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Phylogenetic Inference of Colony Isolates Comprising Seasonal *Microcystis* Blooms in Lake Taihu, China

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Abstract Blooms of the toxin-producing cyanobacterium, *Microcystis* spp., are an increasingly prevalent water quality problem and health hazard worldwide. China's third largest lake, Lake Taihu, has been experiencing progressively more severe *Microcystis* blooms over the past three decades. In 2009 and 2010, individual *Microcystis* colonies, consisting of four different morphospecies, were isolated and genotyped using a whole-cell multiplex PCR assay. The 16S–23S rDNA-ITS sequences were aligned based on Bayesian inference and indicated that one morphospecies was genetically unique (*Microcystis wesenbergii*) and three were indistinguishable (*Microcystis aeruginosa*, *Microcystis flos-aquae*, and *Microcystis ichthyoblabe*). Microcystin (*mcyB*) genes were detected intermittently in two of the morphospecies while the other two morphospecies lacked the *mcyB* gene in all samples. Water temperature was found to influence bloom formation and morphotype prevalence, and chlorophyll *a* and temperature were positively and significantly correlated with microcystin concentration. Cooler water temperatures promoted toxigenic strains of *Microcystis*. Wind appeared to influence the distribution of morphotypes across the lake, with *M. aeruginosa* and *M. ichthyoblabe* being more susceptible to wind stress than *M. wesenbergii* and *M. flos-aquae*. The results of this study indicated that the blooms were composed of a variety of *Microcystis* morphospecies, with more genotypes observed than can be attributed to individual morphotypes. We conclude that morphology is not a reliable indicator of toxigenicity in Lake Taihu, and caution should be exercised when the *M. aeruginosa* morphotype is present because it is capable of producing MC-LR, the most toxic microcystin isoform.

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Introduction

On a global scale, human-induced eutrophication is a threat to the use and sustainability of aquatic ecosystems. Cyanobacterial harmful algal blooms (CHABs) are occurring at increasing frequencies worldwide due to nutrient over-enrichment and rising water temperatures [45–47]. Under these conditions, cyanobacteria can proliferate to form dense blooms which foul water bodies impact food webs and create large hypoxic zones leading to fish kills and other ecological disruptions [14, 31, 34, 54]. Some cyanobacterial genera also produce potent hepatotoxins and neurotoxins, making these CHABs of particular concern from environmental and human health perspectives.

Microcystis is a widespread, freshwater bloom-forming cyanobacterium. Many strains produce microcystins (MCs), which are cyclic heptapeptides and potent liver toxins, documented in numerous human and animal poisonings over the past two centuries [10, 38, 58]. The major mechanism of acute microcystin toxicity in eukaryotes is inhibition of protein phosphatases 1 and 2A in hepatocytes leading to cellular collapse and liver hemorrhage [7, 36]. Epidemiological studies of the long-term impact of chronic exposure to MCs suggest they play a role in primary liver

cancer and tumor formation [4, 9, 13]. The World Health Organization's provisional guideline for microcystins in finished drinking water recommends less than 1 μg l^{-1}. Thus, from water quality and environmental health perspectives it is critical to be able to determine when *Microcystis* blooms form and whether or not they are toxin producing.

Microcystis is characterized morphologically as coccoid shaped cells possessing gas vesicles, a tendency to aggregate as colonies, which are enclosed in a mucilaginous sheath [20, 29]. *Microcystis* blooms are easily recognizable because of their buoyancy (leading to green paint-like scums) and colonial morphology (Fig. 1). Natural assemblages of *Microcystis* are believed to aggregate in colonies because it enhances buoyancy, increases access to light, and mitigates zooplankton grazing [62]. Historically, *Microcystis* taxonomy was based upon microscopic observations of colony morphologies and individual cell sizes. However, as prior studies have pointed out, *Microcystis* exhibits a high degree of phenotypic plasticity and seemingly identical strains may vary morphologically under varying environmental conditions [42]. As such, the taxonomy of this genus needs to be re-evaluated, preferably using more definitive molecular techniques. In the current study, a whole-cell multiplex PCR assay was utilized to determine the genotype of different *Microcystis* assemblages collected from hypereutrophic Lake Taihu.

Lake Taihu is the third largest freshwater lake in China and is situated in its most populated province, Jiangsu. It supplies drinking water to 38 cities, with over 10 million people dependent on the lake for drinking water, fisheries, and tourism income [17]. The lake is large (2,338 km^2), shallow (mean depth 2.0 m), and well mixed year-round (polymictic), with a hydraulic residence time of ~300 days [5, 64]. For the past three decades, seasonal blooms of *Microcystis* spp. have regularly formed on the lake, usually starting around May and persisting through October. In recent years, the severity of the blooms has increased, culminating in a drinking water crisis in 2007, during which the government had to supply bottled drinking water to 2 million people [50]. Due to this highly publicized event, much attention has been directed at remediating the eutrophication problems of the lake.

Figure 1 Meiliang Bay, Lake Taihu (June 3, 2009). Photo credit: Hans Paerl

The aim of the current study was to assess whether the blooms occurring during the summers of 2009 and 2010 were composed of MC-producing strains of *Microcystis* and whether toxicity could be inferred from colony morphology, or if both toxin- and non-toxin-producing strains of *Microcystis* spp. appeared as multiple morphotypes. To date, systematic identification of the morphospecies comprising the blooms in Taihu has not been carried out on a genetic level.

