FINAL REPORT FOR THE 2020 NORTH CAROLINA SEA GRANT/ALBEMARLE-PAMLICO NATIONAL ESTUARY PARTNERSHIP FELLOWSHIP

CYANOBACTERIAL MICROCYSTIN PRODUCTION AND AEROSOLIZATION DYNAMICS IN THE CHOWAN RIVER-ALBEMARLE SOUND ESTUARY, NC

By: Haley E. Plaas

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ABBREVIATIONS

ASV = Amplicon Sequence Variant

CEEG = Chowan Edenton Environmental Group

CHAB = Cyanobacterial Harmful Algal Bloom

CHLA = CHLorophyll A concentration

C:N = particulate C to N molar ratio

CR-AS = Chowan River - Albemarle Sound estuary

DNA = Deoxyribonucleic Acid

EPA = Environmental Protection Agency (US)

FACS = Fluorescence Activated Cell Sorting

GFF = Glass Fiber Filter

MC = Microcystin

NC = North Carolina

NC-DEQ = North Carolina Department of Environmental Quality

NH_4 = Ammonium

NO_x = Nitrates

PM = Particulate Matter a.k.a. Aerosol

PM_{10} = Particulate Matter with aerodynamic diameters < 10 µm

$PM_{2.5}$ = Particulate Matter with aerodynamic diameters < 10 µm

PN = Particulate Nitrogen

PO_4 = Soluble Reactive Phosphorus

POC = Particulate Organic Carbon

QFF = Quartz Fiber Filter

RAPM = Relative Abundance of the cyanobacterial genera in PM samples

RAW = Relative Abundance of the cyanobacterial genera in Water samples

RH = Relative Humidity

SAL = Salinity

SiO_2 = Silicate

SI = Solar Irradiance

T = air Temperature

TDN = Total Dissolved Nitrogen

TRB = TuRBidity

US = United States

UTC = Coordinated Universal Time

VOC = Volatile Organic Compound

WS = Wind Speed

PROJECT NARRATIVE

CYANOBACTERIAL MICROCYSTIN PRODUCTION AND AEROSOLIZATION DYNAMICS IN THE CHOWAN RIVER-ALBEMARLE SOUND ESTUARY, NC

AUTHORS

Haley E. Plaas[1], Ryan W. Paerl[2], Karsten Baumann[1], Colleen Karl[3], Kimberly J. Popendorf[4], Jill Paxson[5], Malcolm Barnard[1], Naomi Chang[1], Nathaniel Curtis[2], Hwa Huang[2], Olivia Mathieson[2], Joel Sanchez[2], Daniela J Maizel[4], Amy Bartenfelder[1], Jeremy Braddy[1], Nathan S. Hall[1], Karen Rossignol[1], Randy Sloup[1], and Hans W. Paerl[1]

[1]UNC-Chapel Hill
[2]North Carolina State University
[3]University of Miami
[4]Chowan Edenton Environmental Group
[5]North Carolina Department of Environmental Quality

ABSTRACT

The environmental health of the Chowan River-Albemarle Sound (CR-AS) estuary, North Carolina, is threatened by the expansion of harmful cyanobacterial blooms (CHABs) linked to the production of microcystin, a potent toxin associated with liver disease. In addition to obvious negative effects on water quality, recent findings suggest that CHABs also impact air quality via spray aerosol emissions carrying cyanobacterial cells and cyanotoxins. With the NC Department of Environmental Quality and community scientists of the Chowan Edenton Environmental Group, we conducted a field campaign in summer 2020 to 1) investigate the occurrence of toxic cyanobacteria and microcystin in $PM_{2.5}$ in the airshed of the CR-AS and 2) elucidate potential environmental drivers of toxicity in CR-AS CHABs. From June to October, 2020, during peak CHAB season, we sampled $PM_{2.5}$ continuously and collected interval water samples to carry out objectives. Results revealed that bacterial communities measured in $PM_{2.5}$ and water samples were ecologically distinct, but several amplicon sequence variants (ASVs) overlapped, suggesting direct water to air transfer. Notable genera of aquatic cyanobacteria were recorded in $PM_{2.5}$, including *Dolichospermum sp.* and *Microcystis sp.*. However, throughout the entire campaign, microcystin concentrations in water remained low ($< 1 \mu g\ L^{-1}$), and accordingly, no microcystin was detected in $PM_{2.5}$ samples. Using a series of linear regression models, several environmental variables were found to be associated with the enrichment of cyanobacterial genera in $PM_{2.5}$ and with increased ambient $PM_{2.5}$ mass concentrations, suggesting a linkage between CHABs and decreased air quality in the region. Findings reveal previously unrecognized interactions between water and air quality and underscore the need to further investigate such interactions to promote ecosystem and human health in the CR-AS and beyond.

INTRODUCTION

The Chowan River-Albemarle Sound (CR-AS) is a key recreational, fisheries, and residential region of coastal North Carolina (NC). The CR, whose basin spans large portions of Virginia and NC, flows into the Albemarle-Pamlico estuary, the second largest estuarine system in the United States. Since the early 1980's, the CR-AS has faced recurrent harmful cyanobacterial blooms (CHABs).[1] Early research establishing nutrient-bloom thresholds during this period led to management actions (specific nitrogen [N] and phosphorus [P] input reductions) that successfully mitigated the blooms.[2,3] However, over the past decade, blooms have reemerged and are expanding, as CHABs originating in the upstream CR are now proliferating in the AS and appearing in its other tributaries (Fig. 1), including the Perquimans, Little, and Pasquotank Rivers.[4]

Specifically, mixed cyanobacterial assemblages of known microcystin (MC) producers, chiefly *Dolichospermum sp.,* and *Microcystis sp.,* have been more frequently reported in the CR-AS.[5] MC is a potent liver toxin,[6] and following prolonged or acute exposure, MC can accumulate in human tissues, leading to liver damage, tumor promotion, and even liver cancer.[7–9]. During summer 2019, the North Carolina Department of Environmental Quality (NC-DEQ) reported concentrations of MC as high at 620 $\mu g\ L^{-1}$, which is more than 60 times the US Environmental Protection Agency's (EPA) recommended limits for swimming water.[10]

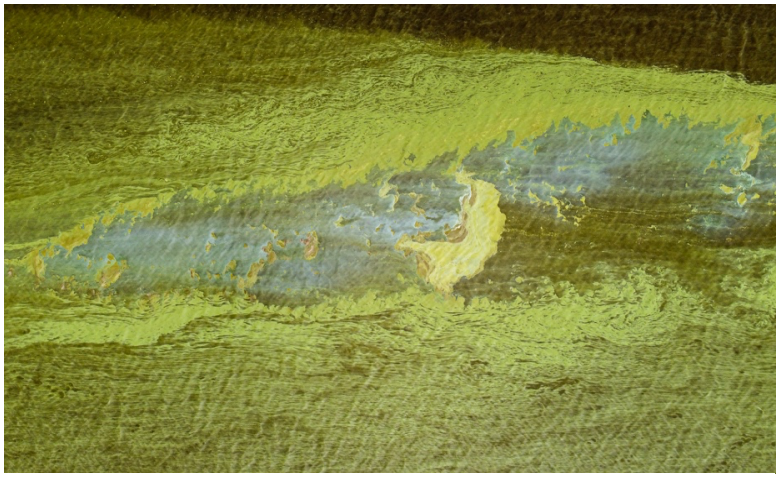

Figure 1. A drone-based image of a cyanobacterial bloom, primarily composed of *Dolichospermum sp.* in the CR. Photo captured by Abe Loven on July 7th, 2020.

In addition to obvious negative effects on water quality, recent findings suggest that CHABs also impact air quality via emissions carrying cyanobacterial cells and cyanotoxins.[11] Specifically, when air bubbles burst at the water-air interface via breaking wave action or human activities such as boating, droplets are directly ejected into the air, generating spray aerosol carrying cells and biogenic compounds from the water column.[12] People are most likely to come into contact with aerosolized CHAB compounds during recreational water activities, but this aerosol can persist into communities onshore.[13–15] At present, it is unclear to what extent CHAB compounds are aerosolized in the airshed of the CR-AS and how this is impacting respiratory health in the area. Beyond the suspected health effects associated with exposure to cyanotoxins, the inhalation of particulate matter (PM) with aerodynamic diameters $\leq 2.5\ \mu m$ ($PM_{2.5}$) also pose significant health risks, regardless of particle composition.[16–18] Therefore, it is crucial to better understand the occurrence, physicochemical properties, and environmental fate of airborne CHAB cells and toxins in order to safeguard public health in this region and beyond. Accordingly, this study evaluated associations between CHABs and local air quality and investigated potential environmental drivers of toxicity in the CR-AS.

METHODS

In summer 2020, a field campaign was conducted to generate environmental data. From June 11th to October 1st, 2020, continuous $PM_{2.5}$ samples and interval water samples were collected for targeted analyses of microbial community composition, bacterial cell counts, and the concentration of MC, chlorophyll a and key nutrient analytes. Corresponding air and water quality metadata were also recorded to perform statistical analyses.

Study sites

Two sites were selected along the CR to deploy aerosol samplers, supporting equipment, and collect environmental samples. Site A was located at the Chowan Beach subdivision near Arrowhead Beach (36.212593 N, 76.715342 W) of the upper CR, and Site B at Pembroke Creek near the mouth of the CR in the western most part of the AS in an embayment near the town of Edenton (36.057583 N, 76.620208 W). Both sites, located on private docks, were graciously provided by community scientists with the Chowan Edenton Environmental Group (CEEG). Sites were chosen due to their variable locations along the CR-AS estuarine gradient, proximity to known cyanobacterial bloom hotspots, proximity to the waterfront (<1 m), proximity to recreational/residential areas where people are more likely exposed, access to electricity, and overall security for the long-term, autonomous deployment of the equipment (Fig. 2A).

Field Collection Methods

Aerosol sampling

Aerosol (a.k.a. PM or $PM_{2.5}$) was sampled continuously via the installation of two, high-volume $PM_{2.5}$ samplers (Tisch Environmental, Polyurethane Foam Sampler, Model #1000D-BL) deployed at each site. Samplers were secured on docks, <1 m from the water and <2 m above the water (Fig. 2B). $PM_{2.5}$ was size fractionated via a cyclonic inlet and samples were impacted onto 102 mm pre-combusted quartz fiber filters (QFF). Prior to deployment, samplers were calibrated by the EPA and passed all requirements. Instruments were deployed from June 11th to October 1st, 2020 and ran daily from 07:00-19:00 UTC autonomously for the entirety of the sampling period, other than August 3rd to August 12th, when samplers were stored indoors for the duration of Hurricane Isaias. Single $PM_{2.5}$ samples were integrated on QFF over each sampling period, which ranged from 8-14 days (Fig. 2C). Between sampling periods, QFF were swapped, stored, data was downloaded, and filter cassettes were cleaned with methanol wipes. Each sampling period, a dynamic field blank was collected in a complementary field cassette housed within the sampling apparatus.

Alongside the high-volume sampler, real-time $PM_{2.5}$ mass concentrations were measured continuously with a nephelometer fitted with a $PM_{2.5}$ size-selective inlet cyclone (Thermo Scientific MIE pDR-1500). Nephelometers were housed under each high-volume sampler, and readings were resolved to every 7 minutes (Fig. 2B). Meteorological data was recorded via a single Davis Weather Station deployed at Site B, with readings resolved to every 5 minutes. Meteorological parameters recorded include air temperature, precipitation sum, relative humidity, solar irradiance, and wind direction and speed. Air quality metadata was downloaded in the field from each piece of equipment between sampling periods.

