

Article

Flow Cytometric Analysis of Freshwater Cyanobacteria: A Case Study

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Abstract: Eutrophication is a process that occurs due to the excessive accumulation of nutrients, primarily nitrogen and phosphorus, from natural and anthropogenic sources. This phenomenon causes cyanobacterial overgrowth, which over time leads to cyanobacterial harmful algal blooms (CHABs) that affect public drinking water sources and water sites with recreational usage. The rapid detection of bloom-forming cyanobacteria in freshwater bodies is critical in order to implement prevention strategies. Cyanobacteria contain phycobiliproteins such as phycoerythrin and allophycocyanin as part of the phycobilisome that allows autofluorescence. In this study, samples from 36 freshwater bodies in 14 New Jersey counties were collected and analyzed using flow cytometry with forward-scatter phycoerythrin and allophycocyanin parameters. Pure cultures of *Synechococcus* sp. IU 625, *Cylindrospermum* spp. and *Microcystis aeruginosa* were used as references. The results revealed that 17 out of the 36 analyzed sites contained all three references and related species. Seven sites showed *Microcystis* and *Cylindrospermum*-like species, while four sites indicated *Microcystis* and *Cylindrospermum*-like species. Six water bodies showed *Cylindrospermum*-like species, and two sites showed *Microcystis*-like species. Polymerase chain reaction (PCR)-based assays further confirmed the flow cytometric results. The findings from this study suggest that flow cytometry could potentially serve as a rapid method for freshwater cyanobacteria detection and screening.

Keywords: cyanobacteria; flow cytometry; cyanobacterial harmful algal blooms (CHABs)

1. Introduction

Cyanobacteria, also known as blue green algae, are some of the earliest dated oxygen-releasing prokaryotes, originating about 3.5 billion years ago [1]. Their size ranges from 0.5 to 50+ μm , and they may be composed of different cellular arrangements, such as unicellular *Synechococcus* spp., filamentous *Cylindrospermum* spp., and colonial *Microcystis* spp. Cyanobacteria can be found in marine and freshwater environments, including lakes, ponds, rivers, brackish water and water reservoirs. Moreover, these bacteria can be terrestrial, colonize in volcano ashes, and even colonize in the human gut. Cyanobacteria are able to survive and outcompete other biological species in eutrophied water bodies and, consequently, can over-grow nutrients such as nitrogen and phosphorus, leading to the formation of algal blooms. Climate change as well as human disruptions such as agriculture, industrial and recreational activities, and sewage effluent all lead to eutrophication [2]. The bloom masses may cover the water surface, lowering the dissolved oxygen and causing hypoxia in the water. Although not all cyanobacteria are toxic, many possess the ability to release toxins and produce secondary metabolites known as cyanotoxins, including hepatotoxins, neurotoxins and dermatotoxins, which are toxic to other cells, organisms, fish, animals and humans. Cyanobacterial harmful algal blooms (CHABs) are considered a significant concern to the ecosystem. In addition, their toxins can be very dangerous to the public, who utilize the water for drinking and recreational purposes. Cyanobacterial toxicity can vary from one bloom to another, where specific species can secrete a certain toxin or even

multiple species can release different cyanotoxins [3,4]. Intracellular toxins for most cyanobacteria are not secreted unless an environmental stress factor induces cell lysis [4,5].

Approximately 80% of human exposure to cyanotoxins is from the ingestion of contaminated water [6,7]. The United States Environmental Protection Agency (USEPA) lists several cyanotoxins on the candidate list of contaminants that primarily affect public drinking water, including microcystin, cylindrospermopsin and the anatoxin-a group. Microcystins, primarily found in fresh and brackish water, causes toxicity to the liver by triggering liver inflammation and/or liver hemorrhage due to cytoskeletal damage induced by inhibition of serine/threonine-specific protein phosphatases [8,9]. Anatoxin-a involves the binding of its toxin to a neuronal pre-synaptic acetylcholine receptor, where nicotine receptors are mimicked, thus, causing neurological effects such as paralysis and even death from respiratory arrest [10–15]. Other cyanobacterial neurotoxins include saxitoxin (also known as paralytic shellfish toxin) and non-proteinous amino acid β -methylamino-L-alanine (BMAA) [16–19]. Cylindrospermopsin (CYN), a cytotoxin with five variants or analogues found further below the water surface, can have its toxin released extracellularly in up to 90% of the total CYN content in the water column [4,13,20–25]. The toxic mechanism of cylindrospermopsin involves protein synthesis inhibition, which leads to kidney and liver failure [9]. It can also induce oxidative stress and can cause cell-cycle arrest, apoptosis or necrosis in various cell lines [26]. The World Health Organization (WHO) has established a recommended limit for microcystin-LR of 1.0 $\mu\text{g/L}$ in drinking water. Most countries follow this WHO guideline, but some have minor variations that can range from 1.0 to 1.5 $\mu\text{g/L}$ [27]. Children are at greater risk due to their body size to weight ratio. USEPA has provided separate guideline limits for children younger than six years old of 0.3 and 0.7 $\mu\text{g/L}$ for microcystin and cylindrospermopsin, respectively [12,28].

