllum* no-choice experiment, fish offered *Myriophyllum* after being offered *Lyngbya* generally consumed *Myriophyllum* (paired t-test; $T_{11} = 5$, $P < 0.001$) (Fig. 2.4). The lone exception to this pattern was the large fish treatment (t-test mean= 0; $T_3 = -1.69$, $P = 0.19$).

Fish generally avoided agar pellets made from fish feed and lettuce containing *Lyngbya* (ANOVA, $F_{3,12} = 156$, $P < 0.001$) (Fig. 2.5). In fact, fish avoided pellets containing $\geq 17\%$ (by biomass) *Lyngbya* while readily consuming pellets lacking this cyanobacterium (t-test mean= 0; $T_3 = -48.48$, $P < 0.001$).

DISCUSSION

In general, grass carp did not consume *Lyngbya* despite conducting several experiments that manipulated available whole plant diets, fish density, fish size, and percent composition of ground *Lyngbya* in pellet diets.

Grass carp have been studied extensively for their preference and ability to consume multiple types of aquatic macrophytes (166 publications cited in review by Dibble and Kovalenko 2009). Some studies have documented that grass carp consume filamentous green algae (*Cladophora* sp., *Chara* sp., *Nitella* sp., and *Pithophora* sp.), especially after preferred macrophytes are eliminated, but that grass carp avoid consuming some species entirely (Avault 1965, Lembi et al. 1978, Lewis 1978, Pine et al. 1989, Colle and Shireman 1994). For example, *Spirogyra* sp. is avoided because of the texture of the alga making it difficult to ingest (Prowse 1971). There is mixed evidence that grass carp can consume different species of filamentous algae, whereas only one unreplicated study documented that grass carp control the filamentous cyanobacterium

Lyngbya (Zolczynski and Smith 1980). In that study grass carp stocked at a rate of 74 fish per hectare over four y and a partial draining of the lake showed 100% control of *Lyngbya*. However, this study did not document consumption of *Lyngbya* by grass carp. Since their study was uncontrolled and conducted in a single pond, it is unclear what factors mediated the loss of *Lyngbya* from this system. However their study did not document grass carp consumption of *Lyngbya*.

Water quality managers and pond owners currently need an effective control agent for the spread of invasive *Lyngbya*. Given that herbicide applications are not practical for some waterbodies (e.g., drinking water reservoirs), it is important to evaluate the role of biological control agents, like grass carp, for controlling nuisance filamentous algae (Shireman and Smith 1983). For this reason and because use of grass carp is broadly promoted by consultation, managers, and public agencies, it is critical to rigorously determine if grass carp can consume *Lyngbya* (Wright and Reeves 2004). Studies have shown that temperature has a large impact on grass carp consumption, and when water temperatures reach above 16°C fish feed more intensely and less selectively (Stroganov 1963). As all of our experiments were conducted when the water temperature was >22°C, we would expect carp to be less selective and more likely to consume a wide range of available food offered to them, including not preferred species. Also, since fish were fasted for 2-3 d prior to each experiment, our results should be considered conservative regarding grass carp feeding preferences. Results from several mesocosm experiments indicate that, grass carp generally avoided *Lyngbya* while they actively consuming palatable *Muriophyllum* or lettuce. When given no choice and only offered *Lyngbya*, grass carp did not consume the filamentous cyanobacterium.

Although grass carp are recommended to be stocked at high densities for controlling filamentous algae (62-125 grass carp per ha, Wright and Reeves 2004), our relative stocking densities were substantially higher than this level (five or ten fish per 3.45m² mesocosm = 14,488-28,977 fish per ha). Given that grass carp did not consume *Lyngbya* at these extreme stocking rates, it is unlikely that the current recommended stocking densities will be sufficient to control *Lyngbya* blooms effectively. However, field tests are needed to confirm this hypothesis given that herbivory is only one method of control that could be mediated by grass carp.