Materials and Methods

Sample Collection and Processing

Sampling transects of the entire northern half of Lake Taihu were conducted six times during the period June 22, 2009 to July 27, 2009 and six times from June 3, 2010 to June 21, 2010 in which 1 L depth integrated water column samples (n=96) were collected using a 2-m long, 10-cm wide tube with a one-way valve from eight sites and from any blooms (n=16) encountered between sites (Fig. 2). The microbial assemblage of each sample was photographed using a Zeiss (NY, USA) Axiovert 135 microscope at ×40 to ×400 magnification. Also, a portion of each sample was filtered onto Whatman (NJ, USA) GF/F filters (25 mm) for chlorophyll and carotenoid photopigment analysis by HPLC [49] and onto Pall (MI, USA) Supor-200 membrane filters (47 mm, 0.2 μm) for subsequent DNA extraction. Additionally, for whole-cell PCR genotyping, individual colonies of *Microcystis* spp. from select samples were diluted in dH_2O, photographed, and then picked using micro-tip Pasteur pipettes. Extra care was taken to minimize disaggregation of colonies during transfer as they were rinsed in dH_2O and then finally stored at −20°C in sterile PCR tubes containing 20 μl of 1× TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). A total of 29 colonies were isolated for genotyping (*Microcystis aeruginosa* = 10, *Microcystis flos-aquae* = 9, *Microcystis wesenbergii* = 5, *Microcystis ichthyoblabe* = 5) from Meiliang Bay to the north and from the western basin. Environmental parameters (water temperature, dissolved oxygen, pH, and turbidity) were collected from each site with a YSI 6600 multiprobe sonde (OH, USA). The southern half of the lake was not sampled because it is dominated by macrophytes and

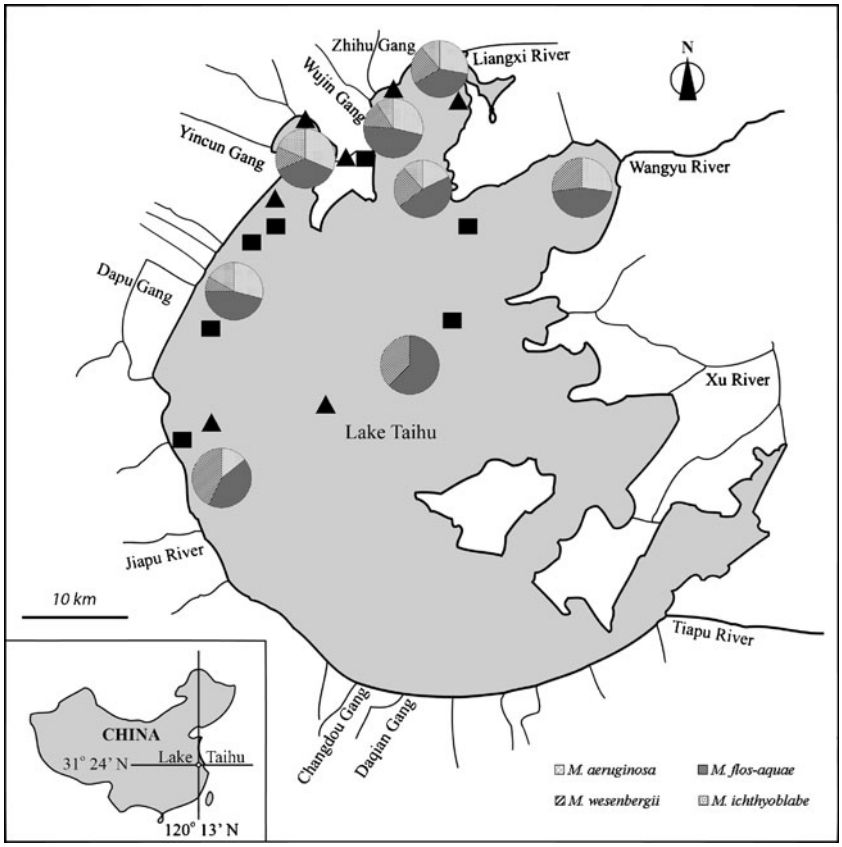

Figure 2 Map of Lake Taihu sampling sites. *Pie graphs* represent the total *Microcystis* morphospecies composition of each sample station compiled from 12 transects (96 samples) during June and July, 2009, and June, 2010. *Triangles* indicate locations of blooms in 2009, *squares* indicate locations of blooms in 2010

rarely supports *Microcystis* blooms [64]. Lastly, data on wind speed and direction were collected at 10-min intervals using a Davis meteorological station located near the southeastern shore of Meiliang Bay.

DNA Preparation and PCR Amplification

Prior to PCR amplification, sample tubes underwent three freeze–thaw cycles and then were vortexed for 1 min to break apart the colonies. A multiplex PCR was carried out consisting of the following oligonucleotides: CSIF and ULR for 16S–23S rDNA-ITS amplification for species identification, heretofore termed the ITSc primer set [26, 27], MSR-S1F and MSR-S2R for 16S rDNA amplification for additional species comparisons [40], and *mcyB*-F and *mcyB*-R to determine potential toxicity based on the presence or absence of the microcystin synthetase *mcyB* gene [8]. Each PCR reaction consisted of: 2 μl of cell solution, 10 μl 5× Colorless GoTaq Flexi Buffer (Promega, WI, USA), 5 μl $MgCl_2$ (25 mM), 2 μl of forward (10 μM) and reverse (10 μM) primers, 1 μl dNTP mix (10 mM each), 0.4 μl GoTaq DNA Polymerase (5 U μl^{-1}), and dH_2O to a volume of 50 μl. Reactions were carried out on a Techne (NJ, USA) TC-512 thermocycler with the following conditions: initial denaturation at 94°C for 2 min, followed by 20 cycles of 94°C for 1 min, a 1-min annealing step with T_A decreasing 0.5°C each cycle from 62°C to 52°C, and extension steps of 1 min at 72°C and a final elongation step of 5 min at 72°C. The amplification product sizes were approximately 410, 550, and 800 bp for MSR, ITSc, and *mcyB*, respectively.

Cloning and Sequencing

Each band was extracted from the gels and purified using a Qiaquick Gel Extraction kit (Qiagen, CA, USA). The DNA was ligated into a pCR2.1 TOPO vector (Invitrogen, CA, USA) and transformed into TOP10 *Escherichia coli* per the manufacturer's instructions. The cells were grown overnight on selective media plates at 37°C. For each positive transformant selected, 12 replicates were forward and reverse sequenced using M13F and M13R primers on an ABI 3130 DNA sequencer.