Current treatments for algal bloom include the use of algaecides and chlorine, but they have been shown to cause cell lysis and, subsequently, the secretion of toxins [29,30]. Furthermore, while microfiltration and ultrafiltration have been shown to be effective for the prevention of intracellular toxins with up to 98% removal of *Microcystis aeruginosa*, they are ineffective for the removal of extracellular toxins [13,31]. Recently, researchers have looked at using cyanophages, host-specific bacteriophages that have the ability to infect species-specific cyanobacteria, as a possible biological prevention approach for cyanobacterial blooms. Further knowledge of cyanophages can aid in the reduction of bloom-forming cyanobacteria and increase water quality and the health of the ecosystem [32].

Finally, there is an immediate need for rapid detection of harmful algal blooms worldwide in order to avoid and or halt further issues and reduce the need for chemical treatments that ultimately cause cell lysis and toxin secretion in water bodies. Currently, implemented detection methods contain some limitations. For instance, phenotypic identification through microscopy can be time-consuming, especially with high volumes of samples for monitoring purposes. Molecular identifications such as polymerase chain reaction (PCR) are more accurate in terms of specificity and sensitivity; however, they are not cost effective and cannot detect single cells [33]. Moreover, biochemical and immunoassays can be utilized, but require a field expert, expensive equipment, and may result in false positives [34]. Flow cytometry analysis may be an effective tool for analyzing routine environmental water samples for the prevention of algal blooms [31]. Cyanobacteria contain phycobilisomes, which are composed of autofluorescent pigments known as phycoerythrin (PE), phycocyanin (PC), and allophycocyanin (APC) [35,36]. Flow cytometry detects these natural pigments in photosynthetic microorganisms, including PE and APC [31,36]. Previous reports have discussed the utilization of flow cytometry to study cyanobacteria and monitor microbes in drinking water sources [37,38]. This study aims to determine the cyanobacterial profiles of freshwater bodies by comparing water samples to the reference cyanobacteria with flow cytometry. *Synechococcus* sp. IU 625, a unicellular cyanobacterium, *Microcystis aeruginosa*, and *Cylindrospermum* spp. have been commonly used as cyanobacterial algal bloom indicators [4,11] and are included in this study as reference cyanobacteria.

2. Materials and Methods

2.1. Water Sample Collection and Processing

Water samples from 36 water bodies (250 mL per site in Nalgene bottles) in New Jersey (Figure 1) were obtained by the New Jersey Department of Environmental Protection (NJDEP) in summer (May to August), 2015. These water bodies are utilized for recreational purposes, drinking water, or water supply reservoirs. The Global Positioning System (GPS) coordinates as well as water temperature (°C), pH and dissolved oxygen (mg/L) were measured and recorded on site. Water samples were collected one meter below the surface.



The 36 Tested Water Body Sites in New Jersey

Symbol #	Site Name	County	Symbol #	Site Name	County	
1	Atlantic City Reservoir	Atlantic	19	NJLM 1034	Monmouth	
2	NJLM 1286 (Shadow Lake)	Bergen	20	NJLM 1045 (Cifrese Lake)	Morris	
3	Deverson Lake	Burlington	21	Lake Morski Oko	Morris	
4	Lake Pachoango		22	Prosperstown Lake	Ocean	
5	Lake Pemberton		23	Green Turtle Lake	Passaic	
6	Mt. Misery Lake		24	Alloway Lake	Salem	
7	Mirror Lake		25	Parvin Lake		
8	NJLM 0315		26	Rainbow Lake		
9	NJLM 0754		Cumberland	27	Great Gorge Lake	Sussex
10	Hands Mill Pond			28	Hunts Pond	
11	Union Lake	29		Laidlaws Pond		
12	Cooper Lake	Gloucester	30	Mashipeacong Pond		
13	Iona Lake		31	NJLM 0213		
14	Franklinville Lake		32	NJLM 0378		
15	McCarthy's Lake		33	Silver Lake		
16	NJLM 0489	Hunterdon	34	Upper East Highland Lake		
17	Arnwell Lake		35	Watchu Pond		
18	Roosevelt County Park Lake	Middlesex	36	Deer Park Pond	Warren	

Figure 1. The 36 water body sites in New Jersey collected from and analyzed in this study. The locations are labeled on the map with the corresponding counties listed.

2.2. Filtration

A Thermo Scientific™ Nalgene™ (Thermo Scientific, Rochester, NY, USA) vacuum filtration unit with polycarbonate membrane filters (Sterlitech Corporation, Kent, WA, USA) was used for filtration. The water samples were thoroughly mixed and serial filtered through 30 and 0.4 μm membrane filters, sequentially.

