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Article

Coexistence of *Synechococcus* and *Microcystis* Blooms in a Tropical Urban Reservoir and Their Links with Microbiomes

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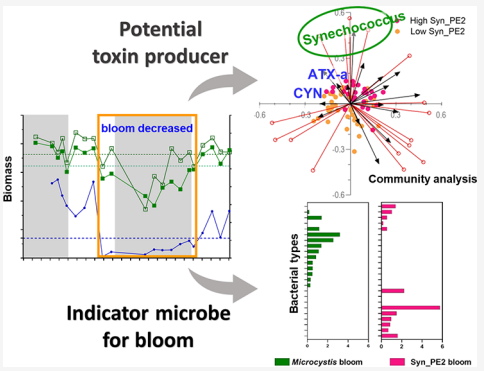
ABSTRACT: Bacteria play a crucial role in driving ecological processes in aquatic ecosystems. Studies have shown that bacteria−cyanobacteria interactions contributed significantly to phytoplankton dynamics. However, information on the contribution of bacterial communities to blooms remains scarce. Here, we tracked changes in the bacterial community during the development of a cyanobacterial bloom in an equatorial estuarine reservoir. Two forms of blooms were observed simultaneously corresponding to the lotic and lentic characteristics of the sampling sites where significant spatial variabilities in physicochemical water quality, cyanobacterial biomass, secondary metabolites, and cyanobacterial/bacterial compositions were detected. *Microcystis* dominated the upstream sites during peak periods and were succeeded by *Synechococcus* when the bloom subsided. For the main body of the reservoir, a mixed bloom featuring coccoid and filamentous cyanobacteria (*Microcystis*, *Synechococcus*, *Planktothricoides*, *Nodosilinea*, *Raphidiopsis*, and *Prochlorothrix*) was observed. Concentrations of the picocyanobacteria *Synechococcus* remained high throughout the study, and their positive correlations with cylindrospermopsin and anatoxin-a suggested that they could produce cyanotoxins, which pose more damaging impacts than previously supposed. Succession of different cyanobacteria (*Synechococcus* and *Microcystis*) following changes in nutrient composition and ionic strength was demonstrated. The microbiomes associated with blooms were unique to the dominant cyanobacteria. Generic and specialized bloom biomarkers for the *Microcystis* and downstream mixed blooms were also identified. Microscillaceae, Chthoniobacteraceae, and *Roseomonas* were the major heterotrophic bacteria associated with *Microcystis* bloom, whereas Phycisphaeraceae and Methylacidiphilaceae were the most prominent groups for the *Synechococcus* bloom. Collectively, bacterial community can be greatly deviated by the geological condition, monsoon season, cyanobacterial density, and dominant cyanobacteria.



KEYWORDS: *cyanobacterial blooms, microbiome, Microcystis, Synechococcus, cylindrospermopsin*

1. INTRODUCTION

Freshwater algal blooms, often led by nuisance cyanobacteria (Cyanophyceae), are known to alter the stability of aquatic ecosystems and degrade water quality for drinking, recreational, fishery, and agricultural purposes.[1] Harmful algal blooms attributed to anthropogenic pollution and global warming have increased worldwide at higher frequency, greater intensity, and longer duration.[2] For decades, a great deal of effort has been made to understand and predict the start and end, duration, and severity of harmful blooms, but the focus has been given primarily to abiotic variables that determine phytoplankton growth, i.e., nutrients, pH, temperature, light, and water stratification. Interactions between autotrophic cyanobacteria and heterotrophic bacteria were largely unaccounted for in most of the earlier studies because it was impossible to characterize the entire bacteria community in water using conventional approaches such as morphology identification.

High-throughput sequencing has transformed the way microorganisms are studied[3] including cyanobacterial bloom microbiota. Like cyanobacteria, heterotrophs are also affected by the same environmental factors.[4] However, indications about the resilience of heterotrophs to environmental changes and their consequences on phytoplankton are less explored. Recently, studies on the bacterial community thriving along with the cyanobacterial bloom have been gaining particular interest, especially with respect to how they contribute to key ecosystem processes. The suite of coexisting bacteria could have either positive or negative impacts on the bloom through population succession, nutrient cycling, toxin degradation, or

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cyanolytic activity.[5−8] Laboratory and field studies revealed similar bacteria groups attached or adjacent to cyanobacterial aggregates,[5−7,9,10] creating a microenvironment named the phycosphere. Within this interactive space, cyanobacteria may release compounds required by heterotrophs such as oxygen, nitrogen compounds, and dissolved organic carbon by photosynthesis and cell lysis,[5,10,11] whereas the return services from heterotrophs include removing reactive oxygen species and providing readily available nutrients and CO_2 by material mineralization.[9,12]

Our recent surveillance of six reservoirs in Singapore identified *Microcystis* and *Synechococcus* as the most prevalent coccoid cyanobacteria in Singapore, whereas other noncoccoid taxa made up 16−56% of the community. *Microcystis*, having a cell size of 2−7 μm and colony size 3−40 mm in diameter,[13] is widely recognized as the most successful genus in freshwaters[14] and also the causative species for many toxic blooms.[15] This plankton usually forms buoyant colonies in the wild, facilitating cells to position vertically for light and nutrient access and sheltering them from external stresses.[16] *Synechococcus* is generally the most abundant picophytoplankton (<2 μm) in freshwater systems.[17] Interspecies competition between *Microcystis* and *Synechococcus* has been documented as a function of nutrient gradients, and laboratory cocultures have shown marked inhibition between the two blooms.[18,19] The future prominence of toxic cyanobacterial bloom has been predicted by many climate change models.[20,21] Picocyanobacteria such as *Synechococcus* can easily adapt to diverse environments of different light, temperature, salinity, and nutrient levels.[22−24] However, what is known about this taxon has largely been derived from marine or coastal strains, whereas freshwater picocyanobacteria are considered friendly species as they seldom form toxic blooms.[25] Our recent work in several local reservoirs has registered high densities of *Synechococcus* and identified their ability to produce cylindrospermopsin and anatoxin-a,[26] which was previously not known for the taxa.

In this study, we tracked the development of blooms in an estuarine reservoir located in the equatorial region and dominated by *Microcystis* and *Synechococcus*. Bloom episodes were monitored through changes in physicochemical water quality, secondary metabolites (cyanotoxins and taste and odor compounds), cyanobacterial biomass and composition, and bacterial community assemblage. The following research questions are addressed: (1) What are the factors driving bloom diversity and intensity? (2) Does bacterial community composition vary with different dominant cyanobacteria? (3) Can we determine major producers of cyanotoxins using community assemblage analysis?

