Co-occurring microorganisms regulate the succession of cyanobacterial harmful algal blooms

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Abstract

Cyanobacterial harmful algal blooms (CyanoHABs) have been found to transmit from N₂ fixer-dominated to non-N₂ fixer-dominated in many freshwater environments when the supply of N decreases. To elucidate the mechanisms underlying such “counter-intuitive” CyanoHAB species succession, metatranscriptomes (biotic data) and water quality-related variables (abiotic data) were analyzed weekly during a bloom season in Harsha Lake, a multipurpose lake that serves as a drinking water source and recreational ground. Our results showed that CyanoHABs in Harsha Lake started with N₂-fixing Anabaena in June (ANA stage) when N was high, and transitioned to non-N₂-fixing Microcystis- and Planktothrix-dominated in July (MIC-PLA stage) when N became limited (low TN/TP). Meanwhile, the concentrations of cyanotoxins, i.e., microcystins were significantly higher in the MIC-PLA stage. Water quality results revealed that N species (i.e., TN, TN/TP) and water temperature were significantly correlated with cyanobacterial biomass. Expression levels of several C- and N-processing-related cyanobacterial genes were highly predictive of the biomass of their species. More importantly, the biomasses of Microcystis and Planktothrix were also significantly associated with expressions of microbial genes (mostly from heterotrophic bacteria) related to processing organic substrates (alkaline phosphatase, peptidase, carbohydrate-active enzymes) and cyanophage genes. Collectively, our results suggest that besides environmental conditions and inherent traits of specific cyanobacterial species, the development and succession of CyanoHABs are regulated by co-occurring microorganisms. Specifically, the co-
occurring microorganisms can alleviate the nutrient limitation of cyanobacteria by remineralizing organic compounds.

Keywords
CyanoHABs; Bloom succession; Microbiome; Metatranscriptomics; Nitrogen limitation

1. Introduction
Cyanobacterial harmful algal blooms (CyanoHABs) are occurring with increasing frequencies and impacted areas globally as a result of eutrophication and global warming (Huisman et al., 2018). Recognized as important environmental hazards, CyanoHABs deteriorate the water quality of various aquatic environments by increasing water turbidity (Scheffer et al., 1993), depleting dissolved oxygen (Rabalais et al., 2010), and producing a variety of toxic secondary metabolites (Carmichael, 2001). Many cyanobacterial genera can cause CyanoHABs in freshwater ecosystems; these include N₂-fixing (diazotrophic) taxa, such as Anabaena, Cylindrospermopsis, and Nodularia (Schindler et al., 2008), and non-N₂-fixing taxa, such as Microcystis, Planktothrix, and Oscillatoria (Paerl and Otten, 2013).

Nitrogen availability is a known regulator for the CyanoHAB community structure (Lu et al., 2019). It is generally assumed that N limited condition favors the growth of N₂-fixing cyanobacteria, whereas, when N supply is ample, non-N₂-fixing cyanobacteria would outcompete slow-growing N₂-fixing taxa (Paerl and Otten, 2016). However, many blooms have been reported to start with N₂-fixers when N was replete and then transited into non-N₂-fixing genera under low N concentrations (Beversdorf et al., 2013; Lu et al., 2019). This “opposite scenario” has been attributed to direct/indirect transfers of N from N₂-fixers to non--N₂-fixing cyanobacteria (Beversdorf et al., 2013; Lu et al., 2019).

Many recent studies have suggested the important role of microorganisms in regulating CyanoHABs (Brauer et al., 2015; Woodhouse et al., 2016; Wang et al., 2020). Nutrient recycling by the co-occurring microorganisms (i.e., the microbiome of CyanoHABs) has been shown to supply a large proportion of nutrients to cyanobacteria when the allochtonous supply is low (Christie-Oleza et al., 2017). Moreover, microbiome communities have also been found to provide key limiting micronutrients (i.e., iron and vitamins) to cyanobacteria (Croft et al., 2005; Amin et al., 2015; Xie et al., 2016).

CyanoHABs also host a diversity of cyanophages; these “cyanobacteria grazers” are often strain-specific (Gerphagnon et al., 2015) and can shift cyanobacterial community structures by selectively removing certain cyanobacterial taxa. On the other hand, cyanobacteria have evolved defensive mechanisms against cyanophage infection, such as antivirus genes (Makarova et al., 2011; Rohrlack et al., 2013) and CRISPR-Cas systems (Kuno et al., 2014).

The succession of cyanobacterial taxa may be also due to species-specific responses of cyanobacteria to seasonal changes of temperature and light (Oberhaus et al., 2007; Paerl et al., 2011). For example, warm temperatures (28–32 °C) tend to favor the growth of
Microcystis (1.6 divisions day\(^{-1}\)) over Anabaena (1.25 divisions day\(^{-1}\)) (Nalewajko and Murphy, 2001). Planktothrix can thrive over a broader temperature range and maintain a high growth rate at lower light intensity than the aforementioned cyanobacterial genera (Oberhaus et al., 2007).

Although the impacts of individual environmental and biotic factors on cyanobacterial growth are becoming clear (Huisman et al., 2018; Li et al., 2020), their interplay in driving cyanobacterial species transitions during CyanoHABs remains largely unknown. Elucidating mechanisms governing CyanoHAB development and species succession is critical to understand the ecology of CyanoHABs, which knowledge is essential to guide wise management strategies that can help to prevent and/or mitigate CyanoHABs pollution.

To address this knowledge gap, metatranscriptomes of cyanobacteria and their microbiome as well as in situ environmental variables were examined weekly over a four-month period (June to September) in Harsha Lake, a water reservoir in Ohio, USA. Harsha Lake has been set as a CyanoHAB research station, which has been experiencing CyanoHABs in the past decades. The CyanoHAB event in Harsha Lake started with Anabaena bloom in early Spring and followed by Microcystis and Planktothrix bloom (Chen et al., 2017; Lu et al., 2019).

