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3 **A review of the global ecology, genomics, and biogeography of the toxic cyanobacterium,**
4 ***Microcystis* spp.**

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7 Matthew J. Harke¹, Morgan M. Steffen^{2*}, Christopher J. Gobler³, Timothy G. Otten⁴, Steven W.
8 Wilhelm⁵, Susanna A. Wood⁶, Hans W. Paerl⁷

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10 ¹ Department of Earth and Environmental Sciences, Lamont-Doherty Earth Observatory, Columbia University,
11 Palisades, NY 10964

12 ² James Madison University, Department of Biology, 951 Carrier Dr. Harrisonburg, VA 22807

13 ³ Stony Brook University, School of Marine and Atmospheric Sciences, 239 Montauk Hwy, Southampton, NY
14 11968

15 ⁴ Oregon State University, Department of Microbiology, Nash Hall 226, Corvallis, OR 97331

16 ⁵ University of Tennessee, Department of Microbiology, 1414 West Cumberland Ave., Knoxville, TN 37996

17 ⁶ Cawthron Institute, Private Bag 2, Nelson, New Zealand and Environmental Research Institute, University of
18 Waikato, Hamilton, New Zealand

19 ⁷ University of North Carolina at Chapel Hill, Institute of Marine Sciences, 3431 Arendell Street, Morehead City,
20 NC 28557

21 * Corresponding author: Morgan M. Steffen, steffemm@jmu.edu

Abstract

This review summarizes the present state of knowledge regarding the toxic, bloom-forming cyanobacterium, *Microcystis*, with a specific focus on its geographic distribution, toxins, genomics, phylogeny, and ecology. A global analysis found documentation suggesting geographic expansion of *Microcystis*, with recorded blooms in at least 108 countries, 79 of which have also reported the hepatatoxin microcystin. The production of microcystins (originally “Fast-Death Factor”) by *Microcystis* and factors that control synthesis of this toxin are reviewed, as well as the putative ecophysiological roles of this metabolite. Molecular biological analyses have provided significant insight into the ecology and physiology of *Microcystis*, as well as revealed the highly dynamic, and potentially unstable, nature of its genome. A genetic sequence analysis of 27 *Microcystis* species, including 15 complete / draft genomes are presented. Using the strictest biological definition of what constitutes a bacterial species, these analyses indicate that all *Microcystis* species warrant placement into the same species complex since the average nucleotide identity values were above 95%, 16S rRNA nucleotide identity scores exceeded 99%, and DNA-DNA hybridization was consistently greater than 70%. The review further provides evidence from around the globe for the key role that both nitrogen and phosphorus play in controlling *Microcystis* bloom dynamics, and the effect of elevated temperature on bloom intensification. Finally, highlighted is the ability of *Microcystis* assemblages to minimize their mortality losses by resisting grazing by zooplankton and bivalves, as well as viral lysis, and discuss factors facilitating assemblage resilience.

Introduction

Blooms of toxic cyanobacteria have become a common occurrence in water bodies worldwide. One of the most pervasive bloom-forming cyanobacteria in freshwater ecosystems is *Microcystis*. In temperate systems, this organism overwinters in the benthos and during the summer rises to the epilimnion where it can accumulate to form blooms and scums on the water surface (Reynolds and Rogers, 1976; Ibelings et al., 1991). Blooms of *Microcystis* generally occur when water temperatures exceed 15°C (Okino, 1974; Reynolds et al., 1981; Jacoby et al., 2000) and the occurrence of blooms has been linked to anthropogenic nutrient loading (Perovich et al., 2008; Dolman et al., 2012). Many *Microcystis* strains can produce the potent hepatotoxin microcystin, and thus persistent blooms pose a risk to those who use impaired water resources for drinking water supplies, recreational activities, and fisheries. Microcystins are the only cyanotoxins for which the World Health Organization has set drinking and recreational water standards and are typically the only cyanotoxins screened for by municipal management agencies (Chorus and Bartram, 1999; Hudnell et al., 2008). As global climate changes, the occurrence and intensity of *Microcystis* blooms is expected to increase (Paerl and Huisman, 2008; Michalak et al., 2013; Paerl and Otten, 2013).

This review synthesizes the current state of knowledge regarding *Microcystis*; focusing on its geographic distribution, toxin production, phylogeny, and structural genomics. How these factors influence the ecology of this globally significant cyanobacterium is discussed and a series of knowledge gaps are identified and a list of high priority research topics are provided.

Geographic distribution

The cosmopolitan cyanobacterium *Microcystis* has been reported to bloom on every continent except Antarctica (Zurawell et al., 2005). Over the past decade there has been an expansion in the awareness of toxic cyanobacterial blooms and reports of these events (O'Neil et al., 2012; Paerl and Paul, 2012). To provide an update on the global geographic distribution of *Microcystis* blooms, a literature search for records from 257 countries and territories was conducted. Reports of *Microcystis* blooms were found for 108 countries (Figure 1, Table S1). Many of the countries without reported incidents were small island nations, such as those in the Pacific region. The number of reports per country varied markedly with North American, Australasian, and European countries having many hundreds of records, whereas accounts from developing countries were often scarce or from only a single study. Occurrence rate or specific sites of blooms within each country are not reported, as this is likely a representative function of the extent and intensity of monitoring and research programs (and their geographic locations) in each country rather than a true reflection of bloom prevalence. Where *Microcystis* blooms were identified, it was also investigated whether there were associated reports of toxins. Confirmation of microcystins associated with blooms was identified for 79 countries. In some cases, there was conclusive evidence that *Microcystis* was the producer, e.g., strains of *Microcystis* were isolated, cultured, and toxin production confirmed, or molecular techniques such as screening for microcystin synthetase (*mcy*) genes were used. In many instances, these steps were not undertaken and it is plausible that other cyanobacteria present in the blooms (e.g., *Planktothrix* or *Dolichospermum/Anabaena*) were the producers. These scenarios have not been differentiated in Figure 1. In one instance (Niger), the evidence for microcystin production was based on symptoms in a mouse bioassay. In all other studies, chemical or biochemical methods were used

to identify the toxins. The analysis suggests an expansion of *Microcystis*, as previous documentation noted less than 30 countries (Zurawell et al., 2005), demonstrating that *Microcystis* has proliferated and dominated phytoplankton communities in a wide range of freshwater ecosystems in both temperate and tropical climates.

Toxins

Many cyanobacterial species produce natural compounds that are toxic (cyanotoxins) to other organisms, including mammals. Cyanotoxins exhibit a wide range of toxicities, including hepatotoxicity, nephrotoxicity, neurotoxicity, and dermatotoxicity. *Microcystis* is most well-known for its ability to produce the hepatotoxin microcystin (Bishop et al., 1959) and has been studied globally for many decades. However, data on the production of other cyanotoxins by this genus are scarce or preliminary. Here, these other compounds are mentioned briefly and the remainder of this section focuses on microcystins.

