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Alternative Methods for Analysis of Cyanobacterial Populations in Drinking Water Supplies: Fluorometric and Toxicological Applications Using Phycocyanin

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Abstract

The management of cyanobacteria and potential exposure to associated biotoxins requires the allocation of scarce resources across a range of freshwater resources within various jurisdictions. Cost effective and reliable methods for sample processing and analysis form the foundation of the protocol yielding reliable data from which to derive important decisions. In this study the utilization of new methods to collect, process and analyze samples enhanced our ability to evaluate cyanobacterial populations. Extraction of phycocyanin using the single freeze thaw method provided more accurate and precise measurements (CV 4.7% and 6.4%), offering a simple and cost-effective means to overcome the influence of morphological variability. In-vacuo concentration of samples prior to ELISA analysis provided a detection limit of 0.001 μg·L^{-1} MC. Fractionation of samples (<0.2 μm, <2.0 μm, <50 μm, WLW and BFC) influenced our interpretations and improved our ability to establish a causative relationship between phycocyanin and microcystin levels in two aquatic systems with distinctly different cyanobacterial populations. In a *Microcystis* spp. dominant system Log MC (ng·L^{-1}) = −0.279 + (1.368 ∗ Log PC (μg·L^{-1}) while in an *Aphanizomemon* spp. dominant system Log *MC* (ng·L^{-1}) = 0.385 + (0.449 ∗ Log *PC* (μg·L^{-1}). These methods and sampling protocol could be used in other aquatic systems across a broader regional landscape to estimate the levels of microcystins.

Keywords

Cyanobacteria, Size Fractions, Fluorometry, Monitoring, Phycocyanin Extraction

1. Introduction

Cyanobacteria have gained attention from professionals and the public in light of the biotoxins they produce. Regulatory agencies responsible for recreational and drinking water supplies can utilize contact and/or consumption advisories to inform citizenry on the risks associated with exposure to cyanotoxins. To assess and quantify this risk, a variety of monitoring programs and protocols have been developed tailored to specific objectives. Within this paradigm efficient allocation of personnel and financial resources becomes paramount, and can be guided via a metric based framework including those for recreational waters [1] [2] and drinking water supplies [3] [4]. Metrics developed using improved techniques, including fluorometry, can be compared to existing metrics to determine efficacy. Fluorometric pigment analysis using phycocyanin may replace the need for cell counts and calculations of biovolume to describe cyanobacterial biomass [1] [3] [4] [5] [6] [7]. Previous studies describing correlative relationships between cyanobacterial biomass and toxin levels suggest that community composition [8] [9] [10] [11] [12], relative abundance [3] [13] and physical size [9] [12] [14] of the cyanobacterial species within the population influence the results. We questioned how existing sample collection and processing techniques in combination with novel field and laboratory methods would improve our ability to describe cyanobacterial populations and quantify associated risks. The purpose of this study was to evaluate 1) the single freeze-thaw (SFT) method for the extraction of phycocyanin prior to fluorometric analysis, 2) sample preparation using in-vacuo evaporation prior to toxin analysis and 3) the use of size fractionated samples to describe cyanobacterial populations by conducting seasonal surveys of cyanobacterial populations in two surface water supplies.

2. Materials and Methods

2.1. Studied Sites

Samples were collected from two lakes (Lake Attitash, Amesbury, MA and Lake Cochichewick, North Andover, MA) subject to similar weather patterns, being geographically located within 20 miles of each other (**Figure 1**). Lake Attitash is a secondary water supply to the town of Amesbury, MA and heavily used as a primary contact recreational water. The lake is 369 ha with a maximum depth of 9 m (average depth of 3.4 m), and a watershed with heavy residential development. Lake Cochichewick is the primary water supply to the town of North Andover, MA and a secondary contact recreational water. The lake is 575 ha with a maximum depth of 15 m (average depth of 7.7 m), and a watershed with agricultural land and light residential development.

2.2. Sampling

Samples for analysis were collected on a bi-weekly or weekly basis from July-October 2016 from the deep site between the hours of 10 AM and 3 PM. An integrated whole lake water sample (WLW) was collected using a 13 mm I.D.

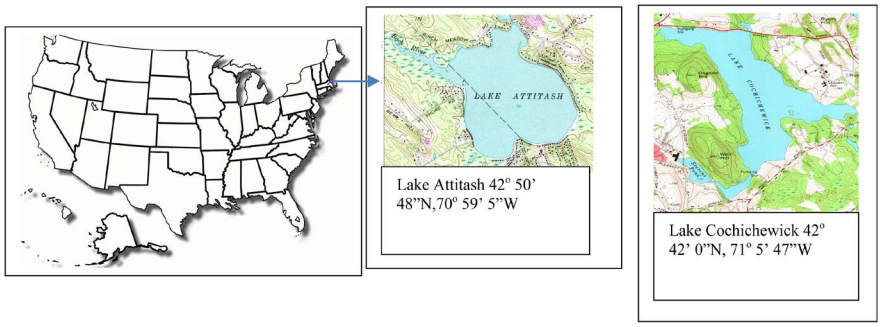

Figure 1. Map of United States of America and location of sampling sites in Massachusetts: Lake Attitash and Lake Cochichewick.

clear weighted vinyl tubing lowered to 3 m depth, cinched, and pulled upwards from the bottom. The sample was dispensed into a 500 mL darkened amber bottle. Approximately 100 mL of the WLW sample was passed through a 53 um ring net, and the filtrate collected in a 125 ml darkened amber bottle. Approximately 2 - 60 mL aliquots of the WLW sample was poured into a HSW 50 mL luer lock syringe fitted with a Swinnex filter holder containing either a 2.0 µm 25 mm diam. TTTP filter or a 0.22 µm 25 mm diam. GSWP filter, whereupon samples were filtered and 3 - 20 mL filtrate samples collected. All samples were placed in the dark and chilled during transport. After transport, the samples were gently mixed for a minimum of 30 s and triplicate subsamples (5 mL) were removed with a 5 mL pipette, and placed in darkened, labeled microvials. A bloom forming cyanobacteria (BFC) isolate was collected as a net plankton tow (NET), using a Students Plankton net (15 cm diam. 53 µm mesh), lowered to a 3 m depth and pulled upwards at a rate of 0.5 $m \cdot s^{-1}$. The sides of the plankton net were rinsed with lake water and the NET sample was allowed to concentrate and fill the cod end (approx. 250 mL). The NET sample was placed in a darkened amber container for a minimum of 2 h to initiate the process of respiration with resultant increase in positive buoyancy [15]. After this time, the sample was gently mixed for a minimum of 30 s. and triplicate subsamples (approx. 60 mL) were placed into three plankton separation devices or Pocket ZAPPRs™ that consisted of a darkened portion on the top and a clear portion on the bottom [16] [17]. The samples were allowed to separate for 30 min, whereupon the BFC isolate formed in the upper 5mL of the darkened portion of the devices due to a continued increase in positive buoyancy and the zooplankton concentrated in the clear portion of the devices due to positive phototaxis [18]. The BFC isolates were removed from the air-water interface with a 5 mL transfer pipette and placed into a darkened, labeled microvial. If a surface scum or surface bloom was observed a 125 mL surface grab sample was collected from the densest area of the scum (bloom) at the scum (bloom)/water interface and placed in a darkened amber container and chilled. The sample was gently mixed for a minimum of 30 s. and triplicate subsamples (5 mL) were removed with a 5 mL pipette and placed in darkened, labeled microvials.