Phylogenetic Analyses

The forward and reverse sequences were imported into the software program Sequencher (v.4.8) and assembled into contigs using the dirty data algorithm with a minimum match percentage of 99% and a minimum overlap of 20 bases. Ambiguities were manually corrected by reviewing individual chromatograms. Transformants resulting in mul-

tiple contigs were excluded from further analyses as they were presumed to contain DNA from more than one individual colony. Contigs were exported and phylogeny inferred based on a Bayesian framework using the program MrBayes v.3.1.2 [16, 23, 52]. Analysis was carried out using a general time reversible substitution (GTR) model with gamma distributed variation run for 2 million generations with a sampling frequency of every 10th generation and a burn-in of 40,000 trees. Analyses were run in parallel and the resulting consensus trees were based on 207,820 tree iterations and shared identical positions, with each having a probability greater than 99%. Phylograms were viewed in TreeView (v.1.6.6). For comparison, a second phylogram of the ITSc sequences was generated using ClustalX v.2.0 [32] and the neighbor-joining method with 1,000 bootstrap replicates and rooted with a microcystin-producing strain of *Planktothrix rubescens* PCC 7821 [53]. The tree was viewed in Dendroscope (v.2.7.4) and exhibited the same clustering patterns as the Bayesian inferred phylogram (data not shown). Multiple pairwise alignments were carried out on the amino acids derived from the *mcyB* sequences using the VectorNTI AlignX program. Predicted protein structures for the *mcyB* genes were generated on the Phyre server [28] and viewed in the 3D Molecule Viewer program of VectorNTI. The binding pocket signatures of the adenylation domain sequences were identified as previously described [59].

Statistical Analyses

Kendall Tau non-parametric rank analyses were conducted to assess the correlation of total microcystins with water temperature, total chl *a*, pH, turbidity, and dissolved oxygen from all samples (16 blooms and 96 sample stations).

Nucleotide Sequence Accession Numbers

All *mcyB* and ITSc sequences determined in this study have been deposited in GenBank under accession numbers HQ625391–HQ625426.

Results

Morphological Observations

On a seasonal basis, the colony morphologies observed were variable. In 2009, three different *Microcystis* morphospecies were observed (Fig. 3). Morphotype 1 was identified as *M. aeruginosa* and consisted of medium to large colonies irregularly shaped, being generally long (100–3,000 μm) and narrow (100–940 μm), densely packed with individual cells, each being 3–4 μm in diameter, and no visible mucilaginous sheath. Morphotype 2 was identified as *M. flos-aquae* and consisted of small- to medium-sized (100–1,175 μm diameter) round to semi-round colonies, densely packed with slightly smaller individual cell diameters of 2.5–3.5 μm, and no visible mucilaginous sheath. Morphotype 3 was identified as *M. wesenbergii* and consisted of medium- to large-sized colonies irregularly to lobate shaped being generally longer (100–2,115 μm) than wide (100–1,175 μm), loosely packed with larger individual cell diameters (5–6 μm) and possessing a thick mucilaginous sheath. In 2010, a fourth morphotype was observed and identified as *M. ichthyoblabe*. It consisted of medium- to large-sized colonies with diameters 300–1,200 μm, round to semi-round in shape, composed of a single layer of cells with diameters 3–4 μm, and with no visible mucilaginous sheath. The *M. ichthyoblabe* morphotype was only observed in 2010 and never in 2009. Conversely, the *M. wesenbergii* morphotype was not observed in 2010. With regard to the spatial distribution of the *Microcystis* morphotypes across the lake, all morphotypes were distinguished at least once at all sampling sites; with the exception of the lake center, in which *M. aeruginosa* and *M. ichthyoblabe* were never observed (Fig. 2). Additionally, the *M. ichthyoblabe* morphotype was not detected in the southwest and northeast sampling sites.

Wind Data

Anemometer data collected from a monitoring station in the northern bay (Meiliang Bay) indicated that the average wind direction during the study period was from the west–northwest (123.4°) at an average wind speed of 3.56 m s^{-1} (minimum=0.0, maximum=16.2 m s^{-1}).

Statistical Analyses

The average water temperatures differed significantly from 2009 to 2010, with 2010 being considerably cooler. In 2009, the average water temperatures ranged from 28.1–30.5°C over the course of the sampling period, whereas in 2010 average water temperatures ranged from 21.6–27.1°C. Temperature influenced *Microcystis* bloom formation and morphotype prevalence. *M. wesenbergii* colonies were only observed at water temperatures greater than 27°C and *M. ichthyoblabe* colonies were only observed at temperatures less than 28°C. While the *M. wesenbergii* and *M. ichthyoblabe* morphotypes were always non-toxic (lacking *mcyB* genes), a mixture of toxic and non-toxic strains of *M. aeruginosa* and *M. flos-aquae* were observed at all temperatures (22–31°C). The *M. flos-aquae* morphospecies was the dominant taxa among all groups and at all temperatures (Fig. 5). Even though the *M. aeruginosa* and *M. flos-aquae* morphotypes