There are few reports of *Microcystis* producing neurotoxins. For instance, there is a single report of four *Microcystis* strains isolated from three Japanese lakes producing the neurotoxic anatoxin-a (Park et al., 1993) with several of the strains also producing microcystin. Since this finding has not been replicated in the past two decades of intensive cyanobacterial research, it remains possible that the anatoxin-a measured in these cultures was derived from a co-cultured microbe. Similarly, an isolate of *Microcystis* from a lagoon in São Paulo (Brazil; SPC 777) was reported to produce a range of paralytic shellfish poison (PSP) neurotoxins (Sant'Anna et al., 2011). Upon sequencing the genome of the isolate however, no saxitoxin biosynthesis genes were identified casting significant doubt that *Microcystis* was truly the causative agent. Although the study of β -N-methylamino-L-alanine (BMAA), has become a somewhat contentious issue (Holtcamp, 2012; Otten and Paerl, 2015), studies also suggest that

the majority of cyanobacteria, including *Microcystis*, may produce BMAA (Cox et al., 2005), whereas many other investigators have failed to identify this compound (Faassen, 2014). BMAA is a non-protein amino acid which that has been linked to neurodegenerative diseases, including amyotrophic lateral sclerosis, Parkinson's, and Alzheimer's Disease (Cox and Sacks, 2002; Bradley and Mash, 2009; Banack et al., 2010; Holtcamp, 2012). The exact mode of BMAA toxicity is still under investigation, with both acute and chronic mechanisms indicated (Lobner et al., 2007). Perhaps the most methodical investigation to date was conducted by Réveillon et al. (2014), who reanalyzed a number of cyanobacterial isolates, including *M. aeruginosa* PCC 7806, that were reported as BMAA producers. Notably, using highly sensitive and specific hydrophilic interaction chromatography coupled to tandem mass spectrometry (HILIC-MS/MS), they failed to detect BMAA in any cultures. Both free and bound forms of the closely related isomer, 2,4-diaminobutyric acid (DAB) were detected in all cultures, and may be the compound identified as BMAA in previous studies using other analytical approaches. Beyond direct toxins, *Microcystis* has also been shown to produce compounds that act as endocrine disruptors; while not lethal to fish, these compounds regulate genetic elements associated with sexual maturity and differentiation (Rogers et al., 2011).

Microcystin was originally identified as Fast-Death Factor (Bishop et al., 1959), but was renamed microcystin a few years later (Konst et al., 1965). Microcystins are cyclic heptapeptides which contain a unique β -amino acid, Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid; Rinehart et al., 1988; Tillett et al., 2000). Microcystins generally contain two conventional D-amino acids in positions one and six, a D-erythro- β -methylaspartic acid in position three, and position seven is often *N*-methyldehydroalanine (Tillett et al., 2000). To date, over 100 different microcystin congeners have been characterized (Puddick et al., 2014), mostly

due to substitutions of variable L-amino acids in positions two and four, although modifications have been reported for all amino acids (Rinehart et al., 1994; Puddick et al., 2014). Microcystin congeners are named according to the single letter code of the amino acids incorporated at positions two and four; e.g., microcystin-LR contains leucine (L) in position two and arginine (R) in position four.

Different microcystin congeners vary in toxicity from essentially non-toxic (e.g., [(6Z)-Adda⁵] microcystin-LR, LD₅₀ > 1,200 µg kg⁻¹) to highly toxic (e.g., microcystin-LR, LD₅₀ = 50 µg kg⁻¹; Rinehart et al., 1994). Toxicity is manifested as an irreversible covalent bond formed between the toxin and protein phosphatases, especially in hepatocytes, which leads to subsequent cell structure damage (Goldberg et al., 1995; Maynes et al., 2006; Feurstein et al., 2009; Feurstein et al., 2010) and can result in liver disease as well as nephrotoxicity (Milutinović et al., 2003). This toxicity has led the World Health Organization (WHO) to propose a drinking water guideline of 1 µg l⁻¹ for the common hydrophilic variant microcystin-LR (WHO, 2003).

Numerous fatalities and severe poisonings of livestock, pets and wildlife have been attributed to microcystin-containing *Microcystis* blooms (reviewed in Stewart et al. (2008)). Reports of human illness from microcystins are also well documented, with major exposure routes including direct consumption of drinking water and accidental ingestion of water or skin contact during recreational use of waterbodies (Ressom et al., 2004; Falconer, 2005). A less widely reported exposure route is via inhalation that may result from recreational activity in the vicinity of blooms (Wood and Dietrich, 2011). One of the most severe cases of human poisoning occurred in Brazil in 1996, when a bloom of *Microcystis* in a drinking reservoir contaminated the water supply of a dialysis treatment clinic with microcystins resulting in 56 fatalities (Azevedo et al., 2002). A promising potential therapy utilizing cholestyramine to competitively bind

microcystins and facilitate their excretion was successfully used to treat a dog suffering cyanotoxicosis and is a promising avenue for future research (Rankin et al., 2013).

Microcystin concentrations have been determined in cultures and environmental samples using a variety of methods including protein phosphatase inhibition assays, enzyme-linked immunosorbent assay (ELISA), chemical derivatization with gas chromatography-mass spectrometry analysis, and high performance liquid chromatography (HPLC) coupled to either ultra-violet, photodiode array detector or mass spectrometry detection (Spoof, 2005; Sangolkar et al., 2006). Choice of the most appropriate analytical method requires consideration regarding sensitivity, specificity, and associated consumables and equipment costs. For instance, ELISA broadly detects all microcystin congeners, but provides no information on which specific congeners are present, however the equipment required to perform the assay is minimal. Variations in detection methodology in concert with different starting materials (i.e., dried, filtered, wet samples) and methods of microcystin extraction make comparisons of toxin concentrations among waterbodies and between studies challenging. Nevertheless, it is clear that microcystin concentrations can reach extremely high levels during *Microcystis* blooms worldwide. For example, levels reached 7,300 $\mu\text{g g}^{-1}$ dry weight (dw) in China (Zhang et al., 1991), 7,100 $\mu\text{g g}^{-1}$ dw in Portugal (Vasconcelos et al., 1996), 4,100 $\mu\text{g g}^{-1}$ dw in Australia (Jones et al., 1995), 19.5 mg l^{-1} in Japan (Nagata et al., 1997) and 36 mg l^{-1} in New Zealand (Wood et al., 2006).

Factors that regulate production of microcystin and the potential ecophysiological role of the toxin for *Microcystis* have been topics of intense scientific research in recent decades. Early studies focused on factors commonly associated with the formation and senescence of blooms such as temperature, nutrients, and light. Such studies primarily used laboratory cultures

and only observed relatively minor (3- to 4-fold) shifts in microcystin production. Another popular hypothesis as discussed below (*Grazing* section) is that microcystins act as feeding deterrents for predators such as zooplankton and fish (Jang et al., 2003; Jang et al., 2004). However, phylogenetic analysis suggests that the genes responsible for microcystin synthesis pre-date the eukaryotic lineage (Rantala et al., 2004). More recently, factors such as chelation of metals (Humble et al., 1997; Sevilla et al., 2008), intraspecies communication (Schatz et al., 2007), colony formation (Gan et al., 2012), and protein-modulation (Zilliges et al., 2011) have been implicated as potential functions for microcystin. A brief review of studies in these areas is given below.

Culture-based studies have shown that microcystin concentrations are generally highest between 20 and 25°C (van der Westhuizen and Eloff, 1985; Watanabe and Oishi, 1985; van der Westhuizen et al., 1986; Codd and Poon, 1998; Amé and Wunderlin, 2005). Dziallas et al. (2011) provided further evidence regarding the influence of temperature on microcystin production by incorporating gene expression assays. These authors found that the fraction of microcystin-producing *M. aeruginosa* were significantly lower at 32°C than at 20 and 26°C, although microcystin concentrations increased at these higher temperature (26 and 32°C). Temperature has also been shown to alter ratios of microcystin congeners. Using batch cultures and a natural population of *M. aeruginosa* kept at 20°C, microcystin-LR was predominately produced, whereas at 28°C the ratio of microcystin-LR and microcystin-RR remained constant (Amé and Wunderlin, 2005).

