Problem and Research Objectives:

There were two major goals of this research: 1) to investigate the spatio-temporal distribution of potentially toxin-producing cyanobacteria in the Charles River during the growth season, and 2) to investigate the influence of nutrients on overall growth limitation of phytoplankton in the Charles River, in parallel with relative influence on potentially toxic species of cyanobacteria.

Methodology:

Sample collection and experimental setup

Samples were collected from four stations in the Charles River biweekly from mid-June to the end of October (a total of 9 times) in 2015 (Figures 1, 2).

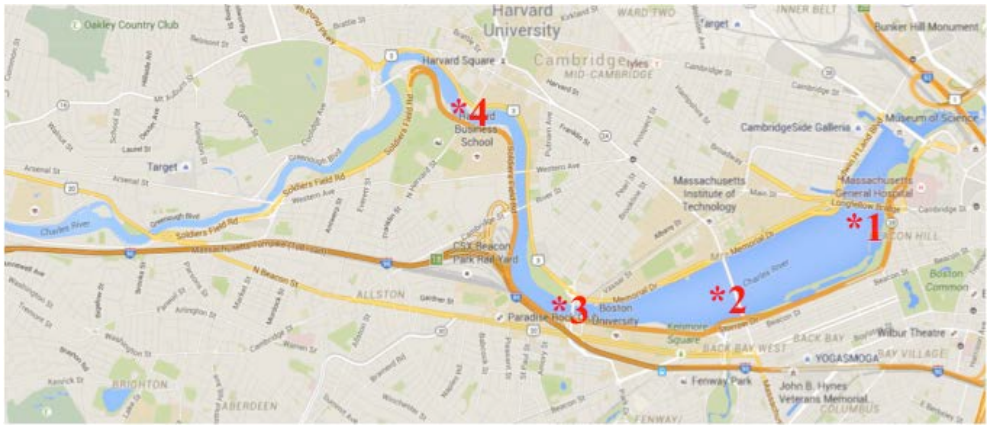

Figure 1. Sampling locations on the Charles River.

The sampling was collected from a small outboard motor boat, in collaboration with the Charles River Watershed Association (CRWA). The samples were collected with a water sampler (Wildco) into acid washed polycarbonate bottles, kept in a cooler with blue ice and immediately brought back to UMass Dartmouth. The water was then filtered to preserve materials for several analyses. For determination of chlorophyll *a* and dissolved inorganic nutrient concentrations, 50 mL of the water sample was filtered on GF/F glass fiber filters that were blotted dry, then frozen in clean microcentrifuge tubes at -20°C. The flow-through was saved in a clean microcentrifuge tube that was frozen at -20°C, to be processed for measurements of inorganic nutrients. For determination of the composition and abundance of potentially toxic cyanobacteria, water was filtered on 0.2 µm membrane filters to collect DNA. The filters were placed in sterile bead beater tubes and immediately frozen at -20°C. In addition, samples were preserved for microscopic observation by preserving 30 mL with Lugol's solution in polyethylene bottles. Subsamples were also filtered on GF/F filters for collection of material for determination of microcystin in the particulate matter. The GF/F filters were frozen in foil packages at -20°C.

Nutrient amendment bioassays were conducted monthly for water sampled from two of the stations. A total of five bioassay experiments were conducted (June, July, August, September, October), of which the June experiment was considered a pilot study (data not shown). For each experiment, 300 mL of water was distributed to 24 acid-washed polyethylene bottles from each site. Six of these bottles per site received either no nutrient additions (control), nitrogen (added as nitrate+ammonium; N), phosphorus (P), or N+P. Triplicates of each treatment from each site were incubated at the light intensity of 55 µmol

m^{-2} s^{-1} ("low light"), and the second set was incubated at 129 µmol m^{-2} s-1 ("high light") in incubators at a 15:9 Light:Dark cycle at 25°C, using color temperature 6500K (daylight) light bulbs. The bottles were incubated for a total of 72 h (3d), and the bottles were then removed from the incubation and contents preserved for the same analyses as samples collected during the seasonal time series. We also initiated culture isolates by inoculating samples from blooms into BG-11 or Z80 media, to attempt to enrich non-N_2 fixing or N_2 fixing cyanobacteria, and kept the vials in natural light on a lab window in ambient room temperature. The attempts were successful and we obtained enrichment cultures of *Anabaena* and *Aphanizomenon* spp. (see below, Fig. 3)