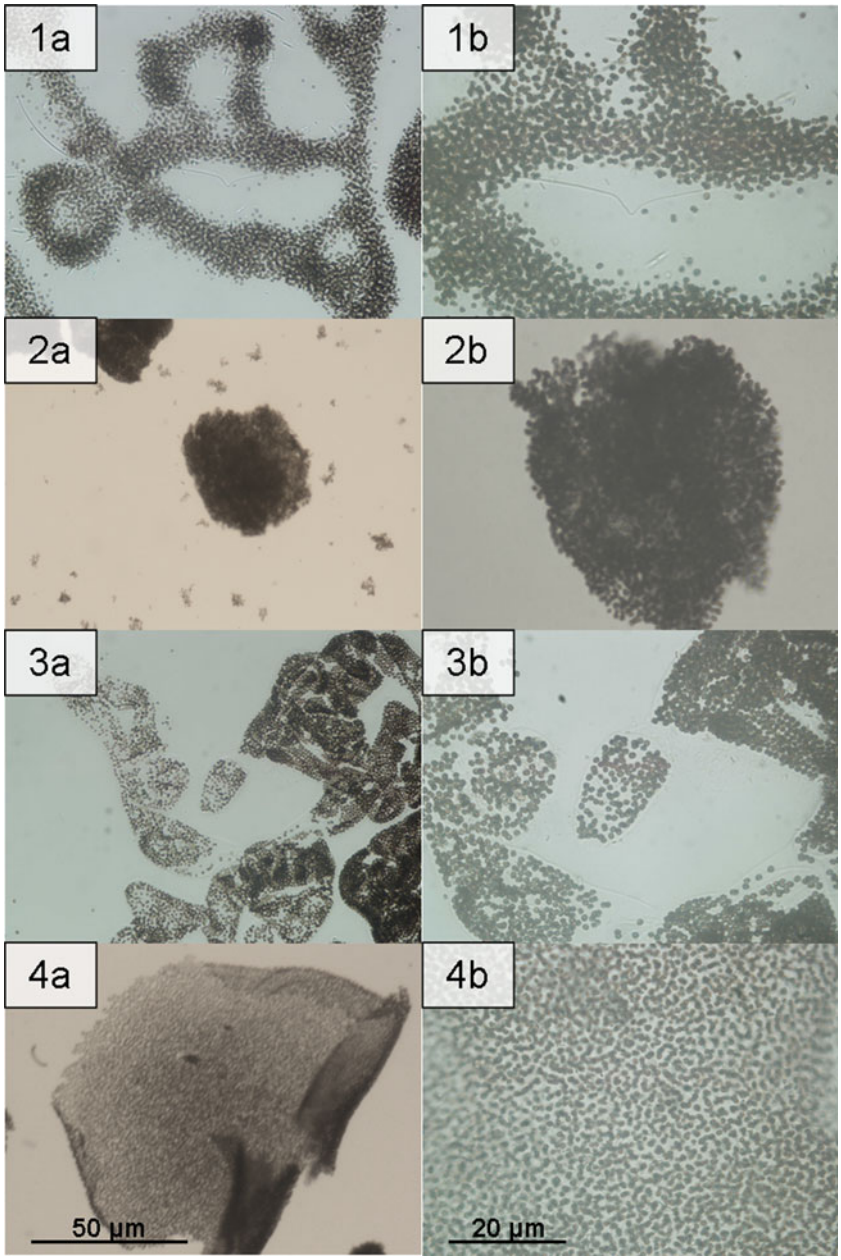

Figure 3 Microscope images of the four *Microcystis* morphospecies comprising the Taihu summer blooms at two different magnification levels (**a**=×200, **b**=×400). *Sample numbers* indicate the following morphospecies designations: (*1*) *M. aeruginosa*; (*2*) *M. flos-aquae*; (*3*) *M. wesenbergii*; (*4*) *M. ichthyoblabe*

were observed at a range of temperatures, dense surface blooms were not observed until the water temperature exceeded 23°C. Based on HPLC analyses, total chlorophyll *a* (chl *a*) ranged from 2.48–294.79 µg l^{-1} (med= 16.72 µg l^{-1}, geomean=19.48 µg l^{-1}, SD=1.92) in 2009, and from 2.73–997.1 µg l^{-1} (med=12.50 µg l^{-1}, geomean= 17.68 µg l^{-1}, SD=3.11) in 2010. Non-parametric rank analyses using Kendall tau correlations indicate that temperature is positively correlated at the 95% confidence level with microcystin concentration (τ=0.216, p value=0.003) and chl *a* is positively correlated with microcystin concentration (τ=0.452, p value<0.001). Dissolved oxygen, pH, and turbidity were not found to be significantly correlated with microcystin concentration. The correlation between MCs and chl *a* is dampened by the presence of other phytoplankton groups representing a fraction of the total chl *a* measurements, such as *Anabaena* spp. and *Synechocystis* spp., which have been observed to be strong competitors of *Microcystis* spp. in Lake Taihu [65]. However, chlorophyll *a* has previously been recognized as a good indicator of *Microcystis* biomass in Lake Taihu because it is the dominant algae during the summer blooms and often accounts for over 95% of phytoplankton [6, 68]. These findings were corroborated based on our own microscopic observations where the most abundant (on a biovolume basis) phytoplankton group encountered was cyanobacteria

(*Microcystis* spp. and *Anabaena* sp.) and to a lesser extent green algae (*Chlorella* sp. and *Pediastrum* sp.). Additionally, the geometric means of various carotenoid pigments measured from all samples (n=112), as determined by HPLC analysis, indicate that the lake's phytoplankton composition was predominately cyanobacteria (zeaxanthin=1.15 μg l^{-1}, myxoxanthophyll=0.31) and green algae (chlorophyll b= 1.01 μg l^{-1}), with smaller proportions of cryptomonads (alloxanthin=0.43) and diatoms (fucoxanthin=0.26 μg l^{-1}) [35]. There was no evidence of dinoflagellates in any samples as this group was never viewed under the microscope and the diagnostic pigment peridinin was not detected.