2.3. Semi-Quantitative Analysis of Bloom-Forming Cyanobacterial Isolates

Subsamples of fresh BFC isolates for qualitative analysis were transferred to a Sedgewick-Rafter counting cell and enumerated under a compound microscope at 100×. The composition of the BFC isolate was determined after at least 200 organisms were identified to genus. Dominance was noted and relative abundance (%) was calculated. Single genus dominant samples (>60%) and mixed assemblages (combined abundance > 60%) were recorded.

2.4. Flourometric Analysis

Fluorometric analysis was conducted on samples within 2 hrs of collection (fresh) and after extraction. PC was extracted using a SFT at −20˚C for a minimum of 6 h, thawed in a warm water bath, and equilibrated to a temperature between 20˚C - 24˚C. PC concentrations were quantified using a two channel handheld Aquafluor fluorometer (Turner Designs 2013) using a 3 mL sample of distilled water as background. Within 20 min of equilibrating to a temperature between 20˚C - 24˚C, a 3 mL subsample was removed from the previously frozen < 0.2 μm, <2.0 μm, <50 μm, WLW, BFC and scum/bloom sample and placed into a methacrylate cuvette. The filled cuvette was placed in the fluorometer and readings ($\mu g \cdot L^{-1}$) were taken and recorded using channel A (PC). Samples were diluted with distilled water as needed to obtain readings within the range of the fluorometer. PC (excitation at 595 nm, emission at 670 nm) was standardized using freeze-dried powder (Beagle Bioproducts Lot PC-H-1002/413 +/− 12 ppb).

2.5. Toxicological Analysis

A subsample of approximately 1.8 mL was removed from the previously frozen <0.2 μm, <2.0 μm, <50 μm, WLW, BFC and scum/bloom samples and transferred into a labeled Fisherbrand 2.0 mL polypropylene graduated centrifuge tube with locking lid, pre-weighed using an O'Haus Adventurer balance set to 0.0000 g, and a Thermo Scientific Finnpipette F2 fitted with a FINNTIP. The filled centrifuge tube was then reweighed, recorded and the initial sample volume calculated. The samples were vortexed using a VWR Scientific for 10 s at a setting of "4", sonicated in a warm bath (40˚C) for 3 min on high power in a Fisher Scientific Ultrasonic Bath and re-frozen at −80˚C. This process was repeated two more times for a total of three freeze-thaw-vortex-sonicate cycles. Upon completion, samples either remained at existing concentration, were diluted or concentrated. For dilutions, 50 μL subsamples were removed using an Oxford Bench mate pipet fitted with a Fisherbrand Redi-Tip and placed in a 2.0 mL polypropylene graduated centrifuge tube with locking lid. Various volumes of Mill-Q water were added to achieve the desired dilutions and the sample vortexed using a VWR Scientific, for 30 s at a setting of "4". Samples to be concentrated were evaporated in vacuo using a Savant DNA 120 Speed-Vac Concentrator until the total sample volume was approximately 0.90 mL, 0.36 mL, or 0.18

mL, representing 2×, 5× and 10× concentrations, respectively. To achieve a 20× concentration, two 10× samples were prepared simultaneously (vial #1 and vial #2), whereupon the contents of vial #2 was transferred to vial #1 using a ThermoScientific Finnpipette F2 fitted with a FINNTIP, and then reduced to approximately 0.18 mL. Samples were reweighed, recorded, and the final sample volumes and concentration factors were calculated. Toxin analysis for total microcystins was conducted using Envirologix EP-022-HS Quantiplate Kit for Detection of Microcystins-High Sensitivity: Low Level of Detection by ELISA plate = 100 pg·mL^{-1} High Level of Detection by ELISA plate = 1200 pg·mL^{-1}. Readings were taken using a Bio-Tek Instruments Inc. El-800 Universal Microplate Reader Primary 450 nm Reference 630 nm and interpreted using KC Junior software. The standard curve was calculated in Sigma Plot using a 4 point logistic regression. Values for all fractions were reported as total microcystins representing dissolved + particulate microcystins, except for the <0.2 μm fraction representing only dissolved microcystins.

2.6. Statistical Analysis

Data were log transformed as needed to meet the requirements for parametric analysis, including analysis of variance, Tukey's pairwise comparison, Pearsons product moment correlation analysis and linear regression analysis. Proportional data were arc-sine transformed prior to analysis using T-tests. Non-parametric analysis included Kruskal-Wallis ANOVA on ranks and Spearman rank correlation as needed [19]. All analyses were conducted in Sigma Plot Version 12, dated 2010 [20].

3. Results and Discussion

3.1. Single Freeze-Thaw Method

The single freeze-thaw (SFT) method was used prior to fluorometric analysis of PC to facilitate sample storage, minimize error and maximize pigment extraction. Having demonstrated that phycobilins can be stored at −80˚C for 6 months without degradation [21], this storage protocol has been used [22] [23] prior to extraction of pigments for fluorometric analysis. Sources of error in the extraction process relating to sample composition and morphological variability have been suggested [21] and quantified [24]. Horvath, *et al.* [24] found that the coefficient of variation (CV) was species dependent and highly variable, generally being lowest after the first freeze-thaw cycle and decreasing depending on Chl-*a* concentration. For example, at Chl-*a* of 5 μg·L^{-1}, the CV values varied by 15 fold, including 0.93% for *C. raciborskii*, 2.86% for *A. spiroides* and 14.3% for *A. flos-aquae*, while at Chl-*a* of 10 μg·L^{-1} the results varied by ten-fold, including 1.80% for *C. raciborskii*, 14.3% for *A. spiroides*, 20.2% for *A. flos-aquae*. In our study comparing the CV for fresh and SFT samples, the results were less variable (**Table 1**), even though we observed distinct cyanobacterial populations in each lake. In Lake Attitash, the CV for <2.0 μm, <50 μm and WLW size fractions

Table 1. Coefficient of variation for fresh and extracted (SFT) samples of various size fractions in Lake Attitash and Lake Cochichewick. Italicized values represent standard error of the mean (SEM).

Lake Attitash	Coefficient of Variation (%)									
	<2.0 μm		<50 μm		WLW		BFC		All	
Fresh	8.8	*3.3*	4.9	*0.7*	3.9	*0.9*	4.8	*1.1*	5.6	*0.9*
Extracted	7.6	*1.1*	4.7	*0.9*	1.7	*0.4*	4.9	*0.8*	4.7	*0.5*
Lake Cochichewick	Coefficient of Variation (%)									
	<2.0 μm		<50 μm		WLW		BFC		All	
Fresh	4.5	*1.2*	6.2	*1.3*	20.2	*8.6*	5.9	*1.2*	9.2	*2.3*
Extracted	7.2	*1.9*	6.4	*0.9*	7.5	*1.5*	4.6	*1.1*	6.4	*0.7*

decreased, while the CV of the BFC fraction had a slight increase. In Lake Cochichewick, the CV for the WLW and BFC size fractions decreased, while the <2.0 μm and <50 μm fractions increased. For all samples combined we observed a decreased CV in both lakes using the SFT method from 5.6% to 4.7% in Lake Attitash and from 9.2% to 6.4% in Lake Cochichewick.