Water Sampling

At interval dates between defined $PM_{2.5}$ sampling periods (Fig. 2C), water was collected at each site from the shoreline, using a bucket to collect grab samples from the top 0.3 m of the water column. Water was collected first thing in the morning (prior to 9:00 UTC) and dispensed into pre-cleaned (acid washed then flushed with CR water) 20 L carboys, and transported to Site B for processing by community scientists from the CEEG in the field. All CEEG scientists were briefed on a study-specific handout and completed sample processing training prior to handling of

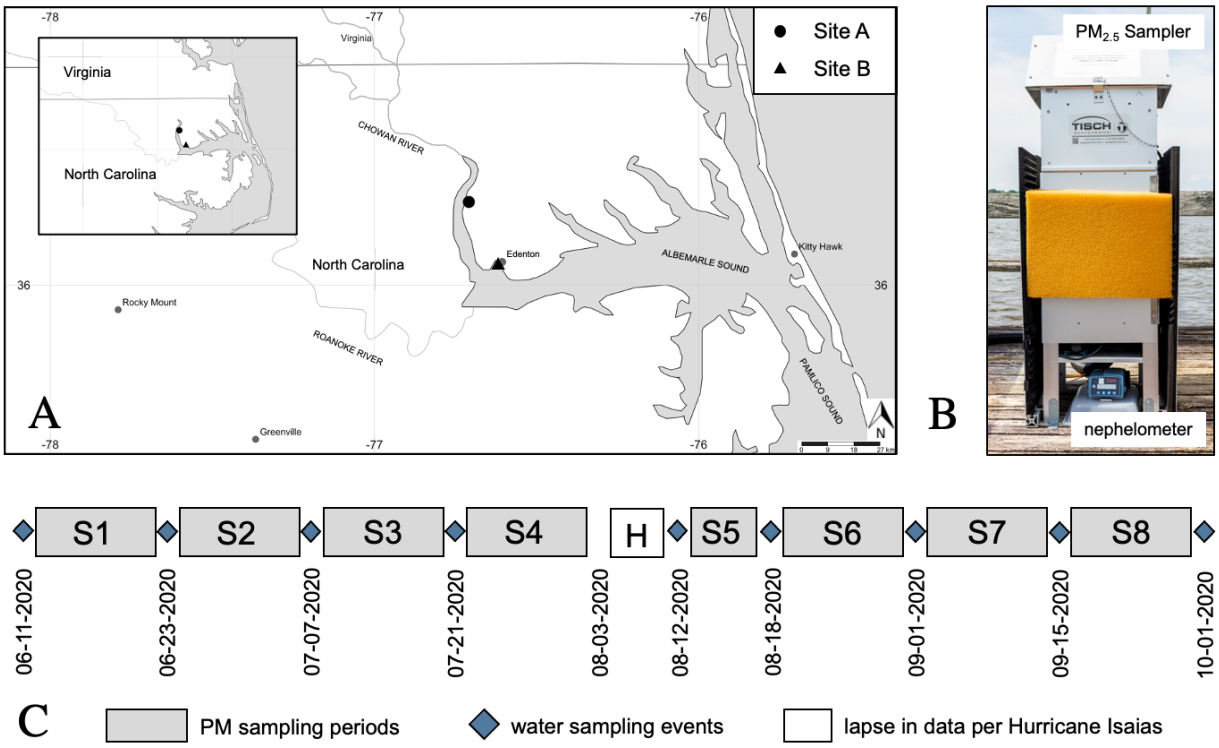

Figure 2. A) Map of the study sites where $PM_{2.5}$ samplers were deployed and water samples were collected within the CR-AS basin. This figure was created with https://www.simplemappr.net (accessed January 2022). B) A picture of the $PM_{2.5}$ equipment set-up, with nephelometer housed underneath and noise muffler surrounding the high-volume sampler. Photo by: Abe Loven. C) A schematic demonstrating the data collection timeline, with $PM_{2.5}$ sampling periods spanning 8-14 days, and water sampling occurring in between. Data was not collected from August 3rd to 12th, 2020, per the storage of all equipment during Hurricane Isaias, which made landfall on August 4th, 2020 in NC.

samples. Water samples were stored via several processes specific to the method for each data end point (see analytical methods). Water quality metadata was collected with a YSI Sonde at the time of water sampling. Triplicate readings were collected at a depth of < 1 m and averaged. Parameters recorded include dissolved oxygen concentration, pH, salinity, turbidity, and water temperature.

Analytical Methods

Phytoplankton Biomass Determination. Chlorophyll a, as an approximation of overall phytoplankton biomass, was measured by vacuum filtering known volumes of sample onto Whatman glass fiber filters (GFF) in the field. Triplicate samples were stored in pre-combusted

foil packets and transported in the dark on ice and frozen at −20 °C until extraction using a tissue grinder in 90% acetone during low-light conditions. Chlorophyll a concentration was measured with a fluorometer calibrated with pure chlorophyll a standards (Turner Designs, Sunnyvale, CA, USA).[19]

Nutrient Determination. Nutrients were vacuum filtered in the field through pre-combusted GFF into acid-washed flasks. Both were collected in triplicate. Filters stored on petri dishes and filtrate stored in 50 mL falcon tubes were transported in the dark on ice to be kept at −20 °C until analyses. Filtrates were analyzed for dissolved nutrient concentrations, including nitrate plus nitrite (NO_x), ammonium (NH_4), soluble reactive phosphate (PO_4), silicate (SiO_2), and total dissolved nitrogen (TDN). Filtrates were analyzed via ion chromatography (Lachat Instruments QuickChem 8000 flow injection autoanalyzer). Filters were analyzed for particulate/intracellular nutrients including particulate organic carbon (POC) and particulate nitrogen (PN). Particulate nutrients were quantified by high-temperature combustion (Costech ECS 4010 analyzer). Detailed method numbers and standard protocols for all nutrient analytes can be found in Paerl et al., 2020.[20]

Bacterial Cell Counts and Community Composition Determination. Samples for the determination of bacterial and small (<100 µm) phytoplankton cell counts were fixed in the field by pipetting 5 mL of sample into small culture tubes, pre-loaded with 50 µL of 1% glutaraldehyde. Samples were transported in the dark on ice and stored at -80°C until analyses. Counts were obtained by flow cytometry using a Guava EasyCyte (Millipore) equipped with blue and red excitation lasers. Bacterial counts were determined using SYBR Green I staining and small phytoplankton were distinguished based on autofluorescence based on blue and/or red-light excitation.

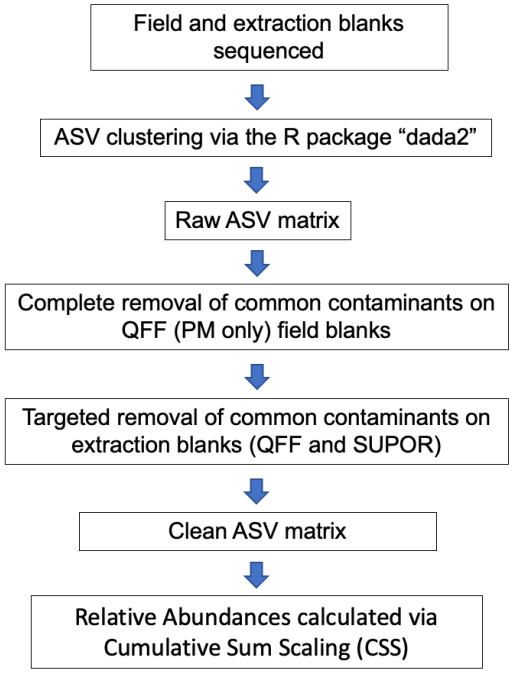

Figure 3. Workflow of the 16S sequence data cleaning.

Both $PM_{2.5}$ and water samples were processed for downstream analyses of microbial DNA. $PM_{2.5}$ samples were removed from sampler filter cassettes in the field in a portable glove box, and ½ of the QFF was carefully removed with pre-cleaned scissors and stored in a combusted foil packet for DNA analyses. Water samples were collected by vacuum filtering known volumes of sample onto Pall membrane filters in the field and stored in DNA-free centrifuge tubes. Samples were transported in the dark on ice and stored at -80°C until analyses. DNA was extracted using phenol/chloroform and quantified using a Qubit 3.0. The phenol/chloroform method was selected to maximize DNA yields from QFF $PM_{2.5}$ samples.[21] Primers targeting the v4-v5 region of the 16S rRNA gene (515F and 926R)[22] including CS1 and CS2 linker sequences, were used to generate amplicons via PCR. Amplicons were sequenced using the Illumina platform (MiSeq 300 PE; UIC Genome Research Core) and sequence analysis was performed in RStudio. Amplicon sequence variants (ASVs) were determined using the 'dada2' algorithm to infer the biological sequences present. Sequence variants were assigned taxonomic ranks analyses of populations using 'phyloseq'.[23] ASVs

identified on QFF field blanks were labeled contaminants and completely removed from analyses, but high frequency ASVs on the box blanks were identified as contaminants via 'decontam'[24] and removed (Fig. 3). Relative abundances of ASVs were calculated using cumulative sum scaling and visualized via 'phyloseq' at several taxonomic levels.

Microcystin Quantification. Both $PM_{2.5}$ and water samples were processed for downstream analyses of microbial DNA. $PM_{2.5}$ samples were removed from sampler filter cassettes in the field in a portable glove-box, and ½ of the QFF was carefully removed with pre-cleaned scissors and stored in a pre-combusted foil packet for MC analyses. Water samples were collected in triplicate by vacuum filtering known volumes of sample onto GFF in the field with storage in pre-combusted filter packets. $PM_{2.5}$ and water samples were analyzed for concentrations of eight congeners of MC (MC-LA, -LF, -LR, -LW, -LY, -WR, -YR, and D-Asp-MC-LR), which are commonly reported in North America.[25] $PM_{2.5}$ samples were extracted from ½ the QFF while water samples were extracted from GFF, both in known volumes of 80% HPLC-grade MeOH. MC congeners were quantified using reverse phase high pressure liquid chromatography coupled with triple quadrupole mass spectrometry (LC-MS/MS) using methods adapted from Gambaro et al., 2012.[26] Detailed LC-MS/MS specifications and method settings can be found in Gaston et al., 2021.[27] Leucine enkephalin acetate (ENK, Sigma Aldrich L9133) was used in each sample as an internal standard to measure extraction efficiency and ultimate recovery, and MC congeners were quantified from a calibration curve generated from a series of commercially available MC standards (Enzo Life sciences). Final MC concentrations are reported by individual congeners and as bulk MC, calculated by adding the values of each congener.

NC-Department of Environmental Quality (DEQ) Microcystin Analyses. In addition to the $PM_{2.5}$ and water samples which were analyzed for MC content, several samples were also provided by the NC-DEQ for MC congener analyses. At present, the NC-DEQ Ambient Monitoring System routinely monitors several stations for MC along the CR-AS and its tributaries. However, the NC-DEQ utilizes ELISA kits to quantify bulk MC concentration and no congener-specific data had been generated prior to this study. Gaining insight on the specific congeners in addition to the cyanobacterial genera linked to MC production was important for this study because congeners have variable toxicities,[28] which can become enriched in aerosol differentially.[29] Therefore, in collaboration with the NC-DEQ, we quantified eight congeners of MC from eight stations on the CR-AS (M390000C, M610000C, D94900000, D8950000, D8356200, D995000N, D995000C, and D995000S – Fig. 4) on four sampling dates spanning June-September, 2020.

Figure 4. The NC-DEQ's Ambient Monitoring System (AMS) stations in the CR-AS used for this study (red circles). Blue circles are other AMS stations not used for this study.

Statistical Analyses

All data cleaning, statistical modeling, related calculations, and visualizations were conducted in RStudio, primarily using the packages 'dplyr', 'stats', and 'ggplot'(other than the ChemTax matrix analyses, for which code in MatLab had already been written). All scripts can be found on H. Plaas' public GitHub account, with a repository dedicated to this project (https://github.com/haleyplaas/CR-AS_2020). Raw data files are available upon request.

Data Cleaning. When studying bioaerosols, one of the most challenging tasks is determining signal from noise, due to extremely low DNA concentrations present in PM samples.[30,31] Therefore, it is crucial to collect and analyze field and extraction blanks, in an attempt to distinguish contaminant taxa from sample taxa.[32] To identify contamination on samples, we collected and sequenced one representative PM field blank (QFF), an extraction blank for PM (QFF), and an extraction blank for water (membrane) (Fig. 3). All ambient $PM_{2.5}$ data was vetted for outliers using z-scores, and additional days with abnormal readings as result of *known* external sources of PM, such as fireworks and nearby military testing, were removed from analyses.

Statistical Testing. Shannon and Inverse Simpson alpha-diversity indices were used to examine differences in species richness and evenness between PM and water microbial communities, coupled with Student's T-tests to determine statistical significance. To evaluate associations between several environmental variables, a series of univariate linear regression models were used. A Wilcoxon signed rank test was used to evaluate the differences in ambient $PM_{2.5}$ concentrations in defined periods during and outside of bloom activity. The CHAB period was defined as June 23rd to July 21st, 2020, when estimated chlorophyll a concentrations were highest (> 15 ug L^{-1}) on average, corresponding with visible surface scums at both sites (Fig. 1) and satellite imagery from CyanoTracker's *Sentinel2* remote-sensing algorithm reporting a CHAB.[33]

RESULTS

Comparison of PM and Water Bacterial Communities

In $PM_{2.5}$ and water samples, 3423 and 4854 distinct ASVs were respectively detected. Of these, 201 ASVs overlapped, including 8 cyanobacterial genera, suggesting direct water to air transfer of bacterial communities. Overall richness and diversity were comparable in both $PM_{2.5}$ and water bacterial communities (Shannon-Wiener index $t = -0.68$, $p = 0.506$; Inverse Simpson index $t = -0.66$, $p = 0.517$), but communities were ecologically distinct (Observed $t = -8.75$, $p < 0.001$). This is likely due to sources of bacteria other than water communities in $PM_{2.5}$, such as soils, trees, and swamps.

Over the entire campaign, $PM_{2.5}$ samples were dominated by Proteobacteria (41.3 ± 6.1%), followed by Firmicutes (22.2 ± 6.0%), Bacteroidetes (12.5 ± 3.4%), Actinobacteria (9.1 ± 2.5%), Planctomycetes (4.2 ± 2.2%), Cyanobacteria (3.3 ± 2.9%), and Acidobacteria (2.5 ± 1.3%). Only 1.8% of ASVs were unable to be assigned a class, and all other classes comprised ≤1% of PM samples throughout the entire study period. Aquatic communities were also dominated by Proteobacteria (36.7 ± 3.2%), but were instead followed by Actinobacteria (19.3 ± 2.6%), Bacteroidetes (16.3 ± 3.4%) , Cyanobacteria (6.7 ± 2.2%) , Planctomycetes (5.2 ± 1.0%), and

Verrucomicrobia (5.1 ± 1.1%). Only 3.5 % of water ASVs were unable to be assigned a class, and all other classes comprised ≤1% of water samples (Fig. 5).