Phylogenic Inference Based on rDNA and *mcyB* Sequences

The 16S rDNA sequences obtained from the MSR primers were found to lack variation, making meaningful comparisons between different *Microcystis* strains and species impossible. The MSR primers proved to be suitable for detecting the *Microcystis* genus, but because they amplified within a highly conserved region of the 16S ribosome, intra-species variation could not be assessed. These findings are in agreement with previous findings which observed upward of 99% sequence similarity in the 16S rRNA of several *Microcystis* species [37]. The 16S rDNA sequences amplified using the MSR primers on 10 *Microcystis* isolates from 2009 were aligned and found to be 100% identical to each other (data not shown). For this reason the MSR primer set was excluded from additional analyses in 2010. Conversely, the sequences obtained from the ITSc primers provided markedly improved species/strain differentiation; with all four morphospecies sharing only 91.4% nucleotide identity (524 bp) with one another. The phylogram created by Bayesian inference utilized the ITSc sequences from the 2009–2010 Taihu isolates (n=23) along with eight additional reference *Microcystis* strains acquired from the Pasteur Culture Collection (PCC), the Canadian Phycology Culture Collection (CPCC), and the University of Texas at Austin (UTEX) (Fig. 4). Only 23 of the original 29 isolates were included in the phylograms because three failed to amplify and three were found to contain more than one ITSc sequence and were omitted. For comparison, a second phylogram was generated utilizing the neighbor-joining method with 1,000 bootstrap replicates and rooted with a microcystin-producing strain of *Planktothrix rubescens* PCC 7821 (data not shown). Both phylograms were congruent, with each comprised of two nodes with four primary branches. Only one of the branches was in close agreement between morphotype and genotype (monophyletic), while the other groupings consisted of a mixture of genotypes and morphotypes (polyphyletic). The *M. wesenbergii* isolates were found to cluster tightly together forming a distinct clade. Additionally, all of the *M. wesenbergii* isolates lacked the *mcyB* gene. Two of the *M. ichthyoblabe* isolates clustered together separately, with an additional two *M. ichthyoblabe* isolates clustering with toxic and non-toxic strains of *M. flos-aquae* and *M.*

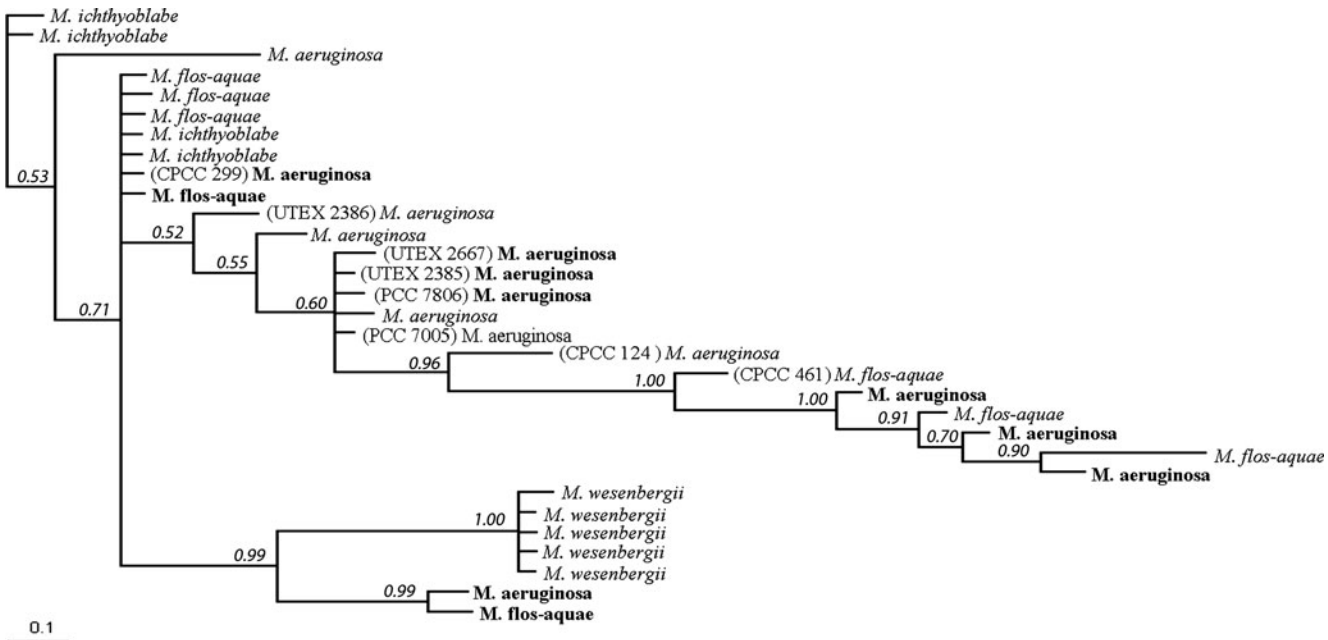

Figure 4 Bayesian inference of phylogeny for ITSc sequences from 2009 and 2010 aligned with eight cultured *Microcystis* reference strains using a GTR model with gamma distributed rate variation. Strains in *bold* possess *mcyB* toxin gene. Bootstrap values are indicated at the nodes. *Scale bar* corresponds to 0.1 changes per nucleotide

aeruginosa. The *M. ichthyoblabe* morphospecies all lacked *mcyB* genes, whereas one of the Taihu *M. flos-aquae* isolates associated with this grouping possessed the *mcyB* gene and three other *M. flos-aquae* isolates did not possess the *mcyB* gene. The *M. aeruginosa* morphotypes (both potentially toxic and non-toxic strains) clustered with *M. flos-aquae* morphotypes (both potentially toxic and non-toxic strains) interchangeably. It has been postulated that *M. aeruginosa* and *M. flos-aquae* are actually the same species, with the former being a toxic variant and the latter a non-toxic variant. The 16S–23S rDNA ITSc sequences obtained in this study suggest the *M. aeruginosa* and *M. flos-aquae* morphotypes may be the same species, but both morphotypes include potentially toxic and non-toxic strains.