2. METHODS

2.1. Sampling and Water Quality Analysis. The study site is a coastal reservoir in Singapore constructed in 2011 and used as a storage of urban stormwater from an unprotected catchment that includes residential, commercial, and nonpollutive industrial areas. The surface area of the reservoir is 1.11 km^2, and the elevation of the catchment is 0−53 m. It is adjacent to a former landfill that was closed in 1999. Sampling was conducted in 2019 (January 23 to November 27) to compare the nonbloom and bloom periods. The sampling stations selected were evenly distributed along the "L-shaped" reservoir to better study the entire waterbody. Three stations were located in the main body of the reservoir (Stations 1, 2, and 4) and one in the upstream area (Station 3). Water samples were collected biweekly from two depths (30 cm below the surface and 30 cm above the bottom) when higher chlorophyll-a concentrations were observed, i.e., January to May (bloom observed upstream) and July to November (bloom detected at the downstream area).

A total of 168 samples were collected from 21 sampling trips; all samples were tested for 36 water quality or bloom-related parameters. Physical parameters, such as water temperature (Temp), pH, conductivity (Cond), dissolved oxygen (DO), salinity (Sal), total dissolved solid (TDS), and turbidity (Turb), were measured with a multiparameter probe (EXO2, YSI). Macronutrients including total and dissolved organic and inorganic carbon (TC, TOC, TIC, DC, DOC, DIC), total nitrogen (TN), nitrite (NO2), nitrate (NO3), ammonium (NH4), total phosphorus (TP), orthophosphate (PO4), and sulfate (SO4) were analyzed using a TOC analyzer and high-performance liquid chromatography (HPLC). Analysis details can be found in Supporting Information SI methods B. Cyanotoxins, i.e., microcystin (MC), cylindrospermopsin (CYN), anatoxin-a (ATXa), and homoanatoxin-a (HATXa), were analyzed with LC−MS/MS,[27] whereas concentrations of the olfactory compounds 2-methylisoborneol (MIB), geosmin (GSM), β-cyclocitral (BCyclo), and β-ionone (BIonone) were detected with GC−MS/MS.[28] Amounts of antecedent rainfall 7 and 30 days (Rain7 and Rain30) prior to the sampling events were obtained from the historical daily climatic record (Singapore National Environment Agency, http://www.weather.gov.sg/climate-historical-daily/).

2.2. qPCR and Amplicon Sequencing. Cyanobacteria were monitored using three methods: quantitative real-time PCR (qPCR), flow cytometry (FCM), and next-generation sequencing. The total abundance of cyanobacteria was determined with a qPCR assay (CYAN) developed previously.[29] Because significant levels of MC and CYN (average concentrations >2.5 μg/L) were detected in the reservoir, the main producers of the toxins, *Microcystis* (MIC) and *Raphidiopsis* (RAPH), were also determined with qPCR.[29] To quantify species producing toxic and nuisance cyanobacterial metabolites (i.e., MC, CYN, and MIB), three qPCR assays were established in this study targeting genes involved in metabolite syntheses: MC synthetase gene (mcyE), CYN amidinotransferase (cyrA), and 2-MIB cyclase gene (MIBg). Picocyanobacteria were enumerated with flow cytometry (FCM) following methods established earlier.[26] Three groups of *Synechococcus* were monitored on the basis of their photosynthetic pigment compositions, namely, the phycocyanin (Syn_PC), high phycoerythrin (Syn_PE1), and low phycoerythrin (Syn_PE2) groups. The bacterial community assemblage, including cyanobacteria, was analyzed with amplicon sequencing of the 16S rRNA V4 region using primers 515F and 806R[30,31] on a MiniSeq Sequencing platform (Illumina Inc.). The raw sequencing reads were run through the Mothur pipeline[32] with details as reported before.[29] The final operational taxonomy units (OTUs) with a 97% cutoff of sequence similarity were annotated using the Silva database version 132. OTUs that contributed <0.05% in all the samples were culled to reduce sequence artifacts. Finally, the downstream beta diversity analyses including biota and/or environment matching (BEST), similarity percentages (SIMPER), permutational MANOVA (PERMANOVA), and principal coordinate analysis (PCOa) were calculated using Bray−Curtis dissimilarity in the PRIMER v7 software (Primer-e). Addi-

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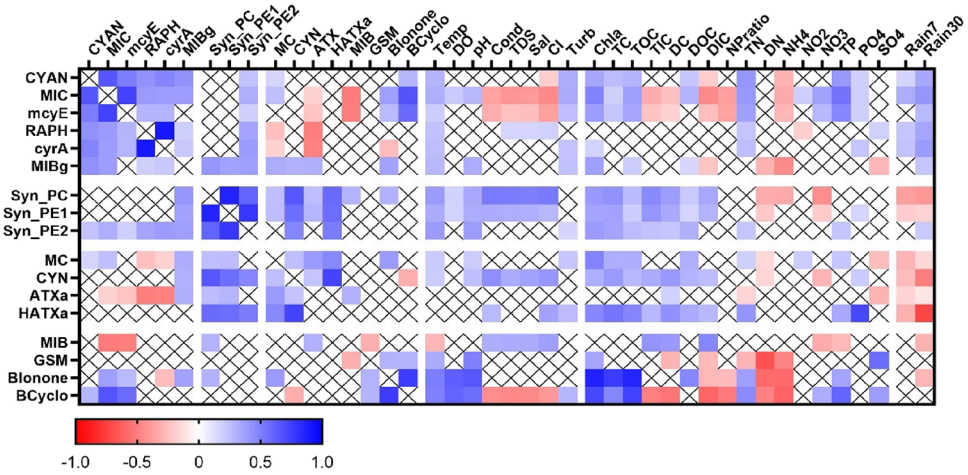

Figure 1. Heat map of Pearson's correlation coefficient matrix of cyanobacterial biomasses and secondary metabolites and environmental variables (nutrients, physical water quality, and amount of rainfall) measured in this study. Blue boxes represent positive correlations, whereas red boxes indicate negative correlations. Only significant correlations ($p < 0.05$) are shown.

tional details on the analytical methods and assays developed are provided in the Supporting Information (SI method).

2.3. Correlation and Association Network. Testing of data normality was performed with skewness and Shapiro–Wilk analysis, whereas data transformation and normalization were achieved via log and a two-step transformation to normality[33] using the SPSS Statistics 23 (IBM Corp) prior to all parametric analyses. The relationships among the environmental and biological parameters and possible spatial–temporal disparities were probed with Pearson's correlation and *t* test. To reduce data complexity caused by a large number of parameters, critical parameters contributing to data variability and sample similarity were identified through principal component analysis (PCA) using Prism 9 (GraphPad Software).

