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Isolation of Picocyanobacteria (Order *Synechococcales*) and Occurrence of the Cyanotoxin Anatoxin-A in a Shallow Mesotrophic Pond

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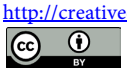

Abstract

We have verified the use of a serial filtration method to isolate picocyanobacteria for analysis. We used eDNA metabarcoding to confirm the picocyanobacteria as members of the Order *Synechococcales*, Genus *Cyanobium,* specifically *Cyanobium* 6307. Fluorometric analysis using accessory pigments phycocyanin and phycoerythrin described periods of excess biomass, where the net growth rate model confirmed these conditions. The total anatoxin-a concentrations in the picocyanobacterial sample ranged from 0.0074 - 6.41 $\mu g \cdot L^{-1}$ representing a 40-fold difference over the entire sampling season. Sampling frequency of every three days appeared to be an important factor in capturing these changes in anatoxin-a concentration. During a period of excess biomass, we were able to establish a linear correlation between cyanobacterial biomass and Anatoxin-a concentrations.

Keywords

Picocyanobacteria, Anatoxin-a, eDNA, Phycocyanin, Phycoerythrin

1. Introduction

Cyanobacteria are known to be a diverse and widely distributed group of bacte-

ria that occupy a wide range of ecological niches. Their populations can be described using cyanobacterial accessory pigments and size-structure analysis [1] where cell size and organization are important parameters. The cyanobacteria produce secondary metabolites that have been shown to be toxic to various receptors, commonly termed cyanotoxins, making them a threat to human and ecological health. The most commonly cited cyanotoxins include microcystins (and their variants), cylindrospermopsin, and anatoxin-a. These cyanotoxins are often associated with various genus and/or species of the cyanobacteria, allowing for broad generalizations related to exposure potential [2].

Anatoxin-a is a neuroactive compound, that represents an acute exposure profile. Anatoxin-a is most commonly associated with bloom-forming cyanobacteria (BFC's) in the Order *Nostocales* [2] [3] including *Dolichospermum flos-aquae* [4] [5] [6] and *Cuspidothrix issatschenkoi* [7] [8] [9] [10]. Among the cyanobacteria the single celled picocyanobacteria (Order *Synechococcales*) are commonly found in both marine and freshwater systems [11]-[16]. They can be operationally defined [17]. As that portion of the cyanobacterial population is less than 5 μm in size [14]. Within the past ten years, the "picos" have been the focus of intensive research to verify their toxin production and role in exposure pathways including food webs and aerosols in particular microcystin (MC) and *B*-methyl-L-alanine (BMAA) [12] [14] [15] [18]. Most recently anatoxin-a (ATX) has been identified in several freshwater ponds [19] and more specifically within their picocyanobacterial populations [20]. Recent genomic analysis has confirmed that commonly found *Synechococcus* species can produce cyanotoxins including anatoxin-a [21].

Existing cyanobacterial monitoring programs can vary somewhat between local, state and federal agencies, depending on program objectives, funding availability, staff training requirements, field equipment and other considerations. A monitoring program that has been developed specifically for cyanobacteria is the cyano Casting Program that uses floatation for the collection of bloom-forming cyanobacteria and serial gravity filtration for sample collection of "edible" cyanobacteria, but does not extend the population continuum to picocyanobacteria by using pressure filtration.

The objective of this project was to verify the methods necessary to isolate and analyze the continuum of cyanobacterial populations and the accompanying anatoxin-a concentrations in a freshwater system with a focus on picocyanobacteria. We propose to verify an easy and effective field and laboratory method to isolate picocyanobacteria for analysis, yielding samples that can meet relatively rigorous quality assurance/quality control requirements. We also wanted to expand the use of cyanobacterial accessory pigments by including phycoerythrin, considered a signature pigment for picocyanobacteria. While we anticipated a wide range of anatoxin-a concentrations to be observed within the picocyanobacterial population, we wanted to verify the range and duration of exposure to this toxin. Finally, we wanted to determine if eDNA barcoding could be used as a tool to provide "value-added" information to our interpretations of exposure potential to cyanotoxins.

2. Materials and Methods

2.1 Field Sampling

Collection of replicates of whole lake water (WLW) and net (NET) water samples was conducted on a (minimum) weekly basis extending from May to October from the shoreline (maximum depth 1 m) from, Lower Mill Pond, Brewster, MA (Figure 1(a) and Figure 1(b)) (Latitude: 41.73˚N; Longitude: −70.11˚W) as previously described [18]. Size fractionation using floatation, serial filtration with gravity and serial filtration with pressure were used for sample collection and processing. Collection and processing of three of the samples, specifically the bloom-forming cyanobacteria (BFC), whole lake water (WLW) and <50 µm samples are included in an existing quality assurance project plan [22]. Therefore an addendum [23] was required for collection and processing of additional size fractions, specifically the <10 µm, <5 µm and <0.2 µm samples (Figure 2). The <10 µm filtrate will be collected using gravity filtration by passing a volume of <50 µm filtrate through a 10 µm nylon mesh ring net. The <5 µm filtrate will be collected using serial filtration with pressure by placing a volume of <10 µm filtrate