Analytical methods

Chlorophyll *a* concentration was measured fluorometrically using previously published methods (Moisander et al. 2010). Briefly, the samples were thawed, then 5 mL of 90:10 acetone:water solution (vol:vol) was added. Samples were briefly sonicated to break the cells using a tip sonicator, then kept in the freezer overnight. The samples were vortexed again, and the extract was then cleared by filtering it through a GF/F filter attached to a syringe filter, then read using a Turner 10-AU fluorometer, and diluted for the measurement if necessary. The dissolved nutrient analyses have been arranged to be conducted at the laboratory of Dr. Brian Howes (School of Marine Science and Technology, UMass Dartmouth). DNA was extracted using previously published methods (Moisander et al. 2008). Briefly, a Plant Minikit (Qiagen) was used with modifications. The first buffer from the kit was first added (lysis buffer), and the samples then underwent three freeze-thaw cycles (10 min at -80°C, then 5 min at 65°C). The samples were then subjected to homogenization in a bead beater (Mini-Beadbeater-8, full speed for 2 min). 45 µL of Proteinase-K solution was added, and the tubes were then incubated for 1 h at 55°C. The rest of the steps followed the manufacturer's protocol. We conducted quantitative polymerase chain reaction (qPCR) to quantify the presence of *Microcystis aeruginosa* in the system through the summer, using previously published methods (Moisander et al. 2012). According to reports from the CRWA, and our microscopic observations, however, the dominant bloom-forming cyanobacteria were *Anabaena* spp. and *Aphanizomenon* spp., both N_2 fixing cyanobacteria that are potentially toxic. Since these reports and observations suggested *M. aeruginosa* was not present or its abundances were lower than the two other cyanobacteria, it was relevant to shift the focus to these major bloom forming species that are also potentially toxic. We conducted PCR targeting the N_2 fixation gene *nifH* to characterize the major N_2 fixing components in the blooms.

Principal Findings and Significance:

The data from the seasonal time series showed that there was an increase in overall phytoplankton biomass at the downstream stations 1 and 2 until mid-September, after which the biomass rapidly decreased to early season levels (Figure 2). These two stations, located in the wider, more slow flowing part of the river (Figure 1), developed the highest phytoplankton biomass of the four stations. Stations 3 and 4 that were located in the narrow part of the river had a more similar pattern, with a relatively stable maximum biomass from mid-August to mid-September. These data suggest that the downstream, wide portion of the river was more susceptible for developing bloom conditions during the late summer than the upper, riverine sections.

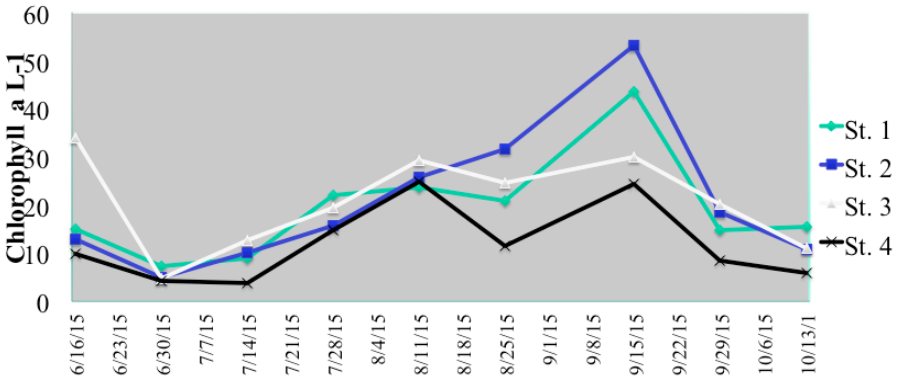

Figure 2. Chlorophyll *a* concentration (L^{-1}) in the Charles River through the summer and fall of 2015 at the four stations.