The relationship among cyanobacteria and heterotrophs was analyzed with an association network using only robust correlations with Spearman's correlation coefficient (ρ) > 0.5 and p value < 0.05. The network, consisting of 111 cyanobacterial OTUs and 1688 heterotrophic OTUs, was visualized with the open-source interactive software Gephi.[34] The topology of the resulting network was described by a set of parameters containing the average node connectivity, average path length, diameter, cumulative degree distribution, clustering coefficient, and modularity following the Fruchterman–Reingold algorithm setting and then displayed in undirected figures. Spearman's correlation was determined in the R environment (http://www.r-project.org) using vegan[35] and igraph[36] packages.

3. RESULTS

3.1. Environmental Characteristics and Water Quality. A summary of physiochemical water quality, biological characteristics, and cyanobacterial metabolites can be found in Table S1. Located in the equatorial region, the reservoir's water temperature varied only slightly (28.3 ± 0.72 °C) throughout the period of study. Despite relatively uniform temperatures, the local climatic condition is under the influence of two major monsoon winds—the Northeast Monsoon (December to March) and Southwest Monsoon (June to September)—and two intermonsoon seasons in between (IM1 and IM2). High TN (2.80 ± 1.24 mg/L), TP (0.12 ± 0.08 mg/L), and Chla (124.60 ± 89.85 μg/L) concentrations and Carlson's indices (73.1 ± 5.9) indicated that the reservoir was under eutrophic to hypereutrophic states.[37] The mass ratio of N/P (28.94 ± 16.26) indicated that phosphorus was the limiting nutrient compared to nitrogen using the Redfield ratio. The water salinity fell within the freshwater range (salinity = 0.25 ± 0.09 ppt) along with four samples reaching the marginal range (>0.5 ppt).

For cyanotoxins, the detection rates were 100% for MC and CYN (2.81 ± 5.56 and 3.31 ± 4.42 μg/L), but for ATXa (0.30 ± 0.48 μg/L) and HATXs (0.53 ± 0.47 μg/L), only 62 and 48% of the samples showed values above the quantification limits. The earthy and muddy taste and odor compounds, 2-MIB (40.92 ± 24.3 ng/L) and GSM (12.75 ± 28.5 ng/L), were oscillating around their odor threshold concentrations (OTCs).[38] Two other olfactory compounds, β-cyclocitral (1310 ± 2076 ng/L) and β-ionone (1358 ± 1215 ng/L), exhibited much higher concentrations that were about 9 and 185 times those of the OTCs. Spatiotemporal trends of measured variables are illustrated in Figures S1 and S2.

3.2. Environmental Factors Associated with Cyanobacterial Blooms. The Pearson's correlations between environmental factors and bloom-related parameters (cyanobacterial biomasses and secondary metabolites) are summarized in Figure 1. It was found that nitrogen (TN) and phosphorus (TP) were positively correlated with CYAN, MIC, RAPH, and *Synechococcus* Syn_PE2 but not with Syn_PC and Syn_PE1. Low N/P conditions favored the growth of *Microcystis* (MIC), which was the most dominant genus in the water system. Nitrate (NO3) was positively correlated with MIC, but the opposite was found for Syn_PC and Syn_PE1. The levels of dissolved salts or ionic strength in water (Sal, Cond, TDS, Cl) also impacted phytoplankton composition, where positive associations were seen with Syn_PC, Syn_PE1, and RAPH whereas negative correlations were formed with *Microcystis*.

For cyanotoxins, MC was positively correlated with its producer, *Microcystis*, Syn_PC, and Syn_PE1 as well as TN, NO2, and TP. However, CYN did not correlate with *Raphidiopsis*, commonly recognized as the CYN producer in freshwater bodies.[39] In fact, positive correlations between CYN and *Synechococcus* were found, as well as between PO_4 and

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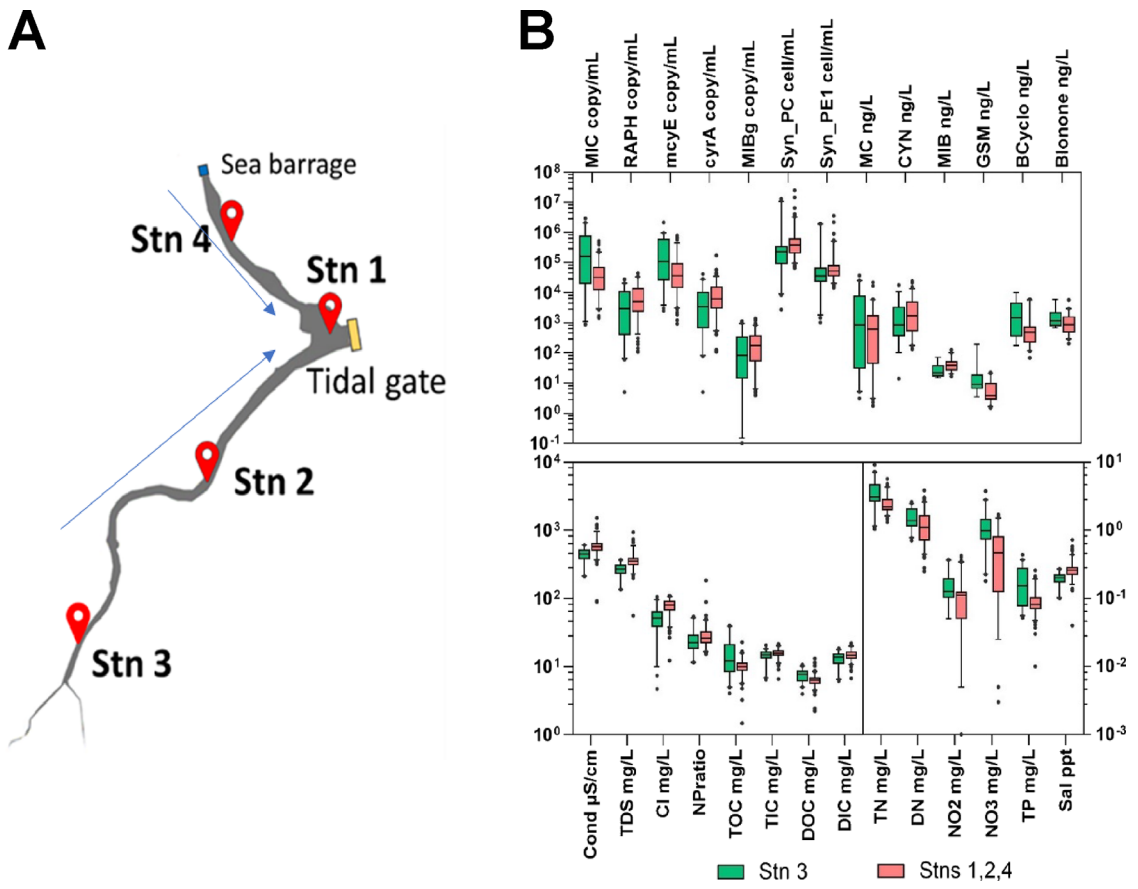

Figure 2. (A) Reservoir map and sampling locations. Water level is regulated through the tidal gate operation. Arrows indicate the direction of water flow. (B) Boxplots of variables showing spatial differences between upstream (Station 3) and downstream reservoir sites (Stations 1, 2, and 4). The upper and lower whiskers indicate the 5th and 95th percentiles, and vertical lines indicate the medians.