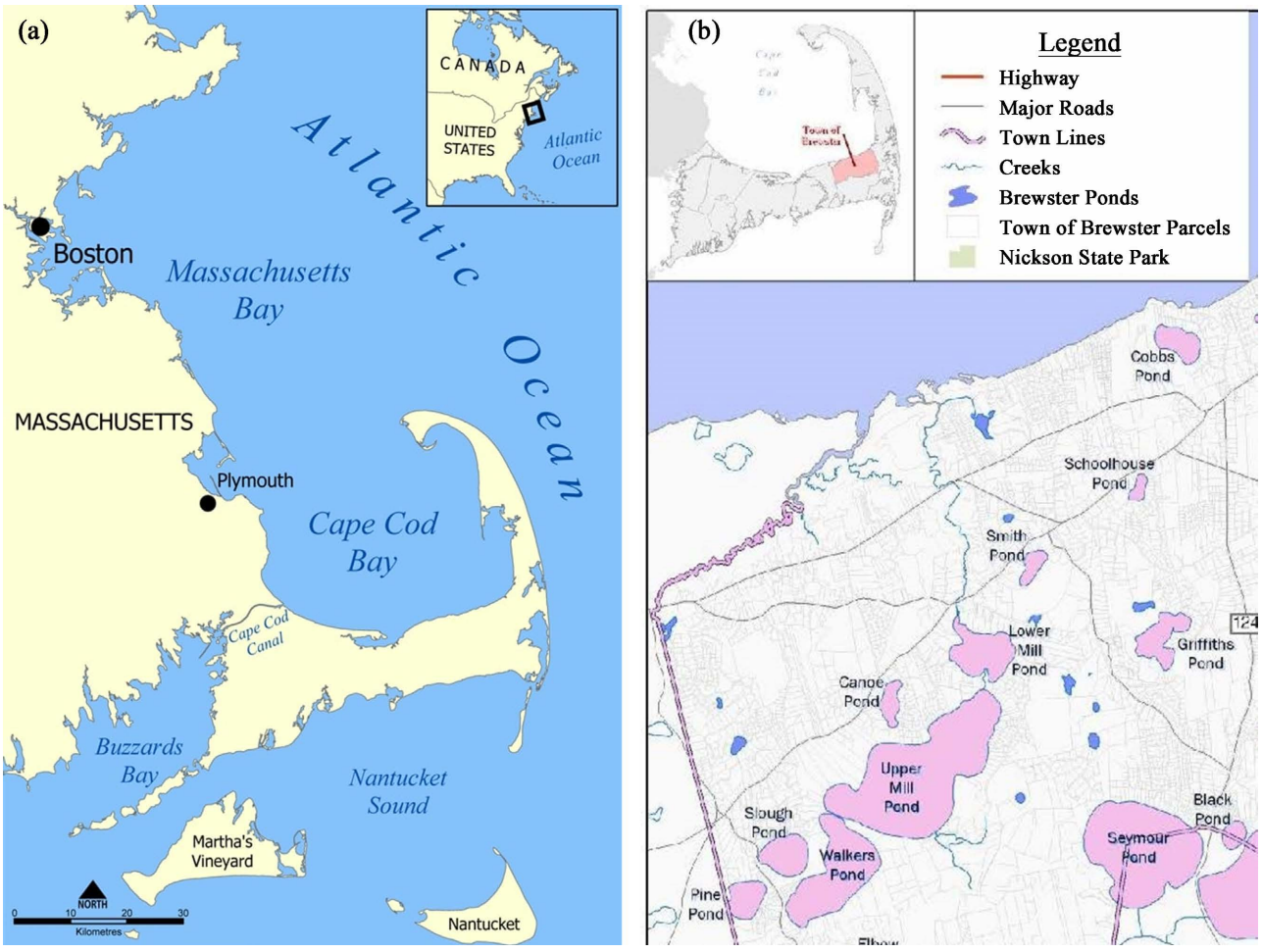

Figure 1. (a) Location of study site in Massachusetts USA; (b) Location of study site, Lower Mill Pond, in Brewster, Massachusetts.