The *M. aeruginosa* and *M. flos-aquae* morphospecies were observed to sometimes possess the *mcyB* gene, whereas it was never detected in the *M. wesenbergii* and *M. ichthyoblabe* morphotypes. The *M. aeruginosa* morphospecies possessed the *mcyB* gene in 57% of colony isolates (n=7) and *M. flos-aquae* contained the *mcy*B gene in 29% of isolates (n=7). Overall, the *mcyB* gene was detected in 26.8% of all transect samples (n=112) spanning the eight sampling stations and the 16 blooms during the 2009 and 2010 sampling periods. In order to validate *mcyB* primer sensitivity, the samples were also amplified using primers (mcyE-F2 and MicmcyE-R8) that amplify a shorter (247 bp) region of the microcystin synthetase *mcyE* gene encoding for the glutamate-activating adenylation domain [60]. Similar PCR sensitivity was achieved, with the *mcyE* primers amplifying in 31.3% of the samples (n=112). Temperature appeared to influence the prevalence of toxigenic strains, with the majority of *mcy* gene positives being observed at cooler temperatures. At water temperatures below 26°C, the *mcyB* gene was detected in 40.4% of the samples (n=52) and the *mcyE* gene was detected in 50% of the samples (n=52). However, at water temperatures above 26°C, the *mcyB* and *mcyE* genes were only detected in 15% of the samples (n=60). These results indicate that the *mcyB* primers have similar sensitivity as the *mcyE* primers and cooler water temperatures may promote toxigenic strains of *Microcystis*.

Analysis of *mcyB* Functionality

The general structure of microcystins is cyclo-D-Ala-X-D-MeAsp-Z-Adda-D-Glu-Mdha, where X and Z are variable L-amino acids [51]. The adenylation domains within *mcyB* directly impact the microcystin variants produced. With regard to the X amino acid, C-type domains activate mainly Arg, whereas B-type domains often activate Leu [11]. The most common microcystin variant is microcystin-LR, which possesses L-Leu at the X position and L-Arg at the Z position. Recombination between the first module of mcyB1 and *mcyC* is linked to the production of different microcystin isoforms, such as MC-RR, which possesses L-Arg at both X and Z amino acid positions. False negatives can occur if PCR primers are used which amplify within the variable condensation and adenylation domains of mcyB1 and *mcyC*. This concern over false negatives is why the use of some *mcyB* primers for detecting toxigenic genotypes has recently been called into question [2, 19]. However, the primers used in this study flank the mcyB1 region prone to recombination and mutation events and can be used to detect toxigenic variants possessing both the mcyB1 (B-type) and mcyB1 (C-type) adenylation domains. A multiple pairwise alignment of the *mcyB* sequences from the *M. aeruginosa* and *M. flos-aquae* isolates revealed that they share 95.3% of identity nucleotide positions (92.6% of amino acid identity) and that they both consist of mcyB1 (C-type) adenylation domains (Supplemental Fig. S1). Analysis of the binding pocket signatures of these adenylation domains indicated that the *M. aeruginosa* morphotype encodes L-Leu and L-Arg, whereas the *M. flos-aquae* morphotype only encodes for L-Arg. Therefore, the *M. aeruginosa* morphotypes are capable of producing the microcystin variants MC-LR and MC-RR, whereas *the M. flos-aquae* morphotype can only produce MC-RR. This was important to distinguish because microcystin toxicity is dependent not only on the concentration of the toxin, but also on which isoforms are present, with MC-LR being 10-fold more toxic than MC-RR [15]. Lastly, a pairwise alignment of the amino acids encoding the highly conserved c-phycocyanin-a gene (*cpcA*) from all *Microcystis* sequences available in GenBank (n=153), revealed that *M. wesenbergii* possesses a unique region of that gene (Supplemental Fig. S2). The *cpcA* gene was observed to be highly conserved across all other *Microcystis* species with each strain sharing 95% or greater amino acid similarity with the consensus translation. However, *M. wesenbergii* only shared 86.7% amino acid similarity with the consensus *Microcystis cpcA* sequence.

Discussion

Role of Water Temperature and Wind on Morphotype Prevalence and chl *a* Concentration

Previous studies have identified water temperature as a key variable regarding *Microcystis* abundance, toxicity, and morphology [24, 33]. The *Microcystis* spp. in Lake Taihu followed this trend as well, with different morphospecies blooming at different water temperatures. Cell buoyancy has been shown to decrease with decreasing temperature [57]. However, because depth-integrated samples were collected, temperature-induced variation of buoyancy is

unlikely to have affected chl *a* values and morphological assessments. Thus, the generally low chl *a* values observed when temperatures were below 23°C (Fig. 6) indicate that cell densities were reduced throughout the water column and not just visibly absent from the water surface due to reduced buoyancy.

There appears to be a threshold temperature for the *Microcystis* blooms in Taihu, in 2010 there were no blooms at or below 21°C, and then several appeared at multiple locations when lake water temperatures reached 23°C. However, when the water temperature dropped back to 21°C no new blooms were initiated, and blooms were not re-initiated until the water temperature again rose above 23°C. This observation may be attributable to changes in growth rate and/or buoyancy at different temperatures. While the *Microcystis* cells continue to divide at temperatures below 21°C, they do so at a reduced rate. Previous studies have demonstrated *M. aeruginosa* growth rates are more than double at 30°C than what they are at 20°C [25]. However, the peak biomass, as determined by total chl *a*, was at water temperatures 26–27°C (Fig. 6). Around this temperature the *Microcystis* diversity is also at its highest, with all four morphotypes co-occurring. The finding that *Microcystis* diversity is highest in this mid-temperature range was anecdotally observed in a previous study that measured the chl *a* content in Taihu over a period of 8 years. In that study, the average peak chl *a* was ~56 μg l^{-1} and occurred in June, and not in July or August when lake water temperatures are at their maximum [69, 70]. These findings imply that variation in water temperature leads to *Microcystis* niche partitioning in Lake Taihu.