In PM, the relative abundance of cyanobacteria ranged from 0.34 to 11.9%, with the maximum occurring at Site B in June during sampling period 2 (S2). Notably, this co-occurred with a CHAB in the CR, and coincided with maximal chlorophyll a readings and peak cyanobacterial relative abundance in water samples (9.47%) at Site B.

On average, many (30.82 ± 13.36%) of the cyanobacterial communities reported in $PM_{2.5}$ were unable to be assigned at the genera level, again suggesting a large influence of sources other than local spray aerosol on bacteria in $PM_{2.5}$. However, several notable genera of waterborne cyanobacteria were recorded in $PM_{2.5}$, including average: *Cyanobium sp., Dolichospermum sp., Aphanizomenon sp., Microcystis sp., Chroococcidiopsis sp., Obscuribacterales sp.,* and *Tolypothrix sp.*. The detection of these genera in $PM_{2.5}$ was highly variable across the season. This suggests that controls on their relative abundance in $PM_{2.5}$ is greatly influenced by seasonal factors. Interestingly, *Cyanobium sp.* relative abundance in PM was highest during the bloom period, when other cyanobacterial genera out-competed *Cyanobium sp.* in water samples and thus its relative abundance in water was lowest. *Obscuribacterales sp.* relative abundance in $PM_{2.5}$ was low most of the campaign, but spiked considerably in the sampling period immediately following Hurricane Isaias, in early August of 2020 (S5).

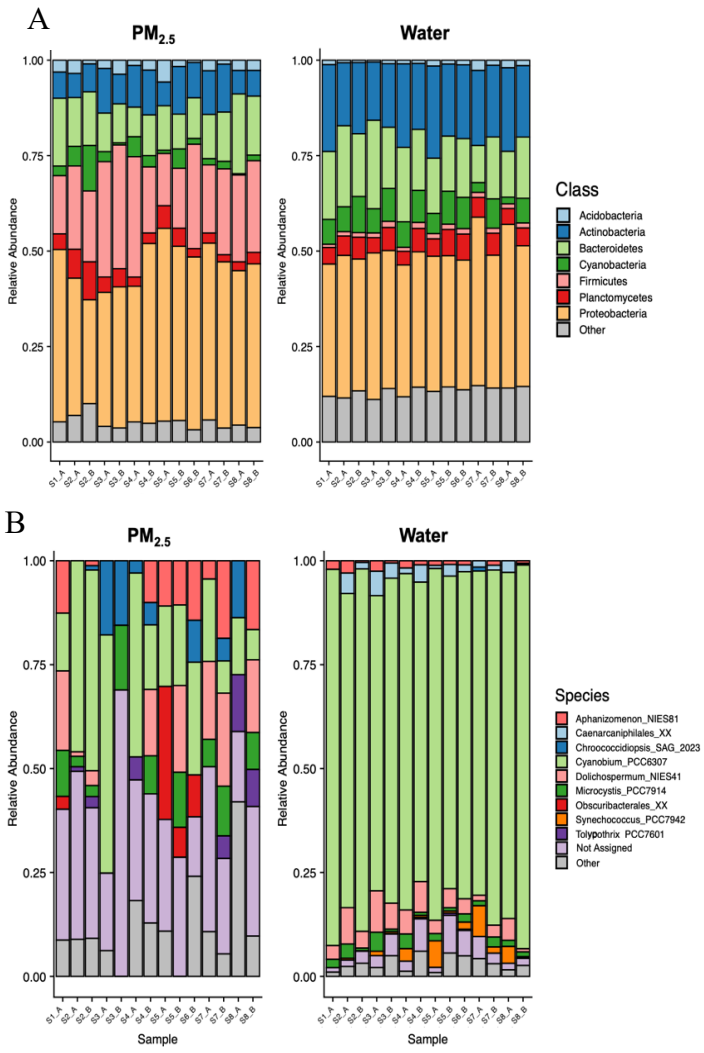

Figure 5. A) Profiles of the most abundant bacterial communities in $PM_{2.5}$ and water, resolved to the taxonomic level of class. B) Profiles of the most abundant cyanobacterial communities in $PM_{2.5}$ and water samples, resolved to the taxonomic level of genera. In the x-axis, S1-S8 denote the sampling period, listed in order of occurrence. _A denotes samples collected at Site A. _B denotes samples collected at Site B.

In water, cyanobacterial communities throughout the entire season were recorded with much less variability, and dominated by *Cyanobium sp.* (80.9 ± 6.6%), followed by *Dolichospermum sp.* (4.8 ± 2.7%), *Caenarcaniphilales sp.* (2.3 ± 1.8%), *Synechococcus sp.* (2.0 ± 2.4%), and *Microcystis sp.* (2.0 ± 1.2%). The highest relative abundances of *Aphanizomenon sp., Dolichospermum sp.*, *Caenarcaniphilales sp.*, and *Microcystis sp.* in water were all recorded during the bloom period (S2-3). In years preceding this study, *Microcystis sp.* had been one of the primary cyanobacterial genera reported in the CR-

AS, especially in association with high concentrations of MC, but in summer 2020, the abundance of *Microcystis sp.* remained relatively low (<5%).

Microcystin Congeners in PM and Water Samples

Throughout the entire summer of 2020, and in great contrast from the previous summer, all quantifiable MC concentrations in water remained low (< 1µg L^{-1}). These results agree with the NC-DEQ's reports and low occurrences of *Microcystis sp.*. Accordingly, no MC was detectable in any PM samples collected during summer 2020, but we did quantify several congeners of MC in water samples at both Sites A and B and several NC-DEQ monitoring stations (Table 1). At Sites A and B, three distinct congeners were quantified on 5 sampling days from June-October, including MC-LA, -LR, and -LF (Fig. 6). MC concentration on any single sampling day were highest on July 7th at site A (17.2 ng L^{-1}) in co-occurrence with a surface scum, but concentrations were recorded on the same order of magnitude into October, not corresponding with any distinct bloom or visible biomass. We suspect this may be the result of a lag-effect due to residual MC in the system per its long half-life in freshwater systems,[34] rather than active production of MC occurring later in the season. MC-LA was the most commonly reported congener at Sites A and B, which is notable because its aerosolization factor is 2000, the highest of all MC aerosolization factors currently known, making it nearly 2.5 times as likely to be enriched in aerosol than MC-LR, and 200 times as likely to be enriched in aerosol when compared to less hydrophobic congeners like MC-RR.[29]

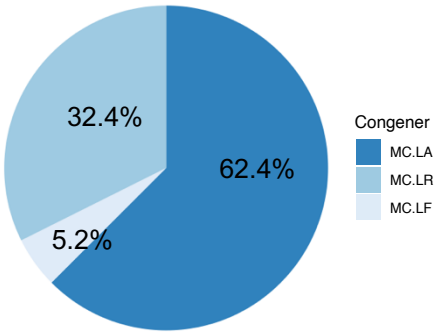

Figure 6. The relative abundance of MC congeners quantified at sites A and B.

Table 1. Average MC concentration calculated during each sampling period at Sites A and B in PM and water samples. All values are reported in ng L^{-1}; a - indicates no data recorded.

Sampling Period	Site	Bulk MC PM	Bulk MC Water	MC-LA	MC-LF	MC-LR
S1	A	0.0	2.07	2.07	0.0	0.0
	B	-	6.36	6.36	0.0	0.0
S2	A	0.0	10.66	2.07	0.0	8.60
	B	0.0	6.36	6.36	0.0	0.0
S3	A	0.0	8.60	0.0	0.0	8.60
	B	0.0	1.36	0.0	1.36	0.0
S4	A	0.0	0.00	0.0	0.0	0.0
	B	0.0	1.36	0.0	1.36	0.0
S5	A	0.0	0.00	0.0	0.0	0.0
	B	0.0	3.57	3.57	0.0	0.0
S6	A	-	0.0	0.0	0.0	0.0
	B	0.0	3.57	3.57	0.0	0.0
S7	A	0.0	0.0	0.0	0.0	0.0
	B	0.0	0.0	0.0	0.0	0.0
S8	A	0.0	1.93	1.93	0.0	0.0
	B	0.0	7.31	7.31	0.0	0.0

For the NC-DEQ MC data, overall MC concentrations were also low (< 1µg L^{-1}), but we quantified four congeners from June to September at six of eight stations (Table 2). Early in the season (June and July), MC was measured at stations upriver, but as the bloom progressed downriver later in the season (August and September) MC was not detected at these stations in the CR but instead was measured at stations across the AS. The maximum bulk concentration of MC (580 ng L^{-1})

occurred at the eastern most station in the AS in September. These congeners measured in the AS were also distinct in their composition from the early season MC measured in the upper CR. While early measurements primarily consisted of MC-LA and MC-LR, these later measurements consisted of MC-LR, MC-YR, and MC-WR. This change in congener composition is important due to variable toxicities between congeners,[35] and could be the result of several processes. The shift in MC congeners is most likely linked to changes in phytoplankton community composition due to seasonal succession or increasing salinity but could also be because of other spatiotemporal factors like changes to nutrient availability.

Table 2. Average MC concentrations recorded at each NC-DEQ site from each monitoring trip in summer 2020. All values are reported in ng L^{-1}.

Station	Bulk MC	MC-LA	MC-LR	MC-LY	MC-YR	D-Asp-MC-LR	MC-LF	MC-LW	MC-WR
D8356200	**131.08**	**18.96**	**102.26**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
Jun	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Jul	131.08	18.96	102.26	0.00	0.00	0.00	0.00	0.00	0.00
Aug	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Sep	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D8950000	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
D94900000	**15.51**	**0.00**	**15.51**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
Jun	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Jul	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Aug	15.51	0.00	15.51	0.00	0.00	0.00	0.00	0.00	0.00
Sep	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D995000N	**9.80**	**9.83**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.40**
Jun	9.80	9.83	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Jul	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Aug	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	**0.40**
Sep	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D995000C	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
D995000S	**3.03**	**3.03**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
Jun	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Jul	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Aug	**3.03**	3.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Sep	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
M610000C	**448.69**	**9.06**	**37.75**	**0.00**	**67.37**	**0.00**	**0.00**	**0.00**	**343.57**
Jun	9.10	9.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Jul	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Aug	256.29	0.00	21.17	0.00	0.00	0.00	0.00	0.00	209.74
Sep	448.69	0.00	37.75	0.00	67.37	0.00	0.00	0.00	343.57
M390000C	**579.38**	**0.00**	**50.94**	**0.00**	**91.19**	**0.00**	**0.00**	**0.00**	**455.57**
Jun	3.30	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3.30
Jul	455.57	0.00	0.00	0.00	0.00	0.00	0.00	0.00	455.57
Aug	145.45	0.00	0.00	0.00	0.00	0.00	0.00	0.00	131.48
Sep	579.38	0.00	50.94	0.00	91.19	0.00	0.00	0.00	437.25
Max recorded	**579.38**	**18.96**	**102.26**	**0.00**	**91.19**	**0.00**	**0.00**	**0.00**	**455.57**

Environmental Drivers of Cyanobacterial Communities in PM and Water

Several environmental parameters were associated with the occurrence of toxic cyanobacterial genera in $PM_{2.5}$. All environmental factors used as predictor variables which were found to be statistically associated with an outcome of interest can be found in Table 3, including ammonium (NH_4), chlorophyll a concentration (CHLA), particulate C to N molar ratio (C:N), Microcystin concentration (MC), nitrate plus nitrite (NO_x), particulate matter a.k.a aerosol (PM), soluble reactive phosphate (PO_4), particulate Nitrogen (PN), relative abundance in PM samples (RAPM), relative abundance in water samples (RAW), relative humidity (RH), salinity (SAL), solar irradiance (SI), silicate (SiO_2), total dissolved nitrogen (TDN), air temperature (T), turbidity (TRB), and wind speed (WS). Specifically, we examined potential environmental drivers on the relative abundance of *Dolichospermum sp.* and *Microcystis sp.* in $PM_{2.5}$. Understanding the environmental transport and fate of these genera are particularly relevant regarding public health because *Dolichospermum sp.* was the main CHAB former in summer 2020, and a metagenomic

analyses revealed that *Microcystis sp.* possessed MC encoding genes (*mcyA-mcyE*). These findings are explored in a complementary study conducted by co-PI R. Paerl (NCSU) and are being prepped for publication. Although no MC was quantified in $PM_{2.5}$ in the CR-AS in summer 2020, to investigate factors still potentially influencing cyanotoxin inhalation, environmental controls on the aerosolization of toxin-producing cyanobacteria were examined, as cyanotoxins are typically found intracellularly.[36]

Table 3. Results from a series of univariate linear regressions examining the association between **air** quality outcomes of interest and several environmental parameters.