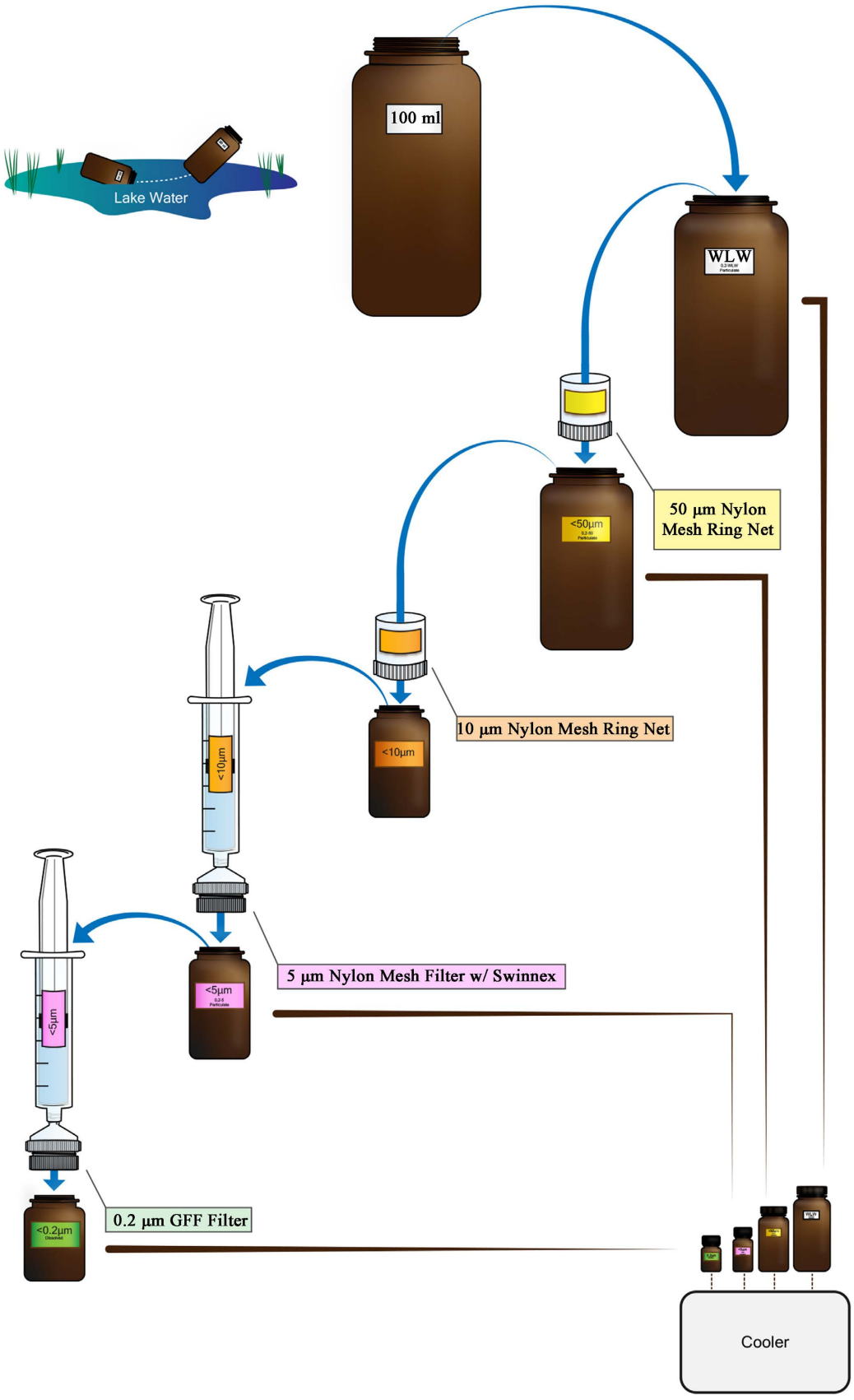

Figure 2. Serial filtration using gravity and pressure for the collection of (pico)cyanobacterial samples.

into a clean, plastic syringe fitted with a Swinnex filter holder passing through a 5 μm nylon mesh filter. The <0.2 μm filtrate will be collected using serial filtration with pressure by placing a volume of <5 μm filtrate into a clean, plastic syringe fitted with a Swinnex filter holder passing through a 0.2 μm prefiltered, precombusted 25 mm GF/F filter. Samples were stored at −4˚C prior to cyanotoxin and fluorometric analysis. Samples for 16S meta barcoding analysis were collected by passing a known volume of either NET, WLW or <5 μm water samples through a Sterivex filter (0.2 μm pore size) using a 50-mL syringe and stored at −80˚C prior to analysis.

2.2. Biomass Quantification of (pico)cyanobacteria

All water samples were prepared, preserved, stored and analyzed according to procedures outlined in the QAPP. Prior to fluorometric analysis, the hand-held fluorometry units were calibrated [24] for both phycocyanin (PC) and phycoerythrin (PE), accessory pigments primarily associated with cyanobacterial (phycocyanin) and picocyanobacterial (phycoerythrin) biomass. The whole lake water sample (WLW) was fractionated (<50 μm, <10 μm, <5 μm, <0.2 μm) according to the project QAPP and samples frozen for a single freeze-thaw extraction (SFT) prior to fluorometric analysis using the handheld Fluoroquik™ fluorometer (excitation wavelengths 595 nm for phycocyanin and 545 nm for phycoerythrin) using a quantitation limit for phycocyanin (PC) at 3 $\mu g \cdot L^{-1}$ and phycoerythrin (PE) at 0.1 $\mu g \cdot L^{-1}$.

As a measure of quality assurance/quality control (QA/QC) cyanobacterial biomass, the 16s meta barcoding results were used to determine if there was crossover from the WLW and/or Net samples into the <5 μm sample that had been collected (**Figure 3(a)**) where an acceptable level of 10% crossover of cyanobacteria (*Nostocales*) from the WLW and/or NET to the <5 μm sample *(Synechococcales)* was used to validate the picocyanobacterial sample. The number of reads was used as a measure of (pico)cyanobacterial abundance.

2.3. Anatoxin-a Quantification in (pico)cyanobacteria

The samples for cyanotoxin analysis were collected, prepared and stored following the protocol that was used for fluorometric analysis with two additional freeze-thaw cycles where samples were vortexed on high speed for 3 minutes followed by sonication for 3 minutes to complete the triple freeze-thaw extraction process as previously described [18]. Water samples were prepared for enzyme-linked immunosorbent assay (ELISA) anatoxin-a (ATX) analysis at full strength (Und), diluted with Milli-Q water (*i.e.* 1:5) or subjected to vacuum centrifugation (*i.e.* 20×) as previously described (Leland and Haney 2018). Samples from Lower Mill Pond required all three preparation techniques. Triplicate (n = 3) anatoxin-a concentrations and standard error of the mean (SEM) are reported for Lower Mill Pond unless otherwise noted by not reporting SEM. The detection limit for the ELISA analysis [25] was 0.15 $\mu g \cdot L^{-1}$.