Although it is difficult to extrapolate limited wind data across the whole of Lake Taihu, anemometer data collected in the northern bay (Meiliang Bay) allowed us to speculate that besides the lake center, the western basin and northeastern embayment may be subjected to higher wind stresses than other sampling sites as there is less land and physical structure to buffer winds from the northwest there. This suggests that the *M. aeruginosa* morphotype, which was not detected in the lake center, and the *M. ichthyoblabe* morphotype, which was not observed in the southwestern basin, the lake center, or the northeastern embayment, are more susceptible to disaggregation from wind shear, with *M. ichthyoblabe* being the most fragile of all four morphospecies. We also hypothesize that *M. flos-aquae* (small, round, and compact) and *M. wesenbergii* (thick mucilaginous sheath) were the only *Microcystis* morphospecies observed in the lake center because they are less susceptible to wind shearing. This hypothesis is supported by the fact that *M. aeruginosa* colonies have previously been shown to be susceptible to disaggregation from turbulent mixing [39]. In *Microcystis*, the inability to maintain colonial form is detrimental in well-mixed waters because larger aggregates are better able to reach the surface waters, and therefore the euphotic zone, than individual cells [63].

Phylogenetic Analyses and Inferred Relationships

Due to the high degree of phenotypic plasticity exhibited by *Microcystis* spp., any given species/strain observed under one set of conditions may appear markedly different when exposed to other contrasting, environmental conditions [1]. For example, based on colony morphology, it was hypothesized that the *M. ichthyoblabe* morphotype may actually be an early stage transitional form of the *M. flos-aquae* morphotype. If that were true, then the *M. ichthyoblabe* morphotypes would form initially as a single plane of cells and then over time divide three-dimensionally to create a mature *M. flos-aquae* colony possessing greater depth and cell density. This hypothesis is in part supported by a previous study in which the *M. flos-aquae* morphotype was distinguished as a type of *M. ichthyoblabe* [66]. The ITSc sequences of the *M. ichthyoblabe* isolates failed to adequately distinguish *M. ichthyoblabe* from *M. flos-aquae*. Two of the *M. ichthyoblabe* isolates appear to be genetically distinct from *M. flos-aquae*, whereas two other *M. ichthyoblabe* isolates were highly similar to toxic and non-toxic *M. flos-aquae* isolates from Taihu. With regard to determining species delimitation between *M. aeruginosa* and *M. flos-aquae* morphotypes, although they appear as morphologically distinct assemblages, they were shown to be highly similar genetically.

The merger of five different morphospecies (*M. aeruginosa*, *M. ichthyoblabe*, *Microcystis novacekii*, *Microcystis viridis*, and *M. wesenbergii*) into a single species of *M. aeruginosa* has been proposed because although morphologically distinct, they possess a high degree of DNA homology [43]. The results from this study did not corroborate this suggestion. Instead of including *M. wesenbergii*, the results from this study suggest *M. flos-aquae* would be a more appropriate addition to the condensed *M. aeruginosa* grouping. *M. wesenbergii* was found to be morphologically and genetically distinct from any other *Microcystis* morphotype. This finding is further supported by comparing the c-phycocyanin genes within this genus. Whereas the *cpcB* and the *cpcBA* intergenic spacer regions are highly conserved across all *Microcystis* species, *cpcA* in *M. wesenbergii* is markedly different (Supplemental Fig. S2). Additionally, *M. wesenbergii* is easily distinguishable because it tends to have the thickest mucilaginous sheath of all *Microcystis* species. An analysis of the polysaccharides comprising the sheaths of *M. aeruginosa*, *M. flos-aquae*, and *M. viridis* reveals a composition of neutral sugars rhamnose, fucose, xylose, mannose, glucose, galactose, and uronic acid. However, in *M. wesenbergii* the mucilaginous sheath is only composed of uronic acid [12].

These data contrast the suggestion that *M. wesenbergii* should be included in the proposed *M. aeruginosa* condensed taxonomic grouping.

Prevalence of *mcyB* Genotypes

Aside from influencing *Microcystis* morphology and prevalence, temperature also affected the proportion of potential microcystin producers, with cooler temperatures favoring *mcyB* possessing strains. The prevalence of MC-producing genotypes has been observed to be exponentially correlated with chl *a* at temperatures below 27°C [22]. Likewise, MC concentrations have been shown to be higher at 20°C than 30°C, suggesting a physiological trade-off between growth rate and toxin production [67]. Over time, population turnover has been observed to occur within *Microcystis* blooms resulting in non-toxic genotypes superseding toxin-producing strains later in the season [3]. Therefore, chl *a* was hypothesized to be a useful proxy for toxicity in early season *Microcystis* blooms, but likely less indicative of toxicity in late season blooms. The peak in MC concentration did not correspond to the peak in biomass (chl *a*), but instead was correlated with the peak *in M. aeruginosa* and *M. flos-aquae* prevalence (Figs. 5 and 6). These morphospecies were identified to frequently possess the *mcyB* gene, and therefore toxicity is dependent on their presence and not directly correlated to total biomass of all *Microcystis* spp. in Taihu. This finding was somewhat contrary to the hypothesis that microcystins would be highest at lower temperatures. Instead, because there is a balance between non-toxic genotypes at both temperature extremes, the peak in chl *a* occurred when all four morphospecies were present, causing a dampening effect between the correlation of microcystins and chl *a*. The correlation between chl *a* and MCs may also be impacted by the fact that some strains of *Anabaena* spp. produce MCs and this organism was observed in 29.5% (n=112) of the samples viewed under the microscope. Real-time quantitative PCR with *Anabaena* specific *mcy* primers will need to be utilized in order to determine if this organism is likely contributing to the MC load in Taihu. Whether or not the increase in MCs observed at higher water temperatures is attributable to the *Anabaena* sp. present or due to increased *mcy* gene expression in the *Microcystis* strains has yet to be elaborated.