The availability of nutrients is a major factor controlling the proliferation of *Microcystis* (see Ecology - Nutrients section). However, their role in regulating microcystin production or whether microcystin may play a role in improving access to nutrients is less well defined. In

batch cultures of axenic *Microcystis*, microcystin production decreased in proportion to cell division when the culture became nitrogen (N) limited, suggesting that microcystin production is controlled by environmental effects related to the rate of cell division (Orr and Jones, 1998). Similarly, using continuous cultures under either N (Long et al., 2001) or phosphorus (P) limitation (Oh et al., 2000), a linear relationship was also observed between microcystin production and growth rate. Downing et al. (2005) suggested that considering a single nutrient in isolation was an oversimplified approach and found microcystin quotas to be positively correlated with nitrate uptake and cellular N content, and negatively correlated with carbon fixation rate, phosphate uptake, and cellular P. They concluded that microcystin quotas were controlled by variables other than growth rate, with N having the most significant effect. In support of this, Harke and Gobler (2013) observed that under conditions of low inorganic N, many of the peptide synthesis genes in the microcystin synthetase cassette (*mcvABCDEF*) were downregulated and microcystin content per cell decreased when cells were N limited. Furthermore, increases in exogenous N concentrations have been associated with increases in microcystin (Van de Waal et al., 2009; Horst et al., 2014; Scott et al., 2014; Van de Waal et al., 2014). Increases in microcystin concentrations or expression of individual *mcv* genes during N limitation have also been observed (Ginn et al., 2010; Pimentel and Giani, 2014) suggesting a more complicated relationship or perhaps strain to strain variability.

Transcription of *mcv* genes in *Microcystis* is thought to be regulated via a bidirectional promoter that is located between the *mcvA* and *mcvD* genes. The promoter contains sequence motifs for both the DNA binding proteins Fur (ferric uptake regulator) and *ntcA* (global nitrogen regulator): observations that support the hypotheses that nitrogen and possibly iron may influence microcystin synthesis (Martin-Luna et al., 2006; Ginn et al., 2010; Neilan et al., 2013).

Exploring the nitrogen link further, Kuniyoshi et al. (2011) observed that increased 2-oxoglutarate levels (a signal of the C to N balance in cells) increased the binding affinity of *ntcA* to these promoter regions.

Light has also been investigated as a factor controlling microcystin synthesis. For instance, Kaebernick et al. (2000) demonstrated that light intensity affects microcystin synthase expression, whereby increases in transcription occurred between dark and low light (16 μmol of photons $\text{m}^{-2} \text{s}^{-1}$) and between medium and high light (31 and 68 μmol of photons $\text{m}^{-2} \text{s}^{-1}$; respectively). Phelan and Downing (2011) found a strong correlation between microcystin concentration and growth rate under high light (37 μmol photons $\text{m}^{-2} \text{s}^{-1}$) conditions for *Microcystis aeruginosa* PCC 7806 and suggested a possible role for microcystin in protection against photo-oxidation. There is also some evidence to suggest microcystins may allow *Microcystis* to acclimate to high light and oxidative stress (Zilliges et al., 2011). Alexova et al. (2011b) showed that microcystins bind to proteins under high light and during periods of oxidative stress. Interestingly, oxidative stress is often brought about by supersaturated oxygen conditions produced by vigorously photosynthesizing surface blooms themselves. Paerl and Otten (2013) suggested that under these conditions, microcystins act as protectants against cellular damage during active surface bloom formation. Indeed, microcystin production is often highest during early nutrient-replete phases of the bloom (Davis et al., 2010), when photosynthetic oxygen production is maximal (Otten et al., 2012). Using a DNA microarray based on the genome of *M. aeruginosa* PCC 7806, Straub et al. (2011) demonstrated that the biosynthesis of microcystins occurred primarily during the light period, although this has been disputed by Penn et al. (2014) who found microcystins were produced throughout the day/night cycle in natural populations of *Microcystis*. One possible explanation for the often

observed disconnect between microcystin concentration and *mcy* transcript abundance is that toxin may be bound to proteins and therefore not detectable by standard methods (Meissner et al., 2013).

There is also mounting evidence that microcystins may be involved in cell-to-cell signaling. For instance, Dittmann et al. (2001) identified a microcystin related protein (*mrpA*) that shares similarities with proteins thought to be involved in quorum-sensing in *Rhizobium*. This protein was only present in a wild-type culture but not an inactivated mutant and was responsive to light, with a rapid decline of transcription under high light conditions. Kehr et al. (2006) provided further evidence for this process by demonstrating interactions between microcystin and the lectin microvirin (MVN) isolated from *Microcystis aeruginosa* PCC 7806. MVN is believed to be involved in the aggregation of single *Microcystis* cells into colonies (Kehr et al., 2006). Schatz et al. (2007) found that the release of microcystin from lysed cells into the extracellular environment induced a significant upregulation of *mcyB* and an accrual of microcystins in remaining *Microcystis* cells. Additionally, Gan et al. (2012) found that high concentrations of extracellular microcystins significantly enhanced *Microcystis* colony size, and that microcystins induced the production of extra-cellular polysaccharides. This contributed to cell colony formation and upregulated genes related to its synthesis. Using field-based studies (Wood et al., 2011) showed that *Microcystis* sp. can ‘switch’ microcystin production on and off. Field and experimentally induced 20-fold changes in microcystin quotas within a five-hour period were observed in concert with up to a 400-fold change in *mcyE* expression (Wood et al., 2011; Wood et al., 2012). In both studies the changes in microcystin quotas were associated with increased *Microcystis* densities (i.e., scum formation, in this case defined as a thin (ca. 3 mm) layer of cells of the lake surface) and were not caused by a shift in the relative abundance of

toxic/non-toxic genotypes. Wood et al. (2012) suggest that this upregulation could either indicate a cell-to-cell signaling role for microcystins (although no increase in extracellular toxins was observed in their mesocosm study), or be a response to stress caused by rapid changes in other bloom-related variables (e.g., pH, light, oxidative stressors) that are mutually correlated with scum formation. Hypotheses regarding the role of microcystin in quorum sensing require that microcystin be exported from the cell. Using ^{14}C tracers to track the location and fate (either exported from the cell or metabolized) of microcystin under various light conditions, Rohrlack and Hyenstrand (2007) found no evidence of export or intracellular breakdown under these conditions, suggesting these theories need further investigation. Moreover, protein location prediction (Yu et al., 2010) of the single transporter gene (*mcyH*) in the microcystin synthetase gene cassette (Pearson et al., 2004) suggests microcystin is transported to the periplasmic space, not extracellularly.

Collectively these studies highlight the complexity in understanding the regulation and ecological role of microcystins in *Microcystis*. It seems plausible that there may be multiple triggers and the toxin could serve several functions for *Microcystis*, or that microcystin is a regulatory molecule linked to multiple cell processes (Wilhelm and Boyer, 2011). Culture-based studies using non-colony forming populations have provided the foundation for much of the current knowledge, however, often only one parameter is changed while others are maintained at optimal levels. There is a pressing need for results of laboratory-based studies to be validated in the field. Increased understanding of the regulation of microcystins in the environment may ultimately help in identifying the times of greatest toxicity and health risk.

Genomics and phylogeny

Genomics of Microcystis spp.