Abiotic factors (i.e., N and P) have been suggested to play significant roles in initiating and sustaining a CyanoHAB event (Lu et al., 2019). Although the co-existing bacterial taxa have been identified (Zhu et al., 2019), little is known about the importance of the co-occurring bacterioplankton in regulating a CyanoHAB event and the succession of cyanobacterial dominance. We hypothesized that the resource exchange between cyanobacteria and their associated microbiome may favor the growth of certain cyanobacterial taxa (measured by biomass), which in turn regulates cyanobacterial bloom development and succession.

2. Materials and methods

2.1. Sample collection and processing

Surface water samples (~0.5 m depth) were collected weekly from June to September 2015 (15 sampling dates, 28 samples) in Harsha Lake at the site of a drinking water treatment plant intake (EFLS site, 39.037°, −84.138°) using a plastic water jug, which had been pre-washed with 5% hydrochloric acid. Water temperature (Temp), pH, dissolved oxygen (DO), electrical conductivity (EC), and turbidity were measured in situ using a multi-parameter sonde (YSI, 650 MDS, OH, USA).

Water samples were immediately placed in a cooler with ice packs and transported back to the laboratory. Within approximately 5 h, for molecular detection, 100–200 mL of water sample was filtered using a Durapore polyvinylidene fluoride filter (0.45 μm, MilliPore, Foster City, CA). Cells that were collected on 0.45 μm filters were stored in 1.5-mL Lysing Matrix A tubes (MP biomedicals, Irvine, CA, USA) that contained 600 μL buffer RLT plus solution with RNase inhibitor (QIAGEN, Chatsworth, CA, USA). For microcystin detection, 15 mL of water sample was filtered using a Whatman™ membrane filter (0.8 μm pore size, Marlborough, MA). Both the filtrate and the filter were collected for each sample in two replicates. The filters were frozen immediately at −80 °C until processed. Filtrates were collected in sterile conical centrifuge tubes and frozen at −20 °C for nutrient and
extracellular microcystin analyses. The community structure of cyanobacteria was identified to genus level under a 400 × magnification using a Nikon microscope (Nikon Corp., Japan) following taxonomic instructions (Thorp and Covich, 2009).

2.2. Nutrient and microcystin analysis

Nutrients were measured according to standard procedures described in the Ohio Environmental Protection Agency (Ohio EPA) methods (https://www.epa.state.oh.us/ddagw/labcert) using the Lachat Autoanalyzer (Quickchem 8000, Hach Co, USA). Briefly, nitrate (NO$_3^-$) and nitrite (NO$_2^-$), and soluble reactive phosphorus (SRP) were measured based on the automated hydrazine reduction method (Ohio EPA Method 4500), and colorimetry method (USEPA method 365.1), respectively. Total organic carbon (TOC) and total nitrogen (TN) were measured using unprocessed raw water samples following Ohio EPA Method 335.2, and USEPA Method 351.2, respectively. Total phosphorus (TP) concentrations were determined using unprocessed raw water samples according to USEPA Method 365.4.

Microcystins (MCs) concentrations were measured from the stored samples using the MC-ADDA Enzyme-Linked Immunosorbent Assay (ELISA) kit (Abraxis, Warminster, PA) according to the manufacturer’s protocol (Chen et al., 2017). This ELISA kit detects MCs by quantifying the β-amino acid ADDA (all-S all-E)-3-Amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid. Water samples were following three times standard freeze-thaw cycles before MCs measurement.

2.3. RNA extraction and sequencing

RNA was extracted from frozen filters following a procedure described previously (Lu et al., 2019). Briefly, filters were thawed and homogenized with a Mini-Beadbeater-16 (BioSpec Products, Inc., Bartlesville, OK) for 30 s twice, and then centrifuged under room temperature at 10,000 g for 3 min. The supernatant was transferred to a new sterile microcentrifuge, and RNA was extracted and purified using an AllPrep DNA/RNA kit (QIAGEN, Chatsworth, CA, USA) according to the manufacturer’s protocol. The genomic DNA contamination was removed using a TURBO DNA-free kit and according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA). The RNA quality was assessed using an Agilent 2100 bioanalyzer (Agilent Tech. Inc., Santa Clara, CA). Ribosomal RNA (rRNA) removal, reverse transcription, and library preparation were performed as described previously (Lu et al., 2019) prior to 300 bp paired-end sequencing on the Illumina MiSeq system. Raw reads were deposited in the National Center for Biotechnology Information (NCBI) short read archive database under accession number SRR12458594-SRR12458621.

2.4. RNA-seq data processing

Low-quality reads (Phred score < 15, reads < 50 bp, and adaptor contaminants) were removed using Trimmomatic version 0.39 (Bolger et al., 2014). Ribosomal RNA (rRNA) reads were removed using SortMeRNA version 2.1 (Kopylova et al., 2012) and non-rRNA reads were used in downstream analyses. The taxonomic affiliation was assigned using the Kaiju software version 1.7.2 with default parameters (Menzel et al., 2016). A resampling procedure was performed before running the Kaiju software based on the sample that
has the lowest number of sequences to ensure results were comparable between samples. To examine the transcriptomic patterns of cyanobacterial populations, non-rRNA reads were first non-redundantly mapped to three reference cyanobacterial genomes, including Microcystis aeruginosa NIES 843 (acc. # NC_010296.1), Planktothrix agardhii NIVA CYA 126/8 (acc. # CM002803.1), and Anabaena sp. 90 (acc. # GCA_000312705.1) using STAR software version 2.7.3 (Dobin et al., 2013). These three cyanobacterial species were the most abundant cyanobacteria identified in Harsha Lake (Lu et al., 2019).

Sequences that did not map to any of the three cyanobacterial genomes were subsequently mapped to six complete or draft reference genomes of cyanobacterial phages, including Microcystis phages Ma-LMM01 (acc. # GCA_000870225.1), MACPNOA1 (acc. # GCA 004320245.1), MaMV-DC (acc. # GCA_001505175.1), and Ma_Me-ZS1 (acc. # GCA_003865555.2). Anabaena phage A-4L (acc. # NC_024358.1), and Planktothrix phage PaV-LD (acc. # NC_016564.1).