2.3. Flow Cytometry

Flow cytometric assays were conducted using a MACSQuant® Analyzer, calibrated with MacsQuant calibration beads (Miltenyi Biotec, Inc., San Diego, CA, USA). The three standard reference cultures *Microcystis*, *S. IU 625* and *Cylindrospermum* spp. with Optical Density (OD_{750nm}) of 0.591, 0.521 and 0.640, respectively, were run through the flow cytometer at fixed voltage settings with various dilutions. The water samples for each site and the <30 μm filtrates were run through the flow cytometer, with the same voltage settings as the standards. The low flow rate and mix sample options were selected for this analysis. The voltage channels for forward scatter (FSC), side scatter (SSC), B2 phycoerythrin (PE), R1 allophycocyanin (APC) were 250 V, 200 V, 800 V, and 275 V, respectively. Each uptake contained 100 μL of the sample. The analysis was carried out using FSC and PE gating strategy with the software FlowJo vX.0.7 (Tree Star, Inc., Ashland, OR, USA).

2.4. Molecular Assays

Chelex DNA extraction was conducted for all collected water samples. Polymerase chain reaction (PCR) was carried out with CYA359F/CYA781R, the primer set that amplifies cyanobacterial 16S rRNA genes [39], to confirm the flow cytometry results. MSF/MSR, a primer set to detect the presence of Microcystin Synthetase A Gene (*mcyA*) in the Cyanobacterial Genus *Microcystis* was also included [40]. A 1% agarose gel electrophoresis was used to visualize the PCR products. PCR products were subsequently sequenced with an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Multiple Sequence Comparison by Log-Expectation (MUSCLE) 3.8 was utilized to analyze the 16S rRNA genes of *Cylindrospermum* spp., *Microcystis* spp., and *Synechococcus* spp. [41].

3. Results

3.1. Water Collection Sites

Water properties were recorded for all 36 sites (Table 1). Among all the water samples collected and tested, the water temperature ranged from 16.97 (NJLM 0213) to 30.9 °C (Alloway Lake). The pH ranged from 4.09 (Mt. Misery Lake) to 9.75 (Cooper Lake), with the majority of sites between 6 and 9. The dissolved oxygen ranged from 1.66 (NJLM 0213) to 19.1 mg/L (Cooper Lake).

Table 1. Water properties including temperature, pH and dissolved oxygen of the 36 sites.

Site Name	Water Temp (°C)	pH	Dissolved O ₂ (mg/L)
Atlantic City Reservoir	20.73	7.16	9.95
NJLM-1286 (Shadow Lake)	28.49	9.18	14.64
Deverson Lake	26.84	5.03	5.97
Lake Pachoango	29.35	6.79	11.96
Lake Pemberton	28.72	6.38	10.21
Mt. Misery Lake	18.41	4.09	6.35
Mirror Lake	21.65	5.89	7.13
NJLM-0315	22.35	6.24	7.05
NJLM-0754	22.95	6.92	9.81
Hands Mill Pond	28.08	4.44	7.65
Union Lake	27.29	7.48	9.2
Cooper Lake	24.88	9.75	19.17
Iona Lake	24.89	6.33	5.07
Franklinville Lake	26.78	5.53	3.09
McCarthys Lakes	26.45	6.27	8.21
NJLM-0489	30.09	9.38	10.77
Amwell Lake	28.25	8.7	9.97
Roosevelt County Park Lake	29.2	8.73	13.42
NJLM-1034	20.32	6.95	9.87
Cifrese Lake (NJLM-1045)	28.17	9.2	10.18
Lake Morski Oko	22.64	7.0	6.57
Prospertown Lake	27.43	6.64	7.44
Green Turtle Lake	28.89	8.92	8.92
Alloway Lake	30.9	8.03	9.59
Parvin Lake	27.8	6.86	8.17
Rainbow Lake	28.1	6.83	7.11
Great Gorge Lake	26.57	9.24	8.89
Hunts Pond	26.67	8.25	8.95
Laidlaw Pond	27.01	8.43	8.1
Mashipacong Pond	26.56	6.2	7.04
NJLM-0213	16.97	6.03	1.66
NJLM-0378	25.8	6.21	2.65
Silver Lake	26.7	6.39	3.32
Upper East Highland Lake	26.87	8.51	7.92
Watchu Pond	25.17	6.83	3.69
Deer Park Pond	28.2	7.15	6.81