The first *Microcystis* genome was sequenced from the toxic isolate *M. aeruginosa* NIES - 843 (Kaneko et al., 2007), followed shortly by that of *M. aeruginosa* PCC 7806 (Frangeul et al., 2008). As late as 2015, only two *M. aeruginosa* genomes have been closed, however, the number of draft genomes has subsequently increased, as strains isolated from diverse locations have been sequenced. To date, 15 draft genomes are available, sequenced from strains isolated in Japan (Kaneko et al., 2007; Okano et al., 2015), the Netherlands (Frangeul et al., 2008), China (Yang et al., 2013; Yang et al., 2015), and Brazil (Fiore et al., 2013) accompany a collection of draft sequences from Humbert et al. (2013) for isolates from Canada, the Central African Republic, France, the United States, South Africa, Australia, and Thailand. Genomes range in size from 4.26 Mbp (*M. aeruginosa* PCC 9806) to 5.84 Mbp (*M. aeruginosa* NIES 843). Previous studies have highlighted genetic diversity between species of *Microcystis*, for example between the potentially toxic *M. aeruginosa* and nontoxic *M. wesenbergii* (Harke et al., 2012), and sequencing of such species may reveal important insight into the divergent ecological strategies that may exist between strains, potentially driven by each strain's unique flexible genes.

The use of targeted genomics (e.g., PCR/QPCR, amplicon and shotgun sequencing) for detection, quantification, and phylogenetic analysis of *Microcystis* in the environment has rapidly expanded in recent years. The most frequent targets of these techniques include the microcystin synthetase gene operon, cyanobacterial and *Microcystis*-specific 16S rRNA or c-phycocyanin photopigment genes (*cpcBA*) (Ouellette and Wilhelm, 2003; Otten et al., 2015), and genes involved in nutrient transport and metabolism (Harke et al., 2012). Much of this work has centered on the characterization of toxic versus nontoxic populations that occur simultaneously

or consecutively throughout the bloom season (Rinta-Kanto et al., 2005; Ha et al., 2009; Baxa et al., 2010; Wood et al., 2011). Similarly, these tools have bolstered the ability to identify organism(s) responsible for toxin production, even in mixed phytoplankton communities (Dittmann and Börner, 2005; Rinta-Kanto and Wilhelm, 2006; Gobler et al., 2007; Steffen et al., 2014b). Recent efforts have resulted in a better understanding of the factors that drive *Microcystis* growth and/or toxicity in the environment, including the role of macronutrients such as phosphorus and nitrogen (Davis et al., 2009; Rinta-Kanto et al., 2009; Sevilla et al., 2010; Harke et al., 2012), micronutrients such as iron (Sevilla et al., 2008; Alexova et al., 2011a), and rising global temperatures (Davis et al., 2009) and carbon dioxide (CO₂) concentrations (Van de Waal et al., 2011).

The combined impact of the availability of *Microcystis* genome information and the application of high-throughput sequencing and targeted genetic analyses has marked a transition to global genomic studies of *Microcystis* ecology and physiology, in both laboratory and field studies. To date, the number of studies employing the genomes of strains NIES-843 and PCC 7806 as type strains far exceeds the usage of the other 13, more recently sequenced genomes. *M. aeruginosa* NIES-843 has been used for transcriptomic (Harke and Gobler, 2013; Penn et al., 2014; Steffen et al., 2014a; Steffen et al., 2015), proteomic (Alexova et al., 2011b), and metabolomic studies (Steffen et al., 2014a), as has *M. aeruginosa* PCC 7806 (Straub et al., 2011; Penn et al., 2014; Makower et al., 2015; Meissner et al., 2015; Sandrini et al., 2015). These studies have provided insight into the nutritional ecology, responses to changing CO₂, and toxin production, among others factors.

As with many other currently sequenced genomes, a large number of the 12,000+ predicted genes across *Microcystis* strains remain uncharacterized. Probing these putative coding

sequences with bioinformatic tools for protein prediction and functional/pathway analysis in targeted studies is needed to provide new insight into the genetic response of *Microcystis* to environmental parameters. For instance, Harke and Gobler (2013) identified a number of genes designated as hypothetical which were highly responsive to growth on high molecular weight organic matter, suggesting *Microcystis* may have unique capabilities to use organic compounds for nutrition. These findings emphasize the need for future genetic function studies such as insertional mutagenesis (Alberts et al., 2002), as employed by Pearson et al. (2004) studying the function of the microcystin transporter *mcyH*, to characterize the role of these hypothetical genes.

The development and increasing availability of high-throughput sequencing technologies has made it possible to generate read libraries containing millions of sequences, well-beyond the scale of traditional clone libraries. The application of this technology to *Microcystis* blooms has thus far been limited to understanding the relationships between bacteria associated with bloom-forming organisms and environmental conditions (Tang et al., 2010; Wilhelm et al., 2011; Dziallas and Grossart, 2012; Parveen et al., 2013). Extension of these pursuits to functional gene libraries will provide new insights into how bloom communities transport and metabolize nutrients and interact with fluctuating environmental conditions, possibly even revealing the ecological mechanisms promoting bloom formation.

The use of both targeted and global approaches are useful tools for gaining insight as to why *Microcystis* dominates when and where it does and which factors may be most important in controlling toxin production. Advances in sequencing technology have allowed for higher resolution investigations into the unique genetic capability of this organism. Challenges remain due to the highly plastic and mosaic nature of the *Microcystis* genome and the large portion of

predicted genes that remain uncharacterized. Further, methods employed have yet to be standardized leading to difficulty when comparing results. Future effort in this regard is needed, a strong focus should be given to understanding the purpose of microcystin production in *Microcystis*, a central debate due to its toxicity to humans and animals (See Toxin Section).

Phylogeny

The genus *Microcystis* is characterized morphologically by highly buoyant, unicellular, coccoid-shaped cells with a diameter ranging between 1-9 μm (Komárek and Komárková, 2002). It's defining feature, and primary basis for species delineation, is that it exhibits a variety of colonial morphologies consisting of dense aggregations of cells under natural environmental conditions (Figure 2). There are over a dozen recognized *Microcystis* 'morpho-species'. The most commonly observed variants appear to be *M. aeruginosa*, *M. botrys*, *M. firma*, *M. flos-aquae*, *M. ichthyoblabe*, *M. natans*, *M. novacekii*, *M. panniformis*, *M. smithii*, *M. viridis*, and *M. wesenbergii* (Figure 2; Komárek and Komárková, 2002). There is concern, however, that species designations have been overprescribed and that single strains can exhibit multiple morphological characteristics in response to environmental or physiological stimuli (Yang et al., 2006). Based on the established standard that DNA-DNA hybridization (DDH) greater than 70% between two bacteria delineates them as likely belonging to the same species (Wayne et al., 1987), Otsuka and colleagues (2001) proposed the unification of five species of *Microcystis* (*aeruginosa*, *ichthyoblabe*, *novacekii*, *viridis*, and *wesenbergii*) under the formal name '*Microcystis aeruginosa* (Kützing) Lemmermann 1907', with isolate NIES-843 serving as the type strain for this species complex. In their study, DDH was greater than 70% for all species tested, with the two isolates classified as *M. aeruginosa* species actually displaying the lowest similarity (Table 1). Similarly, Kondo and colleagues (2000) used DDH to study nine different strains of

Microcystis identified as *M. aeruginosa*, *M. viridis* and *M. wesenbergii* and all strains exhibited greater than 70% DNA relatedness, providing further evidence for the unification of these species.