Previous studies on the extraction of PC from wet biomass of *Spirulina platensis* [25] [26] and *Anabaena oryzae* [27] have demonstrated the efficacy of the SFT method, where optimal temperatures for freezing (−50˚C) and thawing (25˚C) for a complete (100%) phycocyanin extraction in Tris-Cl buffer (pH 8.1) were demonstrated [27]. Extraction efficiency experiments [21] found that centrifugation, followed by single-freeze thaw and sonication proved to be the most effective method, yielding 100% of the phycobilins in the samples. Experiments using four Nostocales species indicated that the SFT method [24] provided maximum pigment extraction for all species in 0.5 M phosphate buffer when Chl-*a* values were below 20 $\mu g \cdot L^{-1}$. In all of the above referenced studies, extraction efficiencies were determined by comparing PC measurements following repeat freeze-thaw cycles, without recording PC measurements at the beginning of the experiments. Prior to conducting this field study, an experiment was conducted using fresh and frozen WLW samples to evaluate the SFT method across three lakes, where the average frozen/fresh ratio of 1.5 was observed [28]. During our study we observed ratios which varied according to size fraction (**Figure 2**), with the lowest observed values (1.2 and 1.4) in the <2.0 μm and highest (3.4 and 2.9) in the BFC size fractions. In Lake Attitash, significant differences between size fractions (ANOVA, Tukeys pairwise comparison $p < 0.05$) were observed, with the exception of the <50 μm: WLW samples. In Lake Cochichewick, we observed significant differences between three size fractions <2.0 μm: WLW, <2.0 μm: BFC and <50 μm: BFC (Kruskal Wallis ANOVA, $p < 0.05$). The frozen/fresh ratios for the BFC samples from Lake Attitash and Lake Cochichewick ranged between 2.2 - 5.0 and 2.4 - 3.7, respectively, while the ratios for bloom material collected from both lakes (data not shown) fell within the range of the BFC samples.

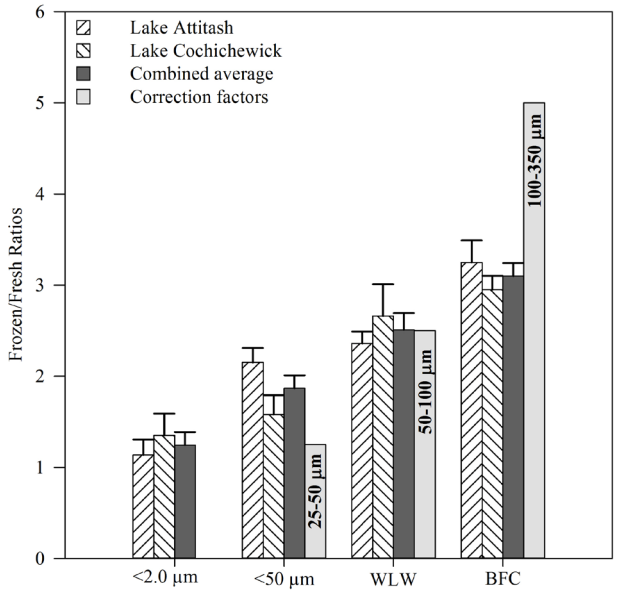

Figure 2. Comparison of observed frozen/fresh ratios for phycocyanin in sample size fractions from Lake Attitash and Lake Cochichewick and suggested *in-vivo* phycocyanin correction factors.

The frozen/fresh ratios observed during this study prompted consideration of their use as correction factors when quantifying cyanobacterial populations using in-situ methods. Correction factors to account for the influence of colony size [29] have been suggested when calibrating *in-vivo* fluorescent devices for quantification of cell densities. Their model calibration and verification study using *Microcystis* found that *in-vivo* PC fluorometry readings needed to be adjusted upwards to account for colony size ranges by factors of 1.02 - 1.25 (25 - 50 µms), 1.25 - 2.5 (50 - 100 µms), and 2.5 - 5.0 (100 - 350 µms). They noted that the model, as developed, applied to spherical colonies and suggested continuous adjustments based on site specific cyanobacterial size distributions. In our study, the observed range of fresh/frozen ratios was similar to the suggested *in-vivo* correction factors (**Figure 2**). There were no differences in the frozen/fresh ratio for size fractions between the lakes, excepting the <50 µm size fraction, where <2.0 µm ($U(35) = 108.00$, $p = 0.068$), <50 µm ($U(35) = 93.00$, $p = 0.022$), WLW ($U(35) = 164.00$, $p = 0.915$) and BFC ($t(35) = 1.253$, $p = 0.109$). Based upon our limited dataset, we could not conclude that sample composition and/or morphological variability affected the CV or the frozen/fresh ratio. These ratios may have the potential to be broadly applied as correction factors for *in-vivo* investigation of aquatic systems with diverse cyanobacterial assemblages. Additional studies on extraction efficiency encompassing shifts in cyanobacterial community composition and dominance would be useful.

3.2. Composition and Relative Abundance of Bloom Forming Cyanobacteria

In this study community composition and relative abundance of bloom-forming

cyanobacteria provided an initial assessment of the populations in each lake and potential for microcystin production. Previous investigations of *Microcystis* dominant systems have used identification to the genus level to describe spatial distributions of cyanobacteria in lakes of varying trophic status [30] and determine co-occurrence of cyanotoxins and taste-and-odor compounds [31]. In a study to quantify species-specific toxigenic cyanobacterial biomass [32], it was suggested that identification to the genus level was sufficient to describe correlations between Chl-*a* and microcystin levels. In Lake Attitash, *Microcystis* spp. was dominant on 7 of 10 sampling dates, while in Lake Cochichewick, *Aphanizomenon* was dominant on 8 of 10 sampling dates, with microcystin-producing assemblages in both lakes (**Table 2**). We anticipated differences in observed microcystin levels between the two lakes, where genus-specific estimates of pigment specific toxicity [33] [34] using phycocyanin [2] could have been applied *a-priori*. Lacking this, we could only evaluate *a posteriori* whether community composition and relative abundance influenced the interpretation of our results.

3.3. Analysis of Size-Fractionated Samples

The PEG model [35] suggests that species follow a process of succession, reflecting the temporal development of composition and abundance within the planktonic community. Where unique genus/species would be represented within each type of sample [8] [9] [10] [11] [12] seasonal patterns [3] [13] could be attributed to distinct cyanobacterial populations differentiated by size [9] [12] [14] as life cycle and composition affect their timing and amplitude. The sampling period for this study consisted of 10 dates on Lake Attitash (July 23-October 6) and Lake Cochichewick (August 22-October 24), using filtration (<0.2 μm, <2.0 μm, <50 μm) and floatation (BFC) to obtain a suite of samples in addition to traditional integrated WLW samples. We used PC and total MC to describe changes in the

Table 2. Dominant bloom-forming cyanobacteria (>60% relative dominance) in Lake Attitash and Lake Cochichewick.