Outcome Variable	Significant Predictor Variables (positively associated)	Significant Predictor Variables (inversely associated)
RA Cyanobacteria in $PM_{2.5}$	RAW *Pseudanabaena****, RAPM *Cyanobium**	
RA *Microcystis* in $PM_{2.5}$	RAPM *Dolichospermum***, *RAMP* N.A. cyanobacterial genera*	*RAPM Cyanobium***, *NH_4**
RA *Dolichospermum* in $PM_{2.5}$	RAPM *Microcystis***, C:N*, RH*, RAPM *Aphanizomenon**	
Ambient $PM_{2.5}$	T***, SI***, SAL**, C:N*, RAW *Pseudanabaena**, RAW N.A. cyanobacterial genera*, RAW *Dolichospermum**, RAW *Caenarcaphilales**	RH**

CHLA = Chlorophyll a, C:N = particulate C to N molar ratio, MC = Microcystin, PM = Particulate Matter, PN = Particulate Nitrogen, RAPM = Relative Abundance in PM samples, RAW = Relative Abundance in water samples, RH = Relative Humidity, SAL = Salinity, SI = Solar Irradiance, T = Air Temperature, TRB = Turbidity, WS = Wind Speed. All predictor variables are listed in order of descending adjusted R^2 and F-statistic values (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$)

The relative abundance of *Microcystis sp.* in $PM_{2.5}$ was positively associated with the relative abundance of *Dolichospermum sp.*, other unassigned cyanobacterial genera in $PM_{2.5}$, and C:N molar ratio in the water, but negatively correlated with the relative abundance of *Microcystis sp.* and NH_4 concentrations in water samples.

In addition to the air quality outcomes, several water quality and bloom-formation outcomes were explored. All water quality outcomes with statistically significant associated environmental factors are found in Table 4. MC concentration and the relative abundance of cyanobacteria, *Dolichospermum sp.*, and *Microcystis sp.* in water samples were examined as outcome variables. *Microcystis sp.* was chosen because it was linked to MC production, and *Dolichospermum sp.* was chosen as it was identified as the dominant genera of the surface scum and bloom which occurred from mid-June through mid-July.

MC was positively correlated with PO_4, CHLA, PN, NH_4, and TDN concentrations in water samples, as well as the relative abundance of *Aphanizomenon sp.* in water (Table 4). The relative abundance of *Dolichospermum sp.*, and therefore the main CHAB in the CR during summer 2020, was positively associated with the relative abundance of *Aphanizomenon sp.*, *Caenarcaphilales sp.*, and *Microcystis sp.* in water samples, solar irradiance, CHLA, NH_4, PO_4, TDN, ambient $PM_{2.5}$ concentrations, and the relative abundance of *Cyanobium sp.* in $PM_{2.5}$. Whereas, it was negatively associated with relative humidity, C:N molar ratio, the relative abundance *Cyanobium sp.* in water, and the relative abundance of *Aphanizomenon sp., Dolichospermum sp.*, and *Microcystis sp.* in

$PM_{2.5}$. *Microcystis sp.* relative abundances were also associated with similar environmental conditions, and these relationships can be found in Table 4.

Table 4. Results from a series of univariate linear regressions examining the association between **water** quality outcomes of interest and several environmental parameters.

Outcome Variable	Significant Predictor Variables (positively associated)	Significant Predictor Variables (inversely associated)
RA Cyanobacteria in Water	Diatom CHLA**, SAL**, CHLA*, T*	NO_x***
RA *Microcystis* in Water	RAW *Aphanizomenon****, NH_4**, TDN**, RAPM *Cyanobium**	RAPM *Microcystis**
RA *Dolichospermum* in Water	RAW *Caenarcaniphilales****, SI**, NH_4** PO_4*, CHLA*, $PM_{2.5}$*, TDN*, RAW *Aphanizomenon**, RAPM *Cyanobium**, RAW *Microcystis**	RAW *Cyanobium***, RAPM *Aphanizomenon**
Microcystin	PO_4***, CHLA**, RAW *Aphanizomenon**, PN*, NH_4*, TDN*	

CHLA = Chlorophyll a, C:N = particulate C to N molar ratio, MC = Microcystin, PM = Particulate Matter, PN = Particulate Nitrogen, RAPM = Relative Abundance in PM samples, RAW = Relative Abundance in water samples, RH = Relative Humidity, SAL = Salinity, SI = Solar Irradiance, T = Air Temperature, TRB = Turbidity, WS = Wind Speed. All predictor variables are listed in order of descending adjusted R^2 and F-statistic values (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$)

Ambient $PM_{2.5}$ Mass Concentration and Bloom Activity

Several factors were also examined in correlation with ambient $PM_{2.5}$ measurements, as shown in Table 3. Temperature, solar irradiation, salinity, C:N molar ratio, and the relative abundance of *Dolichospermum sp., Pseudanabaena sp., Caenarcaphilales sp.,* and unassigned cyanobacterial genera in water samples were all positively correlated with ambient $PM_{2.5}$ mass concentrations (Fig. 7). Chlorophyll a concentrations, wind speed, and the relative abundance of cyanobacteria were also positively correlated with ambient $PM_{2.5}$, but these relationships were not statistically significant ($p < 0.1$). Relative humidity was the only factor which was inversely correlated with ambient $PM_{2.5}$ measurements, which was expected as increased relative humidity is known to increase water condensation on $PM_{2.5}$, increasing aerodynamic diameter and growing particles beyond the range of $PM_{2.5}$.[37] Temperature and solar irradiation are also known to increase $PM_{2.5}$ concentrations, as increased air temperature leads to stagnation of air masses trapping more particles in,[38] and solar irradiation can increase the number of free radicals available in the atmosphere for photochemical and other forms of oxidation, increasing the production of secondary organic aerosol (SOA).[39]

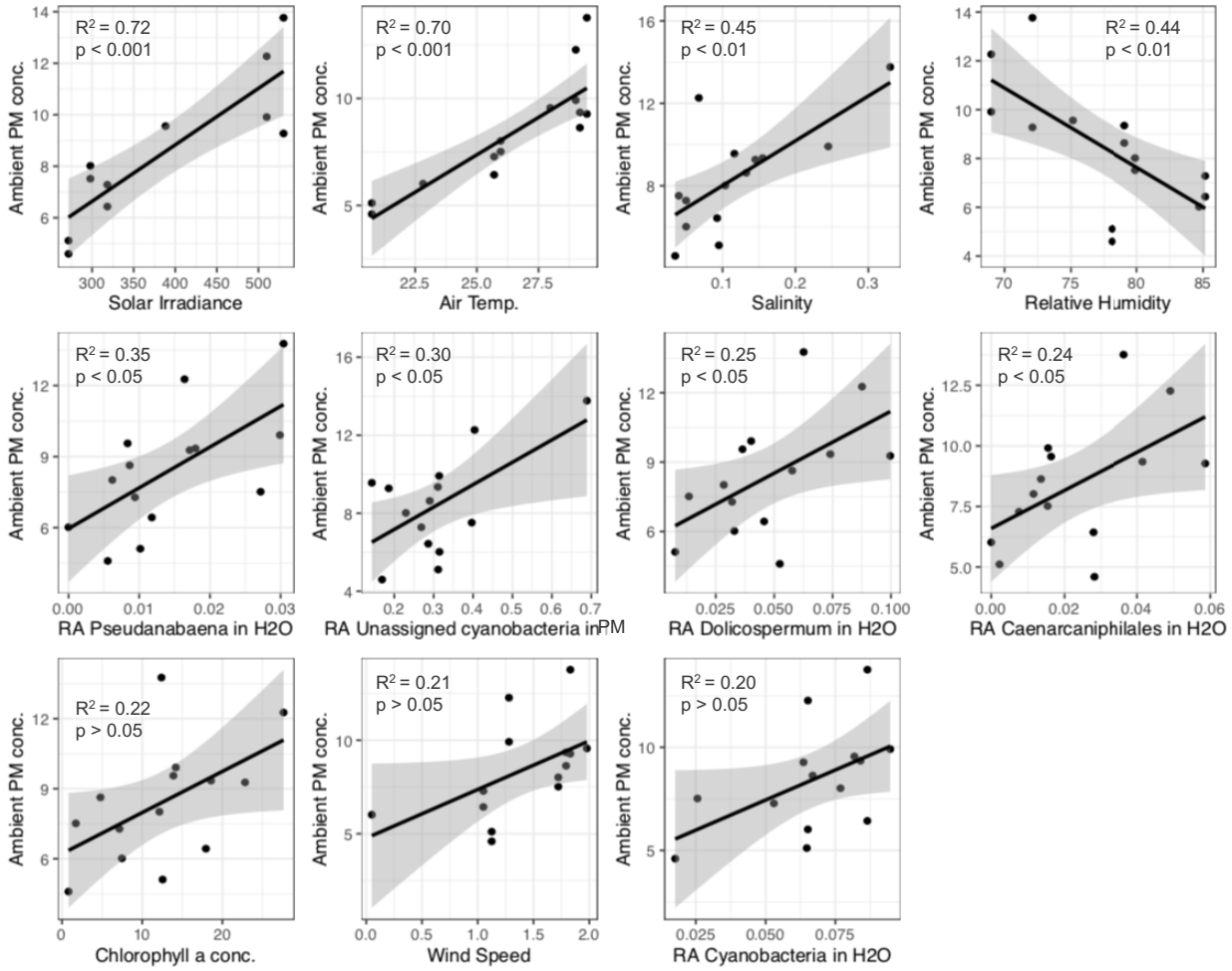

Figure 7. Univariate linear regression plots, examining relationships between ambient $PM_{2.5}$ mass concentrations and several statistically significant predictor variables. Adjusted R^2 and p-values for each regression are denoted on each plot.

When comparing a time-series of ambient $PM_{2.5}$ mass concentrations with bloom activity and interval chlorophyll a measurements, the baseline $PM_{2.5}$ reading appeared to increase slightly (Fig. 8). During the entire field campaign, average diurnal $PM_{2.5}$ readings were 8.06 µg m^{-3}, however during the bloom period, this value increased to 10.5 µg L^{-3}. A Wilcoxon signed rank was used to evaluate the differences in ambient $PM_{2.5}$ concentrations during bloom activity, and it was confirmed statistically that the median value for ambient $PM_{2.5}$ was greater during bloom conditions than the median value for ambient $PM_{2.5}$ during non-bloom conditions ($p < 0.001$). Further, on days when a bloom was present, the ambient $PM_{2.5}$ concentration was more likely to exceed EPA annual $PM_{2.5}$ criteria pollutant standards (> 12.5 µg m^{-3}).

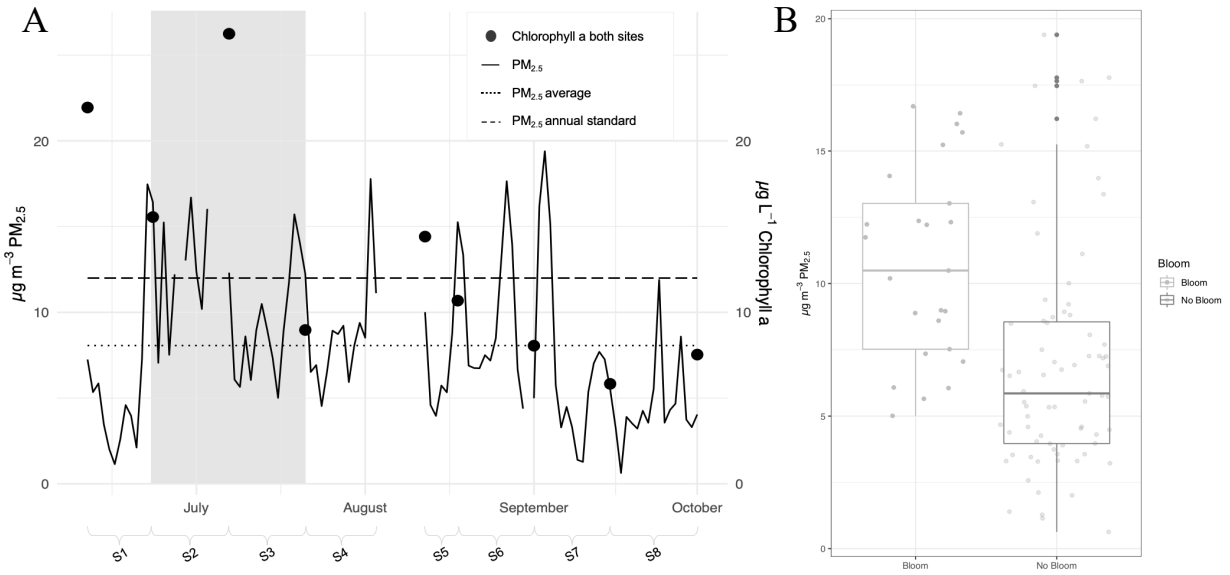

Figure 8. A) A time-series from June-October, 2020, showing daily ambient $PM_{2.5}$ concentrations and intermittent chlorophyll a measurements, collated from two sites on the CR-AS. The PM sampling periods are denoted on the x-axis, and the period defined as a cyanobacterial bloom is shaded in gray. The $PM_{2.5}$ average reported during the entire sampling period was 8.06 µg m^{-3}, and the $PM_{2.5}$ annual standard as regulated by the EPA is 12 µg m^{-3} as of 2021. B) Ambient $PM_{2.5}$ concentrations across the study, grouped by cyanobacterial bloom conditions.