Small grass carp (e.g. 10-13 cm TL) are generally more effective at controlling nuisance aquatic vegetation than larger grass carp because they have higher energy demands and reduce feeding as they grow (Osborne and Sassic 1981, Piplova 2006). Although some studies suggest that smaller grass carp (i.e., 3-9 cm TL) are more likely to consume filamentous algae, including periphyton, (Osborne and Sassic 1981, Watkins et al. 1981, Wright and Reeves 2004), fingerling grass carp begin eating macrophytes when they reach 5 cm TL (Sobolev 1970). However, such small carp are subject to fish and bird predation in many ponds until they are >20 cm TL. Our experiments generally focused on small grass carp, but large stocker size grass carp also were included in one experiment. In all cases, we found that grass carp do not consume *Lyngbya*, except in our large fish treatment, which could have resulted from high variation attributable to mortality in this treatment. Grass carp tend to prefer groups but the large fish treatment replicates contained only two fish per tank. Thus, when one fish died, the remaining fish may have been more stressed.

Lyngbya grows in coarse mats similar to *Pithophora* sp., and should be readily available for the carp to bite and ingest (Prowse 1971). However structural traits of aquatic macrophytes and algae have been shown to be effective deterrents of grazers (Hay et al. 1994, Taylor et al. 2003).

In one study, the polysaccharide sheath of *Lyngbya* was shown to prevent amphipod herbivory (Camacho and Thacker 2006).

Given that grass carp avoided *Lyngbya*, we aimed to determine the mechanism mediating this general pattern by using pellets to vary diet composition independent of texture. Pellets containing ground *Lyngbya* ($\geq 17\%$ biomass) were avoided by grass carp clearly indicating that the physical structure of *Lyngbya* does not prevent its consumption by grass carp. Instead, our data suggest that secondary metabolites may mediate this interaction. Grass carp are known to avoid feeding on macrophytes which produce toxins (Murphy et al. 2002), and *Lyngbya* has been shown to produce saxitoxin in response to amphipod herbivory (Thacker et al. 2005). However, further experiments are needed to determine which secondary metabolites actively deter grass carp consumption of *Lyngbya*.

In conclusion, we demonstrate that grass carp do not consume *Lyngbya* in an experimental setting. Since grass carp do not consume *Lyngbya*, waterbody managers and pond owners interested in managing *Lyngbya* blooms should consider other control methods, such as herbicides, shading, and physical removal. Lastly, it is important to remember that aquatic ecosystems are complex and that a single management option may not be effective in all situations.

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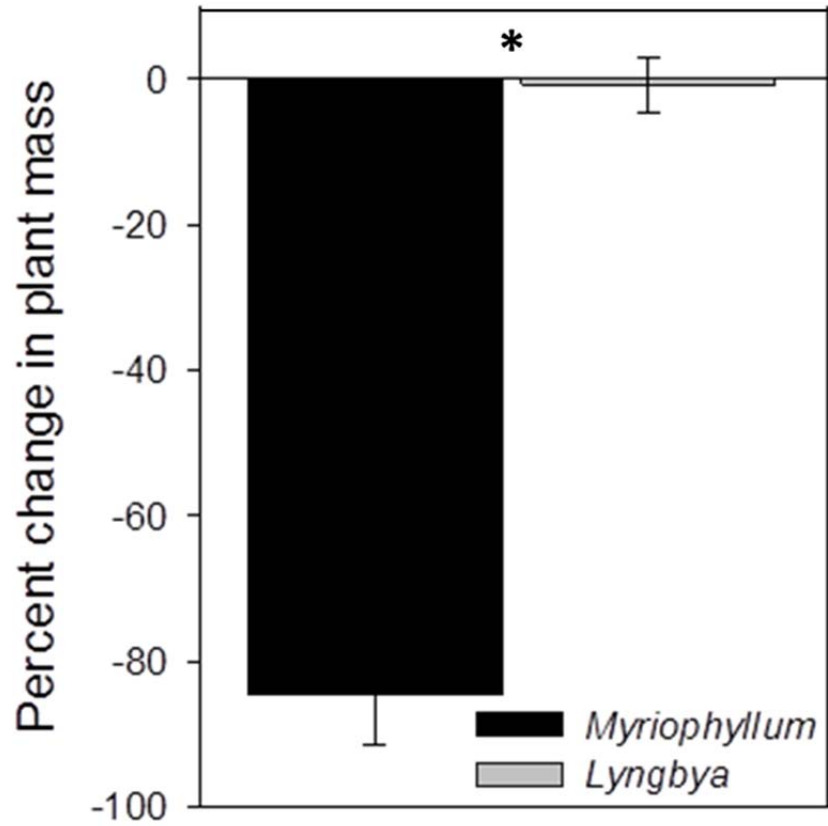