Lake Attitash		Lake Cochichewick	
Date	Taxa	Date	Taxa
23-Jul	*Dolichospermum* spp.	22-Aug	*Aphanizomenon* spp.
28-Jul	*Dolichospermum* spp.	29-Aug	*Aphanizomenon* spp./*Dolichospermum* spp.
6-Aug	*Woronichinia* spp./*Microcystis* spp.	6-Sep	*Dolichospermum* spp.
11-Aug	*Microcystis* spp.	12-Sep	*Aphanizomenon* spp./*Microcystis* spp.
19-Aug	*Dolichospermum* spp./*Microcystis* spp.	20-Sep	*Aphanizomenon* spp.
27-Aug	*Dolichospermum* spp.	26-Sep	*Woronichinia* spp./*Microcystis* spp.
3-Sep	*Microcystis* spp.	3-Oct	*Microcystis* spp./*Aphanizomenon* spp.
11-Sep	*Microcystis* spp.	10-Oct	*Aphanizomenon* spp.
23-Sep	*Microcystis* spp./*Dolichospermum* spp.	18-Oct	*Aphanizomenon* spp.
6-Oct	*Microcystis* spp.	24-Oct	*Aphanizomenon* spp.

cyanobacterial populations [8] [9] [10] [11] [12], as other researchers have previously demonstrated that PC could be used as a preferred measure of biomass [4] [6] [7] for seasonal toxicological investigations [1] [3] [13] [36].

3.3.1. Extracellular Photopigment and Toxin Analysis

The extracellular (dissolved) fraction presents challenges for field practitioners. For fluorometric applications, extracellular PC may be an interference that needs to be accounted for prior to interpretation (Bastien, *et al.* 2011, Zamyadi, *et al.* 2016) [37] [38]. For toxicological applications, extracellular cyanotoxins need to be included in risk assessments (U.S. EPA 2015) [39]. Difficulties for both techniques are the detection and quantification limits that could exclude samples from analysis and limit interpretations.

1) Extracellular Phycocyanin

In an evaluation of PC probes with the Trios fluorometer, [37] found a method detection limit (MDL) and method quantification limit (MQL) of 0.93 $\mu g \cdot L^{-1}$ PC and 3.1 $\mu g \cdot L^{-1}$ PC respectively. Minimum quantification limits for hand-held fluorometers used during this study have ranged from 2 - 4 $\mu g \cdot L^{-1}$ PC depending on manufacturer. This would suggest that measured values less than 2 $\mu g \cdot L^{-1}$ PC be excluded and 4 $\mu g \cdot L^{-1}$ PC potentially unreliable in our analysis. In our study, only 20% of the measurements were greater than 2 $\mu g \cdot L^{-1}$ PC, and 15% were greater than 4 $\mu g \cdot L^{-1}$. The three recordable values, ranging from 5.1 to 8.6 $\mu g \cdot L^{-1}$ PC, were observed in Lake Cochichewick from October 10 - 24 when the PC levels in all size fractions were highest and there was a visible surface scum. The extracellular/total WLW ratios for these three samples ranged from 22% - 28%, with an average of 24%. Bastien, *et al.* [36] previously reported ranges from 1% - 100% with an average of 23%. Application of conservative criteria for detection limits using hand-held fluorometers resulted in a limited database from which to conduct statistical analysis using extracellular PC. We hoped to employ extracellular PC as a surrogate metric for extracellular microcystins where it could have been used within a screening protocol for water supply applications. The previous study [37] was an effort to quantify error associated with extracellular PC and the influence on the interpretation of *in-vivo* fluorometric applications. Given the averages from both studies (23% - 24%) and the range of the observed values, the error could be significant. Further investigation as to whether extracellular PC be considered a source of interference and subtracted as a blank prior to interpretations relating to cyanobacterial biomass should be conducted.

2) Extracellular Microcystins

Currently there is a suite of analytical techniques for the quantification of cyanotoxins [39] based upon available resources and objectives. Commonly used techniques [4] [31] [40] include liquid chromatography/tandem mass spectrometry (LC/MS/MS), high performance liquid chromatography (HPLC), ELISA (enzyme linked immuno-sorbent assay) and protein phosphatase inhibition assay (PPIA) [41]. Method detection limits (MDL) $\mu g \cdot L^{-1}$ MC from previous stu-

dies vary, with LC/MS/MS and HPLC typically 0.01 μg·L^{-1}, PPIA from 0.01 to 0.02 μg·L^{-1} and ELISA from 0.05 to 0.2 μg·L^{-1}. Sample preparation for LC/MS/MS, HPLC and PPIA includes 10× pre-concentration (lyophilization and rehydration) prior to analysis, which reduces the occurrence of "non-detection" results. Reported sample non-detection in previous studies quantifying dissolved microcystins ranged from 4% using HPLC [40], 17% and 22% using LC/MS/MS and ELISA respectively [31], 82% using ELISA [4] and 48% [42] using ELISA. In this study, we used in vacuo speed-vac sample preparation for ELISA providing a detection limit of 0.001 μg·L^{-1} MC, a 10-fold increase in sensitivity over LC/MS/MS, HPLC and/or PPIA. This method allowed for quantification of all our samples as we observed values from 0.008 - 0.053 μg·L^{-1} MC in Lake Attitash and 0.004 - 0.013 μg·L^{-1} MC in Lake Cochichewick. Without the speed-vac method we would not have detected microcystins in any of our dissolved samples, which would have severely restricted our analysis.

Quantification of dissolved cyanotoxins has been represented as a comparison of extracellular (dissolved) toxins to intracellular (particulate) toxins [40] [41] [42] [43] or dissolved toxins to mixed samples (total) toxins [31]. Park, *et al.* [40] used net plankton samples to quantify the dissolved MC/particulate MC ratio, with an average of 32.6% and a range from 0.08% - 180%. Oh, *et al.* [41] found that dissolved MC levels were 28% of the particulate forms in surface grab samples. The extracellular MC/total cellular MC (E/T) values previously observed [42] ranged from 1.6% - 107%, with an average of 7%. Su, *et al.* [43] using samples collected from the water column, observed times when extracellular (eTLR) exceeded intracellular total MC-LR concentrations (iTLR) but did not report the average. Graham, *et al.* [31] quantified the dissolved MC /total MC concentrations in bloom material and observed averages of 8% (ELISA) and 7% (LC/MS/MS) with a maximum of 30% (LC/MS/MS). In Lake Attitash, the dissolved (<0.2 μm) MC/total WLW MC ranged from 4% - 47%, averaged 18%, and reached the maximum (47%) on August 11. During the bloom event (October 6) in Lake Attitash the <0.2 μm MC/total WLW MC was 8% (**Figure 3(a)**). In Lake Cochichewick, the <0.2 μm MC/total WLW MC ranged from 71% - 117%, averaged 95%, and reached the maximum (117%) on August 29. During the bloom events in Lake Cochichewick (October 10 and 18), the <0.2 μm MC/total WLW MC were 72% and 71% respectively (**Figure 3(b)**). The percent contribution of dissolved microcystins to whole lake water in the lakes differed significantly ($U(18) = 0.00$, $p = 0.003$). Comparisons were made between microcystin levels in the dissolved (<0.2 μm) and other size fractions (<2.0 μm, <50 μm, WLW and BFC) as shown in **Table 3**. In Lake Attitash dissolved microcystins were significantly correlated with WLW and BFC size fractions but not with the <2.0 μm and <50 μm size fractions. In Lake Cochichewick dissolved microcystins were significantly correlated with <2.0 μm, <50 μm and WLW size fractions but not with the BFC size fraction. Regression analysis (**Table 4**) confirmed a linear relationship between the dissolved, <2.0 μm (picocyanobacteria) and <50 μm (nanocyanobacteria) size fractions, where dissolved microcystins were more closely