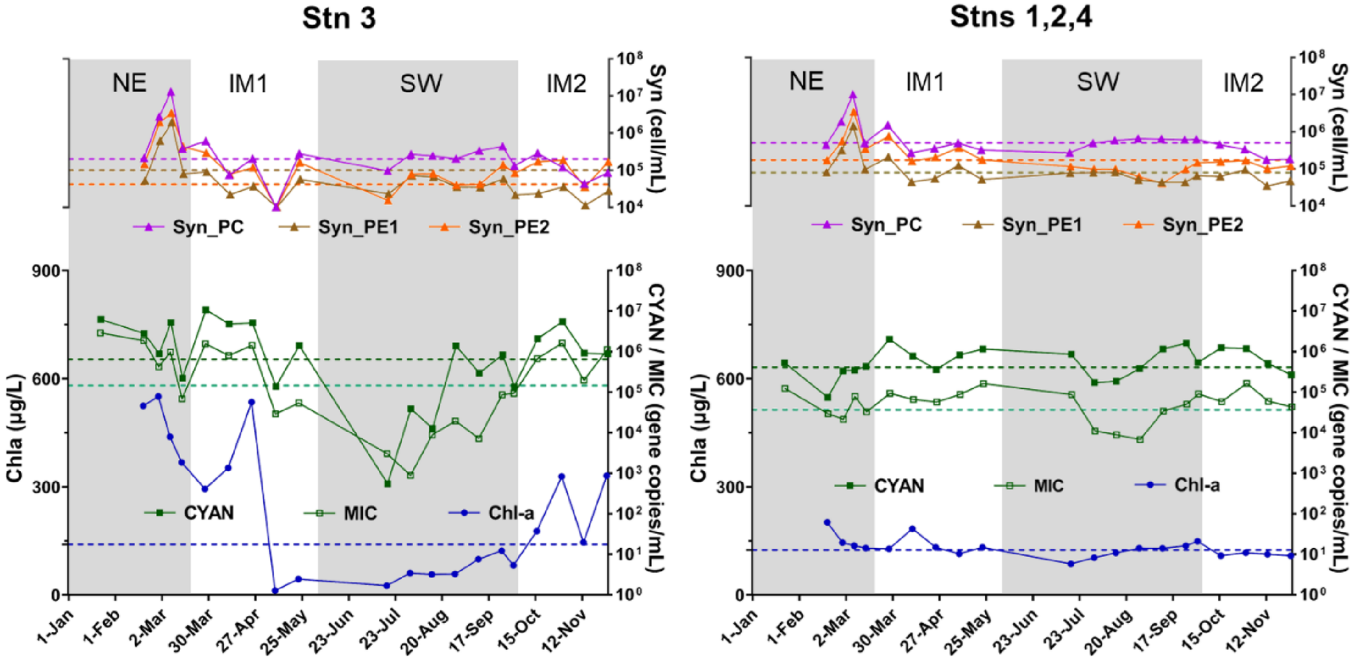

Figure 3. Changes in cyanobacterial biomass at the upstream (Stn 3) vs the downstream sites (Stns 1, 2, and 4) determined by Chla, qPCR (CYAN and MIC), and FCM (Syn_PC, Syn_PE1, and Syn_PE2). Only major taxa (*Microcystis* and *Synechococcus*) are shown. Monsoon (NE, Northeast; SW, Southwest) and intermonsoon seasons (IM1 and IM2) corresponding to the sampling times are indicated by the shaded and clear areas.

carbon (TC, TOC, TIC, etc.). Apart from CYN, concentrations of ATX-a were also positively linked to the abundances of *Synechococcus* populations. When the amount of antecedent rainfall increased, we observed a surge in cyanobacterial

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biomass (except for Syn_PC and Syn_PE1) but a drop in cyanotoxin concentration in the reservoir. High rainfall could increase nutrient inputs, promoting cyanobacterial growth while flushing out cyanotoxins, particularly extracellular toxin, from the reservoir.

The PCA analysis on all parameters showed a significant locale variability between samples collected from the upstream and reservoir sites (Figure S3). Parameters showing significant spatial disparities (t test, $P < 0.05$) are summarized in Figure 2. In general, upstream site had greater levels of organic carbon (TOC, DOC), nutrients (TN, DN, NO2 NO3, TP), *Microcystis*, and secondary metabolites (MC, BIonone, and BCyclo). Conversely, the downstream reservoir sites exhibited greater concentrations of ions (Sal, Cond, TDS, and Cl), *Synechococcus* (PC and PE1), CYN, MIB, and MIBg. We also found that the correlations among cyanobacterial biomass parameters (Chla, MIC, and Syn_PC, PE1, PE2) differed by location. At the upstream location, Chla positively correlated with all cyanobacterial parameters especially *Microcystis* and Syn_PE2 (Pearson's coefficients, MIC = 0.762, Syn_PE2 = 0.733). However, within the reservoir itself, Chla only positively correlated with *Synechococcus* populations, particularly the Syn_PC (Pearson's coefficient = 0.523).

We observed a similar spatial discrepancy when comparing the bacterial community assemblages using dendrograms (Figure S4) of cyanobacterial and noncyanobacterial communities. To eliminate the effect of spatial patterning when studying bloom development, data sets were merged according to upstream and reservoir sites to better present the two bloom types.

3.3. Changes in Cyanobacterial Composition and Abundance and Environmental Parameters during Bloom Development. As the reservoir was under a eutrophic condition during the study period, bloom period was determined by comparing concentrations of Chla and cell number to the geometric means. Cyanobacterial biomasses showed greater fluctuation at the upstream location than in the reservoir (Figure 3). For the upstream section (Stn 3), highly concentrated biomass (average CYAN = 4.6×10^6 gene/mL, Chla = 437.5 μg/L) was detected from the beginning of the study followed by a distinct clearance between May 9 and October 2, coinciding with the first intermonsoon (IM1) and Southwest Monsoon season. This nonblooming interval lasted for 3 months until a gradual increase in Chla was detected in the second intermonsoon period (IM2). The lowest and highest readings for Chla depicted a 50-fold difference, whereas qPCR-measured cyanobiomasses (CYAN, MIC, and RAPH) showed 3−5 log differences. In contrast to the trends observed upstream, drastic variation was absent in the main body of the reservoir (Stns 1, 2, and 4); i.e., there was only a maximum 5-fold difference for Chla and 1−3 log differences for qPCR readings. *Synechococcus* population had more stable dynamics oscillating between 10^4 and 10^6 cell/mL, except for a peak detected in early March, compared to *Microcystis* that varied between 10^3 and 10^7 cell/mL. The variability of other environmental parameters during bloom and nonbloom periods was also illustrated in PCA plots (Figure S5).