The remaining unmapped non-cyanobacterial reads from bacterioplankton, fungi, and microbial eukaryotes were assembled into contigs using Trinity software version 2.8.5 (Haas et al., 2013). The non-cyanobacterial reads were then mapped back to contigs for quantification using the Bowtie2 alignment tool (Langmead and Salzberg, 2012). Putative coding regions and corresponding encoded proteins of assembled contigs were identified and retrieved using TransDecoder software (http://transdecoder.github.io/). Assembled contigs and predicted protein-coding sequences were aligned to the SwissProt database using local DIAMOND BLASTX and BLASTP programs, respectively (Buchfink et al., 2015). The alignment results were further parsed by Trinotate for functional annotation and gene ontology (GO) term assignment. The taxonomic classification of contigs was determined using DIAMOND (Buchfink et al., 2015) against the NCBI nr database. Transcripts were normalized by calculating transcript per million (TPM) using the RSEM program with default settings (Li et al., 2011).

2.5. Statistical analyses

Potential differences in environmental variables between different stages of cyanobacterial blooms (biomass > 10 mg/L) were examined by one-way ANOVA using the vegan package (Oksanen et al., 2013) in R. The transcript data (TPM values) were square-root-transformed, and a Bray-Curtis similarity matrix was constructed to perform clustering. Principle component analysis (PCA) based on Bray-Curtis distance was used for visualization of gene expression patterns among samples. Differentially gene expression analysis of cyanobacterial gene transcripts between the different clusters (based on PCA results) was performed using the DESeq2 package (Love et al., 2014). The false discovery rate (FDR) values were calculated to correct corresponding P values using the Benjamini-Hochberg algorithm (Benjamini and Hochberg, 1995). Gene transcripts with FDR <0.05 and an absolute fold change ≥1.5 were considered as differentially expressed.

Weighted gene co-expression network analysis (WGCNA) was performed to detect modules (a set of co-expressed genes) that are associated with the variations of cyanobacterial biomass using the R package WGCNA (Langfelder and Horvath, 2008). WGCNA analyses were performed separately on matrices of gene transcripts of cyanobacteria (e.g., Anabaena,
Microcystis, Planktothrix), cyanophages, and other non-cyanobacterial microorganisms. Expression matrices were all Hellinger-transformed before WGCNA analysis (Guidi et al., 2006). In WGCNA networks, nodes correspond to gene transcripts, and edges are determined by Pearson pairwise correlations between gene transcripts. WGCNA analysis uses a soft thresholding (p) of the correlation matrix in order to show a scale-free topological network where key nodes are highly connected with others (Zhang et al., 2005). Briefly, pairwise Pearson correlation coefficients between gene transcripts were calculated. A signed adjacency matrix was calculated by raising the absolute value of these Pearson correlation coefficients to the power of p. Topological overlap measure (TOM) was then calculated based on the obtained adjacency matrix. Modules were identified by performing a hierarchical clustering using a distance matrix based on the TOM values. The first principle component was used to represent each module.

For each module, pairwise Pearson correlation coefficients between environmental variables (including cyanobacterial biomass) and identified principal components were calculated. The corresponding P values were corrected for multiple testing by calculating FDR values (Benjamini and Hochberg, 1995). Gene modules that showed significant correlations with variations of cyanobacterial biomass (Anabaena, Microcystis, or Planktothrix) were selected for downstream analyses.

For each selected module, the module membership is measured by calculating Pearson correlations between the relative abundance of gene transcripts and its first principal component. The module significance is measured by the Pearson correlations between the relative abundance of gene transcripts and cyanobacterial biomass. The results of module membership and module significance were presented as a scatter plot. A correlation between the module membership and module significance indicates the potential correlation of the module structure (topology) and the cyanobacterial biomass (i.e., the more a given gene transcript defines the module topology, the more it is correlated to cyanobacterial biomass).

The sparse partial least square (sPLS) analysis was further performed to assess whether the gene transcripts within the selected module could explain the biomass variation of a cyanobacterial taxon using R package mixOmics (Rohart et al., 2017). The prediction power of the model was assessed by calculating correlation coefficients between the predicted and measured values. The significance of the prediction power was assessed by permuting the data 1000 times with leave-one-out cross-validation (LOOCV). In addition, the score of value importance in projection (VIP) was calculated for each transcript within the selected module, which estimates the contribution of each transcript in the sPLS regression. Gene transcripts that had VIP scores >1 were designated as VIP genes and they were considered important for the sPLS prediction of the response variable (e.g., cyanobacterial biomass) (Guidi et al., 2016).

Gene transcripts within each selected module were assumed to have a similar expression pattern across samples and be distinct from those of the other modules (Ghazalpour et al., 2006). Gene ontology (GO) enrichment analysis was performed within individual modules based on the Fisher’s exact test using topGO package (Alexa and Rahnenführer, 2006).
2009) to identify modules that were enriched with gene products in a specific subcellular compartement or biochemical pathway on the domain of biological process (BP).

3. Results

3.1. Temporal variations of active communities and environmental variables

A total of 28 metatranscriptomic libraries were sequenced, cyanobacteria accounted for 50.8% (ranging between 13.7 and 83.8% per library) of the total sequences (Fig. 1a). Active members of cyanobacterial communities went through three distinct successional stages during our study (Fig. 1a). Specifically, *Anabaena* (10.1–29.0% of the total sequences) dominated in June (designated as the ANA stage) but was outnumbered by *Microcystis* (6.7–16.1%) and *Planktothrix* (14.6–27.8%) from July to early August (the MIC-PLA stage). Then, the relative abundance of cyanobacterial sequences significantly decreased (*Anabaena*: 0.8–9.5%; *Microcystis*: 0.3–1.2%; *Planktothrix*: 2.9–4.6%) in late August (the Post-bloom stage; Fig. 1a). The same succession pattern was observed by microscopic measurements of individual cyanobacterial biomass (Fig. 1c). The total cyanobacterial biomass did not change significantly when the ANA stage transitioned to the MIC-PLA stage (one-way ANOVA, *P* > 0.05) and then significantly decreased when entering the Post-bloom stage (one-way ANOVA, *P* < 0.05, Fig. 1d).