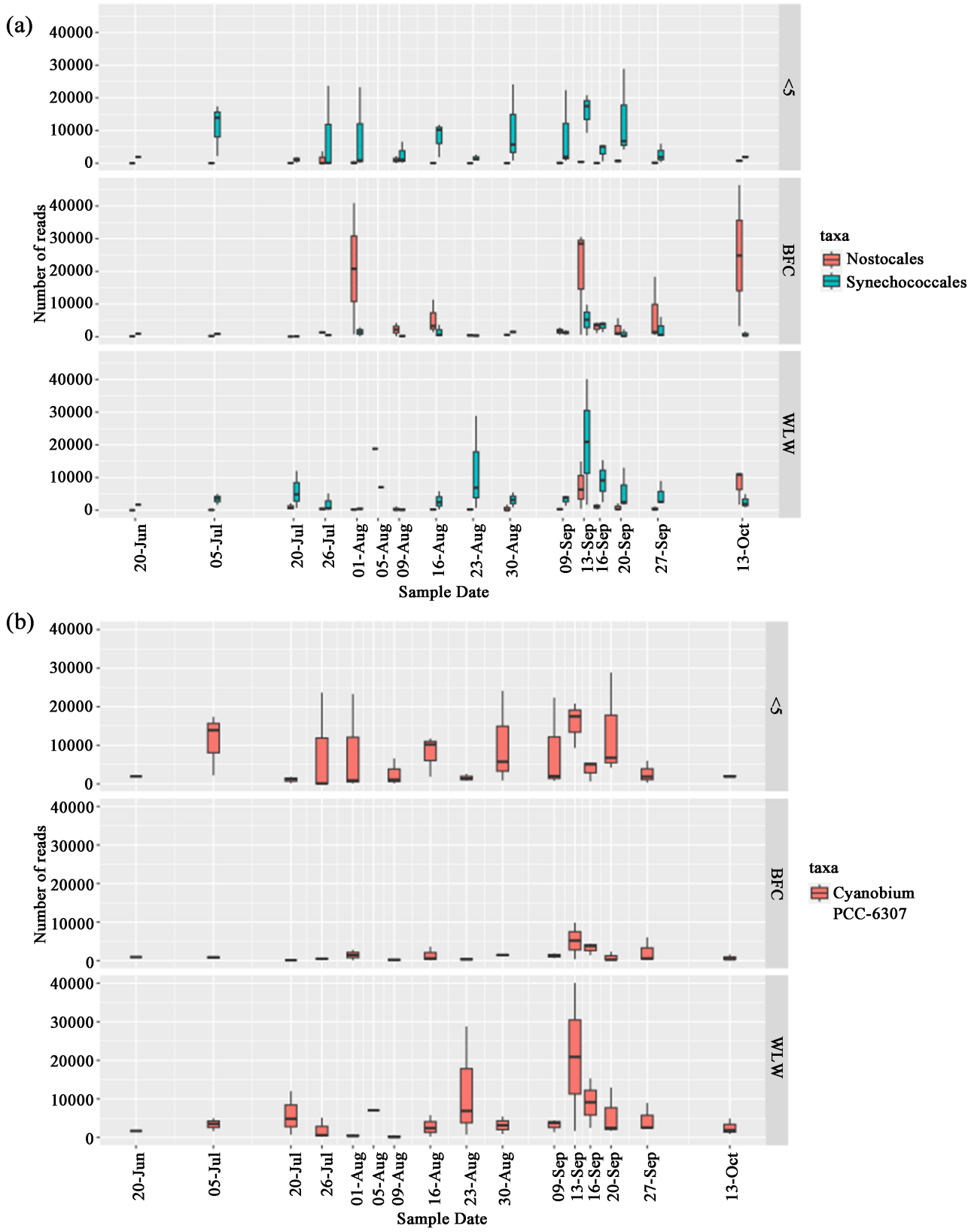

Figure 3. (a) 16s metabarcoding analysis of net (BFC), whole lake water (WLW) and <5 μm (<5) samples from Lower Mill Pond, including taxa (*Nostocales* and *Synechococcales*) and number of reads; (b) 16s metabarcoding analysis of net (BFC), whole lake water (WLW) and <5 μm (<5) samples from Lower Mill Pond, including taxa (*Cyanobium* 6307) and number of reads.

2.4. 16s Metabarcoding

The DNA was extracted from a 0.22-micron filter for each size fraction of water samples collected for this study. The total DNA for each sample fraction was amplified with 16s primer 515 - 926 R. [26]. Amplicons were then sequenced on the Novaseq platform (250 bp paired-end reads). Sequences were trimmed, denoised, and assigned taxonomy with Qiime2 [27]. Each ASV (unique sequences found among all samples) was assigned a taxonomy with the sklearn algorithm using a feature classifier generated from the Silva reference database (v132, 99 OTU). To compare the total number of sequences that were assigned to either *Nostocales* or *Synechococcales* (**Figure 3(a)**) we collapsed the taxonomic assignments down to the level of Order.

2.5. Statistical Data Analysis

Statistical analysis of cyanotoxin and fluorometric data was performed using Sigma Plot Version 14 software. Parametric analysis (Pearson's correlation coefficients and linear regression analysis) were used to describe relationships between variables. For linear regression analysis autocorrelation (Durbin-Watson = 2.0), leverage (Studentized deleted residuals: SDR > 2) collinearity (VIF > 3), and influence (Cooks distance: Cd = 4/n and Difference in Fits: DFFits = 2 × sq. rt. [(p + 1)/ (n − p − 1)] where n = number of observations, p = number of variables (including the constant) were examined. Cyanobacterial biomass daily net growth rates ($\mu \cdot d^{-1}$) were calculated using phycocyanin (PC) concentrations or phycoerythrin (PE) concentrations as previously described [1].