Amplicon analysis of the partial 16S rRNA gene identified 15 genera of cyanobacteria in the reservoir (Figure 4). The top eight genera contributed >90% of the total cyanobacterial reads. They were coccoid taxa *Microcystis* (35.1%) and *Synechococcus* (12.9%), and filamentous taxa *Nodosilinea* (10.7%), Leptolyngbyaceae_ge (8.8%), *Planktothricoides* (7.7%), *Prochlorothrix* (6.5%), *Raphidiopsis* (5.8%), and *Planktothrix* (3.9%). PERMANOVA and ANOSIM tests confirmed the locational dissimilarity between upstream and downstream sites ($p < 0.005$). The SIMPER test discovered that 54% of the dissimilarity (Bray−Curtis) was attributed to five genera: *Microcystis*, *Synechococcus*, *Nodosilinea*, Leptolyngbyaceae_ge, and *Planktothricoides*. Coccoid cyanobacteria were significantly higher in the upstream site compared to their filamentous counterpart, which contributed to a greater portion in the main reservoir's autotrophic community (Table S2).

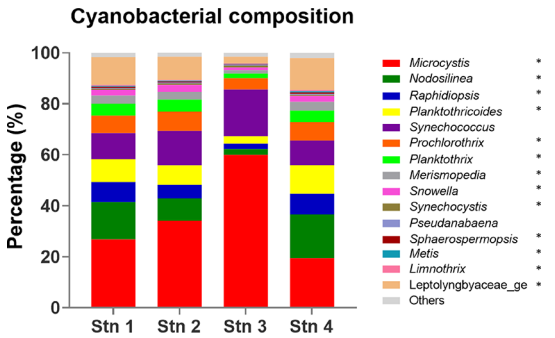

Figure 4. Proportion of various cyanobacterial genera at sampling stations determined based on 16S rRNA gene V4 sequences. Cyanobacterial community compositions showed longitudinal differences. Asterisks denote genera with significant spatial differences (Kruskal−Wallis test, $p < 0.05$).

To investigate which cyanobiomass parameters (Chla, MIC, and Syn) could better reflect changes in the cyanobacterial community as the bloom progressed, samples were sorted into high-concentration and low-concentration groups by comparing the geometric means of the parameters. The discrepancies between high- and low-concentration groups were then determined using one-way ANOSIM and PERMANOVA. Two different bloom patterns were observed in the reservoir. For the upstream, *Microcystis* largely dominated the bloom phase (70.6% of total cyanobacterial reads) but was succeeded by *Synechococcus* during the nonbloom phase (56.8%). The PCOa plot of the cyanobacterial community composition showed a clear-cut division when samples were grouped according to levels of *Microcystis* (Figure 5A). In the reservoir, a mixed community featuring *Microcystis*, *Synechococcus*, and filamentous cyanobacteria *Planktothricoides*, *Nodosilinea*, *Raphidiopsis*, and *Prochlorothrix* was observed. For this mixed community, the only parameter that formed distinct clustering was Syn_PE2 (Figure 5B). In addition to cyanobiomass, we also observed the impact of monsoon seasons on phytoplankton configuration (Figure S6). Within the reservoir, *Synechococcus* was the dominant player during the Northeast Monsoon followed by *Microcystis* and *Snowella* during the IM1 period, *Nodosilinea* during the Southwest Monsoon, and *Prochlorothrix*, *Raphidiopsis*, and *Planktothricoides* for the IM2 period. At the upstream area, the blooming time concurred with the Northeast Monsoon season but disappeared at the onset of the Southwest Monsoon season.

Associations between individual cyanobacterial OTU with cyanobacterial metabolites were also presented in the PCOa plots (Figure 5) using the multiple correlation strategy (Primer-E). Cyanotoxin MC was linked to the occurrence of *Microcystis* OTU (Mic1) that presented in large quantities at the upstream site. Interestingly, we found that high levels of

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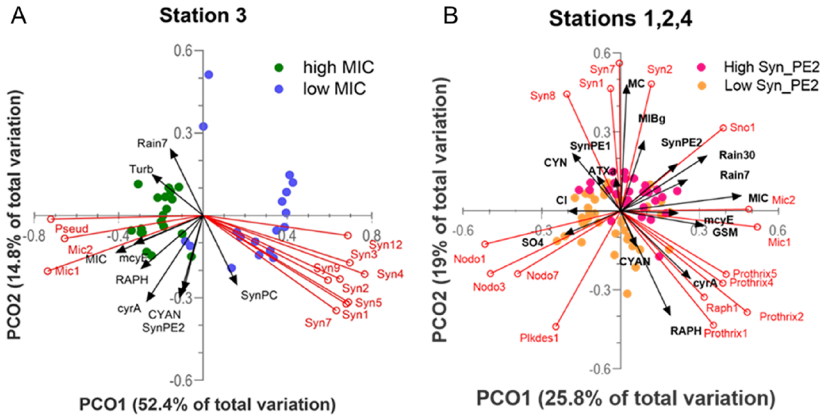

Figure 5. Principal coordinate analysis (PCOa) showing the alterations of cyanobacterial community assemblages at the (A) upstream section and (B) reservoir by different cyanobacterial biomass and concentrations. Each node represents a sample calculated using Bray−Curtis similarity. Environmental variables (black arrows) and cyanobacterial OTUs (red circles) significantly correlated with sample clustering (coefficient of multiple correlation >0.25) are shown. Abbreviations for cyanobacterial OTUs are: Mic, *Microcystis*; Nodo, *Nodosilinea*; Plkdes, *Planktothricoides*; Prothrix, *Prochlorothrix*; Raph, *Raphidiopsis*; Pseud, *Pseudanabaena*; and Syn, *Synechococcus*.