The structure of the active members of the cyanobacterial microbiome (i.e., the composition of eukaryotes, bacterioplankton, and cyanophage) shifted along with the cyanobacterial species succession (Fig. 1b). Eukaryotes accounted for 0.38–7.65% of the total sequences and were dominated by phytoplankton *Bacillariophyta* (0.05–3.2%) and Chlorophyta (0.3–4.5%), and the relative abundance of phytoplankton was significantly higher in the ANA and Post-bloom stages than the MIC-PLA stage (one-way ANOVA, *P* < 0.05; Fig. 1b). Zooplankton sequences only accounted for <0.1% of the total sequences. Bacterioplankton dominated the microbiome; their relative abundance exhibited a consistently increasing trend throughout the three stages (Fig. 1b). The major taxa of bacterioplankton, i.e., *Alphaproteobacteria* (4.7–21.1%), *Betaproteobacteria* (2.5–23.9%), *Gammaproteobacteria* (4.3–23.1%), and *Actinobacteria* sequences (2.0–10.6%), had a significantly higher relative abundance in the MIC-PLA and Post-bloom stages than the ANA stage (one-way ANOVA; *P* < 0.05; Fig. 1b). Viruses only accounted for 0.2–0.9% of the total metatranscriptomic sequences and their relative abundances were not significantly different among the three bloom stages (one-way ANOVA; *P* > 0.05; Fig. 1b).

Environmental variables also exhibited temporal variations among the three succession stages (Fig. S1). Out of the 13 measured variables, total nitrogen (TN, 757–1960 μg/L), total organic carbon (TOC, 7.04–9.89 mg/L), and dissolved oxygen (DO, 5.2–15.5 mg/L) were significantly higher in the ANA stage compared to the MIC-PLA or Post-bloom stages (one-way ANOVA, *P* < 0.05, Fig. S1). pH (7.9–9.6) and microcystin (MC) concentrations (0.15–5 μg/L) were significantly higher in the MIC-PLA stage (one-way ANOVA, *P* < 0.05, Fig. S1). Turbidity (3.1–19.2) was significantly higher in both ANA and MIC-PLA stages compared to the Post-bloom stage (one-way ANOVA, *P* < 0.05, Fig. S1). Temperature (Temp, 23.63–28.59 °C), electronic conductivity (EC, 208.4–250), total phosphorus (TP, 32.7–86.3 μg/L), soluble reactive phosphorus (SRP, 23.1–48.6 μg/L), nitrate (NO₃, 1.33–515 μg/L), and nitrite (NO₂, 0.11–0.7 μg/L) were also significantly higher in the ANA stage compared to the MIC-PLA or Post-bloom stages (one-way ANOVA, *P* < 0.05, Fig. S1).
μg/L), nitrite (NO$_2$, 4.63–41.7 μg/L), and TN/TP (12—48) showed no significant difference among different stages (one-way ANOVA, $P > 0.05$, Fig. S1).

3.2. Transcriptional responses to variations of Anabaena biomass

Differentially gene expression analyses were performed (pairwise comparisons among ANA, MIC-PLA, and Post-bloom stages) to identify genes that were responsive to bloom stage transitions in the categories of nutrient metabolism, gas vesicle, secondary metabolites, and extracellular polysaccharides (EPS) productions. Ten differentially expressed genes (DEGs) were identified from Anabaena sequences; all of them were overexpressed in the ANA stage compared to either of the MIC-PLA or the Post-bloom stages (Fig. 2). Four of the above Anabaena DEGs, were involved in N metabolism ($nitBK$: N fixation; $amtB$: ammonium transport; $nirA$: ferredoxin-nitrite reduction), five were related to gas vesicle synthesis ($gvpA5A6A7FW$), and one was involved in EPS production ($ANA_C13742$, glycosyl transferase related gene; Fig. 2). No MC synthesis gene of Anabaena was overexpressed in the ANA stage compared to either the MIC-PLA or the Post-bloom stages, although direct measurements of MC concentrations were high in the ANA stage (Fig. S1).

Weighted gene co-expression network analysis (WGCNA) was further performed to detect co-expressed genes (modules) that were correlated with biomass variations of specific cyanobacterial taxa. Two gene modules that were found significantly correlated with Anabaena biomass by WGCNA. One of these Anabaena biomass responsive (ABR) modules was from the Anabaena (Ana1) expression network (Ana1, 258 genes in total; Fig. 3a) and the other one was from the noncyanobacteria (NC) expression network (NC8, 59 genes in total; Fig. 3d). The structures (topology) of ABR-Ana1 and -NC8 modules were also correlated significantly with the biomass of Anabaena ($r > 0.60$, $P < 0.05$, Figs. S2a-b). WGCNA further revealed that ABR-Ana1 was also significantly correlated with both N and P variables i.e., TN ($r = 0.54$, $P = 0.04$), TN/TP ($r = 0.91$, $P = 3e-06$) (Fig. 3a), while ABR-NC8 was only significantly correlated with N variables i.e., TN ($r = 0.62$, $P = 0.02$) (Fig. 3d).

Expressed genes within the ABR-Ana1 module were mainly enriched (FDR < 0.05) in genes of GO (gene ontology) terms for photosynthesis, nucleoside monophosphate biosynthesis, translation, and protein-chromophore linkage (Fig. 4a). The expression level of genes of the enriched GO terms in this module underwent dramatic downregulation from the ANA stage to the MIC-PLA stage, and then slightly increased in the Post-bloom stage (lasso regression, Fig. 4a). ABR-NC8 module was mainly enriched in GO terms for photosynthetic electron transport chain, protein-chromophore linkage, and response to herbicide (Fig. 4c). The expression level of these enriched GO terms showed no significant change between the ANA and MIC-PLA stages and was the lowest in the Post-bloom stage (Fig. 4c).