Figure 2.1. Effect of paired diet choice on grass carp consumption of *Myriophyllum* and *Lyngbya*. Error bars represent 95% confidence intervals. * indicates significance of ANOVA at $\alpha=0.05$.

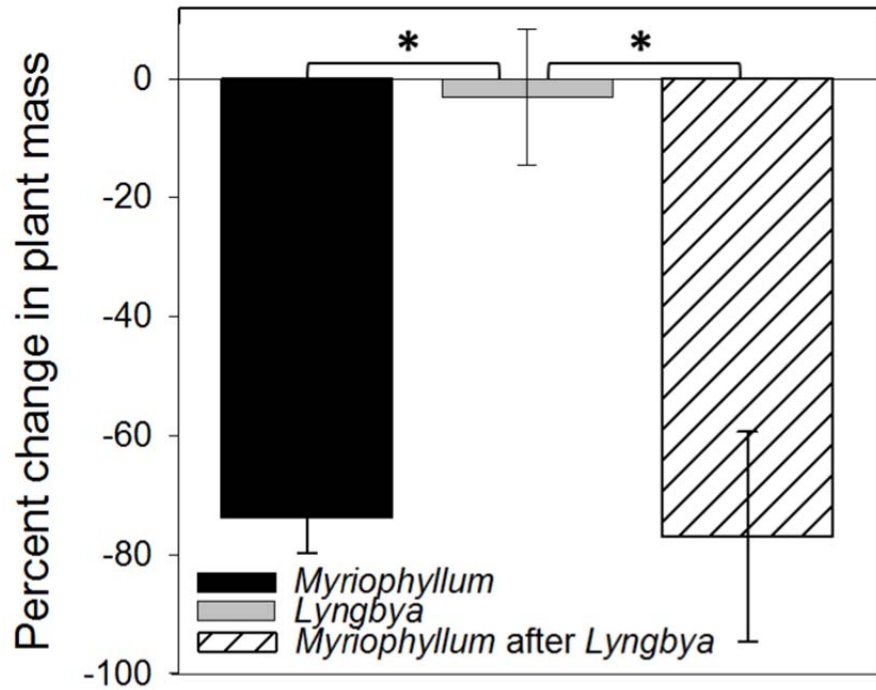


Figure 2.2. Effect of plant type (*Myriophyllum* or *Lyngbya*) on grass carp consumption. *Myriophyllum* or *Lyngbya* were offered separately (no choice). Hashed bar indicates *Myriophyllum* offered to same fish earlier fed *Lyngbya*. Error bars represent 95% confidence intervals. The left bracket indicates the results of an ANOVA, and the right bracket shows the results of the paired t-test. * indicates significance at $\alpha=0.05$.