After alignment with known, previously published culture isolates and environmental samples, sequences recovered from the samples and enrichment cultures fell into several clusters (Figure 3). One of the clades was closely related with previously published *Anabaena aphanizomenoides* and *A. sphaerica* cultures. A second clade fell in a cluster with closest relatives of *Aphanizomenon*. A third cluster containing sequences both from our enrichment culture and a sample from the time series was most closely related with other previously reported *Anabaena* spp. A few *nifH* sequences were present that matched non-cyanobacterial reference sequences, suggesting presence of potentially N_2-fixing heterotrophic bacteria or archaea. This result allowed us to compare the sequences to current quantitative PCR primers and probes we have in our lab and we determined that the Charles River populations are distinct from these. Quantifying the *Anabaena* and *Aphanizomenon* in our samples would thus require developing new qPCR assays. This was beyond the scope of this study but we hope to continue characterizing the samples and the Charles River *Anabaena* and *Aphanizomenon* with follow-up funding.

Figure 3. Phylogenetic neighbor-joining tree showing the relationship of *nifH* sequences from samples collected from the Charles River (CR), with previously published sequences from the public databases (NCBI, GenBank). The CR sequences are shown in red and sequences from our enrichment cultures are in blue.

Although reports from CRWA and our own general microscopic surveys suggested that *Microcystis* was not a dominant cyanobacterium in the blooms, we wanted to check for its presence using quantitative PCR. qPCR specific to the *Microcystis aeruginosa cpc*BA (phycocyanin gene intergenic spacer) was conducted to determine abundances of *M. aeruginosa* on the seasonal time series (Figure 4). Surprisingly, the results showed presence of *M. aeruginosa* at all stations. The highest abundances were detected at stations 1 and 2 – the two downstream sites. The highest abundances in Station 2 occurred during the highest phytoplankton abundances in August and September, but at station 1 the abundance increased already in July (Figure 4). Ongoing work is investigating responses of *Microcystis* in the bioassay experiments under the different nutrient additions.

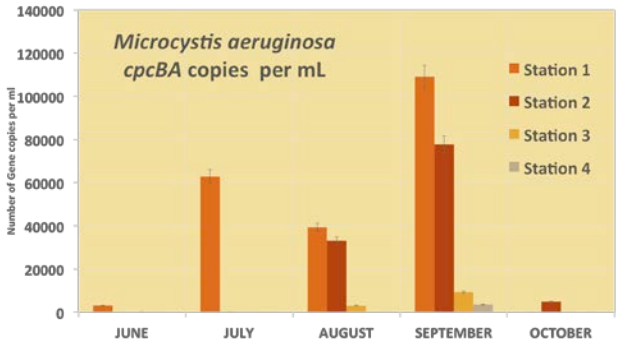

Figure 4. Abundance of *Microcystis aeruginosa* during the summer season of 2015 in the Charles River based on quantitative PCR (copies of *cpc*BA mL^{-1}). The numbers shown are based on preliminary analyses. Each gene copy corresponds to approximately one cell, with the assumption that there is one genome per cell.

The enrichment experiments showed variability in responses to nutrients and light (Figure 5, Table 1). The data show that throughout the season, phytoplankton increase was observed if both N and P were provided. However, the primary nutrient limiting phytoplankton growth varied through the season. P was the primary nutrient limiting phytoplankton growth during early and late season, while N limitation was observed at both stations in August or September (Table 1, Figure 5). Interestingly, N limitation was seen only in low light in August, and only in high light in September, suggesting light and nutrient availability in combination influenced competitive fitness of different phytoplankton species.

Table 1. Summary of nutrient limitations observed under low and high light. Shaded boxes show significant differences based on results from one-way ANOVAs (tested separately for each light level and site).

	Low light			**High light**		
	N	**P**	**NP**	**N**	**P**	**NP**
July station 1						
July station 3						
August station 1						
August station 3						
Sept station 1						
Sept station 3						
Oct station 1						
Oct station 3						