Sparse partial least square (sPLS) regression analysis was further performed to evaluate how well the expressed genes of the identified ABR modules could explain the biomass variation of Anabaena. sPLS regression revealed that genes of the ABR-Ana1 module predicted 23.4% of the variability of Anabaena biomass (LOOCV-$R^2 = 0.234$, Fig. S2c). In addition, 49 out of the 258 ABR-Ana1 genes were identified as VIP genes (sPLS regression, VIP score > 1) (Table S1), which had higher prediction power for Anabaena biomass.

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Five of the VIP genes were affiliated with gas vesicle production, nitrogen fixation, and ammonium transport (Fig. 4b, Table S1). Most of the remaining VIP genes were affiliated with photosynthetic activities, ribosome biogenesis, and translation (Table S1), which were consistent with the results of GO enrichment analysis. The ABR-NC8 module predicted 15.1% (LOOCV-\(R^2 = 0.151\), Fig. S2d) of the variability of Anabaena biomass. Out of the 59 genes in this module, 23 were identified as VIP genes and 8 of the VIP genes were assigned to functional categories including photosynthesis, aspartate oxidase, and ferredoxin (Fig. 4d, Table S2), different from the enriched GO terms (except photosynthetic activities) (Fig. 4c). Taxonomic classification revealed that VIP genes of the ABR-NC8 module were mainly affiliated with both algae (Chlorophyta, Bacillariophyta) and bacteria (Firmicutes, Gammaproteobacteria, Betaproteobacteria, and Actinobacteria) phyla (Table S2).

3.3. Transcriptional response to variations of Microcystis biomass

The number of DEGs of Microcystis (27 genes) nearly doubled that of the Anabaena (Fig. 2) and all of them were overexpressed in the MIC-PLA stage compared to either the ANA or the Post-bloom stages (Fig. 2). Specifically, seven Microcystis DEGs were affiliated with N transformations, including genes for glutamate metabolism (glnA, gltBDX, glsF), arginine decarboxylation (MAE_46810), and amino acid transport (MAE_32490) (Figs. 2a–1 and 2a-2). Six Micorcystis DEGs were related to P metabolism, including those for alkaline phosphatase (ALP) (MAE_30190), sensor histidine kinase (MAE_02100, MAE_31720, MAE_03210), creatininase (MAE_62630), and phosphate-starvation induced ATPase (MAE_43330) (Figs. 2b–1 and 2b-2). Anabaena DEGs did not include any of the microcystin synthesis genes. However, Microcystis DEGs included three microcystin synthesis genes (mcyACE) and their expression level was higher than the other two stages (Figs. 2e-1). This high representation of mcy genes was coincident with the highest MC concentrations measured in the Harsha lake (Fig. S1). Like those of Anabaena, Microcystis DEGs also contained genes for synthesizing gas vesicles (gvpAI, gvpAII, gvpAIII, gvpCFIN, Fig. 2c) and EPS (MAE_06090, MAE_27990, MAE_37160, and MAE_12350, Fig. 2d) and they were overexpressed when Microcystis was dominant (in the MIC-PLA stage).

WGCNA analysis identified five Microcystis biomass responsive (MBR) modules (Fig. 3). Three were from the Microcystis network (Mic4, Mic6, and Mic7; 153 genes in total; Fig. 3b); one was from the microbiome NC2 (219 genes in total; Fig. 3d), and the last one was from the cyanophage network (CP9; 61 genes in total; Fig. 3e). The structures of all five MBR modules were correlated significantly with Microcystis biomass (0.43 < r < 0.85, \(P < 0.05\), Figs. S3a, c, e, g, i). MBR modules were significantly correlated with several environmental variables, including water temperature (\(r > 0.51\), \(P > 0.01\)), \(\text{NO}_2^-\) (\(r > 0.62\), \(P > 0.01\)) and \(\text{NO}_3^-\) (\(r > 0.62\), \(P > 0.01\)) (Fig. 3).

Expressed genes within the MBR-Mic modules (Fig. 5a) were enriched in different (except photosynthesis) GO terms from the ABR-Ana1 module and include those affiliated with pigment biosynthesis, translation, redox homeostasis, ribonucleotide biosynthesis, tRNA aminoacylation, and ncRNA processing. Opposite of the ABR module (Fig. 4a), the expression levels of genes of the enriched GO terms in the MBR-Mic modules consistently
showed an increasing trend from the ANA stage to the MIC-PLA stage and then decreased in the Post-bloom stage (Fig. 5a). Expressed genes within the MBR-NC2 modules (Fig. 5c) were enriched in GO terms of water-soluble vitamin biosynthesis, lipopolysaccharide metabolic process, folic acid-containing compound metabolism, and cellular response to DNA damage stimulus. This list of enriched GO terms and the expression pattern of genes of these enriched GO terms both differed from those of the ABR-NC8 module (Fig. 4c).

sPLS regression analysis found that the three MBR-Mic together predicted 57% of *Microcystis* biomass variation (LOOCV-$R^2 = 0.57$, Fig. S3b, d, f). From the 153 genes of these three modules, 52 were identified as VIP genes. Eight of the VIP genes were affiliated with the CO$_2$-concentrating mechanism (CCM), CO$_2$ fixation, gas vesicle, and Na$^+$/H$^+$ antiporter (Fig. 5b, Table S3). The MBR-NC2 module predicted 57.8% of the *Microcystis* biomass variability (LOOCV-$R^2 = 0.578$, Fig. S3h). Within the MBR-NC2 module, 46 out of 218 genes were identified as VIPs and 5 of them were affiliated with CAZymes, alkaline phosphatase protein, and chemotaxis protein (Fig. 5d, Table S4), different from the enriched GO terms in this module (Fig. 5c). VIP genes of the MBR-NC2 module mainly affiliated with heterotrophic bacteria in the taxa of Alphaproteobacteria and Actinobacteria (Table S4). The MBR-CP9 module predicted 44.3% (LOOCV-$R^2 = 0.443$, Fig. S3j) of the *Microcystis* biomass variability. Five out of 61 MBR-CP9 genes were VIPs and their expression a gradually increasing pattern from the ANA to the Post-bloom stages (Fig. 5e, Table S5). The MBR-CP9 VIPs all appeared to be *Microcystis*-specific, including MaMV-DC (accession number [acc. #] GCA_001505175.1), Ma-LMM01 (acc. # GCA_000870225.1), and MACPNOA1 (acc. # GCA_004320245.1) (Fig. 5f, Table S5).