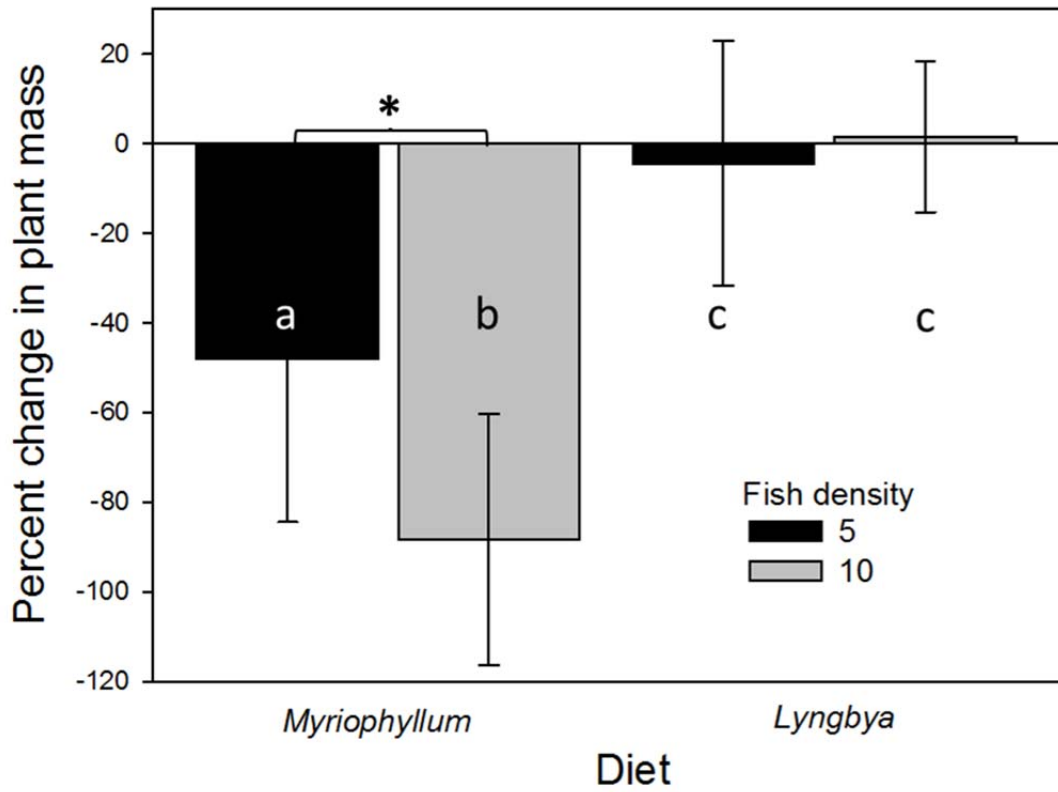


Figure 2.3. Effect of grass carp density (5 (black bars) or 10 (gray bars) fish) on consumption of *Myriophyllum* or *Lyngbya*. Error bars represent 95% confidence intervals. * indicates significance of a paired t-test at $\alpha= 0.05$. Letters indicate grouping based on Tukey's multiple comparison test ($\alpha= 0.05$).