3.2. Filtration Cytometric Analysis of Three Reference Cyanobacteria

Three reference species, namely, *Synechococcus* sp. IU 625, *Microcystis aeruginosa*, and *Cylindrospermum* spp., were measured and analyzed through flow cytometry. The results revealed that *M. aeruginosa* is detectable at an accurate range from an OD_{750nm} of 0.001 and 4.196×10^3 cells/mL up to an OD_{750nm} of 0.591 and 2.0×10^6 cells/mL, based on the phycoerythrin (PE) and allophycocyanin (APC) parameters (Figure 2). *Synechococcus* sp. IU 625 can be accurately detected at an OD_{750nm} range of 0.008 to 0.521 and cell count of 2.787×10^3 to 1.878×10^5 cells/mL (Figure 2). The detectable range of *Cylindrospermum* spp. spans from an OD_{750nm} of 0.001 to 0.640 and cell count of 1.479×10^3 to 7.21×10^5 cells/mL (Figure 2). *M. aeruginosa* contains higher PE and APC intensities when compared with *S. IU 625* and *Cylindrospermum* spp.

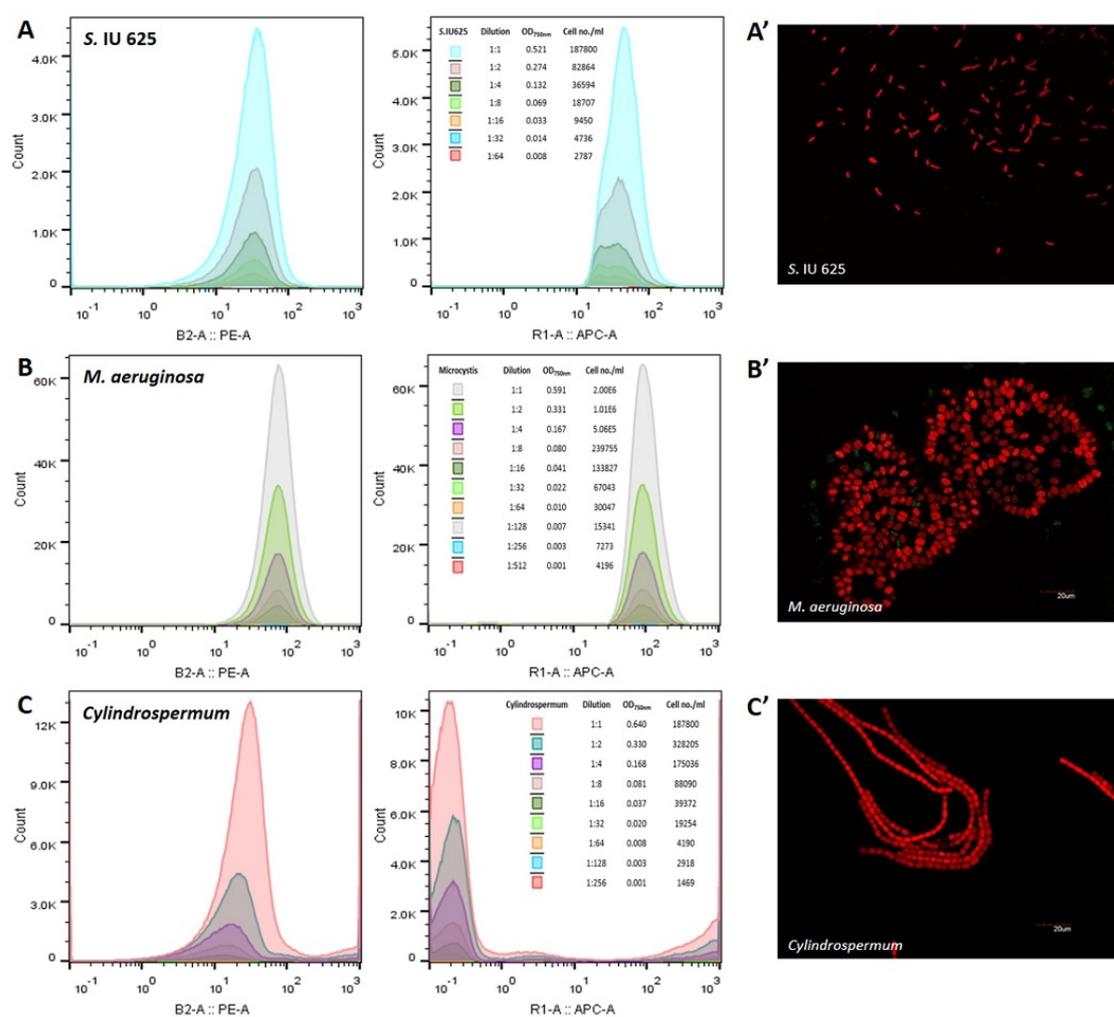


Figure 2. Histograms of phycoerythrin (PE) and allophycocyanin (APC) for three reference cyanobacteria: (A) *Synechococcus* sp. IU 625; (B) *Microcystis aeruginosa*; and (C) *Cylindrospermum* spp. The corresponding fluorescence microscopy images are shown in (A'), (B') and (C') respectively.