With the advent of high-throughput DNA sequencing, it is now tenable to compare microbial genomes *in silico*. The average nucleotide identity (ANI) of conserved genes from two strains of bacteria has been demonstrated to be as robust as DDH for delineating species when using a cut-off for delineation of 95-96% identity or greater (Goris et al., 2007). This metric is also slowly replacing the use of 16S rRNA comparisons to infer phylogeny because it is based on a larger sample of genetic information. Recent studies now suggest that when using 16S rRNA gene sequences to infer phylogeny, the cut-off to distinguish one species from another should be raised from 97% to 98.7% or greater (Stackebrandt and Ebers, 2006; Kim et al., 2014). For this review, ANI alignments were performed on all *Microcystis* genomes sequenced to date using the following parameters: 700 bp minimum length, 70% minimum identity, 50% alignment minimum and fragment options were set to a window size of 1000 bp and step size of 200 bp. Comparisons of the 16S rRNA locus (1489 bp) were made with *M. aeruginosa* NIES-843 as the type strain. Table 1 displays the ANI and 16S rRNA gene sequence similarity of all *Microcystis* genomes sequenced to date and their 16S rRNA gene identity relative to the first fully sequenced *M. aeruginosa* genome and type strain NIES-843, along with additional *Microcystis* strains which have been investigated although not fully sequenced. For comparison, the genomes of other unicellular, but non-*Microcystis* genera are provided. Based on these outlined assumptions, all *Microcystis* species whose genomes or 16S rRNAs have been sequenced to date warrant placement into the same species complex since all ANI values exceeded 95%, 16S identity

scores always exceeded 99%, and DNA-DNA hybridization were consistently greater than 70% (Table 1).

An analogy can be drawn from the bacterial systematics used to characterize *Escherichia coli*. Whole genome sequencing of a number of *E. coli* isolates suggests that the core genome for this species is approximately 47% shared across all strains, and that specific pathovars, such as those inducing uropathogenic or enterohaemorrhagic symptoms, are due to laterally acquired genes/plasmids (Welch et al., 2002; Rasko et al., 2008). In this vain, it is likely that if all *E. coli* strains did not share similar morphological characteristics, then there would be far more species groups assigned to this genus. Similar to *E. coli*, a recent genomic comparison of 12 different strains of *M. aeruginosa* indicated that only about half the genome of a given strain consists of a shared core set of genes (~2,462 core genes, $5,085 \pm 749$ total genes; Humbert et al., 2013). The remainder of each *M. aeruginosa* genome was comprised of genes shared among some but not all of the strains, including a variety of non-ribosomal peptide synthetases (NRPS) and polyketide synthases (PKS) such as those involved in microcystin biosynthesis (See Toxins section) among other genes, and collectively the flexible pangenome appears to be very diverse, consisting of over 12,000 genes identified in only these 12 strains (Humbert et al., 2013). In this context, any two strains of *Microcystis* may exhibit vastly different morphological, physiological or ecological characteristics, likely due to accrued mutations or rearrangements in core genes or variation in the flexible genes they possess owing to widespread horizontal gene transfer. Yet fundamentally at their core, they share the same genes that ultimately identify *Microcystis* as distinct from other bacteria. Hence, they should be placed within the same species complex. The extent to which gene rearrangements and DNA methylation patterns may be influencing *Microcystis* strain ecology and function is unclear. Genome architecture (synteny) between

strains may be considerably different, even if gene content is shared, owing to the diverse array (10% or more of the total genome) of transposable elements and insertion and repeat sequences (Kaneko et al., 2007; Frangeul et al., 2008). As previously mentioned (above), until recently, only one strain of *Microcystis* had been fully sequenced and its genome closed (NIES-843). Using Pac Bio RS II long read sequencing a second strain of *Microcystis aeruginosa* (NIES-2549) has been recently sequenced and its genome closed (Yamaguchi et al., 2015). The completion of this second genome enables, for the first time, a true assessment of genome synteny (i.e., gene order/arrangement) in *Microcystis*. Using the bioinformatics program Gepard (GEnome PAir - Rapid Dotter; Krumsiek et al., 2007) the genome synteny was assessed for these two strains, and for comparison, the genome synteny analysis originally provided by Novichkov et al. (2009) was recreated in order to illustrate the five different patterns of genome rearrangement presently recognized to occur in prokaryotes (Figure 3). This assessment clearly demonstrates that *Microcystis* retains almost zero genome synteny, a finding in stark contrast to the synteny values of 68-86% reported elsewhere (Humbert et al., 2013) that relied only upon relatively short contiguous fragments of draft genomes. Considering that these two *Microcystis* isolates were both classified as *M. aeruginosa*, exhibited 99.66% 16S rRNA identity, shared 3,342/4,282 coding sequences (CDS), exhibited an ANI of 95.95%, and were both isolated from the same water body (Lake Kasumigaura, Japan), it is anticipated that all closed *Microcystis* genomes will exhibit a similar decay pattern in genome synteny. Furthermore, evidence for active genome rearrangement was recently observed for transposase genes that exhibit differential transcription patterns in response to nutrient availability in culture (Steffen et al., 2014a) and in environmental samples (Harke et al., 2015; Steffen et al., 2015). Indeed, these observations imply that regional heterogeneities in drivers of transposable element activity may

lead to localized evolution of genetically similar populations due to rearrangements (or gains / losses) in genomic architecture: in some ways a microbial manifestation of the island theory of biogeography (MacArthur and Wilson, 1967). As in the previous example, the rise of long read sequencing (e.g., Pac Bio RS) that is becoming commonplace in bacterial genomics should help to further resolve these questions.

Whether or not all *Microcystis* morpho-species should be placed within a single *M. aeruginosa* complex could be construed as a purely esoteric question, but doing so could provide additional applied benefits for the scientific, research, and managerial communities. For example, it would simplify the task of microscopic identification and enumeration for public health purposes and remove much of the subjectivity inherent to each taxonomist. More importantly, such a unification of *Microcystis* morpho-species would also counter the widespread belief that certain cyanobacterial species are universally toxic or nontoxic. For example, *Microcystis wesenbergii* and *Aphanizomenon flos-aquae* are frequently cited by water quality managers as being nontoxic species despite documented reports to the contrary (Yoshida et al., 2008).

Ecology

Nutrients

Traditionally, P input reductions have been the focus for controlling cyanobacterial blooms based on the premise that N supplies can be met by N₂ fixation (Schindler et al., 2008). An important distinction between the genus *Microcystis* and several other major bloom forming cyanobacterial genera (e.g., *Dolichospermum/Anabaeana*, *Aphanizomenon*, *Cylindrospermopsis*, *Nodularia*) is that the former is incapable of supplying its N requirements *via* N₂ fixation, while

the latter are capable of doing so (Carr and Whitton, 1982; Potts and Whitton, 2000). This distinction has important ecophysiological and nutrient management ramifications, because growth and proliferation of *Microcystis* are exclusively reliant on either external N sources generated by various human activities (whose natural occurrence can be markedly augmented), including agriculture, urbanization, and industrial pollution or internal regeneration of combined N forms (largely ammonium). While P input controls are still very much at the center of bloom management strategies, an increasing number of freshwater ecosystems are now experiencing expanding blooms of non-N₂ fixers like *Microcystis* and/or *Planktothrix*, despite having such controls in place. This suggests that anthropogenic N inputs play a role in the global proliferation of these organisms (Paerl et al., 2014a). Indeed, in numerous eutrophic systems experiencing both spatial and temporal expansions of *Microcystis* blooms (e.g., Lakes Taihu-China, Erie-USA/Canada, Okeechobee-Florida/USA, Ponchartrain - Louisiana), it has been shown that N enrichment plays a key role in bloom proliferation (Paerl and Huisman, 2009; Chaffin and Bridgeman, 2014; Paerl et al., 2014a; Paerl et al., 2015). Overall, the world-wide proliferation of *Microcystis* appears closely linked to increases in both P and N loading from expanding human activities (Paerl, 2014). This conclusion confirms the changing nutrient limitation paradigm, where N and P co-limitation (and hence the need for N and P nutrient inputs controls) is much more common than previously thought, especially in eutrophic waters (Dodds et al., 1989; Elser et al., 2007; Lewis and Wurtsbaugh, 2008; Conley et al., 2009; Lewis et al., 2011; Paerl et al., 2014b).