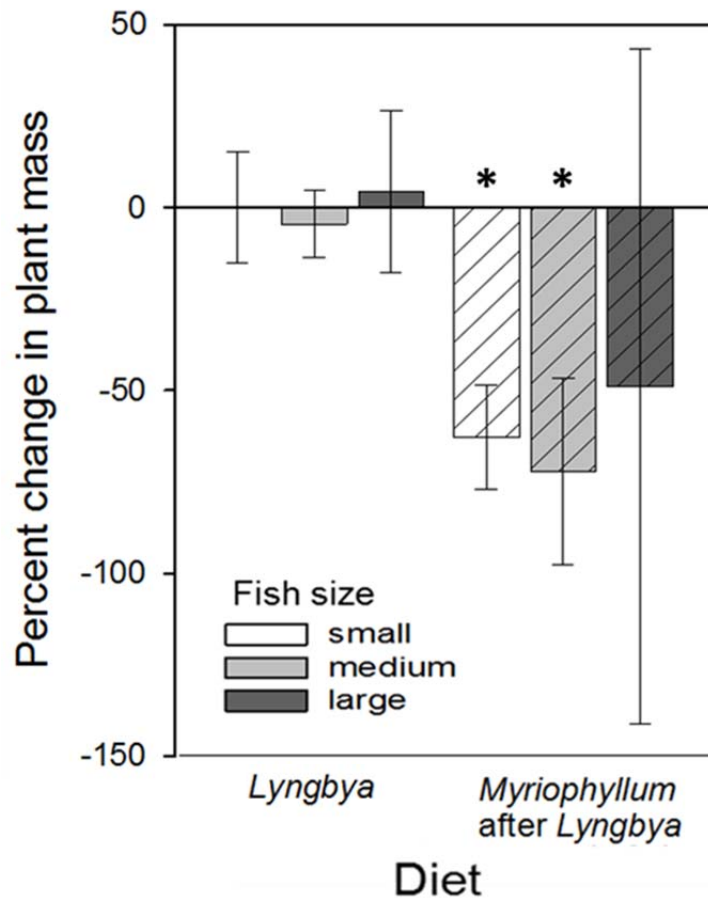


Figure 2.4. Effect of grass carp size (small (7-12cm TL, white bars), medium (14-19cm TL, light gray bars), or large (21-24cm TL, dark gray bars)) on plant consumption. Error bars represent 95% confidence intervals. Hashed bar indicates *Myriophyllum* offered to same fish earlier fed *Lyngbya*. * indicates significance of a t-test ($H_0 = \text{means are equal for same fish fed } Lyngbya \text{ and then } Myriophyllum$) for each treatment at $\alpha = 0.05$.

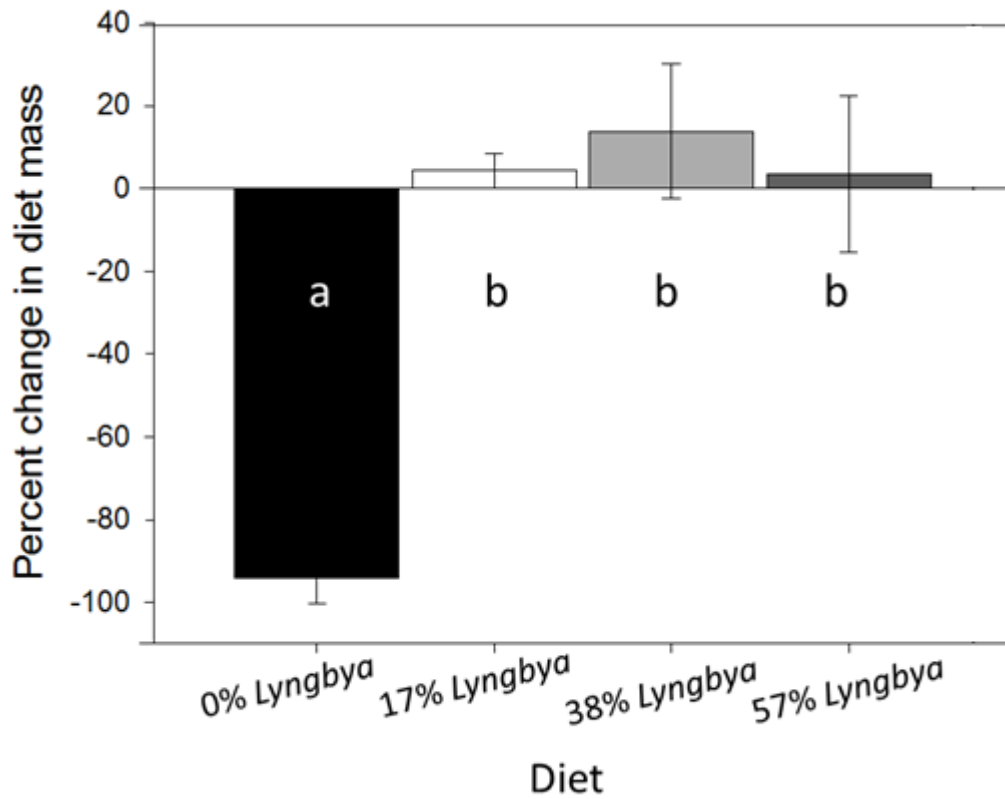


Figure 2.5. Effect of *Lyngbya* (% dry weight) in pellets on grass carp consumption. Error bars represent 95% confidence intervals. Letters indicate grouping based on Tukey's multiple comparison test ($\alpha= 0.05$).