3.4. Transcriptional response to variations of *Planktothrix* biomass

Sixteen *Planktothrix* DEGs were identified across the three bloom stages and were all overexpressed in the MIC-PLA stage compared to either the ANA or Post-bloom stages (Fig. 2). Four of the *Planktothrix* DEGs were N metabolism-related, including genes for glutamate metabolism (gltAD, glnA) and cyanophycin synthesis (cphA) (Fig. 2a). Four *Planktothrix* DEGs were related to P metabolism, including those for P-scavenging (pstAC) and sensor histidine kinase (A19Y_0178, A19Y_2590) (Fig. 2b). Three *Planktothrix* DEGs were related to the synthesis of secondary metabolites, including cyanopeptolin (ociAB) and anabaenopeptin (apnA) (Fig. 2e). As has been found for *Anabaena* and *Microcystis*, *Planktothrix* DEGs again included genes for gas vesicle (gvpANG) (Fig. 2c) and EPS (A19Y_2613, A19Y_1456) productions (Fig. 2d); and all these genes were also overexpressed when *Planktothrix* was dominant (in the MIC-PLA stage).

WGCNA analyses identified three *Planktothrix* biomass responsive (PBR) modules (Fig. 3). One was PBR-Pla12 (136 genes in total; Fig. 3c) from the *Planktothrix* network; one was PBR-NC5 (184 genes in total; Fig. 3d) from the NC network; and the third one was PBR-CP8 (47 genes in total; Fig. 3e) from the cyanophage network. The structures of all three PBR modules were correlated significantly with *Planktothrix* biomass (0.39 < r < 0.58, P < 0.05, Figs. S4a, c, e). Among the PBR modules, the Pla12 module was significantly associated with water temperature (r = 0.57, P = 0.009) and pH (r = 0.53, P = 0.013)
(Fig. 3c), whereas the NC5 (Fig. 3d) and CP8 (Fig. 3e) modules showed no significant correlations with any of the measured 14 environmental variables.

Expressed genes within the PBR-Pla module (Fig. 6a) were enriched in GO terms of ATP biosynthetic process and cellular protein-containing complex assembly, which were different from those of the MBR-Mic and the ABR-Ana1 modules. The expression level of genes of these enriched GO terms within the PBR-Pla module showed a similar expression pattern as those of the MBR-Mic modules (Fig. 5a) but different from those of the ABR-Ana1 module (Fig. 4a).Expressed genes within the PBR-NC5 module (Fig. 6c) were enriched in completely different GO terms (i.e., glycogen metabolic process, regulation of cellular respiration, and viral life cycle) compared to the ABR-NC8 (Fig. 4c) and the MBR-NC2 modules (Fig. 5c). The expression level of genes of these enriched GO terms in the PBR-NC5 module had an increasing trend from the ANA stage to the Post-bloom stage and then kept constant, different from those of the ABR-NC8 and the MBR-NC2 modules.

sPLS regression analysis found that the PBR-Pla12 module predicted 21.1% (LOOCV-$R^2$ = 0.211, Fig. S4d) of the variability in *Planktothrix* biomass. Within the PBR-Pla12 module, 62 out of 136 genes were identified as VIP genes and 6 VIP genes were assigned to CCM, cyanophycin synthesis, nitrate reductase, and gas vesicle production (Fig 6b, Table S6). The PBR-NC5 module predicted *Planktothrix* biomass with high accuracy (LOOCV-$R^2$ = 0.70, Fig. S4e). From 185 genes of the PBR-NC5 module, 45 were detected as VIP genes and 7 VIP genes were affiliated with CAZymes, a chemotaxis protein, and peptidases (Fig 6d, Table S7), which agreed with GO enrichment results (except viral life cycle GO term). VIP genes of the PBR-NC5 module were mainly assigned to heterotrophic bacteria in the taxa of *Actinobacteria* and *Gammaproteobacteria*. The PBR-CP8 module from the cyanophage network only predicted 11.1% (LOOCV-$R^2$ = 0.111, Fig. S4f) of the *Planktothrix* biomass variability. Within the PBR-CP8 module, 12 out of 47 genes were identified as VIP genes and their expression patterns were different from the MBR-CP9 module and showed gradual increases from the ANA stage to the MIC-PLA stage, and then kept constant in the Post-bloom stage (Fig. 6e, Table S8). Among the VIP genes, 2 were affiliated with a *Planktothrix*-specific phage (i.e., PaV-LD, acc. # NC_016564.1) (Fig. 6f).

4. Discussion

Overall, our results suggest that the inherent genetic traits of cyanobacteria and their close interactions with co-occurring microorganisms on essential processes synergistically govern the transition of CyanoHABs (Fig. 7).

4.1. The importance of cyanobacterial traits in bloom succession

The varying physiological and functional traits among cyanobacterial species provide a competitive edge to cyanobacteria over other phytoplankton taxa during resource partitioning and allow them to win dominance and establishing dense blooms in many aquatic environments (Huisman et al., 2018). Like algae and plants, cyanobacteria use CO$_2$ as the carbon source for growth. Dissolved CO$_2$ concentration decrease during bloom development (Verspagen et al., 2014) and thereby increases water pH, which further shifts the equilibrium of inorganic carbon towards bicarbonate and carbonate (Fig. 7) (Verspagen et al., 2018).
To outcompete other phytoplankton for CO\textsubscript{2}, cyanobacteria have evolved a CCM (Carbonic Anhydrase) mechanism that enables cells to take up bicarbonate (Fig. 7) (Price et al., 2008). In the present study, three CCM genes of Microcystis and Planktothrix were detected with high predictive power for cyanobacterial biomass (high VIP scores) (Fig. 3), suggesting a mechanism by which Microcystis and Planktothrix achieve a competitive advantage over other phytoplankton under dense bloom conditions.