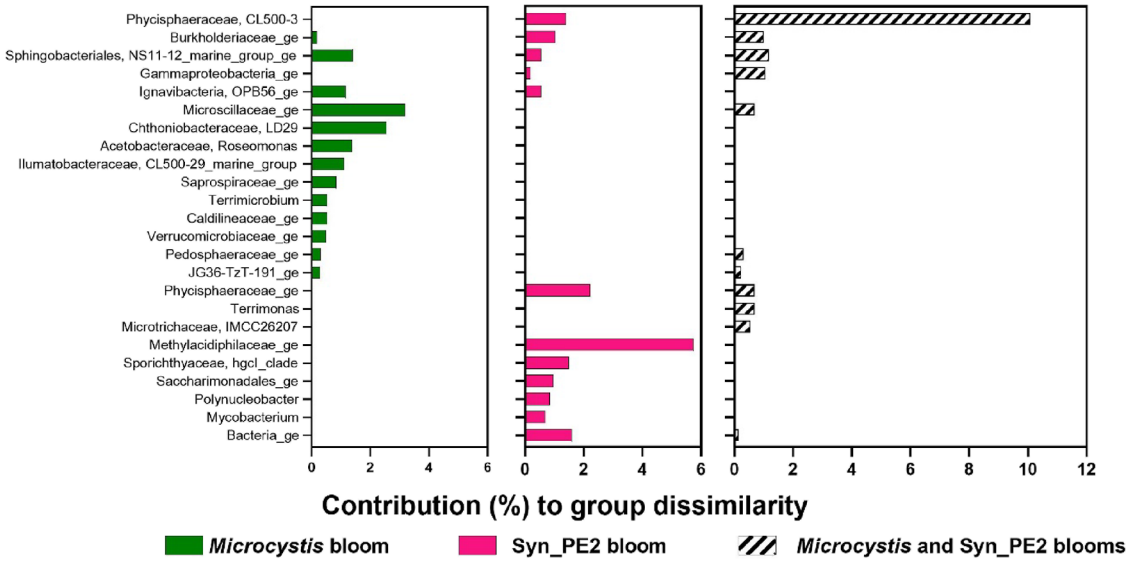

Figure 6. Heterotrophic bacterial OTUs associated with high abundances of *Microcystis* and *Synechococcus* (PE2) bloom concatenated to the level of genus. Percent contribution of each OTU was determined using the SIMPER test with a cutoff at 70%. Nonshaded bars consist of OTUs only significant to one type of bloom, whereas shaded bars include OTUs significant to both bloom types.

the toxins CYN and ATX-a corresponded to the occurrence of *Synechococcus* OTUs (Syn1, Syn7, and Syn8). This is in line with our earlier study showing that *Synechococcus* isolates can produce CYN and ATX-a.[26] Comparing the representative sequences of these OTUs against toxic *Synechococcus* spp. revealed the close phylogenetic relationship between the *Synechococcus* population and the toxic strains (Figure S7).

3.4. Associated Heterotrophic Bacterial Community.

Using the SIMPER test, we identified important bacterial OTUs associated with blooming and nonblooming situations. Analyses were done separately for the upstream *Microcystis* bloom and the reservoir mixed bloom. By pooling observations from the two blooms together in the Venn diagram, 140 bacterial OTUs emerged when cyanobacteria thrived, and 102 OTUs ascended when the bloom lapsed (Figure S8). There were 69 and 50 bacterial OTUs uniquely linked to *Microcystis* and Syn_PE2 blooms, respectively, whereas another 21 OTUs were related to both bloom types. The top 20 bacterial OTUs defining community dissimilarity between the pits and peaks are listed in Figure S9.

A collation of bloom associated bacterial OTUs according to genus classification is illustrated in Figure 6. Bacterial OTUs spawned together with the dominance of *Microcystis* spp., belonging to Microscillaceae (Bacteroidia), Acetobacteraceae (Alphaproteobacteria), Ilumatobacteraceae (Acidimicrobiia), Chthoniobacteraceae (Verrucomicrobiae), OPB56_fa (Ignavibacteria), and Burkholderiaceae (Gammaproteobacteria). In the main body of the reservoir, we saw increases in OTUs from Methylacidiphilaceae (Verrucomicrobiae), Phycisphaeraceae (Phycisphaerae), Sporichthyaceae (Actinobacteria), and Burkholderiaceae (Gammaproteobacteria) coupled with an increase in Syn_PE2. The major heterotrophic bacteria contributing only to changes in the *Microcystis* bloom microbiome were Microscillaceae_ge, LD29 (Chthoniobacteraceae); *Roseomonas*, OPB56 (Ignavibacteria); and CL500-29 marine group (Illumatobacteraceae), whereas for the mixed bloom with high Syn_PE2, the specialist groups Phyci-

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sphaeraceae_ge; Methylacidiphilaceae_ge, hgcl_clade (Sporichthyaceae); Saccharimonadales_ge; and Polynucleobacter were the major players. Four genera contributed similarly to both types of blooms, namely, CL500-3 (Phycisphaeraceae); Burkholderiaceae_ge, NS11-12_marine_group_ge (Sphngobacteriales); and Gammaproteobacteria_ge. Additionally, we also found that a portion of OTUs were stimulated by one type of bloom but shrunken by the other (Figure S8). For example, Microscillaceae, Ilumatobacteraceae CL500-29_marine_group, Chthoniobacteraceae LD29, and Ignavibacteria OPB56 were linked to high *Microcystis* and low Syn_PE2 samples, whereas the opposite was found for Methylacidiphilaceae, Sporichthyaceae hgcI_clade, and *Polynucleobacter*, for which samples showed high Syn_PE2 or low *Microcystis* abundance.

The interaction between cyanobacteria and co-occurring heterotrophic bacteria was also explored with a network analysis illustrated in Figure 7. The network comprised five

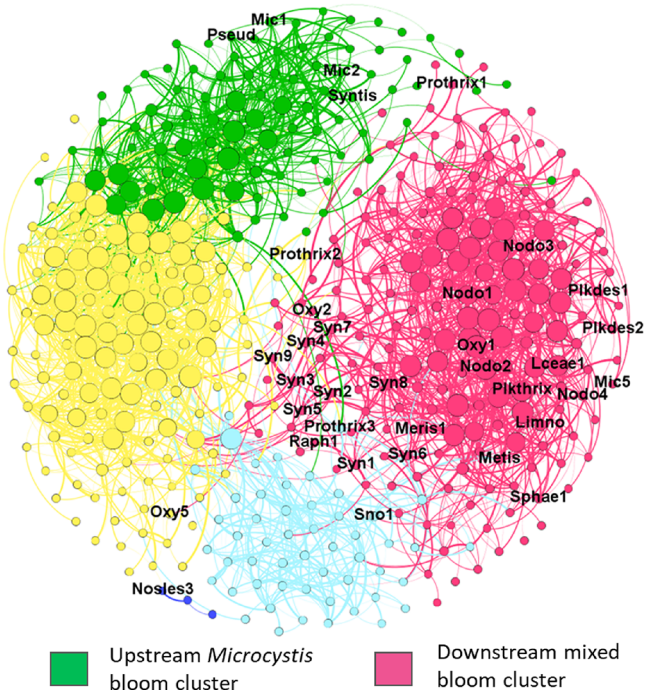

Figure 7. Association network reveals the modular connections between heterotrophic bacteria with different cyanobacteria at the studied site. Each node represents a cyanobacterial/bacterial OTU contributing to at least 5% of the community in any of the samples. The edges represented significant correlations ($p < 0.05$) between nodes with Spearman's $\rho > 0.5$. Cyanobacterial OTUs are labeled in bold text.