DISCUSSION

When examining the outcomes of the univariate linear regression models altogether, a couple trends became apparent.

1) While slightly variable in strength of association, in general, cyanobacteria, MC concentrations, and key genera including *Aphanizomenon, Dolichospermum, Microcystis,* and *Pseudanabaena* are associated with CHLA concentrations and key nutrient analytes including NH_4, TDN, and PO_4, other than NO_x, which was inversely related to cyanobacterial relative abundance. More work must be conducted to understand precise cause-effect relationships. Nutrient bioassays are currently being conducted by the Paerl lab to better understand direct responses of CR-AS algal communities to nutrient additions and limitations.

2) Metagenomic findings of *mcyA* encoding genes possessed by *Microcystis sp.* suggest that *Microcystis sp.* was the only MC producer in the CR in summer 2020, which was previously suspected by empirically unknown. However, we found a strong association between *Aphanizomenon sp.* and MC concentrations, suggesting that *Aphanizomenon sp.* may co-occur or compete with *Microcystis sp.* in the CR-AS. More work is necessary to determine the role of *Aphanizomenon sp.* in *Microcystis sp.* growth and MC production. Thus, *Aphanizomenon sp.* community interactions and nutrient uptake dynamics should also be considered by regulatory agencies when developing rules and regulations to prevent unsafe MC levels in the CR-AS. Further, despite low concentrations of MC recorded in 2020, it is striking that MC was found in association with key nutrient analytes. While it is impossible to assign causation via our study

design and statistical approach, this association could be explained by several processes. Heightened concentrations of nutrients being utilized for MC production, re-suspension of nutrients and MC from sediments, or MC production co-occurring with the cycling of nutrients within CHABs during heightened bloom activity (e.g., denitrification and related processes) could all explain the interactions between MC and nutrients, as outlined in previous work.[40]

3) In direct contrast with our hypothesis, which stated that increased occurrences of cyanobacteria in water would increase occurrences in $PM_{2.5}$, *Dolichospermum sp.* and *Microcystis sp.* relative abundances in aerosol were all inversely related to their relative abundances in water. Further supporting this conclusion, other environmental conditions we found to be associated with bloom activity, such as elevated nutrient concentrations, were also inversely related to relative abundances of these genera in PM. We propose a couple explanations for this: firstly, as result of the physicochemical properties and ecological activity of CHABs. *Dolichospermum sp.* and *Microcystis sp.* can form dense surface scums due to an aggregation of cyanobacterial cells and colonies. Increasing cell density (suspected with increasing relative abundances), often leads to the formation of such colonial and filamentous clusters, disallowing cells from being scavenged by bubbles in surface waters and subsequently aerosolized. Secondly, if aerosolized, such clusters would likely surpass 2.5 μm in diameter, and thus be undetectable via our sampling method. In our future studies, we are correcting this issue with the addition of another sampler running alongside $PM_{2.5}$ measurements to simultaneously collect cyanobacterial communities in PM_{10} (< 10 μm). Because < 1% of cyanotoxins in the field are found in the dissolved form,[41] we suggest that airborne toxins are more likely to be found bound to partial or intact cells, and thus enriched in PM_{10} or larger PM, rather than $PM_{2.5}$. Although PM in these larger size fractions present a lesser respiratory health concern when compared to $PM_{2.5,}$ there is still evidence to suggest the inhalation of cyanotoxins or cells in this size range could impact in the upper respiratory tract and association toxicological impacts are currently unknown. Future work should examine size distributions of aerosolized cyanotoxins, environmental controls on this, and related health ramifications.

One challenge in this study was to assign the origin of cyanobacterial genera in PM samples. The 201 ASVs which overlapped between PM and water communities, including ASVs assigned as *Aphanizomenon sp., Dolichospermum sp.,* and *Microcystis sp.,* suggest a direct water to air transfer of toxic cyanobacterial communities. The most well-understood mechanism of microbial transfer to the atmosphere from aquatic sources is spray aerosolization. Salinity was significantly associated with ambient $PM_{2.5}$ mass concentrations, providing more evidence to suggest presence of spray aerosol in our $PM_{2.5}$ samples, as previous work has demonstrated that salinity increases the mass concentration of spray aerosol.[42,43] However, cyanobacterial relative abundance in water was either inversely or not associated with concentrations in $PM_{2.5}$, and several environmental factors demonstrated to be linked to the production of spray aerosol (such as wind speed), were not significantly associated with $PM_{2.5}$ in our regression models. Therefore, it is unlikely that spray aerosolization was the only mechanism associated with CHAB's impact on air quality in this study.

Another interesting finding from the metagenomic data from co-PI R. Paerl's complementary study revealed that the dominant cyanobacterial genera of the bloom, *Dolichospermum*, possessed geosmin encoding genes. Geosmin is a volatile organic compound (VOC) synthesized by several photosynthetic organisms, including aquatic microbes like cyanobacteria and actinobacteria. [44]Although significantly less toxic than other cyanobacterial metabolites like cyanotoxins,

geosmin, along with other VOCs produced by CHABs, are undesirable in drinking water and aquaculture operations due to their unwanted tastes and odors.[45] VOCs such as geosmin have never been investigated as potential sources of PM, but due to their chemical structure, they are likely candidates for the formation of secondary organic aerosol (SOA),[46,47] i.e., PM that forms from the nucleation or condensation of gas-phase compounds into the particle-phase following atmospheric chemical processing. Provided that ambient $PM_{2.5}$ concentrations were not related to the relative abundance of any bacterial group in PM, but were elevated during CHAB activity and associated with bloom-specific environmental variables including *Dolichospermum* relative abundance, there is evidence to suggest that CHABs are impacting ambient $PM_{2.5}$ concentrations via mechanisms other than just primary spray aerosol formation via bubble-bursting. We suggest the emission of VOCs such as geosmin are possibly contributing to SOA formation in the airspace above CHABs.

A handful of studies over the open ocean have found correlations between increased atmospheric PM and marine phytoplankton activity,[48–50] and specifically the emission of VOCs from marine phytoplankton.[51–53] Further, air quality has been linked to CHABs through the nitrogen cycle, via the atmospheric deposition of bioavailable particulate nutrients, or the use of gaseous N_2 by N-fixing blooms, demonstrating already recognized interactions between CHABs and air pollutants.[54] Herein, we demonstrate the primary aerosolization of CHAB cells in NC and present the first evidence to suggest that freshwater algae and specifically CHABs may impact air quality in the CR-AS region via similar processes to biogenic SOA formed over the open ocean. The role that freshwater CHABs play in regional air quality is complex, but such links are becoming more frequently recognized and important to understand for ecosystem and human health.

We are currently prepping all data for publication in a scientific journal, the current target being *Science of the Total Environment.* Data generated in collaboration with the NC-DEQ is also being prepped for a formal presentation to this key stakeholder, and all raw data is being shared with primary CR-AS and CHAB NC-DEQ points of contact including Jill Paxson, Kristen Slodysko, Tammy Hill, Elizabeth Fensin, and Forest Shepherd. Preliminary findings will substantiate the first data chapter of H. Plaas' dissertation effort at UNC Chapel Hill (UNC-CH), and have led to future funded work in NC and beyond, aimed at further investigating the links between air quality and CHABs.

Beyond the scientific findings of this study, this project supported two UNC students (undergraduate intern Abe Loven and PhD student Haley Plaas), engaged several community members directly through the CEEG, and thousands more indirectly through generated outreach materials through UNC research social media, NCSG *Coastwatch*, and NC Environmental Education's Discovery Lecture Series. In summary, the opportunity provided through this NCSG-APNEP joint fellowship has served as a springboard for the career of UNC-CH doctoral student Haley Plaas. This was the first study to investigate the airborne transport of toxic cyanobacteria in NC, and make a connection between freshwater CHABs and air quality degradation. In collaboration with the NC-DEQ, this work generated the first congener-specific data on MC in the CR-AS, providing key information to the state as MC is increasingly reported across the state and globe.

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CHOWAN EDENTON ENVIRONMENTAL GROUP COMMUNITY SCIENTIST WATER AND AIR QUALITY MONITORING MANUAL

CHOWAN-ALBEMARLE RESEARCH SUMMER 2020

Written by: Haley Plaas, PhD Student
Edited by: Co-PIs Nathan Hall & Hans Paerl
UNC-Institute of Marine Sciences

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I. INTRODUCTION

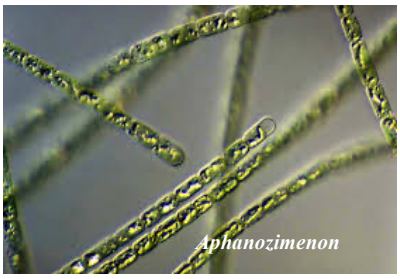
Project Contact Information

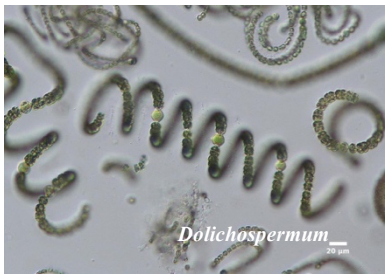
Project Lead:
Haley Plaas, PhD Student, UNC-IMS, email: hplaas@live.unc.edu, phone: (913)-634-9176
Principal Investigator:
Hans Paerl, Distinguished Professor, UNC-IMS, email: hans_paerl@unc.edu
Community Scientist Coordinator:
Colleen Karl, CEEG Chair, email: ckarl2010@gmail.com
Science Communication Intern:
Abe Loven, Undergraduate Student, UNC-CH, email: abeloven@live.unc.edu

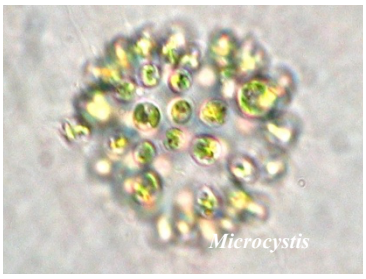
Collaborating Researchers:
Karsten Baumann, Assistant Professor, UNC-CH, email: kaba@email.unc.edu;
Naomi Chang, PhD Student, UNC-CH, email: nychang@live.unc.edu;
Nathan Hall, Assistant Professor, UNC-IMS, email: nshall@email.unc.edu;
Ryan Paerl, Assistant Professor, NCSU, email: rpaerl@ncsu.edu;
Joel Sanchez, PhD Student, NCSU, jjsanche@ncsu.edu

Your role in this research. Thank you for volunteering to help monitor water and air quality in your area. We couldn't perform such a thorough study without your time, generosity, and commitment to protecting our water resources. Community scientist (formerly citizen science) efforts are incredibly important, and help foster a sense of environmental stewardship in the greater community. The data you help us collect is incredibly valuable and necessary. As a community scientist volunteer, you will be filtering water samples and ensuring the security of our aerosol sampling equipment at your respective location along the Chowan River-Albemarle Sound (CR-AS). The suite of air and water quality parameters we are analyzing your samples for are explained later in this manual.

What is a harmful cyanobacterial bloom (or CyanoHAB)? Cyanobacteria are ancient, photosynthetic algae that can thrive in freshwater, estuarine, and marine ecosystems on every continent (even Antarctica!). During the summer, when waters are warm and there is plenty of sunlight, cyanobacteria can bloom into dense, surface scums (Paerl & Otten, 2013a, 2013b). Nutrient-loading from fertilizers and/or untreated sewage in runoff can promote the growth of cyanobacterial blooms (Bullerjahn et al., 2016; Paerl et al., 2011). Harmful cyanobacterial blooms (CyanoHABs) can produce toxic metabolites called cyanotoxins—these toxins are hypothesized to be a result of oxidative stress on the cell. There are several