associated with pico- (Adj, $r^2 = 0.87$, $p < 0.001$) than nannocyanobacteria (Adj. $r^2 = 0.63$, $p = 0.004$).

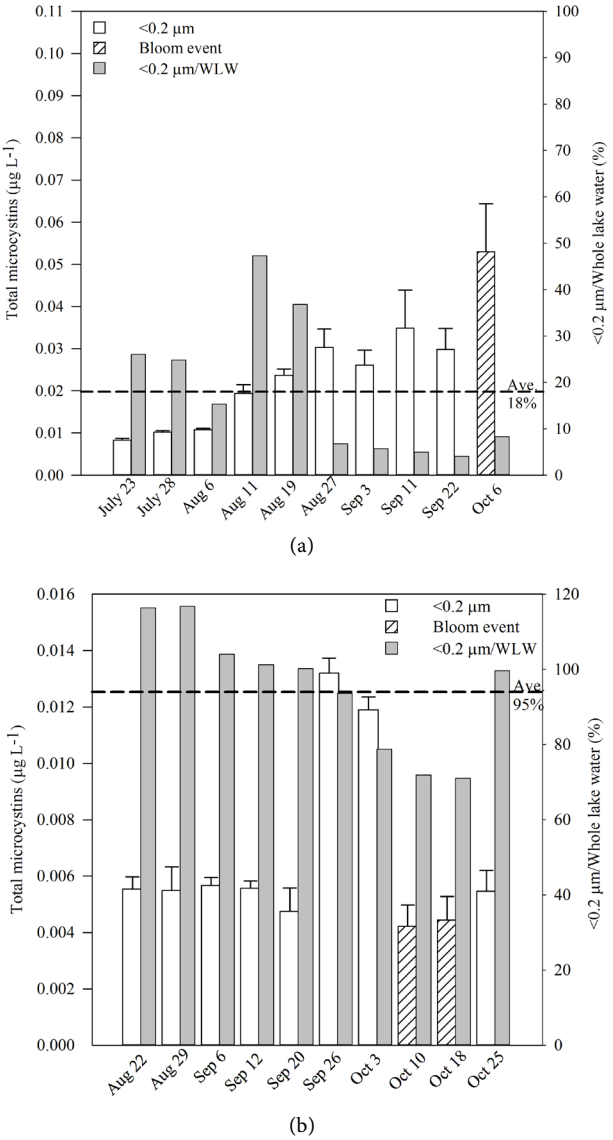

Figure 3. (a) Dissolved microcystins and percentage of dissolved microcystins in whole lake water samples in Lake Attitash; (b) Dissolved microcystins and percentage of dissolved microcystins in whole lake water samples in Lake Cochichewick.

Table 3. Pearsons product moment correlation coefficients of total microcystins ($ng \cdot L^{-1}$) in various size fractions with dissolved microcystins after log transformation. For all sample size fractions n = 10.

Total microcystins	Sample size fraction			
	<2.0 μm	<50 μm	WLW	BFC
Lake Attitash < 0.2 μm	r = 0.582, p = 0.078	r = 0.588, p = 0.074	r = 0.846, p = 0.002	r = 0.785, p = 0.007
Lake Cochichewick < 0.2 μm	r = 0.940, p = 0.00005	r = 0.819, p = 0.004	r = 0.909, p = 0.0003	r = 0.222, p = 0.537

Table 4. Results of linear regressions for dissolved and other size fractions in Lake Cochichewick, where Log Y = a + b * Log X. Microcystin values in ng·L^{-1}.

Lake Cochichewick size fractions							
<2.0 μm				<50 μm			
a	b	Adj. r^2	p	a	b	Adj. r^2	p
−0.0589	1.034	0.87	<0.001	0.161	0.736	0.63	0.004

The highest concentrations of dissolved microcystins that were observed in Lake Attitash and Lake Cochichewick were at the low end of the range of those previously reported [31], where the average (18%) and range of values (4% - 47%) observed in Lake Attitash were similar (7% and 1% - 30%), while the results for Lake Cochichewick were notably different (95% and 71% - 117%) [31]. The results may reflect the differences in cyanobacterial populations and community composition (**Table 2**). In the mid-west study, 74% of bloom material was either a mixed assemblage containing *Anabaena* spp./*Microcystis* spp. or *Microcystis* spp. dominant. This is similar to Lake Attitash where relative dominance in the BFC isolate was mixed assemblages shifting between *Dolichospermum* spp. and *Microcystis* spp. Our correlation analysis revealed that dissolved microcystins were positively associated with total microcystins in the BFC isolate. In Lake Cochichewick, *Aphanizomenon* spp. was dominant in the BFC isolates throughout most of the study period, with mixed assemblages of *Aphanizomenon* spp./*Dolichospermum* spp. noted during the last three sampling dates. In addition, the average proportion of the picocyanobacteria (<2.0 μm) to the WLW biomass (as measured by PC) in Lake Cochichewick was 45%. Linear regression revealed that dissolved microcystins were more closely associated with total microcystins in the <2.0 μm fraction than the <50 μm fraction. Thus, it appears that aquatic systems dominated by picocyanobacteria may tend to have a higher relative amount of dissolved microcystins than systems dominated by bloom-forming cyanobacteria. The instances where samples of dissolved microcystins were greater than total microcystins (>100%) raised questions as to potential sources of these findings. Error introduced during sample processing (sub-sampling and weighing) and breakage of cells during the filtration process may have occurred, affecting the concentrations to an unknown extent. Experiments comparing filtration protocols (syringe versus vacuum) would be useful in this regard.