3.3. Flow Cytometric Analysis of 36 Water Bodies in New Jersey

Samples from a total of 36 water bodies across NJ were collected and analyzed using flow cytometric assays. Three pure cyanobacterial strains, *Cylindrospermum* spp., *Microcystis aeruginosa* and *Synechococcus* sp. IU 625, were included in this analysis. Using a color-gating algorithm with the forward-scatter (FSC) versus phycoerythrin (PE) parameters, the reference cyanobacteria were shown in three populations: *Cylindrospermum* spp. in red, *Microcystis aeruginosa* in orange, and *Synechococcus* sp. IU 625 in blue color (Figure 3).

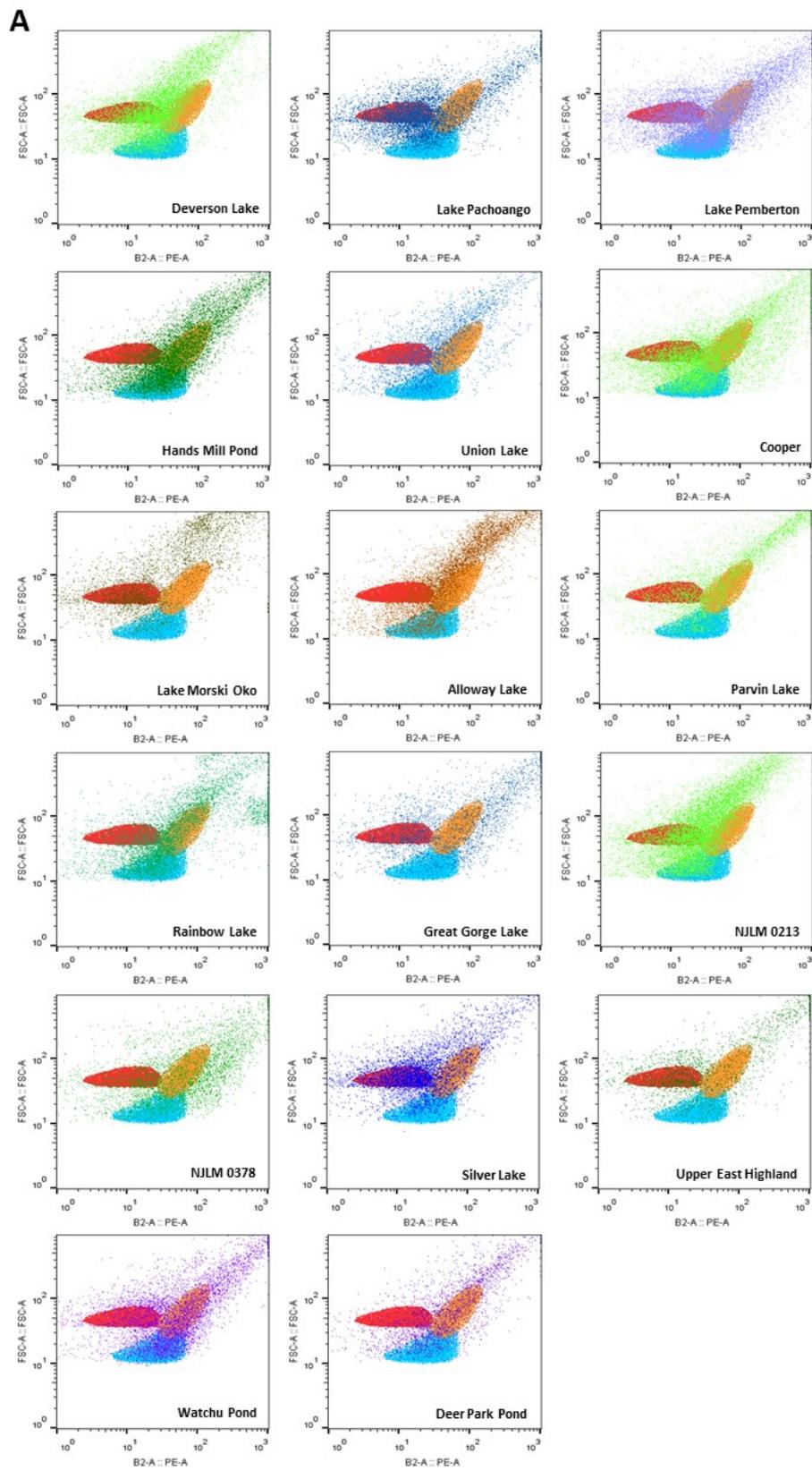


Figure 3. Cont.