Several studies have indicated that, among biologically-available forms of N, reduced N (as ammonium) is generally preferred over oxidized N (nitrate/nitrite) by *Microcystis* as well as other bloom forming taxa (Blomqvist et al., 1994; Hyenstrand et al., 1998; Flores and Herrero,

2005). Therefore, eutrophic freshwater ecosystems that contain relatively high concentrations of reduced N may have a tendency to favor cyanobacterial blooms. This, combined with the fact that most eutrophic systems are highly turbid and potentially light-limited, will favor cyanobacterial blooms that can regulate their buoyancy and vertically migrate in order to access nutrient rich bottom waters (i.e., by sinking) and optimize utilization of radiant energy (by floating as buoyant surface blooms). *Microcystis* is particularly adept at using such a strategy, especially during thermally-stratified summer bloom periods, when bottom waters will be relatively enriched with reduced N, while near-surface irradiance is maximal and reduced N inventories may be depleted.

The ability to migrate vertically also optimizes access to biologically-available P. *Microcystis* is extremely effective in sequestering sources of P, even at low concentrations (Jacobson and Halmann, 1982; Kromkamp et al., 1989; Sbiyyaa et al., 1998; Baldia et al., 2007; Saxton et al., 2012). This strategy is particularly effective in eutrophic, turbid, shallow water systems in which *Microcystis* can rapidly migrate between P-rich bottom sediments and take advantage of periodic sediment resuspension due to wind-mixing. By rapidly adjusting its buoyancy depending on photosynthetic CO₂ fixation versus nutrient acquisition needs, *Microcystis* can maintain dominance. *Microcystis* is capable of intracellular storage of P (polyphosphate bodies), enabling it to survive during periods of P deprivation (Carr and Whitton, 1982) and it is also capable of collecting P on its exterior surface (Saxton et al., 2012). Moreover, *Microcystis* has been shown to upregulate genes to synthesize high-affinity phosphate transporters and alkaline phosphatases that allow it to persist under low P conditions (Harke et al., 2012, Harke and Gobler, 2013). In summary, *Microcystis* is exceptionally good at accessing both N and P via a variety of cellular mechanisms, including buoyancy regulation, cellular

storage, high affinity transporters, and coloniality, which both enhances buoyancy and plays a pivotal role in developing close associations with other microbes, including heterotrophic bacteria, and a range of protozoans (Paerl, 1982).

Despite the fact that *Microcystis* is capable of extracting N and P over a wide range of ambient concentrations, members of this genus do exhibit periods of nutrient limitation, when ambient nutrient levels fall well below saturation. In highly eutrophic Taihu, China (Taihu means “large lake” in Mandarin), where *Microcystis* blooms can account for more than 80% of total phytoplankton community biomass, *in situ* microcosm and mesocosm bioassays indicate that the lake exhibits P limitation during early phases of the blooms, while N limitation characterizes summer bloom conditions (Xu et al., 2013). In most instances combined N and P additions provide the greatest biomass yields (Paerl et al., 2014b; Paerl et al., 2015). This pattern appears to also be present in Lake Erie (Chaffin and Bridgeman, 2014) and Lake Okeechobee (Havens et al., 2001). These results strongly argue for dual nutrient (N and P) input reductions as a best overall bloom control strategy (Paerl et al., 2014a; Paerl et al., 2015).

It has been proposed that reducing N inputs under elevated P conditions may lead to replacement of non-N₂ fixing cyanobacteria such as *Microcystis* with N₂ fixing cyanobacterial bloom species such as *Dolichospermum/Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Nodularia* (Schindler et al., 2008; Schindler, 2012). This possibility has recently been tested using *in situ* mesocosms in Lake Taihu (China) by enriching summer cyanobacterial bloom communities dominated by *Microcystis* with P (as PO₄³⁻) without adding dissolved inorganic nitrogen (DIN; to enhance N limitation) while ensuring sufficient supplies of iron (Fe) and other trace metals. Incubations of up to a month under these conditions failed to induce succession of N₂ fixers over *Microcystis* and no significant increases in N₂ fixation were reported (Paerl et al.,

2014b). In fact, net increases in *Microcystis* biomass were observed during the course of the experiment. This indicated that *Microcystis* was able to effectively compete with N₂-fixing taxa under conditions highly favorable for N₂ fixation (Paerl et al., 2014b). In summary, these findings argue for increased attention to dual nutrient input constraints to deplete the lake of previously loaded nutrients. Once the overall phytoplankton biomass is reduced by these measures, it may be possible to shift to a more P-oriented control strategy, although in more eutrophied ecosystems this may take years to decades to accomplish (Paerl et al., 2014b).

Physical factors

Physical factors, including irradiance, temperature, turbulence, vertical mixing and hydrologic flushing have all been implicated in the potential control of *Microcystis*-dominated blooms (Paerl, 2014). Adequate irradiance is of fundamental importance for maintaining optimal rates of photosynthesis. *Microcystis* colonies exhibit physiological strategies aimed at optimizing photosynthetic production in the highly turbid systems that characterize eutrophic waters during bloom conditions. First and foremost is its ability to regulate buoyancy through the formation and collapse of intracellular gas vesicles (Walsby et al., 1997). When cells are deplete in photosynthate (*i.e.*, following periods of darkness or poor irradiance conditions), cell turgor pressure decreases and gas vesicles can readily form, making cells buoyant. This enables colonies to rise to water surfaces, where photosynthetic rates can be optimized. *Microcystis* is also capable of producing carotenoid and other photoprotective pigments (Paerl et al., 1983), allowing for efficient access to light while minimizing photo-inhibition and photo-oxidation (Paerl et al., 1985). Once photosynthetic needs have been met, the buildup of cellular photosynthate (*i.e.*, ballast) leads to increased cell turgor pressure, causing gas vesicles to collapse and decreasing buoyancy. Using these oscillating processes, cells can optimize

photosynthetic production during the day, while accessing hypolimnetic nutrient pools at night (Walsby et al., 1997).

In many instances, buoyancy compensation by *Microcystis* can overcome light to moderate wind mixing, which enables it to remain in surface waters more readily than other bloom forming taxa that it may be competing with. For example, in Taihu, China, highly buoyant *Microcystis* colonies maintain strong dominance in surface waters during N-limited summer conditions, despite the fact that N₂-fixing genera (e.g., *Dolichospermum/Anabaena*, *Aphanizomenon*) are present during this period. This superior buoyancy and the ability to thrive on regenerated N may contribute to this dominance over radiant energy demanding diazotrophs during N-limitation (Paerl, 2014).