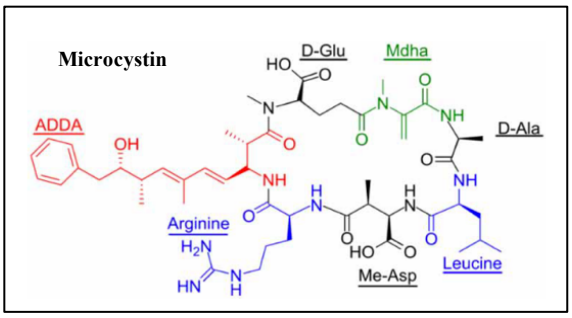




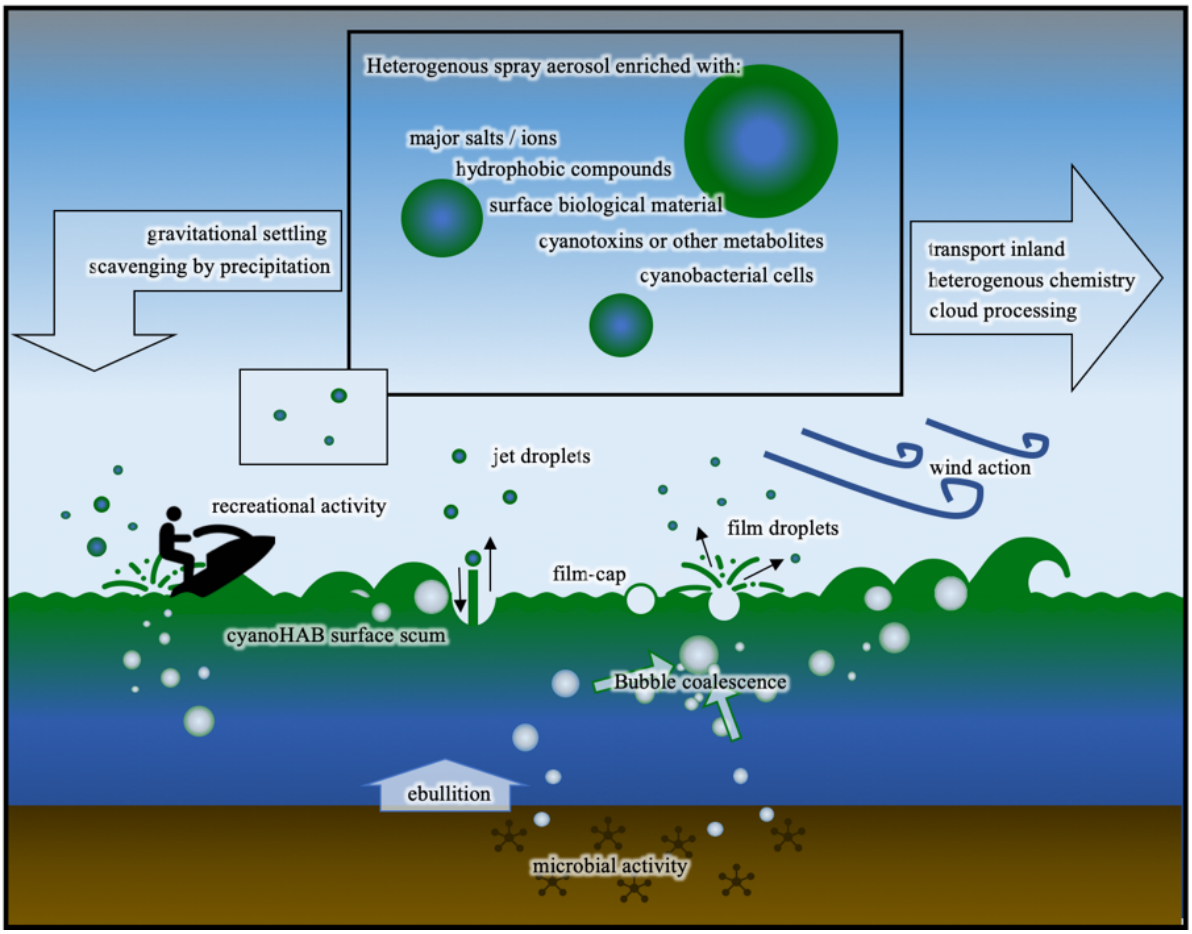


strains of CyanoHABs, but our focus is on *Microcystis,* which has recently been proliferating in the CR-AS. The toxin of concern in the CR-AS is microcystin, a liver toxin frequently

produced by *Microcystis*. Human exposure to microcystin is regulated by the World Health Organization and US Environmental Protection Agency because of its negative impact on human and animal (e.g. pet, livestock, and wildlife) health. The primary route of microcystin exposure is through ingestion, but inhalation is another potential public health concern which is less understood.



What is spray aerosol? Algal cells and their metabolites can be incorporated into aerosol at the water-air interface (May et al., 2018; Murby & Haney, 2016; Yung et al., 2007) . As wind stress or recreational activity generates waves, air bubbles are entrapped beneath the surface. As these bubbles rise, chemical and biological matter in the water column can be adsorbed to the bubbles' surfaces (O'Dowd et al., 2004). When the bubbles reach the surface and pop, a spray is ejected into the air, forming aerosols that may carry cyanobacteria and cyanotoxins. This aerosol can travel for several miles depending on its size, shape, and chemical properties.



The goals of this study are to (1) characterize spray aerosol formed during cyanoHAB conditions to inform potential human exposure to airborne microcystin, and (2) better understand the environmental factors promoting CyanoHAB growth and toxin production. To achieve this, we will collect and quantify (1) microcystin and cyanobacterial DNA in both aerosols and in the water column, and (2) measure environmental parameters in the air and water (e.g. nutrients, chlorophyll a, phytoplankton pigmentation, ambient weather conditions) to understand controls on CyanoHAB growth dynamics, microcystin production, and potential microcystin aerosol formation.

II. WATER AND AIR QUALITY PARAMETERS

Chlorophyll *a* (Chl *a*) is a photosynthetic pigment that is essential for photosynthesis in cyanobacteria (as well as many other algae and plants). It absorbs sunlight and converts it into energy to be used by the plant cell. In water quality monitoring, chlorophyll *a* is easy to measure and is a useful proxy for algal biomass in a water sample. During cyanobacterial bloom conditions, chlorophyll *a* readings, in conjunction with other measurements, can indicate the relative cell concentration of phytoplankton in the sample.

High Performance Liquid Chromatography (HPLC) is a technique with many applications across many fields of research. HPLC separates a mixture of compounds to identify, quantify, and purify the individual components of a mixture, in our case, phytoplankton pigments (Jeffrey et al. 1997). This provides a sense of the types of phytoplankton (algae, bacteria) present in the sample based on the relative abundance of each pigment; for instance, phycocyanin is the pigment responsible for the "blue-green" coloration and nickname of cyanobacteria.

Phytoplankton Morphology is the size, shape, and visible physical characteristics of phytoplankton in a sample. We collect a small sample of water (20 mL), and treat it with Lugol's Iodine solution. This preserves the cells and stains them so that we may look at them under a microscope. The characteristics of the algae we see with microscopy can be paired with our findings from HPLC to reveal more traits about the phytoplankton.

Flow Cytometry (FCM) is a specialized type of cell counting using fluidics without filters or a microscope. It provides a method to both quantify and identify phytoplankton in a heterogenous sample, based upon the specific light scattering and fluorescent characteristics of each cell. It is a useful scientific instrument, as it provides fast, objective and quantitative recording of fluorescent signals from individual cells as well as physical separation of cells of particular interest. Similar to HPLC, it can reveal the relative abundance of phytoplankton pigmentation in a sample but it can do so for individual cells, providing more fine-tuned measurements. It can also count cells, giving us a cell concentration per volume. Sometimes, if a sample is too densely populated, we cannot accurately use FCM, hence the need for multiple methods.

Cyanobacterial DNA will reveal the species of cyanobacteria present in the sample. By extracting DNA from a sample, we can amplify the gene sequences we choose with a PCR (Polymerase Chain Reaction) kit. For this study, we will be amplifying the 16S rRNA sequence from cyanobacteria, which is a segment of the genetic code that is highly conserved across all bacteria. We will also amplify *mcyA*, a gene sequence that is linked to microcystin synthesis when it is actively transcribed. We will be using these samples to look at

cyanobacterial species, but also examine the relative abundance of toxin genes in comparison to measured toxin concentrations. This will help us understand which cyanobacterial species in the Chowan are producing toxins.

Nutrients are the major essential inorganic compounds that cyanobacteria and other phytoplankton use for energy alongside sunlight. Nitrogen (N) and phosphorus (P) are the main two elements needed from nutrients to promote plant growth. Different cyanobacteria can uptake and regulate several variable forms of N and P, some can even use forms from atmospheric gases, but others require more accessible forms, like the type we utilize in fertilizers. Other nutrients required for favorable algal growth are iron, sulfur, potassium, magnesium and micronutrients or cofactors (vitamins). We will analyze dissolved nutrients (in the water) for ammonium (NH_4), nitrate (NO_3), phosphate (PO_4), silicate (Si), and total dissolved nitrogen (TDN). We will analyze the particular nutrients (within biological matter or adsorbed to sediments) from the GF/F, establishing the Carbon to Hydrogen to Nitrogen ratio (CHN). The particulate organic matter (POM) you can see on the filter is made up of living (plankton) and detrital material (fragments of waste, dead organisms). Analyzing the POM and dissolved nutrients suspended in water can reveal the trophic state of aquatic ecosystems.

Microcystin is a cyanotoxin produced by several strains of cyanobacteria; it is amongst the most widespread and frequently detected cyanotoxins (Dawson, 1998). One particularly hardy and widespread genus of microcystin producers is *Microcystis,* which has been detected in the CR-AS in recent summers. Both acute and chronic exposure to microcystin are linked to several adverse human health outcomes. As of 2015, the EPA criterion for microcystin in drinking water is < 0.3 μg/L, and only recently (2019) were guidelines implemented for recreational use of water containing microcystin: <8 μg/L for swimming (US EPA). Last summer, microcystin in the Chowan River was measured in one sample at an alarming 650 μg/L in August and several other samples > 300 μg/L. We are interested in measuring the intracellular (toxin within a cyanobacterial cell) and extracellular (toxin that has been released by the cell through stress or cell death), because this could influence its transport in the environment and aerosolization capability. Furthermore, estuarine gradients are one of the few phenomena demonstrated that cause cyanobacteria to prematurely lyse cyanotoxins into the water column, and thus there may be significant concentrations of both extra/intracellular toxin in our samples. We will analyze the toxin with LC-MS/MS (Tandem Liquid Chromatography-Mass Spectrometry) because it is a high resolution technique that allows us to determine the specific microcystin chemical structures.

Spray Aerosol An aerosol, also referred to as particulate matter or particle, is a solid or liquid suspended in a gas. One of the largest sources of algal and bacterial particles in the atmosphere is spray aerosol (SA), formed at the surface of the ocean, lakes, and rivers, etc. (Aller et al., 2005; De Leeuw et al., 2011). We are interested in quantifying the cyanobacterial DNA and toxins in spray aerosol. The aerosol equipment we are using for this project is on loan from the EPA—so only UNC-CH students and faculty will be operating the equipment. These aerosol samplers operate over long periods of time, so we may ask for your help to make sure the samplers are still operating smoothly and are not bothered during the course of the sampling campaign. If the noise becomes an issue, please let us know and we can adjust our sampling plan accordingly.

Environmental Parameters and ambient weather conditions, including air temperature, water temperature, wind speed, wind direction, wave action, relative humidity, light, and salinity, can influence aerosolization and bloom growth dynamics. We will be recording these factors in order to understand how they may influence ultimate human exposure to CyanoHABs. To measure and report these factors at time of sampling, we will be using YSI Sondes, a meteorological station to collect data on water quality and weather conditions.

III. SAFETY & GENERAL INFORMATION

Do not sacrifice your safety during this process. Some chemicals provided are hazardous and should be treated with care. Some objects in the kits may be sharp and could cut the skin if broken or misused. Some water may be sampled around slippery or unstable docks and shorelines. Some water may contain toxigenic algae or other pollutants to be mindful of touching. Some vials contain hazardous chemicals that should not be touched or ingested, and you must make sure they do not spill in the freezer. No data is worth an accident, or in light of the current global COVID-19 pandemic, exposure to illness. Please take your time, wear PPE, practice caution, and use your best judgement (and common sense ☺).

Many CEEG members have been a part of successful research campaigns for several years, and thus, you already know it is very important that you gather your data precisely, neatly, and accurately! If any small errors occur (which they always do at some point or another), please report this in some notes so we can correct for them upon analysis. I want to reiterate how thankful I am that you are taking the time to volunteer with this study and that I trust your judgement fully. The data you collect is indispensable and makes a large difference in expanding our field campaign.

Safety Guidelines:

1. Volunteers should read the entire manual including specific protocols before beginning sampling. Do not feel overwhelmed, this manual is just available for your reference as needed. Contact Haley Plaas at (913)-634-9176 or hplaas@live.unc.edu if any methods are unclear. Text, email, or call are all welcome.
2. Sample with a partner if your COVID-19 permits, but wear a mask to prevent the spread of any illness. Sampling with a buddy is a safety precaution and reduces errors, allowing everyone to double check each other's work.
3. Keep all equipment and reagent chemicals (specifically Lugol's and the FCM vials with pre-added glutaraldehyde fixative) out of the way of small children or pets. These chemicals are poisonous!
4. In the unlikely event of a chemical accident, contact Haley. It is likely not a big deal, but remain calm and call the American Association of Poison Control Centers at: (800) 222-1222 if necessary. In the event of an emergency, call 911.
5. We recommend that you wear gloves when you conduct all tests. This prevents the samples from exposure to you and also prevents your exposure to chemicals and potentially polluted water containing cyanobacterial toxins. At a minimum, always wash skin with full lather when skin contacts polluted water or chemicals.

6. If sampling from a property that is not yours or is public, always obtain written permission from the landowner or municipal contact. Please be courteous and respectful of individual property rights.