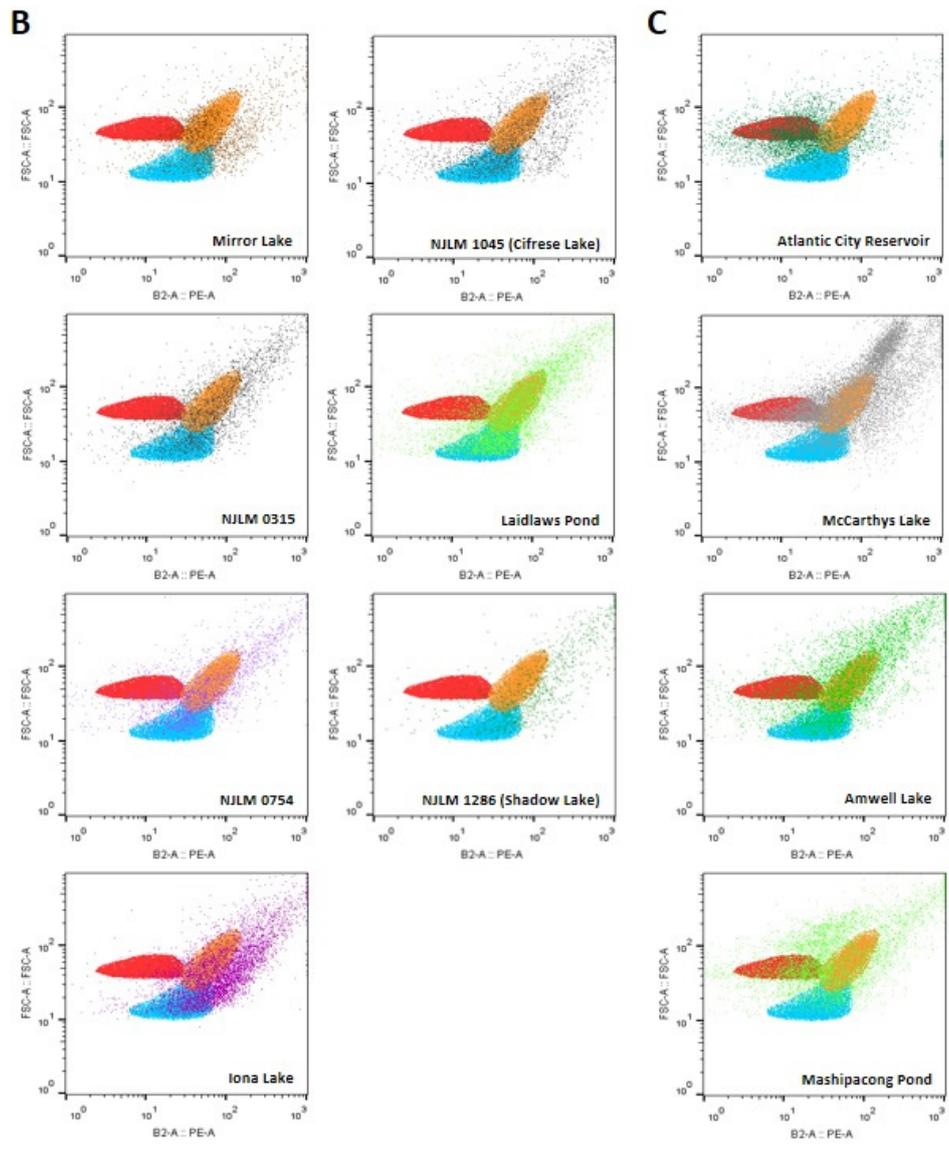


Figure 3. Cont.