Cyanobacteria can produce a variety of secondary metabolites which can have multiple functions in freshwater (i.e., toxins, antibiotics, metal chelators, and odor compounds) (Penn et al., 2014). Like many freshwater lakes (Tang et al., 2018), MC production is a major harmful impact on Harsha Lake during CyanoHABs. MCs can be produced by multiple common freshwater cyanobacterial species, including all three major genera we found in Harsha Lake, i.e., Anabaena, Microcystis, and Planktothrix. However, despite the mixed cyanobacterial community, MC synthesis genes (e.g., mcyABCD) shared by the three MC-producing cyanobacterial taxa were highly expressed only by Microcystis during the MIC-PLA stage (Fig. 6), and their expression levels followed the same trend as microcystin concentrations. This suggests that Microcystis were the main contributors to MCs in Harsha Lake, consistent with previous findings (Chen et al. 2017). Although the ecological roles of these secondary metabolites are not fully resolved, studies have found that MCs may trigger the upregulation of EPS biosynthesis and subsequently enhance Microcystis colony formation (Gan et al., 2012; Tang et al., 2018). The formation of Microcystis colonies might recruit more co-existing bacteria and further promote functional interplay between Microcystis and the co-occurring bacteria (Penn et al., 2014). In line with these studies, the present study observed a simultaneous increase of gene transcripts involved in the synthesis of MCs and EPS in the MIC-PLA stage (Fig. 7), which helps to explain the establishment of Microcystis blooms. MC production has also been suggested to increase the fitness of Microcystis against oxidative stress (Zilliges et al., 2011), which might provide Microcystis fitness advantage over other phytoplankton in the MIC-PLA stage.

Cyanobacterial taxa responded differently to temperature (Otten et al., 2015; Paerl et al., 2016). Studies have found that, at warm temperatures, Microcystis (Nalewajko and Murphy, 2001) and Planktothrix (Oberhaus et al., 2007) have higher maximum growth rates than Anabaena, thus warmer water temperatures would favor the growth of Microcystis and Planktothrix over Anabaena. This cyanobacterial trait also helps to explain the winning of Microcystis and Planktothrix in warmer sampling days (Fig. 1a).

Gas vesicles provide buoyancy to cyanobacterial cells (Pfeifer, 2012). Genes related to the synthesis of gas vesicles have been detected in all three genera of cyanobacteria in Harsha Lake, suggesting that the production of gas vesicles might play a less important role in bloom succession. However, the production of gas vesicles might play a significant role in bloom development, as gas vesicles can help cyanobacterial colonies cover the surface of the lake, which may intercept the influx of light and shade non-buoyant phytoplankton deeper in the water column (Walsby et al., 1997; Huisman et al., 2004; Penn et al., 2014). In addition, cyanobacteria could regulate gas vesicle production to enable cells floating to the surface to exploit light during the day and to allow cells moving to the deeper waters to get nutrients at night (Villareal and Carpenter, 2003).
4.2. The importance of the microbiome in cyanobacterial bloom succession

Close interactions between cyanobacteria and their associated microbiome have been suggested as mutualistic due to their various levels of complementarity (Christie-Oleza et al., 2017). The nutrient cycling between heterotrophic bacteria and cyanobacteria is vital for a functional system, as heterotrophs are usually carbon- and energy-limited, while the cyanobacteria are often limited by inorganic nutrients (Christie-Oleza et al., 2017).

Consistently, in this study, several microbiome gene modules (both non-cyanobacterial and cyanophages) had significant module-trait relationships with cyanobacterial biomass (Fig. 2d). Microbiome modules that were sensitive to cyanobacterial biomass mainly consisted of heterotrophic bacteria. This indicates that cyanobacterial communities may interact more closely with heterotrophic bacteria than with other microbiome microorganisms. Genes encoding CAZymes (i.e., glycoside hydrolase), alkaline phosphatase, and peptidase were mainly involved in the break-down of carbohydrates and complex organic compounds. Several such genes in the microbiome modules had high VIP values in predicting cyanobacterial biomass (Fig. 7), suggesting microbiome bacteria were actively utilizing organic compounds that likely (partly) were released from cyanobacterial cells.

The cycling of nutrients between cyanobacteria and their associated microbiome was also evidenced by the detection of DEGs involved in the glutamine synthetase and glutamine:2-oxoglutarate amidotransferase (GS-GOGAT) in Microcystis and Planktothrix metatranscripts at the MIC-PLA stage (Fig. 2a). This indicates that NH$_4^+$ was supplied to Microcystis and Planktothrix from the microbiome for the active synthesis of N-rich compound glutamine and glutamate (Fig. 7). In addition, a gene from Planktothrix involved in the synthesis of cyanophycin (cphA) displayed large increases in expression at the MIC-PLA stage (Fig. 2a). Cyanophycin serves as a reservoir for newly assimilated N when cyanobacteria are exposed to an excess of N in the environment (Stein, 2015); this compound is consumed by cyanobacteria when exogenous N is depleted (Fig. 7) (Harke et al., 2016). However, nutrient measurement showed that at the MIC-PLA stage, N availability (i.e., TN and TN/TP ratios) was significantly decreased from the ANA stage (Fig. 6), reaching N deficiency (Guildford et al., 2000). These observations suggest that N were actively regenerated from heterotrophic bacteria to alleviate the N limitation to cyanobacteria and sustain the growth of non-N-fixing cyanobacterial species (i.e., Microcystis and Planktothrix in the MIC-PLA stage) during low N supply (Fig. S1).