3.3.2. Bloom-Forming Cyanobacterial Isolates

A novel method has been described using filtration and floatation to isolate BFC's for analysis. Selection of a 53 μm mesh plankton net for the initial separation of BFC's by filtration preferentially collected the portion of the cyanobacterial population that would not be subject to grazing [44] [45] [46] and tend to be buoyant [8] [47] [48]. Floatation has previously been used to isolate *Microcystis* to calculate weight specific toxicity [49] and biomass [23]. The floatation

process utilized an initial 2 h darkened period followed by an additional 30 min darkened period in the separation device. The combined darkened period of 2.5 h agrees well with that previously demonstrated [8] [50] to increase positive buoyancy in gas-vacuolate cyanobacteria. The 30 min floatation period in the plankton separation device (18 cm height) would require an average floating velocity (V_t) of approximately 100 μm·s^{-1} to isolate the cyanobacteria, comparable to floatation velocities of 36 - 88 μm·s^{-1} and 100 - 225 μm·s^{-1} previously demonstrated for *Microcystis* colonies with diameters of 100 and 200 μm respectively [47]. Recent studies [9] [10] [12] using filtration to separate cyanobacteria into size categories for microcystin analysis suggest that the 100 μm size category is responsible for the greatest proportion of total microcystin production [10] [12], microcystin production rates, cell quotas and genotypes [9].

We hypothesized that BFC isolates of populations distributed within the water column could provide similar toxicological profiles to samples collected as accumulations at the surface (*i.e.* bloom conditions), using the ratio of total microcystins to phycocyanin as a measure of pigment specific toxicity. Other researchers have calculated cyanobacterial pigment specific toxicity using chlorophyll-a, where a range from 0.10 to 0.45 ng/ng was observed in *Microcystis* culture experiments [33] and a 90th percentile value of 0.202 μg/μg was used to establish a "worst case scenario" [34] for an alert framework. In Lake Attitash the pigment specific toxicity of the BFC isolates collected on September 11 and October 6 (0.0145 and 0.0135 μg·μg^{-1}, respectively) did not differ while the September 23 isolate (0.0318 μg·μg^{-1}) was significantly higher (ANOVA Tukeys pairwise $p = 0.002$) than values for bloom material collected on October 6 (0.0104 μg·μg^{-1}). In Lake Cochichewick, the pigment specific toxicity in the BFC isolates collected from September 26-October 10 did not differ significantly (ANOVA on ranks $p = 0.108$) from bloom material collected on October 10.

3.4. Seasonal Fluorometric and Toxicological Evaluation of Cyanobacterial Populations

In Lake Attitash, the biomass distributions for the WLW and filtrates (**Figure 4(a)**) followed bimodal patterns while the BFC isolates (**Figure 4(b)**) had a parabolic pattern. Overall, the <2.0 μm, <50 μm fractions and WLW fractions were different from each other, (Tukeys pairwise comparison $p < 0.050$), with filtrates contributing (17% and 74% respectively) significantly different ($p < 0.001$) amounts to the WLW biomass for the entire study period. In Lake Cochichewick, the biomass distributions for the WLW and filtrates (**Figure 4(c)**) and the BFC isolates (**Figure 4(d)**) generally increased from low levels to extended maxima The observed maxima within the size fractions, <2.0 μm, <50 μm and WLW were not significantly different from each other. Overall, the WLW and filtrate biomass were significantly different from each other (Tukeys pairwise comparison $p < 0.050$), however the filtrates (<2.0 μm and <50 μm) were not. The relative of contribution of the <2.0 μm and <50 μm fractions to the WLW

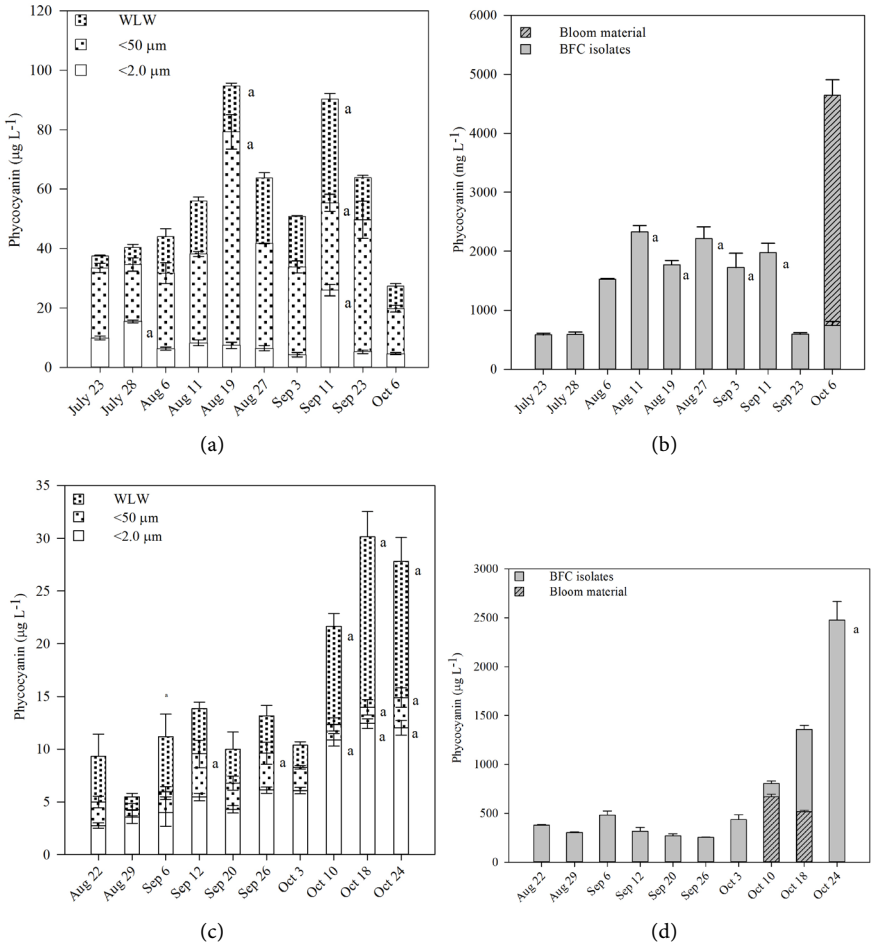

Figure 4. (a)-(d) Phycocyanin levels in Lake Attitash (a)-(b) and Lake Cochichewick (c)-(d) for various size fractions. The letter (a) denotes samples within each size fraction that are the most similar.

biomass (45% and 63% respectively) were significantly different from each other ($p = 0.002$).

In Lake Attitash, the microcystin levels (**Figure 5(a)**) in the <2.0 µm and <50 µm size fractions had concurrent single peaks, while the WLW and BFC isolates (**Figure 5(b)**) had an extended maxima. Overall, toxin levels in the <2.0 µm, <50 µm and WLW fractions were significantly different from each other (Tukeys pairwise comparison $p < 0.050$), with filtrates contributing similar amounts to the WLW toxin levels (27% and 45% respectively). In Lake Cochichewick microcystin levels for the <2.0 µm size fraction and WLW samples (**Figure 5(c)**) generally increased from low levels to extended concurrent maxima while the <50 µm size fractions and BFC isolates had bimodal patterns (**Figure 5(d)**) The observed maxima within the <2.0 µm, <50 µm and WLW size fractions were not significantly different from each other. Overall, the toxin levels in the WLW and filtrates were not significantly different from each other, with the filtrates contributing similar amounts to the WLW toxin levels (94% and 97% respectively).