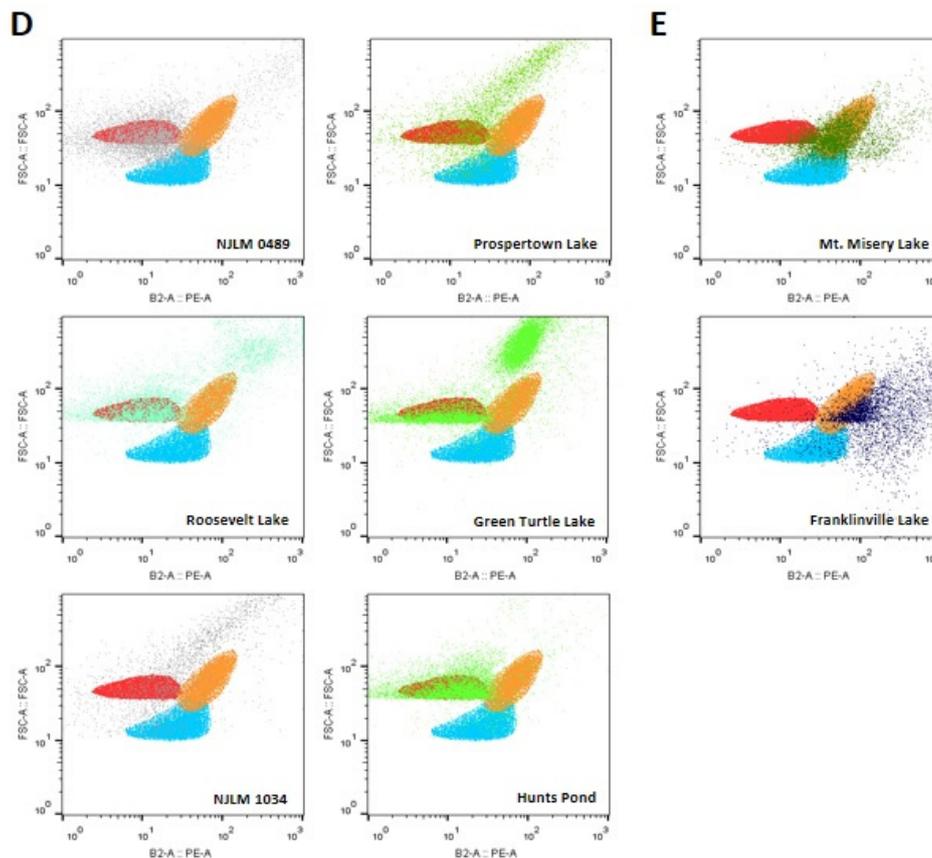


Figure 3. (A) Sites that contain cyanobacteria including all reference species: *Microcystis* spp. (orange), *Synechococcus* sp. IU 625 (blue) and *Cylindrospermum* spp. (red) related species; (B) Seven sites contained cyanobacteria matched to two reference species: *Microcystis* spp. (orange) and *Synechococcus* spp. (blue)-like species; (C) Four sites contained cyanobacteria including *Microcystis aeruginosa* (orange) and *Cylindrospermum* spp. (red)-like species; (D) Six sites contained cyanobacteria including *Cylindrospermum* spp. (red)-like species. (E) Two sites contained cyanobacteria mostly matching to *Microcystis* spp. (orange)-like species.

3.3.1. *Microcystis* spp., *Synechococcus* sp. IU 625 and *Cylindrospermum* spp. Related Species

Flow cytometric analyses showed that 17 water bodies, including Deverson Lake, Lake Pachoango, Lake Pemberton, Cooper Lake, Hands Mill, Lake Morski Oko, Alloway Lake, Parvin Lake, Rainbow Lake, Union Lake, Great Gorge Lake, NJLM 0213, NJLM 0378, Silver Lake, Upper East Highland, Watchu Pond, and Deer Park Pond, contained cyanobacteria including *Microcystis* spp. (orange), *Synechococcus* sp. IU 625 (blue), *Cylindrospermum* spp. (red), and their related species based on the FSC vs. PE profiles (Figure 3A).

3.3.2. *Microcystis* spp. and *Synechococcus* sp. IU 625 Related Species

For seven out of the 36 water bodies, including NJLM 1286 (Shadow Lake), Mirror Lake, NJLM 0315, Iona Lake, NJLM 1045 (Cifrese Lake), Laidlaw Pond, and NJLM 0754, it was revealed that the phytoplanktons in these sites contained cyanobacteria of mostly *Microcystis* spp. (orange) and *Synechococcus* sp. IU 625 (blue) related species, based on the FSC vs. PE profiles (Figure 3B).

3.3.3. *Microcystis* spp., and *Cylindrospermum* spp. Related Species

For four out of the 36 water bodies, including Atlantic City Reservoir, McCarthys Lake, Amwell Lake, and Mashipacong Pond, it was shown that the phytoplankton in these sites contained mostly

Microcystis spp. (orange) and *Cylindrospermum* spp. (red) related species, based on the FSC vs. PE profiles (Figure 3C).

3.3.4. *Cylindrospermum* spp. Related Species

Six out of the 36 water bodies, including NJLM 0489, Roosevelt Lake, and Hunts Pond, revealed primarily *Cylindrospermum* spp. (red) related species (Figure 3D). The results also showed that the cyanobacteria in NJLM 1034, Prospertown Lake, and Green Turtle Lake water bodies contained mainly *Cylindrospermum* spp. (red) related species and an undetermined population, based on the FSC vs. PE profiles (Figure 3D).

3.3.5. *Microcystis* spp. and Related Species

The results showed that Mt. Misery and Franklinville Lake contained cyanobacteria of mostly *Microcystis* spp. (orange) related species, based on the FSC vs. PE profiles (Figure 3E). Franklinville Lake water samples showed another phycoerythrin-rich population.

3.4. Molecular Analysis of Cyanobacteria

Cyanobacteria were detected in all 36 sites (data not shown) [42]. The PCR products were sequenced, and the multiple sequence alignment of the 16S rRNA from the three reference cyanobacterial strains, *Cylindrospermum* spp., *Microcystis aeruginosa* and *Synechococcus* spp., is shown in Figure 4.