clusters characterized by different cyanobacteria. To condense the result for easier interpretation, important bacterial genera of each cluster were determined by summation of eigencentrality of OTUs based on classification at the level of genus (Table S3). The largest of these clusters (red) consisted of cyanobacterial OTUs from *Synechococcus* and filamentous species *Nodosilinea*, *Planktothricoides*, *Planktothrix*, *Limnothrix*, and *Prochlorothrix*. The coupled bacteria groups were CL500-29_marine_group, Caldilineaceae_ge, Methylacidiphilaceae_ge, Pirellulaceae_ge CL500-3, MWH-UniP1_aquatic_group, and Burkholderiaceae_ge. The second cluster (green) concerning bloom-forming cyanobacteria *Microcystis*, *Pseudanabaena*, and *Synechocystis* was found to be associated with NS11-12_marine_group_ge, Alphaproteobacteria_ge, Flavobacterium, Chitinophagaceae_ge, *Rubritepida*, Sphingobacteriales_ge, *Sediminibacterium*, Rubinisphaeraceae_ge, Caulobacteraceae_ge, and Microscillaceae_ge.

4. DISCUSSION

4.1. Influence of Lotic vs Lentic Characteristics. The construction of coastal reservoirs by damming up river mouths or estuaries and separating freshwater and saline water is one of the more popular management strategies for many countries including China, South Korea, and Singapore. These constructed water systems are used for flood control, power generation, or shipping passages and, above all, to enhance freshwater resources for particularly water-stressed cities.[40] These reservoirs typically show combined characteristics of rivers and lakes with obvious water quality gradients from upstream toward the reservoir outlets,[41] which are also evident in the current study. At the upstream section, our studied site resembled a lotic or flowing system, featuring a long narrow shape, shallow (<2 m) and well-mixed water, whereas the downstream lentic section was deeper, stratified with a steadier water flow. The aquatic ecosystem was altered longitudinally, distinguishable by varying physical, chemical, and biological properties. Changing of nutrient limitation is also typical when water traverses from freshwater down an estuarine gradient.[42] Consistently higher N and P contents and N/P ratio were observed at Station 3 suggested that upstream inflow contributed substantial nutrient loadings into the reservoir. These physicochemical factors strengthened the advantage of *Microcystis* in competition with other phytoplankton,[15] making them the prevalent species in that environment.

Moving downstream, *Microcystis* dominance was slowly replaced by a diverse community characterized by nearly equal proportions of coccoid and filamentous taxa based on the ratio of 16S rRNA reads. Although species successions mediated by monsoonal and *Synechococcus* variations were seen, the composition of this mixed Cyanophyceae community was largely maintained throughout the study. Unlike the upstream section, the main body of the reservoir had more steady flow and layers of water that differed in temperature, nutrients, light intensity, and DO. This created ecological niches accommodating various groups of bacteria and cyanobacteria. The higher proportion of filamentous cyanobacteria observed in downstream water may be due to the lower nitrogen availability favoring the growth of nitrogen fixers. This is in line with our observation that all the major filamentous cyanobacteria (*Nodosilinea*, *Planktothricoides*, and *Raphidiopsis*) are capable of fixing nitrogen.[43−45] In terms of coccoid taxa, the increased downstream *Synechococcus* populations might be a dual effect of the lower nitrogen condition. *Synechococcus* has better nitrogen assimilation due to a higher surface-to-volume ratio,[18] whereas *Microcystis* lost colony and buoyancy advantage when nitrogen was limiting.[46]

Besides the different N/P ratio requirement,[47] salt tolerance could be another factor affecting the relative abundances of *Microcystis* and *Synechococcus*. The opposite responses they showed with ionic strength (Sal, Cond, TDS, Cl) suggested that the latter has better tolerance toward high inorganic ion concentrations. *Microcystis* spp. are predominantly freshwater strains with only a few rare strains able to survive in brackish water.[14] *Synechococcus*, however, can be found in both marine and freshwaters.[24] The freshwater species can accumulate cellular compounds such as glucose and glycerol, enabling

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Synechococcus to grow in high-salt waters.[48−50] It is worth noting that nutrients and salt concentration are parameters that can be heavily affected by climatic drivers such as global warming that shift precipitation patterns, runoff pollution, and surface water evaporation rates.[51,52] These advantages can increase *Synechococcus* success in freshwater environments and change the ecosystem.

4.2. *Synechococcus* Can Form Toxic Blooms. The higher MC level found at the upstream site was consistent with the occurrence of *Microcystis* as its main producer. However, unlike MC, CYN correlated neither to the abundance of *Raphidiopsis*, which is widely recognized as the prime producer, nor to the abundance of the CYN biosynthesis gene (cyrA). In place of this missing correlation, significant connections were found, however, between *Synechococcus* and CYN/ATXa, as revealed by the Pearson's correlation analysis on biotic parameters and OTU PCOa analysis. Although the high correlation between *Raphidiopsis* and *cyr*A found in this study could be evidence that *Raphidiopsis* was the main CYN producer as described by other studies,[53,54] it might not be true in the current context. Because CYN-producing *Synechococcus* spp. isolated from this reservoir lack complete cyr genes, it is likely that qPCR targeting the *cyr*A gene cannot detect the presence of toxin producers with the novel toxin pathway. Therefore, using correlations between cyanobacterial taxa and toxin concentration is more suitable as a means to identify potential producers.

In recent years, increased research on freshwater *Synechococcus* has provided valuable information about their distribution. These tiny plankton cells are considered less relevant in bloom management because of their much smaller biovolume compared to microcyanobacteria.[55] Although studies have described marine *Synechococcus* that can inhibit the growth of other phytoplankton (i.e., diatom and filamentous cyanobacteria),[24] a similar investigation on freshwater environment, however, suggested a rather neutral relationship between *Synechococcus* and other cyanobacteria.[55] They are also regarded as harmless species with only a few reports stating their MC producing ability.[56] Nevertheless, we obtained several *Synechococcus* isolates from this reservoir[23] and also a drinking water reservoir in Tehran capable of producing CYN and ATX-a.[57] Taking both the laboratory and field data together, we speculate that *Synechococcus* spp. contributed to the CYN biosynthesis substantially in this reservoir. In addition to the toxicity concern, the persistently bloom-like concentration (average > 1×10^6 cell per mL) detected in our study suggests that *Synechococcus* blooms can be future prevalent phenomena under the climate change scenario,[24] and the blooms will likely be sustained over periods long enough to alter the aquatic biomes. We also observed succession between *Synechococcus* and *Microcystis* following changes in nutrient composition and ionic strength at both upstream and downstream areas. These findings would be imperative for incorporation into bloom models and may provide solutions to revert harmful cyanobacterial blooms to less toxic or concentrated states.