3. Results and Discussion: (Pico)cyanobacterial Detection and Biomass Quantification

The cyanobacteria exhibited two distinct periods of increased biomass resulting in visible surface accumulations and decreased transparency. A review of the range of PC concentrations (**Table 1**) for the WLW was similar to that previously observed (2019-2021) for *Dolichospermum* spp., being confirmed through light microscopy. The range of PC concentrations for the <5 μm sample (**Figure 4(a)**) was typically 50% - 75% lower than those documented for the WLW samples. The "pico" fraction, which has subsequently been identified as *Cyanobium* 6307 using metabarcoding technique (**Figure 3(b)**) began to indicate changes in population biomass on July 26th, being ahead of the WLW sample, verifying the PC-based growth rate model for bloom prediction as excess biomass. The net growth rates for all size fractions (<5 μm, and WLW) were within ranges previously observed [19] therefore we could not conclude that the net growth rates observed for picocyanobacteria were unique to that population. However, the increases we observed did indicate the temporal differences between the WLW (*Dolichospermum*) and pico (*Cyanobium*) populations, each uniquely responding to the variables that influence their net biomass.

A review of the range of PE concentrations (**Table 2**) for the <5 μm and WLW

Table 1. Phycocyanin concentrations (μg·L^{-1}) in water samples from Lower Mill Pond.

	Size fraction					
Date	<0.2 μm	<0.2 μm SEM	<5 μm	<5 μm SEM	WLW	WLW SEM
5-Jul	2.110	0.598	3.440	0.252	6.783	0.939
19-Jul	0.998	0.206	4.623	0.487	8.943	1.619
26-Jul	2.600	0.459	8.880	0.140	11.937	0.659
1-Aug	2.807	0.977	14.773	1.182	77.823	3.795
5-Aug	12.830	2.523	31.263	0.591	47.063	0.930
9-Aug	14.077	0.393	39.580	0.420	66.737	8.044
16-Aug	6.017	1.712	19.417	0.182	69.023	8.582
19-Aug	3.297	1.783	15.467	1.423	62.163	9.051
23-Aug	4.207	1.349	16.507	0.487	51.287	4.826
26-Aug	1.550	0.140	9.363	0.909	19.623	3.197
9-Sep	3.573	1.873	22.330	0.961	119.130	2.799
13-Sep	0.733	0.191	10.547	0.591	69.773	3.090
16-Sep	5.113	0.786	21.913	2.307	115.247	1.708
20-Sep	8.180	1.535	23.230	3.278	142.637	10.419
27-Sep	11.100	2.275	23.227	0.801	60.573	12.002
13-Oct	4.553	0.792	10.203	0.789	31.890	3.683

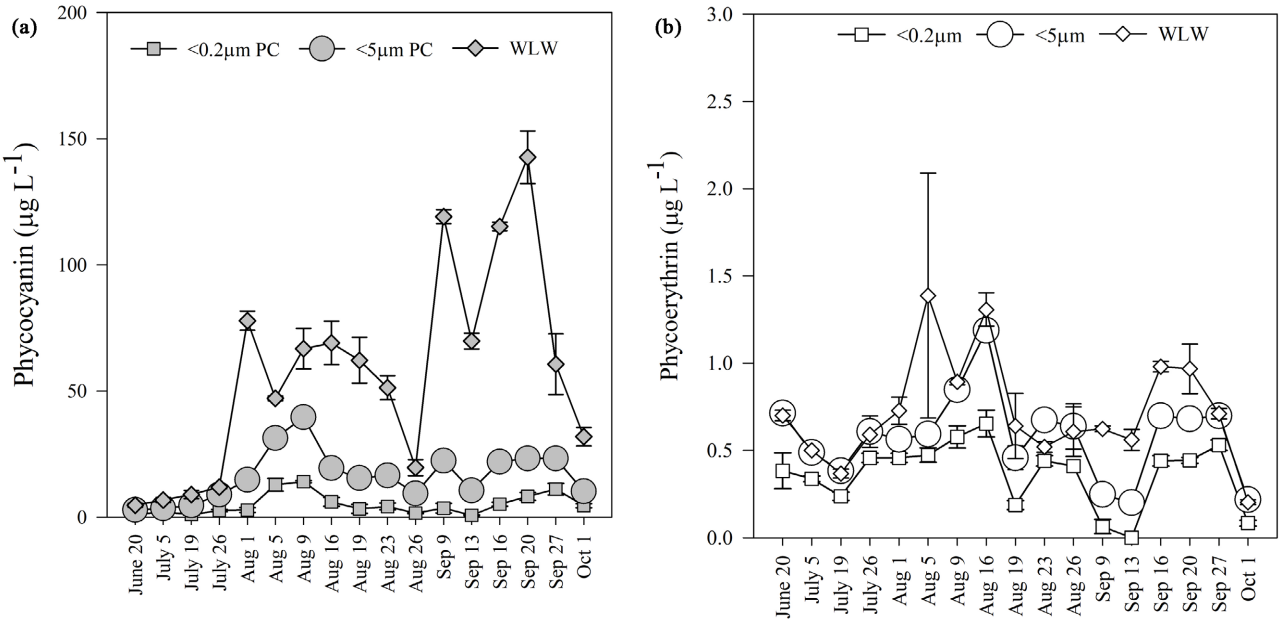

Figure 4. (a) Phycocyanin concentrations for dissolved (<0.2 μm), picocyanobacteria (<5 μm) and whole lake water in Lower Mill Pond; (b) Phycoerythrin concentrations for dissolved (<0.2 μm), picocyanobacteria (<5 μm) and whole lake water in Lower Mill Pond.

Table 2. Phycoerythrin concentrations (μg·L^{-1}) in water samples from Lower Mill Pond.