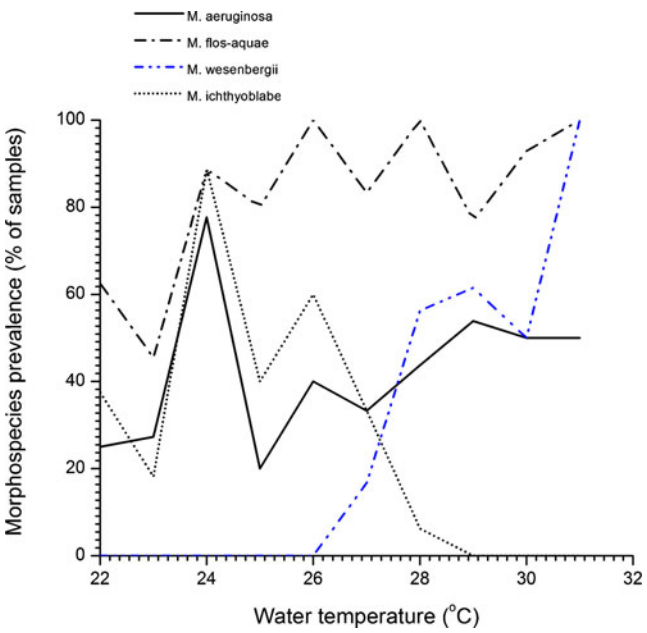

Figure 5 Prevalence of *Microcystis* morphotypes observed from all eight stations sampled during the periods of June and July, 2009, and June, 2010. Prevalence is defined as the total number of sites each morphospecies was detected at divided by the total samples collected at a given temperature and then multiplied by 100

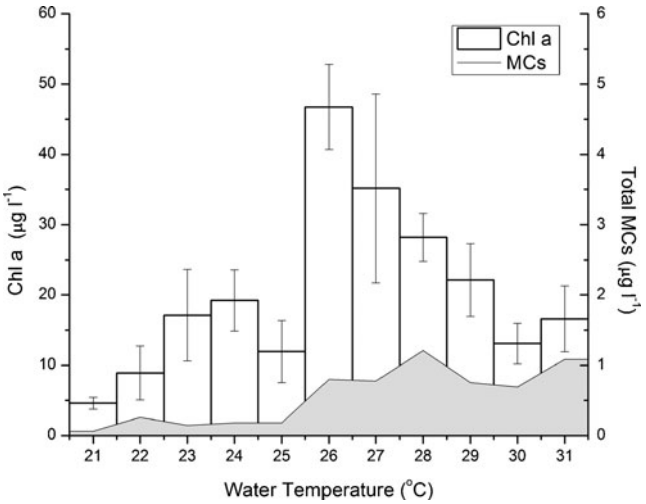

Figure 6 Distribution of chlorophyll *a* (geometric mean) and total microcystins (geometric mean) across Lake Taihu relative to water temperature. *Error bars* indicate 95% confidence intervals

Morphology was not a universal indicator of toxigenicity, as both the *M. aeruginosa* morphotype and the *M. flos-aquae* morphotype consisted of *mcyB* positive and negative strains. This finding is similar to other studies which have concluded that toxicity cannot accurately be determined based on morphology alone as both toxic and non-toxic strains of *Microcystis* may appear identical when viewed microscopically [41, 44]. Conversely, the *M. ichthyoblabe* and the *M. wesenbergii* morphotypes in Taihu consistently lacked the *mcyB* gene and can be considered non-toxic variants [48]. This implies that toxigenicity should be inferred on a system by system basis. Future research on the *Microcystis* spp. comprising the Taihu blooms should address the role of nitrogen and phosphorus (both concentration and molecular form), as

both have been shown to affect bloom potentials and species composition [18, 21, 56].

In summary, within Taihu four different *Microcystis* morphospecies were observed to predominately comprise the algal blooms during the periods of June and July, 2009, and June, 2010. As a caveat, *Microcystis* morphospecies other than those observed in the present study may also occur within the lake at different times of the year, particularly in late fall or early spring. In the present case, more genotypes were detected than were attributable to particular morphotypes based on 16S–23S rRNA ITS sequence analysis. Therefore, it is likely that each *Microcystis* morphospecies is comprised of multiple genotypes. While there is no established threshold for delimiting species based on 16S rRNA sequence identity, it has been proposed, at least among prokaryotes, that organisms sharing greater than 98.7% 16S rRNA sequence similarity and DNA–DNA reassociation values of greater than 70% should be considered of as the same species [30, 55]. In this study, 16S rRNA sequences from all four morphospecies were 100% identical, although there is reason to believe they are not all of the same species. Comparing the consensus 16S–23S rRNA ITS sequences for the *M. wesenbergii* isolates with the consensus 16S–23S rRNA ITS sequence for the *M. aeruginosa* isolates found they share only 96.4% sequence identity. A comparison of *Microcystis cpcA* sequences obtained from GenBank further supports species delimitation, as *M. wesenbergii* is considerably different from other *Microcystis* species. These results suggest that *M. wesenbergii* is a genetically distinct species and should not be incorporated into an *M. aeruginosa* condensed taxonomic grouping as has been previously suggested.

As a whole, due to the high levels of polymorphism exhibited by *Microcystis*, this genus is better described based on functionality (ex. toxic vs. non-toxic strains) than by colony morphology. This research highlighted the fact that neither morphology nor 16S–23S rRNA ITS sequence homology, are reliable indicators of a strain's toxicity. In the present case, we conclude that the *M. wesenbergii* morphospecies in Taihu is indeed a non-toxic strain of *M. wesenbergii*. However, because *M. viridis* closely resembles *M. wesenbergii*, and is a common microcystin-producing variant found in Chinese freshwater ecosystems, it is prudent to exercise caution and assume a bloom to be toxic until proven otherwise. Although screening for the presence of microcystin synthetase genes, as detected by PCR, has been shown to strongly correlate with microcystin production in *Microcystis* spp. [61], direct measurement of microcystins remains the gold standard for health and safety and will provide additional precaution against any other microcystin-producing genera, such as *Anabaena* spp., that may be present in this system.

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