It is well known that vertical stability through stratification and long water replacement times favor cyanobacteria over eukaryotic phytoplankton (Reynolds et al., 1981; Reynolds, 2006); hence, disruption of these conditions can, under certain circumstances, modulate bloom dynamics. Vertical mixing devices, bubblers and other means of destratification have proven effective in controlling *Microcystis* blooms in relatively small lakes and ponds (Visser et al., 1996). However, these devices have limited applicability in large lake, estuarine and coastal waters, because they cannot exert their forces over large areas and volumes (Paerl, 2014).

Increasing flushing rates, i.e., decreasing water residence times or water ages, can also be effective in reducing or controlling bloom taxa; mainly because cyanobacteria exhibit relatively slow growth rates, relative to eukaryotes (Butterwick et al., 2005; Paerl and Otten, 2013). Horizontal flushing, by increasing the water flow, can reduce the time for cyanobacterial bloom development (Mitrovic et al., 2006). While this approach can suppress blooms, inducing these hydrologic changes can be quite expensive and depend on the availability of freshwater supplies

for flushing purposes. Furthermore, water quality managers must ensure that the flushing water is relatively low in nutrient content, so as not to worsen the enrichment problem, especially in long residence time large water bodies, which can retain nutrients and hence have a long “memory” for nutrient inputs. For example, in hypereutrophic Taihu, efforts to reduce *Microcystis*-dominated blooms by flushing this large lake with nearby Yangtze River water reduced the overall residence time in the lake from approximately one year to 200 days, but have not had a significant impact on reducing bloom intensity or duration (Qin et al., 2010). Yangtze River water is exceedingly high in biologically available N and P compounds, making it a nutrient source for further eutrophication. The inflow pattern of Yangtze River water has altered the circulation pattern of Taihu, trapping blooms in the lake’s northern bays, where they were most intense to begin with (Qin et al., 2010). Lastly, few catchments have the luxury of being able to use precious water resources normally reserved for drinking or irrigation for flushing purposes. This is especially true of regions susceptible to extensive droughts (e.g. Australia, Western USA).

Climatic changes, including rising global temperatures, increasing CO₂ levels, altered precipitation patterns, and resultant changes in freshwater discharge or flushing rates have synergistically influenced *Microcystis* bloom dynamics (Paerl and Paul, 2012). Warmer temperatures favor cyanobacterial blooms over eukaryotic phytoplankton taxa because growth rates of the former are optimized at relatively high temperatures (Jöhnk et al., 2008; Paerl and Huisman, 2008, 2009). In addition, warmer global temperatures and changes to precipitation patterns have led to the earlier onset of and longer lasting conditions favoring cyanobacterial blooms (Paerl and Huisman, 2008; Paul, 2008; Paerl and Huisman, 2009; Michalak et al., 2013). Intensification of vertical stratification (Paerl and Huisman, 2009) in combination with

eutrophication also appears to be particularly favorable for development and persistence of *Microcystis* blooms (Jöhnk et al., 2008). With regards to CO₂ levels, *Microcystis* is known to have both high- and low-affinity bicarbonate uptake systems as well as two CO₂ uptake systems (Sandrini et al., 2014). At high pCO₂, *Microcystis* uses its low-affinity bicarbonate uptake systems and increases biomass as well as increasing cellular chlorophyll *a* and phycocyanin content, raising PSI/PSII ratios, and decreasing overall dry weight and carbohydrate content which may improve buoyancy (Sandrini et al., 2015). Steffen et al. (2015) showed that *Microcystis* transcribed its carbon concentration mechanism genes (ccm) at sites across the Western Basin of Lake Erie and proposed that conditions of dense algal biomass, with resultant high-pH and CO₂ limitation, further promote cyanobacterial dominance. *Microcystis* appears to be well adapted to high or low CO₂ concentrations (Sandrini et al., 2015), characteristics that likely permit it to continue to dominate blooms, even as CO₂ concentrations are drawn-down to low levels. Transitions of CO₂ in lakes today due to algal bloom formation and demise (Balmer and Downing, 2011), however, far exceeds anthropogenic changes that will be produced in the future from atmospheric CO₂. Further, the response of other freshwater phytoplankton to changing CO₂ levels has been poorly studied. As such, there remain significant unknowns regarding how rising levels of atmospheric CO₂ will affect future *Microcystis* blooms.

Grazing

The ability of any algal group to form blooms is related to its ability to outgrow competitors and avoid routes of mortality. In aquatic ecosystems, mortality is generally attributed to top-down ecological controls such as grazing and viral lysis (Sunda et al., 2006; Smayda, 2008). *Microcystis* has been shown to experience lower rates of mortality than other algae via grazing by zooplankton and bivalves (Vanderploeg et al., 2001; Wilson et al., 2006). Among the

zooplankton, larger grazers including daphnids and copepods, are generally less capable of grazing *Microcystis* than smaller protozoan species (Fulton and Paerl, 1987; Gobler et al., 2007). While early studies predicted that grazer inhibition may be related to synthesis of microcystin (Arnold, 1971; Fulton and Paerl, 1987; Rohrlack et al., 1999; DeMott et al., 2001), multiple lines of evidence demonstrate this is not the case. Rantala et al. (2008) found that the evolution of microcystin synthesis significantly predated that of metazoans and thus suggested the toxin did not evolve as a grazing deterrent. Meta-analyses of laboratory studies have concluded that while *Microcystis* reduces zooplankton population growth rates, the effects are typically not related to microcystin content of cultures (Wilson et al., 2006; Tillmanns et al., 2008; Chislock et al., 2013). Within an ecosystem setting, Davis and Gobler (2011) quantified grazing rates by multiple classes of zooplankton on toxic and non-toxic strains of *Microcystis* in two ecosystems and found that both microzooplankton and mesozooplankton were capable of grazing both toxic and nontoxic strains with similar frequencies and rates. Incongruence in culture grazing experiments may be due to differential production of other, non-microcystin, secondary metabolites that have not been considered in previous studies.

Beyond microcystin, there is evidence that *Microcystis* colony formation and synthesis of other potential secondary metabolites can act as grazing deterrents. Studies have reported that larger colonies of *Microcystis* are poorly grazed, particularly by smaller crustacean zooplankton (de Bernardi and Giussani, 1990; Wilson et al., 2006), and Yang et al (2006) reported on a strain of *Microcystis* that transformed from uni-cellular to colonial in direct response to small, flagellated zooplankton grazers that could not consume the colonies. Many studies have concluded that *Microcystis* may be a nutritionally inadequate food source for zooplankton (Wilson et al., 2006) and the ability of *Microcystis* to synthesize protease inhibitors such as

aeruginosin and cyanopeptolin may both prohibit digestion of cells and discourage zooplankton grazing (Agrawal et al., 2001; Agrawal et al., 2005).

Outbreaks of *Microcystis* blooms in some lakes in the United States appear to be stimulated in part by the arrival of recently established zebra mussel (*Dreissena* sp.) populations (Vanderploeg et al., 2001; Raikow et al., 2004) and this correlation may be linked to the trophic status of lakes (Sarnelle et al., 2005). While zebra mussel invasions of new ecosystems typically result in significant reductions in all plankton biomass due to intense filter feeding (Caraco et al., 1997), *Microcystis* cells consumed by zebra mussels are typically rejected as pseudofeces from which cells can emerge and regrow (Vanderploeg et al., 2001). Given the ability of zebra mussels to consume both phytoplankton and zooplankton (Jack and Thorp, 2000; Higgins and Zanden, 2010; Kissman et al., 2010), *Dreissena* invasions also effectively eliminate competitors and predators of *Microcystis*. Further, zebra mussels may alter ambient nutrient regimes to favor *Microcystis*. Zebra mussels can increase concentrations of dissolved organic phosphorus (DOP; Heath et al., 1995), and under low P loads zebra mussels may promote *Microcystis* blooms (Sarnelle et al., 2005; Bykova et al., 2006), perhaps via regeneration of organic P given *Microcystis* has the ability to grow efficiently on DOP using alkaline phosphatase (Harke et al., 2012).