General Sampling Guidelines:

1. Protect equipment from prolonged exposure to direct sunlight and extreme temperatures. Store it in the blue boxes provided between sampling times.
2. When collecting a sample, please take your time and follow the steps of each respective protocol. Get familiar with the instructions and procedures before going out in the field. Reference this manual when necessary.
3. Always, ALWAYS homogenize your water sample before pouring. When transferring from the carboy to a graduated cylinder, ensure to mix the carboy thoroughly by gently inverting so that the water is uniform before measuring out the necessary volume for a sample. This is especially important during bloom conditions, when some algae may float or sink in the carboy.
4. Ensure to leave a visible sign/note on the aerosol equipment indicating what the equipment is there for, your contact information, Haley's contact information, and when you will be returning. We do not want to alarm any passersby or attract unwanted attention in case of vandalism.
5. Be careful not to contaminate any samples, especially those for nutrients and DNA processing. Use clean equipment, gloves, and be mindful of other potential contaminants.
6. Carefully seal samples in their respective storage apparatuses, and secure them in Ziploc bags prior to freezing them. Make sure key information like the site, sample number, date, and time collected are recorded on the bags.
7. You may be exposed to cyanobacterial toxins during sampling, and by volunteering, you are agreeing to take full responsibility for your potential exposure to any pollutants that may exist in the sampling region. However, no data or guidelines currently exist to suggest that the air you are breathing will be immediately harmful during bloom events (despite our research goals ☺).

IV. INSTRUCTIONS FOR MONITORING

To reduce any miscommunication on my part, I provided fine-tuned details as if you have never collected a water quality sample before. I apologize in advance for the oversimplification and repetition. The methods provided are what I would follow, but you may find another rhythm that works better for you. All filtration protocols are optimally performed under subdued lighting in an indoor setting to avoid changes in the sample from exposure to sunlight or additional nutrients, but they can also be performed in the field on site if necessary.

Sampling Sites

A. Chowan Beach

- 36°13'33.3"N 76°42'22.7"W

B. Pembroke Creek

- 36°03'31.1"N 76°37'32.4"W

Sample Labeling and Storage Information

Materials:

- Waterproof Sharpie
- Ziploc Bags
- Aluminum Foil Squares
- Microcystin and Nutrient Tubes
- Nutrient (CHN) petri dishes
- DNA centrifuge tubes
- Phytoplankton vials
- FCM vials

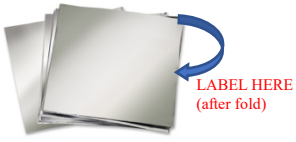
CHN petri dishes

Microcystin and Nutrient tubes

Phyto Vials

FCM Vials

DNA tubes



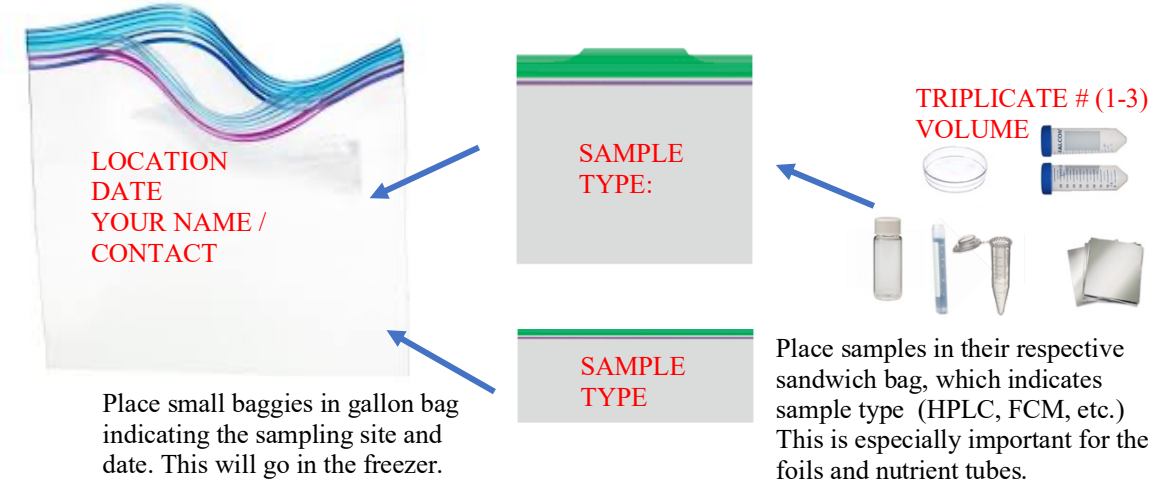
Aluminum Foil Squares

Sample Labeling Scheme: Familiarize yourself with the labeling scheme samples *ahead of time*. This is so that you can be organized during filtration and are familiar with the samples to be collected beforehand. If any information changes at the time of filtration it is OK to alter the label or add additional information. I will ask you to fix any dates that I have incorrectly pre-labeled. The more information, the better, so that upon lab analysis, we get the best sense of when, where, and how the sample was collected. Always use a wide-tipped waterproof sharpie on the foils and if labeling after the fact, be careful not to press greatly on the filter with the sharpie. This could lead to rips in the foil or filter or push the biomass off the filter.

Specific Labeling Information: Note for every type of sample, each apparatus should be labeled with the volume filtered and **a running number scheme to indicate triplicates (i.e. 1-3, 4-6).** Time ON samples vs. time OFF samples can be stored together for the same sampling date, but make sure the time ON samples are the first 3 numbers in the scheme.

To clarify, the first sampling date in June will be the first six samples (1-3 will be the TIME ON water sample, and 4-6 will be the TIME OFF water sample). The next sampling date, two weeks later, will be the next six samples (7-9 for TIME ON, 10-12 for TIME OFF). Make sure the date and time on and time off recorded are reported somewhere on the Ziploc bags.

Try to place the samples back in the bags they came in to keep them organized so I do not confuse samples stored in aluminum foils or falcon tubes since there are multiple types that are stored in those apparatuses. **Volumes are subject to change** based on the amount of particulate matter and biomass in the water sample. If there is too much clogging up the filter so that water is not easily passing, start over, and use your best judgement to reduce the volume filtered. Just make sure you record the final volume on the storage material. When folding the aluminum squares, ensure not to touch the inside of the packet with bare hands. **THE VOLUME, LOCATION, TIME COLLECTED, AND DATE ARE THE MOST CRUCIAL ASPECTS TO RECORD AND MUST BE INHERENT FROM THE LABELS.**



Place small baggies in gallon bag indicating the sampling site and date. This will go in the freezer.

Place samples in their respective sandwich bag, which indicates sample type (HPLC, FCM, etc.) This is especially important for the foils and nutrient tubes.

Reference the "Cheat Sheet" Chart for this information during filtration:

1. **Chlorophyll *a:*** (abbreviate CHLA) Stored in an aluminum foil square. Filtered on 25 mm glass fiber filters (GF/F) on filtration tower. Volume = 50 mL.
2. **HPLC:** (abbreviate HPLC) Stored in an aluminum foil square. Filtered on 25 mm glass fiber filters (GF/F) on filtration tower. Volume = 100 mL x2.
3. **Phytoplankton Morphology:** (no abbreviation necessary – vial is indicative of sample type). Stored in 25 mL vials with 4 mL Lugol's solution to fix phytoplankton. Volume is flexible. This is the only sample that will NOT be stored in the freezer, and should be stored in a dark box at room temperature.
4. **FCM:** (no abbreviation necessary – vial is indicative of sample type). Stored in 5 mL vials with 50 uL fixate, EM grade glutaraldehyde (pre-added).
5. **DNA:** (no abbreviation necessary – tube is indicative of sample type). Filtered on SUPOR filters. Volume = 50 mL.
6. **Nutrients:** (abbreviate NUTS) Filter is stored in in a petri dish, filtrate is stored in a Falcon tube. Filtered on glass fiber filters (GF/F) on the Erlenmeyer flask so that filtrate may be saved. Volume = 50 mL.
7. **Microcystin (MC):** (abbreviate MC) Filter is stored in an aluminum foil, filtrate is stored in two 50 mL Falcon tubes. Filtered on glass fiber filters (GF/F) on the Erlenmeyer flask so that filtrate may be saved. Volume = 100 mL.

Collecting Water Samples

Materials:

- Diaphragm Pump
- Funnel
- Carboy

Collection Protocol:

1. At the water, open the carboy and place funnel at the mouth.
2. Plug the pump in and switch it on. Place the pump in the water (~2 feet beneath the surface) and place the tubing into the carboy.
3. Rinse the carboy with the sample three times. (Place the lid on, swish it around, pour it out, do it again)
4. Fill the carboy with the water sample.
5. Turn off the pump. Secure the lid.

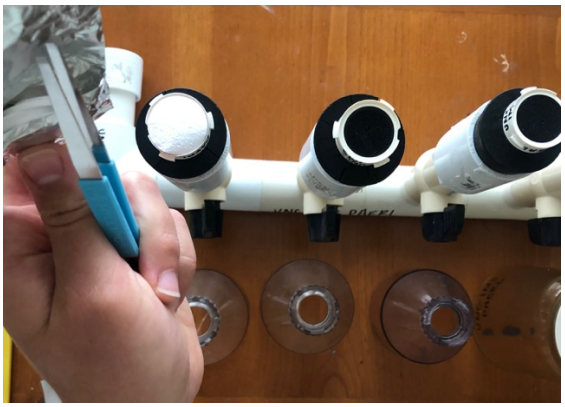
Chlorophyll *a* and HPLC Filtration

Materials:

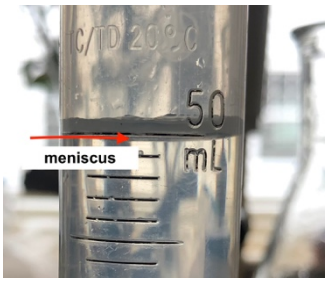
- Vacuum filtration manifold with filtration tower(s) and 25mm bases.
- Vacuum pump (electric)
- Carboy for waste
- Graduated cylinder (50 or 100 mL)
- Forceps
- Whatman GF/F, 25mm
- Aluminum foil packets (see sample labeling information)

Filtration Protocol:

1. Set up your filter apparatus (vacuum pump, towers, waste apparatus, etc). ** see diagram in appendix for correct assembly
2. Wearing gloves, use the forceps to place a GF/F onto the tower base. Secure the tower in place. Ensure that the filter is centered and intact (no visible rips). If you are having issues, turning on the pump helps suction the filter to the base, and you can double check with this to make sure they are secure.



3. Invert the sample bottle gently several times. This mixes the sample so it is uniform.
4. Then pour and measure 50 mL (Chl *a*) or 100 mL (HPLC) in graduated cylinder (with bottom of meniscus touching 50 mL line).

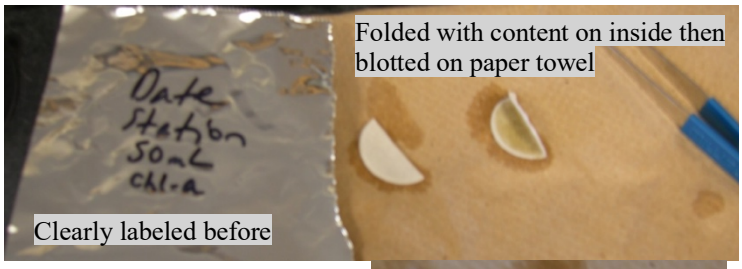


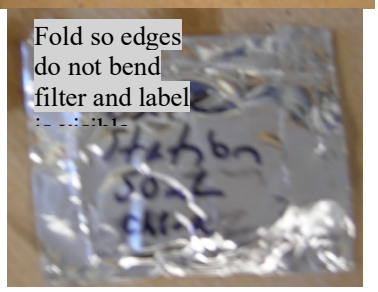
5. Before pouring sample into the tower, be sure the tower and filter are secured, otherwise the sample water will leak. If it does leak, no worries, but you will have start over and get a new filter.
6. Pour the water into the tower. Measure 50 more mL and repeat for the triplicates. You can fill every funnel before turning on the pump.
7. Turn on vacuum source and open valves on filter tower manifold. Vacuum pressure should be around approximately 5 psi. Try not to exceed 8 psi since high pressure may break the pigments.
8. You may have to adjust sample volumes based on conditions or losses of sample (spills / accidents). If you spill water out of the base of the funnel, start over with the same volume. If the water will not go down after ~30 seconds, make sure the waste lid is tight and that water is actually filling. If it is not going through the funnel based on the amount of algae clogging up the filter, reduce volumes and try again. Try cutting the volume in half at first but then by 10 (50→25→15mL) Just make sure to record this on the foil!
9. Close valves as the water finishes filtering and turn off the pump as soon as samples have filtered.

10. Using the forceps, grab a filter and place it on a stack of paper towels (face up, with particulate matter NOT touching the paper towel).