Cyanophages are important predators of cyanobacteria; they can regulate cyanobacteria community structure by removing specific cyanobacterial species (Yoshida et al., 2008; Coloma et al., 2017). Thus, cyanophage infection can lead to dynamics of CyanoHABs of different cyanobacterial genotypes (i.e., cyanophage sensitive vs cyanophage resistant) (Yoshida et al., 2008; Coloma et al., 2017). In the present study, two modules from the cyanophage network showed significant correlations with Microcystis and Planktothrix (but not Anabaena) biomass variation. In addition, our results found that Microcystis biomass decreased when gene transcripts of Microcystis-specific phage increased (Fig. 5). These findings suggest an active role of cyanophage in regulating cyanobacterial shifts. Our results also showed that the expression level of cyanophage genes was significantly lower than the...
expression of Microcystis and Planktothrix genes (Figs. 5 and 6). This indicates that the cyanophages were only able to infect a small portion of the cyanobacterial population (i.e., phage-sensitive populations) (Thingstad and Lignell, 1997; Yoshida et al., 2008).

5. Conclusions

Activities of cyanobacteria and their microbiome were complementary and closely synchronized to facilitate resource recycling. These interactions may contribute significantly to cyanobacterial bloom development and succession. Varied responses to the availability of CO2/bicarbonate and temperature provide Microcystis and Planktothrix competitive advantages over Anabaena under dense bloom conditions; this also contributes to bloom succession from Anabaena to Microcystis/Planktothrix bloom. Moreover, the activities of cyanophages also likely influence the dynamics of cyanobacterial genotypes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments and Disclaimer

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Fig. 1.
Temporal dynamics of (a) cyanobacterial genera and (b) their associated microbiome organisms during CyanoHABs in Harsha Lake (only those organisms with relative abundance > 0.1% were shown). (c) Correlation analysis of cyanobacterial abundance between measurements by metatranscriptomic sequences (relative sequence abundance, %) and microscopically determined biomass (mg/L). (d) PCA analysis of cyanobacterial metatranscriptomes (The dot size is proportional to the total cyanobacterial biomass).
Fig. 2.
Volcano plots depicting differentially expressed cyanobacterial genes in metabolisms of (a) Nitrogen, (b) Phosphorus, (c) Gas vesicle, (d) Extracellular polysaccharide, and (e) Secondary metabolites between different bloom stages (i.e., a1: ANA vs MIC-PLA; a2: MIC-PLA vs Post-bloom; a3: ANA vs Post-bloom). Red and blue dots represent significantly differentially expressed genes (log2 fold change $\geq 1.5$ and $P < 0.05$) between different stages. Text colors are used to indicate genes of different cyanobacteria: Orange, *Anabaena*; Green, *Microcystis*; Blue, *Planktothrix*. The locus tag name (e.g., ANA_C13742) was shown if the gene name is not available.
Fig. 3.
WGCNA networks of (a) Anabaena, (b) Microcystis, (c) Planktothrix, (d) non-cyanobacterial, and (e) cyanophage gene expression matrices. Each network is decomposed into smaller coherent modules (y-axis). Pearson’s correlations between the coherent modules’ eigenvectors and environmental parameters as well as cyanobacterial biomass were calculated. Significant correlations and associated P values are noted. Names of modules that had significant correlations with cyanobacterial biomass are shown in black font.
Fig. 4.
Temporal change of averaged expression levels (Transcripts Per Million) of genes of the enriched GO terms within the (a) Ana1, and (c) NC8 modules. The value importance in projection (VIP) values of genes within the (b) Ana1 and (d) NC8 modules. The size of dots in (b) and (d) is proportional to the VIP value. Genes of interest are pinpointed.
Fig. 5.
Temporal change of averaged expression levels (Transcripts Per Million) of genes of the enriched GO terms within the (a) Mic4, Mic6, Mic7 and (c) NC2 modules. (e) Temporal change of averaged expression levels of high predictive power genes (VIP > 1) within the CP9 module. The value importance in projection (VIP) values of genes within the (b) Mic4, Mic6, Mic7, (d) NC2, and (f) CP9 modules. The size of dots in (b), (d) and (f) is proportional to the VIP value. Genes of interest are pinpointed.
Fig. 6.
Temporal change of averaged expression levels (Transcripts Per Million) of genes of the enriched GO terms within the (a) Pla12 and (c) NC5 modules. (e) Temporal change of averaged expression levels of high predictive power genes (VIP > 1) within the CP8 module. The value importance in projection (VIP) values of genes within the (b) Pla12, (d) NC5, and (f) CP8 modules. The size of dots in (b), (d) and (f) is proportional to the VIP value. Genes of interest are pinpointed.
Fig. 7.
A schematic diagram showing potential interactions between cyanobacteria and their microbiome. DOM, dissolved organic matter; EPS, extracellular polysaccharides; AA, amino acids; ALP, alkaline phosphatase; CAZymes, carbohydrate-active enzymes; TCA, tricarboxylic acid cycle; GS-GOGAT, glutamine synthetase and glutamine:2-oxoglutarate amidotransferase; nifB, nitrogenase cofactor biosynthesis protein; nifK, Mo-nitrogenase MoFe protein; amtB, ammonium transporter; Gln, glutamine; Glu, glutamate; glnA, glutamine synthase; glt genes, glutamate synthases; gdhA, glutamate dehydrogenase; 2-OG, 2-oxoglutarate; Arg, arginine; Asp, aspartate; CA, carbonic anhydrase; ccmK, carbon dioxide-concentrating mechanism protein; rbcS, ribulose-bisphosphate carboxylase. Illustration of the CO2-concentrating mechanism was modified after Huisman et al. (2018). Gray color genes were not detected in the present study, red color genes were either overexpressed or VIP genes of Anabaena in the ANA stage, and blue color genes were either overexpressed or VIPs of Microcystis/Planktothrix in the MIC-PLA stage.