	Size fraction					
Date	**<0.2 μm**	**<0.2 μm SEM**	**<5 μm**	**<5 μm SEM**	**WLW**	**WLW SEM**
5-Jul	0.337	0.017	0.487	0.041	0.500	0.000
19-Jul	0.237	0.023	0.383	0.041	0.367	0.026
26-Jul	0.457	0.026	0.607	0.090	0.590	0.000
1-Aug	0.457	0.026	0.563	0.013	0.727	0.078
5-Aug	0.473	0.041	0.593	0.026	1.387	0.702
9-Aug	0.577	0.064	0.847	0.054	0.893	0.017
16-Aug	0.653	0.077	1.187	0.017	1.307	0.095
19-Aug	0.187	0.026	0.457	0.068	0.640	0.187
23-Aug	0.440	0.030	0.673	0.018	0.520	0.030
26-Aug	0.410	0.000	0.637	0.130	0.607	0.142
9-Sep	0.063	0.041	0.247	0.017	0.623	0.017
13-Sep	0.000	0.000	0.200	0.030	0.560	0.060
16-Sep	0.440	0.030	0.697	0.017	0.980	0.030
20-Sep	0.443	0.017	0.680	0.052	0.967	0.142
27-Sep	0.530	0.030	0.697	0.017	0.710	0.030
13-Oct	0.083	0.017	0.217	0.013	0.203	0.013

samples (**Figure 4(b)**) were surprisingly similar to each other throughout the sampling season, with the exception of the Aug 5th WLW sample, which could be considered an outlier based on sample collection. The maximum concentrations were typically less than 1.5 μg·L^{-1}. Since PE is considered the signature accessory pigment for picocyanobacteria, we could be selecting out that part of the WLW sample which is composed strictly of picocyanobacteria. This data suggests that these PE concentrations may be unique to the cyanobacterial populations in Lower Mill Pond (*i.e. Cyanobium* 6307 and *Dolichospermum* spp.). There were several periods of increased biomass that occurred during similar time periods, the first beginning on July 26th and the second beginning on August 23rd. These periods of rapid net growth (<0.05 day^{-1}) were also accompanied by the appearance of surface accumulations (blooms) and limited water transparency. It appears that PE concentrations could also be used within the framework of net growth rates for excess cyanobacterial biomass and surface accumulation (bloom) prediction.

This project used photosynthetic accessory pigments phycocyanin (PC) and phycoerythrin (PE) to quantify cyanobacterial biomass where PC is cyanobacterial specific and PE is considered a "signature" pigment for picocyanobacteria and potentially other cyanobacterial genus [28] [29]. With the exception of a limited number of dissolved samples, all samples were above the limit of detection

for both PC and PE throughout the sampling season (Table 1, Figure 4(a), Table 2, Figure 4(b)). There was a significant positive correlation between PC and PE concentrations throughout the study ($r = 0.568$, $p < 0.001$). A one-way analysis of variance (ANOVA) of the picocyanobacterial PE/PC ratio (pigment fingerprinting) was not significantly different during the time periods when there was a change in the anatoxin concentrations, suggesting that there was not a shift in community composition. Analysis of cyanobacterial accessory pigments PC and PE have been used in marine environments to aid in the classification to species level [30] [31] and have been documented in both marine and freshwater systems [32]. There exists a plethora of PC data available for freshwater systems while PE data is limited. While PE might be considered more useful in oligotrophic systems where picocyanobacteria are known to dominate, we present here the seasonal range of values that might be expected in a mesotrophic system. The use of phycoerythrin in this study is in contrast to other studies of (pico)cyanobacterial populations in freshwater systems that described the phycoerythrin contribution as "minor" [16].

The 16s metabarcoding confirmed the particulate material collected from the <5 μm sample as belonging to the Order *Synechococcales* (Figure 3(a)) including multiple ASVs for picocyanobacteria being identified among the samples, including sequences belonging to the Genus *Cyanobium*, specifically *Cyanobium* PCC-6307 (Figure 3(b)). During the time periods when there was a change in the pigment concentrations, the metabarcoding suggests that there was not a shift in community composition. Previous studies on the occurrence of *Synechococcus* in brackish [33] [34] and freshwater systems [35] used serial filtration to collect particulate material ranging from 0.22 - 8 μm and 0.22 - 5 μm in size on filters prior to DNA isolation.

4. Results and Discussion: (Pico)cyanobacterial Production of Anatoxin-a

Anatoxin-a was detected in all of the water samples collected throughout the entire sampling season as shown in Table 3. There was as much as a 40-fold difference in concentrations for the samples, ranging from 0.0022 - 1.16 $\mu g \cdot L^{-1}$ in the dissolved (<0.2 μm) sample, 0.0023 - 6.41 $\mu g \cdot L^{-1}$ in the picocyanobacteria (<5 μm) sample and 0.0057 - 8.48 $\mu g \cdot L^{-1}$ in the WLW sample. A seasonal peak with notable (greater than 0.5 $\mu g \cdot L^{-1}$) anatoxin-a concentrations was observed for the dissolved fraction from August 1 - August 16, concurrently with the picocyanobacteria and WLW from July 19-August 16 (Figure 5(a)). A second more modest increase was observed on September 9 for all three sample types (Figure 5(b)). For the remainder of the sixteen (16) sampling events conducted throughout the season, nine (9) had anatoxin-a concentrations that were less than the ELISA detection limit of 0.15 $\mu g \cdot L^{-1}$ (Figure 5(c)). The low concentrations are similar to those previously reported for anatoxin-a concentrations in picocyanobacteria [21] using ELISA analysis.

Table 3. Anatoxin-a concentrations ($\mu g \cdot L^{-1}$) in water samples from Lower Mill Pond.