Previous seasonal studies of *Microcystis* assemblages have documented

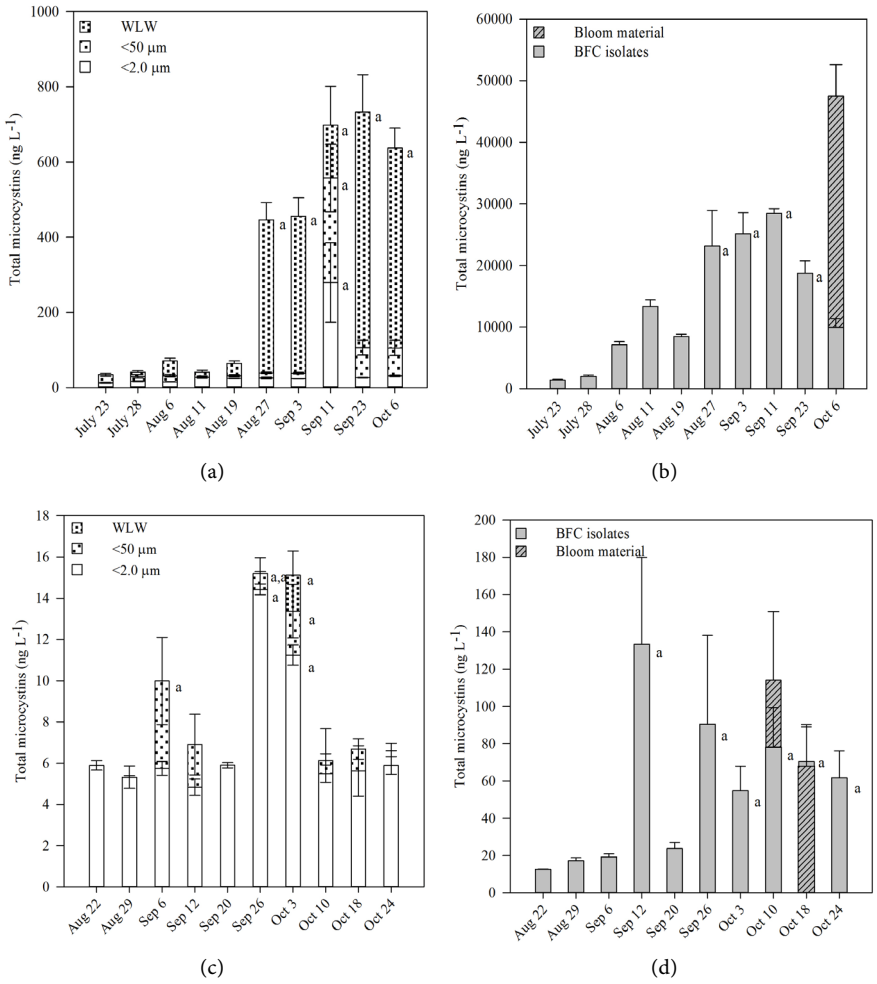

Figure 5. (a)-(d) Total microcystin levels in Lake Attitash (a)-(b) and Lake Cochichewick (c)-(d) for various size fractions. The letter (a) denotes samples within each size fraction that are the most similar.

dynamic temporal variations of phycocyanin and microcystins in WLW samples, where correlation analyses using WLW samples confirmed significant positive correlations between both metrics [3] [13] [36]. Other studies have failed to establish either strong correlations [4] between intra-cellular microcystins, in-vivo fluorescence and cyanobacterial biovolume, or linear relationships between the presence of cyanobacteria and cyanotoxin concentrations [37]. In our study, we could not confirm any correlations using either fresh or extracted WLW samples for either Lake Attitash ($r_s = 0.418$ $p = 0.213$, $r_s = 0.321$ $p = 0.346$) or Lake Cochichewick ($r_s = 0.086$ $p = 0.919$, $r_s = 0.527$ $p = 0.107$) respectively. However, we did observe significant correlations between fresh (**Table 5**) and extracted (**Table 6**) phycocyanin and microcystin levels in both lakes when size fractions (<50 µm, WLW and BFC) were included in the analysis. Inclusion of extracted phycocyanin from bloom material improved our analysis yielding significantly different ($t = 2.00$, $p < 0.001$) linear regressions (**Figure 6**) describing causative relationships between phycocyanin and microcystin levels in Lake

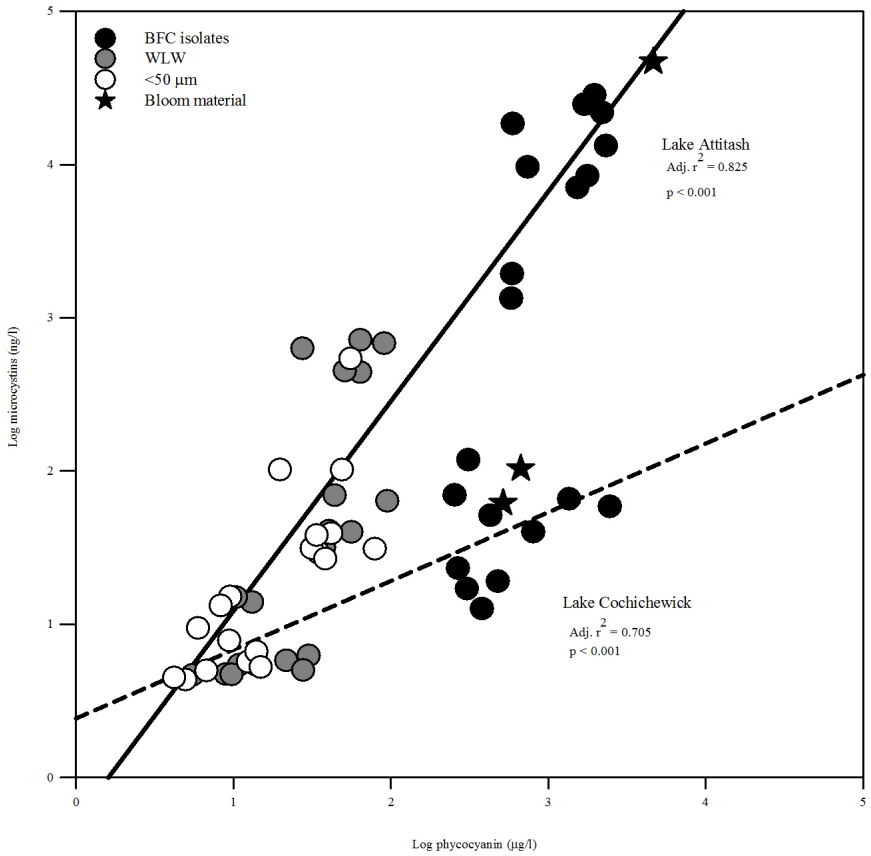

Figure 6. Linear regression between phycocyanin and total microcystins in Lake Attitash (solid line) and Lake Cochichewick (dashed line).

Table 5. Correlation coefficients between microcystins and *in-situ* (fresh) phycocyanin in selected studies. Bold face indicates significance levels at $p < 0.05$.