4.3. Indicator Microbes for Lentic and Lotic Blooms. To the credit of the vast reduction in sequencing cost in the last decade, many studies on cyanobacterial bloom have produced valuable findings on the heterotrophic bacteria associated with blooms. Our observation that phyla Proteobacteria, Planctomycetes, Bacteroidetes, Verrucomicrobia, and Actinobacteria dominated the heterotrophic microbiome during bloom times echoes the common findings from similar studies.[6,58,59] The network analysis and distance-based dissimilarity analysis based on bacterial community structure produced highly congruent results. Earlier reports on cyanobacterial bloom related bacterial communities often focused on a single type of bloom or successions of different genera at staggered periods of a year. In our study, the presences of lentic and lotic types of blooms prevailed simultaneously and were accompanied by distinguishable bacterial consortia of their own. Comparing these two bloom-microbiomes allowed identification of generalist species that fitted two blooms and specialist species that only flourished with *Microcystis* or *Synechococcus*.

Of the specialist species to *Microcystis*, Microscillacaea showed the greatest enhancement during the bloom phase. Reports on Microscillacaea are limited, and their ecological role is unclear. Yet, all the available studies have related this microbe group to *Microcystis* aggregates,[60−63] implying that the heterotrophs possess unique functional potential linked to the proliferation of *Microcystis*. A study on methane oxidation activity within *Microcystis* blooms has shown enriched Microscillacaea in lakes when methane-oxidizing bacteria (MOB) increased.[61] In fact, 10% of the bacterial community in this study was made up of MOB from families of Methylacidiphilaceae, Methylococcaceae, Methylophilaceae, and Beijerinckiaceae (result not shown), pointing to active methanogenesis in the waterbody. Further investigation is needed to test the involvement of Microscillacaea in the natural methane cycle. *Roseomonas* is another genus commonly found with *Microcystis* blooms[10] or within the phycosphere microbiome surrounding *Microcystis* cells.[62,64,65] Many *Roseomonas* spp. are considered clinically important to humans,[66] but their involvement in driving bloom bacterial communities remains unknown. The genus LD 29 from Chthoniobacteraceae was also linked to high concentrations of *Microcystis* in the current work, but it was clustered with *Synechococcus* and filamentous cyanobacteria under the network analysis. Interestingly, studies have shown that this member of Verrucomicrobia could be enriched by *Synechococcus* and *Anabaena* blooms;[5,7,67,68] therefore, we speculate that it is a generalist in different blooms.

Phycisphaeraceae and Methylacidiphilaceae were the most prominent bacteria corresponding to the higher abundance of Syn_PE2, implying that they are potential biomarkers for *Synechococcus* blooms. The Phycisphaeraceae is a family belonging to Planctomycetes, one of the phyla commonly detected during blooms of *Microcystis*, *Anabaena*, *Raphidiopsis*, and *Aphanizomenon*.[5,58,69,70] Studies specifically reporting Phycisphaeraceae have inferred their connections with *Microcystis*[9,71] and *Synechococcus*,[58,72] in agreement with the cyanobacterial assemblage observed in the current study.

The discovery of Methylacidiphilaceae, which was also the largest bacterial family in the system, is atypical for cyanobacterial bloom research. It might be caused by an under-reporting of microbes tied to freshwater *Synechococcus* blooms or the autochthonous characteristics unique to the studied site. Methylacidiphilaceae is more commonly studied as MOB within the phylum of Verrucomicrobia. Representatives from this family are known as the only aerobic methanotrophs not affiliated to Proteobacteria.[61] Inland lakes can emit significant amounts of methane from sediment layers, equivalent to 20% of fossil fuel emissions.[73] Our study site, which is next to a historic landfill,[74] could have nutrient-rich

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sediments releasing methane sources to MOB. The archaeal community in the anoxic sediment layer is believed to be the exclusive source of methane production from lakes.[75] However, field measurements and laboratory culture experiments have indicated otherwise; cyanobacteria including *Synechococcus*, *Prochlorococcus*, *Microcystis*, and *Dolichospermum* are capable of producing methane under oxic conditions.[76] In fact, in our study, 88% of the archaeal methanogens (Methanobacteriales, Methanomicrobiales, Methanosarcinales, and Methanomassiliicoccales) did not correlate to the Methylacidiphilaceae. This points to a possible interaction between *Synechococcus* and Methylacidiphilaceae, as methanogen and methanotroph, demonstrated in the *Microcystis* phycosphere.[61] Nevertheless, the potential involvement between Methylacidiphilaceae and cyanobacteria in the oxic methane cycle is still a subject of further investigation.

Our investigation revealed that bloom-associated microbes deviated closely with the cyanobacterial biomass, dominant species, and monsoon seasons. As the heterotrophic community structure was closely tied with the cyanobacterial assemblage, we speculate that the phototrophs and chemotrophs coexisting in a bloom establish mutualistic bonds to help maintain their fitness in the ecosystem. The findings expand the knowledge of genetic diversity of microbiomes associated with *Microcystis* and *Synechococcus* in equatorial freshwater reservoirs.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.2c04943.

qPCR assay designs and reaction conditions (SI methods); nutrient methods and materials (SI methods B); summary of physiochemical, biological, and cyanobacterial metabolite parameters (Table S1); result of similarity percentages test (SIMPER) (Table S2); top 15 heterotrophic bacterial genera in the networks (Table S3); spatiotemporal variations of cyanobacterial biomasses and secondary metabolites (Figure S1); spatiotemporal variations of water quality parameters (Figure S2); biplot of the principal component analysis (PCA) on environmental and bloom parameters according to sites (Figure S3); dendrogram of cyanobacterial community assemblages (Figure S4); biplot of the principal component analysis (PCA) on environmental and bloom parameters according to stages of blooms (Figure S5); principal coordinate analysis (PCOa) showing the alterations of cyanobacterial community assemblages at the upstream (Figure S6); 16S rRNA gene phylogenetic tree on *Synechococcus* OTUs (Figure S7); Venn diagram showing the number of bacterial OTUs associated with varied bloom types and intensity (Figure S8); and top 20 heterotrophic bacterial OTUs shaping the community differences between the bloom and nonbloom samples (Figure S9) (PDF)

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The manuscript was prepared through contributions of all authors. T.S.H. performed the microbial analyses and wrote the manuscript. G.K.Y.H., H.Y., and K.J.W.K. revised the manuscript. L.R. performed qPCR and network analyses. Y.L. and G.K.C. conducted the metabolite analysis. S.Z.Y. performed flow cytometry analysis. N.H.S. conducted nutrient measurement. Z.D. was involved in data analysis. All authors were involved in site sampling and logistics. All authors have given approval to the final version of the manuscript.

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Notes

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