	Size fraction					
Date	<0.2 µm	<0.2 µm SEM	<5 µm	<5 µm SEM	WLW	WLW SEM
5-Jul	0.0022		0.0023		0.0115	
19-Jul	0.0527	0.0097	0.1600	0.0092	0.1600	0.0286
26-Jul	0.0908	0.0231	0.3000	0.0340	0.5030	0.0636
1-Aug	0.1860	0.0960	1.4980	0.2590	2.9750	0.6110
5-Aug	1.1620	0.3560	6.4100	1.0290	8.4770	1.5470
9-Aug	0.5620	0.2500	3.2850	0.4530	5.6270	1.0530
16-Aug	0.1340	0.0044	0.1570	0.0112	0.1260	0.0055
19-Aug	0.0249	0.0019	0.0318	0.0054	0.0424	0.0015
23-Aug	0.0171	0.0010	0.0304	0.0039	0.0255	0.0010
26-Aug	0.0078	0.0029	0.0500	0.0032	0.0847	0.0001
9-Sep	0.1210	0.0391	0.2240	0.0139	0.4120	0.0212
13-Sep	0.0077	0.0007	0.0113	0.0023	0.0057	0.0014
16-Sep	0.0169	0.0025	0.0074	0.0017	0.0157	0.0012
20-Sep	0.0066	0.0012	0.0096	0.0013	0.0094	0.0008
27-Sep	0.0083	0.0002	0.0092	0.0009	0.0067	0.0013
13-Oct	0.0096	0.0013	0.0109	0.0014	0.0077	0.0005

The seasonal peak was observed over a short time period of ten days, requiring an increased sampling frequency of every three days to capture this event. If sampling frequency had been every two weeks we may have missed this event in its entirety, and if weekly we may have missed the notably high concentrations. Of particular interest were the extremely low concentrations of anatoxin-a observed later in the sampling season when biomass increased again and other surface accumulations were observed on Sept 13th. It is unclear the extent to which the concentrations of anatoxin-a were influenced by community composition, nutrient concentrations, changes in cyanobacterial biomass and/or subject to degradation, where rapid deterioration has been noted due to sunlight, pH and microbial action. When there were significant changes in the anatoxin concentrations, review of the metabarcoding data suggests that there was not a shift in community composition. Thus, we speculate there could have been a change in nutrient concentration or other variables that would trigger the toxigenic switch. Either of these ideas are compelling research topics.

During the time that anatoxin-a was being produced by the picos (*Cyanobium* 6307) at notable concentrations (July 26-August 16), we were able to describe significant correlations between the cyanobacterial biomass and anatoxin-a concentrations, as measured using phycocyanin (**Figure 6(a)**) where Log ATX =

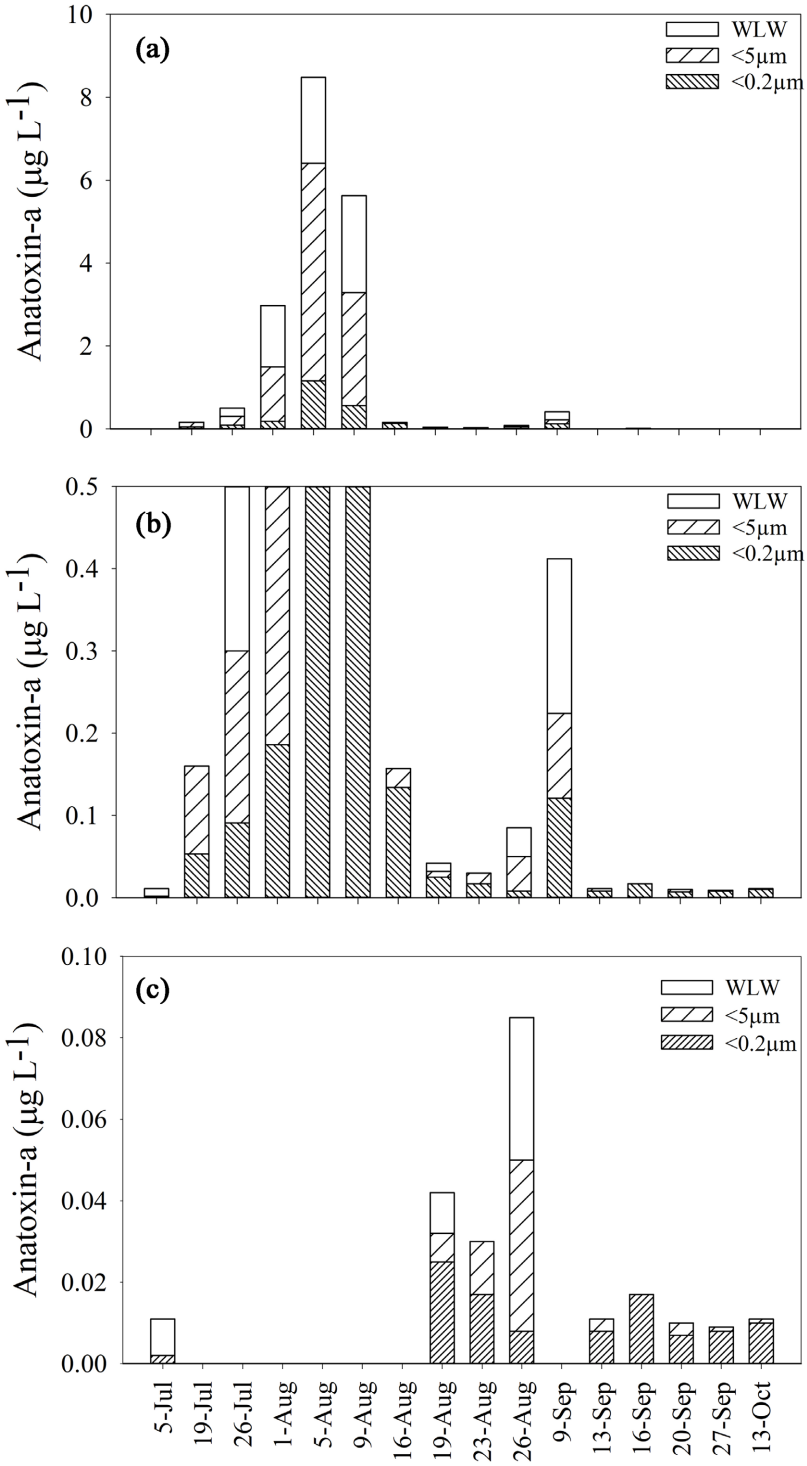

Figure 5. Anatoxin-a concentrations (µg·L^{-1}) in Lower Mill Pond.