Sulejow Reservoir[a]	**r = 0.51**	n = 31	**p < 0.05**
Buckeye Onion Island[b]	**r_s = 0.79**	n = 10	**p < 0.05**
Harsha Main[b]	**r_s = 0.93**	n = 17	**p < 0.05**
Lake Erie[b]	**r_s = 0.85**	n = 24	**p < 0.05**
Lake Attitash[c]	**r_s = 0.816**	n = 30	**p < 0.001**
Lake Cochichewick[c]	**r = 0.754**	n = 22	**p < 0.001**

[a]Izydorczyk, *et al.* 2005. [b]Francy, *et al.* 2016. [c]This study.

Table 6. Correlation coefficients between microcystins and extracted phycocyanin in selected studies. Bold face indicates significance levels at $p < 0.05$.

Lake Erie[a]	**r_s = 0.7633**	n = 45	**p = 0.0000**
Western Basin[a]	**r_s = 0.6274**	n = 25	**p = 0.0008**
Lake Attitash[b]	**r = 0.901**	n = 30	**p < 0.001**
Lake Cochichewick[b]	**r = 0.826**	n = 30	**p < 0.001**

[a]Rinta-Kanto, *et al.* 2009. [b]This study.

Attitash (Adj. $r^2 = 0.825$, $p < 0.001$), Equation (1), and Lake Cochichewick (Adj. $r^2 = 0.704$, $p < 0.001$) Equation (2).

$$\mathrm{Log}MC\left(\mathrm{ng}\cdot\mathrm{L}^{-1}\right) = -0.279 + \left[1.368 * \mathrm{Log}PC\left(\mu\mathrm{g}\cdot\mathrm{L}^{-1}\right)\right] \quad (1)$$

$$\mathrm{Log}MC\left(\mathrm{ng}\cdot\mathrm{L}^{-1}\right) = 0.385 + \left[0.449 * \mathrm{Log}PC\left(\mu\mathrm{g}\cdot\mathrm{L}^{-1}\right)\right] \quad (2)$$

It appears that correlation analyses between phycocyanin and microcystins requires samples representative of a range of conditions, including those collected during bloom conditions or mimicking bloom conditions (*i.e.* BFC isolates). In the two season study of the Sulejow Reservoir [3] the correlation analysis was limited to samples collected during a Microcystis bloom, where approximately 68% of the reported values clustered below 0.50 $\mu g \cdot L^{-1}$ MC and seven samples were less than 3.0 $\mu g \cdot L^{-1}$. In Lake Attitash, the observed WLW maximum for total microcystins was 0.73 $\mu g \cdot L^{-1}$ MC while eight BFC isolates had levels above 3.0 $\mu g \cdot L^{-1}$. In the three lake study spanning two seasons bracketing cyanoHAB events [36], the average values for microcystins in samples ranged from 1.8 to 49 $\mu g \cdot L^{-1}$, very similar to our observed values for BFC isolates and bloom material in Lake Attitash which ranged from 1.4 to 47.5 $\mu g \cdot L^{-1}$ MC. The three season study of Lake Erie [13] presented a data set that spanned "the known range of Microcystis bloom events for Lake Erie, allowing for a thorough profile of the conditions associated with bloom and non-bloom events", where microcystin levels in samples ranged from 0.0004 to 21.7 $\mu g \cdot L^{-1}$ MC. In Lake Attitash, the observed range of total microcystins in the size fractions (excluding bloom material) ranged from 0.026 - 28.5 $\mu g \cdot L^{-1}$ MC.

4. Conclusions

In this study we evaluated alternative methods for analysis of cyanobacterial populations including the single freeze-thaw extraction procedures for phycocyanin, in-vacuo evaporation prior to toxin analysis and the use of size fractionated samples to describe cyanobacterial populations. The single freeze-thaw method, as compared to in-situ measurements, showed a reduction in the CV (%) from 5.6% to 4.7% in Lake Attitash and from 9.2% to 6.4% in Lake Cochichewick. Average measurements of phycocyanin increased from 1.3 fold to 3.2 fold on both lakes after freezing depending on the size fraction. The observed range of fresh/frozen ratios was similar to previously suggested *in-vivo* correction factors to account for error associated with morphological variability. The in-vacuo concentration of samples prior to ELISA analysis provided a detection limit of 0.001 $\mu g \cdot L^{-1}$ MC, a 10-fold increase in sensitivity over LC/MS/MS, HPLC and/or PPIA. In both lakes the maximum dissolved microcystin levels were observed prior to bloom events. In Lake Attitash, the dissolved (<0.2 μm) MC/total WLW MC averaged 18% and reached the maximum (47%) on August 11, prior to the bloom event (8%) on October 6. In Lake Cochichewick, the <0.2 μm MC/total WLW MC averaged 95%, and reached the maximum (117%) on August 29, prior to bloom events 72% and 71% on October 10 and 18. The results

suggest that aquatic systems dominated by picocyanobacteria may tend to have a higher relative amount of dissolved microcystins than systems dominated by bloom-forming cyanobacteria. An enhanced description of cyanobacterial populations using size fractionated samples appears to have improved our analysis where significantly different ($t = 2.00$, $p < 0.001$) linear regressions described causative relationships between phycocyanin and microcystin levels in the study lakes. This relationship in Lake Attitash, as a *Microcystis* spp. dominanted system, was described as Log MC ($ng \cdot L^{-1}$) = −0.279 + (1.368 ∗ Log PC ($\mu g \cdot L^{-1}$) and in Lake Cochichewick, as an *Aphanizomemon* spp. dominanted system, was described as Log MC ($ng \cdot L^{-1}$) = 0.385 + (0.449 ∗ Log PC ($\mu g \cdot L^{-1}$).

Based upon our results, we feel that the improved fluorometric and toxicological methods tested in this study improved our ability to describe diverse cyanobacterial populations. Cyanobacterial samples processed using the single freeze thaw method appear to have provided a more accurate and precise measurement of phycocyanin, offering a simple and cost-effective means to overcome the influence of morphological variability. The in-vacuo concentration prior to ELISA analysis allowed for quantification of toxins for the entire study period for both lakes, many of which were below existing detection limits. Application of this method would facilitate future studies quantifying dissolved toxins, aid in the management of high quality aquatic systems, and quantify toxin levels prior to potential exceedances above the low level (0.3 $\mu g \cdot L^{-1}$) drinking water health advisory. The analysis of size fractions allowed us to characterize distinct cyanobacterial populations appearing to possess unique fluorometric and toxicological profiles, influencing our interpretations and improving our ability to establish a causative relationship between phycocyanin and microcystin levels in two aquatic systems with distinctly different cyanobacterial populations. Additionally, as BFC isolates provided samples representative of those collected during bloom conditions, inclusion of these samples in a monitoring protocol could potentially eliminate the need for multi-year surveys. Additional studies using these methods should be conducted on aquatic systems in different locations and with diverse cyanobacterial community compositions to determine if similar correlations could be established. Our findings in this study suggest that fluorometric analysis using phycocyanin could become a more powerful tool to rapidly provide resource managers estimates of toxin levels thereby facilitating the decision making process and ultimately reducing risk of exposure to microcystins.

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Conflicts of Interest

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

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