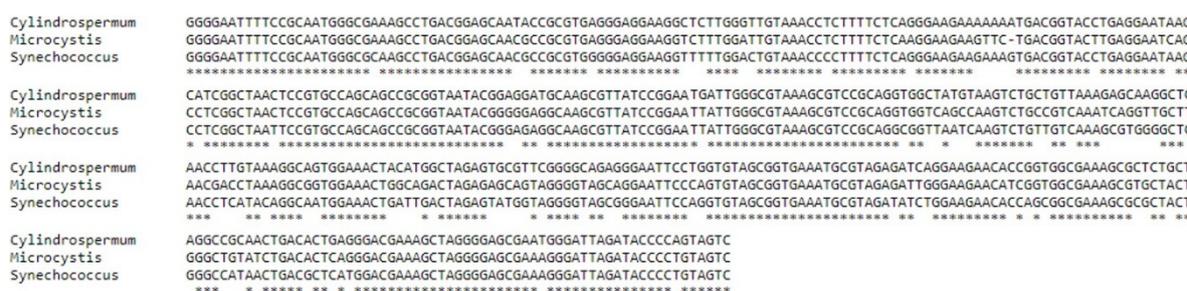


Figure 4. Multiple sequence alignment results of 16S rRNA genes from *Cylindrospermum* spp., *Microcystis* spp., and *Synechococcus* spp., by MUSCLE 3.8 [41].

4. Discussion

In this study, water samples from 36 water bodies throughout 14 New Jersey counties were analyzed via flow cytometry. The water chemistry, including water temperature (°C), pH, and dissolved oxygen, was recorded by NJDEP, as seen in Table 1. Among all sites, NJLM 0213 contained the lowest water temperature and dissolved oxygen, with 16.97 °C and 1.66 mg/L, respectively. The highest water temperature was 30.9 °C from Alloway Lake. The highest pH and dissolved oxygen was found in Cooper Lake, with 9.75 and 19.1 mg/L, respectively. The lowest pH was 4.09 from Mt. Misery Lake. The optimal pH range for cyanobacteria is between 6 and 9 [4]. Most of the sites analyzed fall within that range, allowing cyanobacteria to grow and possibly accumulate (Table 1). Dissolved oxygen is a water property that is crucial where there are concentrations of less than 4 mg/L, which can be considered hypoxic to the water ecosystem [43,44]. Four sites from Sussex County, NJLM 0213, NJLM 0378, Silver Lake and Watchu Pond, contained very little dissolved oxygen, with 1.66, 2.65, 3.32 and 3.69 mg/L, respectively (Table 1), and showed the presence of all three reference *Microcystis* spp., *Synechococcus* sp. IU 625, *Cylindrospermum* spp. and related species as expected (Figure 3A). All three cyanobacterial reference species were found in wide ranges of temperature (16.97 °C to 30.9 °C), pH (4.44 to 9.75) and dissolved oxygen (1.66 to 19.17 mg/L). *Microcystis* spp. was detected in the environment with pH as low as 4.09.

The water profile of each of the 36 New Jersey water sites was obtained via flow cytometric analysis in about 4 to 5 min, per sample. Thus, this study further confirmed that flow cytometry

can be used to rapidly process, screen and accurately detect a large volume of environmental water samples. The profile of different cyanobacteria species can be directly detected in real time without staining or lysis, due to cyanobacterial autofluorescence. Thus, flow cytometry allows early preventive measurements to be implemented and allows for a more cost- and time-efficient routine sampling method. As seen in Figure 3, cyanobacteria were successfully detected in all 36 collected water sites when analyzed by flow cytometry. Most of the cyanobacteria or phytoplankton species were mainly phycoerythrin-rich. It is important to note that reference cyanobacterial cultures were maintained in optimal conditions, which may cause them to exhibit different pigment levels when compared with the same species in lakes. Furthermore, PCR-based assays were conducted as a complementary method to confirm the flow cytometric analysis results [42,45]. The primers utilized were to amplify 16S rRNA genes for each reference cyanobacterium [39]. A microcystin synthetase primer was also included for cyanotoxin detection. The results from PCR-based assays indicated the presence of three reference cyanobacteria in all sites, but no microcystin was detected in any of the testing water samples.

Finally, this study highlighted the use of flow cytometry for the rapid detection of cyanobacteria species in 36 New Jersey water bodies. Flow cytometry is an efficient, rapid routine water sample testing method [46], so a larger volume sampling of freshwater bodies can be achieved. A flow cytometer with sorting capability could further isolate various phytoplankton taxa from mixed environmental samples in water bodies [47]. In addition, portable flow cytometers are available for the samples to be processed on site for more accurate analyses. In summary, flow cytometry could be used as an accompanying method for the rapid detection of cyanobacteria.

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Conflicts of Interest: The authors declare no conflict of interest.

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