−1.574 + (1.259 * Log PC), Adj. $r^2 = 0.714$, $p < 0.0001$ and phycoerythrin (**Figure 6(b)**) where Log ATX = −1.493 + (1.220 * Log PE), Adj. $r^2 = 0.8327$, $p < 0.0001$.

For this study using metabarcoding, DNA was extracted from a 0.22-micron filter for each size fraction of water samples. Total DNA for each sample fraction was amplified with 16s primer 515 - 926 R [26]. A previous study on cyanotoxins

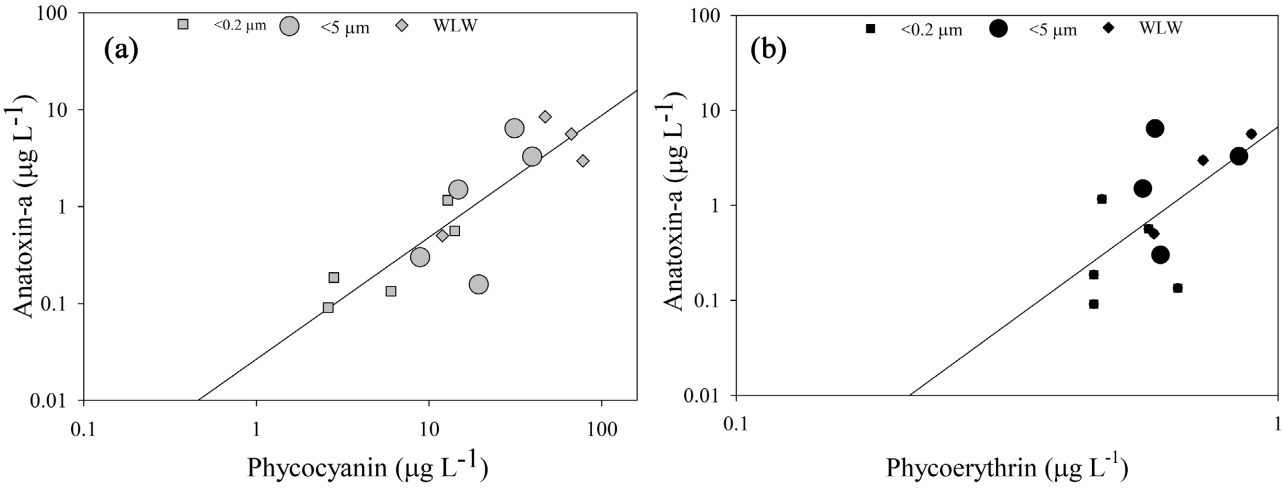

Figure 6. (a) Correlation analysis of (pico)cyanobacterial biomass and anatoxin-a in Lower Mill Pond, July 26-Aug 16. Adj. r^2 = 0.714, $p < 0.0001$, Log ATX = −1.574 + (1.259 * Log PC); (b) Correlation analysis of (pico)cyanobacterial biomass and anatoxin-a in Lower Mill Pond, July 26-Aug 16. Adj. r^2 = 0.833, $p < 0.0001$, Log ATX = −1.493 + (1.2220 * Log PE).

and the occurrence of the picocyanobacteria *Synechococcus* in a tropical freshwater system [21] used flow cytometry, culture techniques and visual inspection, followed by genome completeness and purity checks using CheckM. Serial filtration is an inexpensive technique that can be easily used, while flow cytometry requires trained personnel and relatively expensive equipment, suggesting that serial filtration could be considered a preferred technique.

5. Conclusions

Our investigation has demonstrated that serial filtration using gravity and pressure is an effective field and laboratory method to isolate picocyanobacteria for analysis, yielding samples that can meet relatively rigorous quality assurance/quality control requirements. We were able to collect samples representing distinct cyanobacterial populations to the Order level, specifically *Nostocales and Synechococcales*, as verified using eDNA metabarcoding techniques.

Fluorometric analysis using cyanobacterial accessory phycocyanin and phycoerythrin in this mesotrophic system proved to be a useful technique for describing periods of excess biomass in the picocyanobacterial population. Both pigments provided data necessary to quantify and anticipate this excess, using the net growth rate model. The ratio between these two pigments (PE/PC) appears to be a useful metric to describe picocyanobacterial populations, where the concentrations observed during this study may be applied to other mesotrophic systems.

The range of anatoxin-a concentrations for *Synechococcales* that were documented during this study appears to be unique, given the assumptions regarding planktonic *Nostocales*. During this study, we were able to identify the picocyanobacteria to the genus level using eDNA metabarcoding techniques, where *Cyanobium* 6307 was the major contributor to that total biomass. The 40-fold differences in anatoxin-a concentrations observed throughout the course of this

study, along with the fluorometry and eDNA results, suggest that variables including nutrient availability and other "toxin switches" were influencing anatoxin-a production. While we were able to affirm a linear correlation between cyanobacterial biomass (as measured using phycocyanin and phycoerythrin) and anatoxin-a during times of the highest anatoxin-a concentrations, inclusion of these other variables could improve our analysis.

Acknowledgements

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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