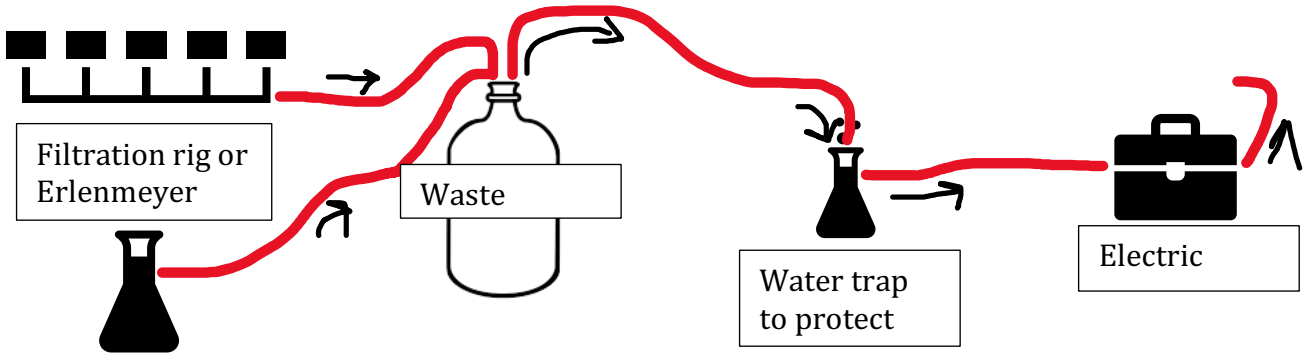
11. Using the forceps, carefully fold filters in half on the paper towel. Try not to touch the sample with the forceps, use the white, untouched edges to do so. The particulate matter should be folded *inside* the filter (like a taco or empanada!) and make the best half circle possible when you fold over. An uneven fold will allow samples to leach out. The folded edges should be even to prevent filter matter from rubbing off onto the towel or foil. **note: You will get the hang of using the wetness from the filter with the paper towel to fold. I usually drag the filter along the paper towel with my forceps so that it sticks and then I can easily fold the top over to make my filter "taco". You can also use two pairs of forceps if this works best for you.
12. Place folded filter between paper towels and gently blot dry by gently pressing it with a PVC roller, like rolling out dough gently. This squeezes the excess water onto the paper towel.

13. Place the folded filter in its designated labeled foil. You can place both HPLC filters in the same packet. Fold each open edge of the foil to make a closed packet but try not to fold the filter itself. Again, if sample volume was different than intended (e.g. due to heavy biomass/ loss of sample) report this on the packet.
14. Place the foil in its respective Ziploc bag. Once the bag is filled with the triplicate samples, place the sealed bag in a freezer ASAP or on ice.





**Filtration Diagram



Phytoplankton Morphology Sample Preparation

Materials:

- Three 25 mL glass vials
- Lugol's Solution
- Pipet / Eye dropper

Phytoplankton Sample Fixing Protocol:

1. Invert sample bottle gently several times. This mixes the sample so it is uniform.
2. Carefully pour the sample into the vial. Leave a bit of headspace.
3. Using the eyedropper and wearing gloves, put 4 mL of Lugol's solution into the vial.
4. Seal the vial.
5. Store sample in designated Tupperware at room temperature.

FCM Sample Preparation

Materials:

- Three 5 mL FCM vials + glutaraldehyde fixative (pre-added)
- Tupperware storage

FCM Sample Fixing Protocol:

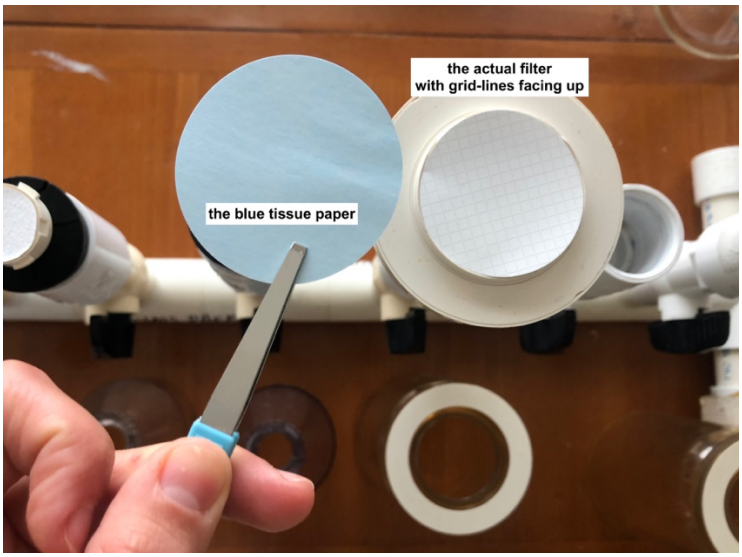
1. Add 5mL of sample to vial with eye-dropper. Invert tube several times.
2. Adjust the label your tube if the date / volume / anything significantly deviates from the original label.
3. Leave vials at room temp, in the dark for at least 10 min.
4. Put vials in the freezer within their Tupperware.

Cyanobacterial & Microbial DNA Filtration

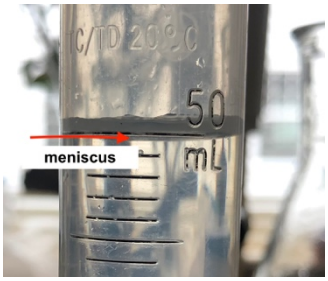
This procedure mirrors much of the chl a / HPLC filtration procedure but **crucially requires the use of different filters, 47 mm PALL SUPOR filters with 47 mm filter towers.*

Filtration Protocol:

1. Set up your filter apparatus (vacuum pump, towers, waste apparatus, etc).
2. Use forceps to place a SUPOR filter onto the tower base. The blue tissue paper is NOT a part of the filter. Remove this from either side of the white filter. Some SUPOR filters have a grid on them. If this is the case, place the grid facing up.



3. Secure the tower in place. Ensure that the filter is centered and intact (no visible rips). If you are having issues, turning on the pump helps suction the filter to the base, and you can double check with this to make sure they are secure.
4. Invert the sample bottle gently several times. This mixes the sample so it is uniform.
5. Then pour and measure 25 mL in graduated cylinder (with bottom of meniscus touching 25 mL line).



6. Before pouring sample into the tower, be sure the tower and filter are secured, otherwise the sample water will leak. If it does leak, no worries, but you will have start over and get a new filter.
7. Pour the water into the tower. Measure 25 more mL and repeat for the triplicates. You can fill every funnel before turning on the pump.
8. Turn on vacuum source and open valves on filter tower manifold. Vacuum pressure should be around approximately 5 psi. Try not to exceed 8 psi since high pressure may break the pigments.
9. You may have to adjust sample volumes based on conditions or losses of sample (spills / accidents). If you spill water out of the base of the funnel, start over with the same volume. If the water will not go down after ~30 seconds, make sure the waste lid is tight and that water is actually filling. If it is not going through the funnel based on the amount of algae clogging up the filter, reduce volumes and try again. Try cutting the volume by 10 (25→15mL) Just make sure to record this on the foil!

10. Close valves as the water finishes filtering and turn off the pump as soon as samples have filtered.
11. Before pouring sample into the tower, be sure the tower and filter are secured, otherwise the sample water will leak. If it does leak, no worries, secure the tower then restart with another measured volume.
12. Turn on vacuum source and open valves on filter tower manifold. Vacuum pressure should be around approximately 5 psi. Try not to exceed 8 psi since high pressure may break cells.
13. Once all the water has gone through the funnel, switch off the vacuum, and close the tower valves. Remove the funnels.
14. Using two pairs of clean forceps, carefully fold the filters in half on the filter base. Try not to touch the central material with forceps; instead use the white, untouched edges to fold them. The particulate matter should be folded *inside* the filter (like a taco or empanada!) and make the best half circle possible when you fold over. **Use two pairs of forceps to achieve multiple folds so that the filter will fit in the DNA tube.**





15. Securely place folded filter (probably 2-4 folds) into a prelabeled screw cap or snap cap plastic tube. Make sure the cap is fitted properly.
16. Place tubes into the freezer as soon as possible, in their designated baggie.

Nutrients and Microcystin Filtration

This procedure mirrors much of the chl a / HPLC filtration procedure but **crucially requires the use of the Erlenmeyer flask instead of filtration rig so that you can save the filtrate.*

Materials:

- Single 250 mL Erlenmeyer flask with filtration tower(s) and base(s) (small-25 mm)
- Vacuum pump (handheld or electric)
- Graduated cylinder (50 or 100 mL)
- Forceps
- Whatman GF/F glass fiber filters, 25mm
- Nutrient tubes and petri dishes (see sample labeling information)

Filtration Protocol:

1. Set up your filter apparatus (vacuum pump, tower, flask, waste apparatus, etc.)**. Make sure the Erlenmeyer flask is clean. You will be collecting the filtrate that ends up in the flask.
2. Wearing gloves, use the forceps to place a GF/F filter onto the tower base. Secure the tower in place. Ensure that the filter is centered and intact (no visible rips). If you are having issues, turning on the pump helps suction the filter to the base, and you can double check with this to make sure they are secure.
3. Invert the sample carboy gently several times. This mixes the sample so it is uniform.

4. Then pour and measure 50 mL in the graduated cylinder (for nutrients, but 100 mL for microcystin) (with bottom of meniscus touching the 50 or 100 mL line). You may have to adjust sample volumes based on conditions or losses of sample (spills / accidents). Reduce volumes if filters are bright green and you are unable to filter 50 mL. Try cutting the volume in half at first but then by 10 (50→25→15mL) Just make sure to record the final volume!

5. Before pouring sample into the tower, be sure the tower and filter are secured, otherwise the sample water will leak. If it does leak, no worries, but you will have start over and get a new filter.

6. Turn on vacuum source. Vacuum pressure should be around approximately 5 psi. Try not to exceed 8 psi since high pressure may break the pigments.

7. Turn off the vacuum as soon as the sample has filtered. Unscrew the funnel.

8. Using the forceps, grab a filter and place it directly onto the bottom side of the petri dish for nutrients, the aluminum foils for microcystin (face up, with particulate matter NOT touching either apparatus).

9. After collecting the triplicate filters for nutrients and microcystin, take off the filter tower, and carefully pour the filtrate into the designated Falcon tubes. Leave a bit of headspace for expansion during freezing: each tube should hold ~40-45 mL of filtrate. It does not have to be exact.

10. Seal the Falcon tubes, petri dishes, and foil samples in their respective bags.

11. Once the bag is filled with the triplicate samples, place the sealed bag in a freezer ASAP or on ice.

Cleaning and storing your equipment

Between TIME ON and TIME OFF carboys, you must DI rinse and sample rinse the equipment. This is not a full cleaning, rather, rinse the towers, graduated cylinders, forceps, and Erlenmeyer flask with DI water 3 times, then 3 times with the next water sample itself. This will ensure no cross contamination between TIME ON and TIME OFF samples.
At the very end of your filtration:
Materials:

- Bottle with acid solution
- Bottle with DI water
- Spray bottle for acid solution
- Spray bottle for DI water

- Paper towels

Cleaning Protocol:

1. Set up a make-shift paper towel drying rack next to a sink.
2. Fill the spray bottles with their respective solutions.
3. Rinse the inside of the equipment in question (i.e. a flask or graduated cylinder) with the acid. Pour down the sink with the water running to dilute. Do this 3 times.
4. Rinse the inside of the equipment with the DI water 3 times in the same fashion.
5. Set on the drying rack to dry.
6. Rinse the filtration rig with hot water (can be from the sink)
7. Dispose of all liquid in the waste carboy.
8. Disassemble the equipment necessary, and once completely dry, store in a safe, room temperature, dry area away from direct sun.

V. SUMMARY

Final Remarks: Thank you for your invaluable volunteer time to make this research a success. Please reach out to Haley Plaas at any time over the course of this study with questions, concerns, or recommendations. Some details that were not mentioned previously:

1. You can be reimbursed for gas money for any travel associated with your water sampling trips at your request.

2. Your contribution to this work will be noted in the acknowledgements section of all resultant publications and professional presentations.

3. In addition to the research this summer, an undergraduate science communication intern will be working closely with me to shoot a short documentary about the impact of CyanoHABs on the environment and livelihood in eastern North Carolina. All outreach products (videos, etc.) will be available for the CEEG to utilize through media platforms.

4. I am happy to attend future CEEG meetings to discuss our ultimate findings, the implications of this research, and further collaboration!

Thanks again, and I look forward to working with you and learning much about the Chowan from you this summer!

VI. FILTERING CHEAT SHEET

SAMPLE	FILTER TYPE	FILTER APPARATUS	VOLUME	BLOT / FOLD ON PAPER TOWEL?	STORAGE
Chlorophyll a	25 mm GF/F	Filter rig	50 mL	YES	Aluminum foil packet -> freezer
HPLC	25 mm GF/F	Filter rig	100 mL x 2	YES	1 Aluminum foil packet -> freezer
Particulate (intracellular) MC	25 mm GF/F	Erlenmeyer flask	100 mL	YES	Aluminum foil packet -> freezer
CHN	25 mm GF/F	Erlenmeyer flask	50 mL	NO	Petri dish -> freezer
NUTS	Filtrate	Erlenmeyer flask	~ 45 mL*	n/a	50 mL Falcon tube -> freezer
Dissolved (extracellular) MC	Filtrate	Erlenmeyer flask	~ 90 mL* (45 mL *in two tubes)	n/a	50 mL Falcon tubes -> freezer
DNA	47 mm SUPOR (grid up, no blue)	Filter rig	25 mL	NO	DNA tube -> freezer
Phytoplankton	n/a	n/a	~25 mL* + 4mL Lugol's	n/a	Vial -> tupperware -> room temperature
FCM	n/a	n/a	5 mL, add water with eye droppers	n/a	5mL vials -> tupperware -> freezer

These can be done in a row. Pour filtrate at end.

* volume does not need to be exact. Otherwise assume volume must be accurate and recorded.

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