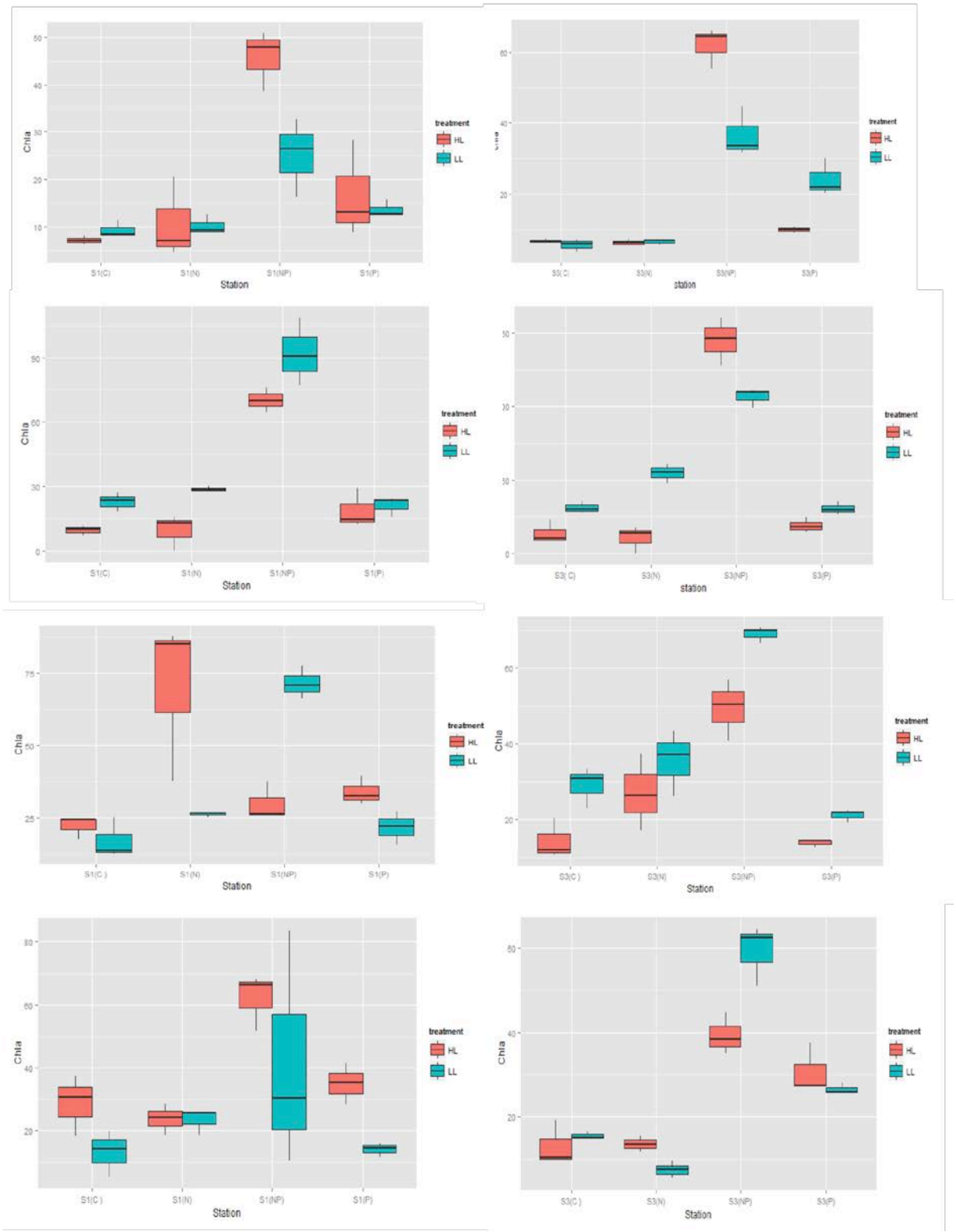

Figure 5. Chlorophyll *a* results from the bioassay experiments shown with box plots. Left panels: station 1; Right hand side panels: Station 3. July, August, September, and October experiments are shown from top to the bottom. C, control; N, nitrogen addition; P, phosphorus addition; NP, N+P addition; LL, low light; HL, high light (Kaushik et al., in prep).

Figure 6: Field Sampling on the Charles River

References

Kaushik K, Cianciola E, Moisander PH. In preparation. Influence of nutrients and light intensity on cyanobacterial blooms in a temperate urban river. To be submitted to Harmful Algae

Moisander PH, Beinart RA, Hewson I, White AE, Johnson KS, Carlson CA, et al. 2010. Unicellular cyanobacterial distributions broaden the oceanic N-2 fixation domain. Science 327:1512-1514.

Moisander PH, Beinart RA, Voss M, Zehr JP. 2008. Diversity and abundance of diazotrophic microorganisms in the South China Sea during intermonsoon. The ISME Journal 2:954-967.

Moisander PH, Cheshire LA, Braddy J, Calandrino ES, Hoffman M, Piehler MF, et al. 2012. Facultative diazotrophy increases *Cylindrospermopsis raciborskii* competitiveness under fluctuating nitrogen availability. FEMS Microbiology Ecology 79:800-811.