Although ecological and evolutionary processes are traditionally assumed to occupy different timescales, a wave of recent studies has demonstrated overlap and reciprocal interplay of these processes (Thompson, 1998; Carroll et al., 2007; Hendry et al., 2007; Post and Palkovacs, 2009). For example, multiple studies have found that a diverse array of zooplankton that are regularly exposed to dense *Microcystis* blooms are generally more adept to grazing on and growing during blooms than naïve populations that do not encounter *Microcystis*. This

suggests a genetic shift occurs in wild zooplankton populations towards populations able to graze *Microcystis* (Hairston et al., 1999; Sarnelle et al., 2005; Davis and Gobler, 2011; Chislock et al., 2013). While filter feeding bivalves may ultimately also be capable of such adaptation (Bricelj et al., 2005), this possibility has yet to be explored.

Microbial Interactions

As a largely colonial bloom forming genus, *Microcystis* has numerous complex interactions with both bacteria and protists (protozoans, microalgae, fungi; Paerl, 1982; Paerl and Millie, 1996; Shen et al., 2011; Shao et al., 2014). These interactions can be both intimate, such as is the case of microbes attached to or existing within *Microcystis* colonies, or more diffuse for microbes co-occurring in time and space. Regarding bacteria associated with colonies, during the decline of a *Microcystis* bloom, Parveen et al. (2013) found colonies to be depleted in *Actinobacteria*, but enriched in *Gammaproteobacteria* and changes in temperature may shape associated bacterial communities (Dziallas and Grossart, 2012). While many of the functional roles of *Microcystis*-bacterial associations remain unknown, it is clear that such associations can be both mutually beneficial as well as antagonistic with regard to their effects on growth potentials, viability and mortality of *Microcystis* and associated microbes. It has been noted that photosynthetic performance and growth rates of epiphytized *Microcystis* cells and colonies are often higher than bacteria-free or axenic cultures (Paerl, 1982; Paerl and Millie, 1996), indicating a mutualistic, if not symbiotic properties of such associations. Paerl and Millie (1996) speculated that while heterotrophic bacteria associated with bloom-forming cyanobacteria (e.g., *Dolichospermum/Anabaena*, *Microcystis*) clearly benefitted from the organic matter produced by the cyanobacteria, the cyanobacteria benefitted from organic matter decomposition, CO₂ production, and nutrient (N, P, Fe and trace metals) regeneration provided by associated

heterotrophs, which can include bacteria and protozoans. Amoeboid protozoans have also been found actively grazing *Microcystis* cells inside colonies (Paerl, 1982). While grazed *Microcystis* cells clearly result in a loss of cyanobacterial biomass, ungrazed cells in these colonies displayed higher rates of photosynthetic growth than cells in colonies that were not grazed by the protozoans (Paerl and Millie, 1996). This suggested that nutrient recycling associated with grazers may have benefitted those cells that escaped grazing, indicating a positive feedback of grazers on “host” colonies (Paerl and Millie, 1996; Paerl and Pinckney, 1996). The extent to which microcystins and other secondary metabolites produced by host *Microcystis* colonies play a role in establishing and mediating such mutually-beneficial associations remains unknown, but this is an important area for research into biotic factors mediating cyanobacterial blooms in aquatic ecosystems.

Over the last two decades, several groups have demonstrated the ability of heterotrophic bacteria to degrade microcystins (Bourne et al., 1996; Cousins et al., 1996; Park et al., 2001). Since the initial characterization of this process (Bourne et al., 1996; Bourne et al., 2001), organisms capable of microcystin degradation have been identified in blooms worldwide, including lakes in North America (Mou et al., 2013), Asia (Park et al., 2001; Saito et al., 2003; Zhu et al., 2014), Oceania (Bourne et al., 2001; Somdee et al., 2013), South America (Valeria et al., 2006), and Europe (Berg et al., 2008). This relatively recent discovery may have important implications for biological management of toxic blooms in freshwater systems (Ho et al., 2006; Ho et al., 2007).

Interactions with viruses

The presence of viruses in environmental samples dates to the initial observations and independent discovery of bacteriophage by Twort (1915) and d’Herelle (1917). Since that time

there have been recurring observations of the potential role of viruses as mortality factors for different populations including a variety of freshwater microbial populations (Wommack and Colwell, 2000; Wilhelm and Matteson, 2008).

Indeed, viruses that may constrain cyanobacterial blooms have long been a “holy grail” for microbial ecologists (Safferman and Morris, 1963, 1964). Indeed much of the early work in virus ecology was dedicated to the idea that bacteriophage might be used to mitigate or even control harmful cyanobacterial bloom populations in the environment (Safferman and Morris, 1964). Chief amongst these efforts, the study of two virus types (LPP-1 and SM-1) were of interest, especially as the later was reported to include the bloom-producer *Microcystis aeruginosa* NRC-1 amongst putative hosts (Safferman and Morris, 1967).

One of the major conclusions of early microbial-viral research was that there was rapid selection for resistant phenotypes of cyanobacteria in the environment. This rapid selection has been considered one of several models of the ongoing evolutionary race between viruses and their hosts: a concept described by the “Red Queen Theory” (Van Valen, 1973) where hosts continually evolve to become resistant to infection and viruses must continue to adapt to infect the population. Like the character of the Red Queen in *Through the looking-glass and what Alice found there* (Carroll, 1917) who states “it takes all the running you can do, to keep in the same place”, viruses and hosts continue to be selected for in a manner that makes their applied use for bloom control, at best, difficult. However, recent efforts point to components of viruses (e.g., lysins) as future targets for the biological control of *Microcystis* blooms, although such efforts will require significant research before they can be realized.

Studies on the potential impact of viruses on *Microcystis* remained at an effective standstill until a virus particle infecting multiple strains of *Microcystis* was described (Tucker and

Pollard, 2005; Yoshida et al., 2006). As part of this effort, Yoshida et al. (2006) sequenced the genome of a virus (Ma-LMM-01), and subsequently (along with other research groups) designed PCR and qPCR primers to study the viruses in various natural systems (Takashima et al., 2007a; Takashima et al., 2007b; Yoshida et al., 2007; Yoshida et al., 2008; Yoshida et al., 2010; Rozon and Short, 2013). Although distributed at high abundances (e.g., over 250,000 per ml) in the Bay of Quinte (Lake Ontario; Rozon and Short, 2013) and consistently detected in the presence of blooms, the virus does not appear to cause senescence of dense blooms. This is also apparent from recent metatranscriptomic studies. Steffen et al. (2015) demonstrated ongoing phage infections of *Microcystis* (based on the presence of virus-specific gene transcripts) in the face of relatively dense *Microcystis* populations, whereas Harke et al. (2015) observed upregulation of phage defense genes in *Microcystis* populations in Lake Erie, USA, in response to P-loading. In a recent survey of more than 1,000 genomes, *Microcystis* was found to contain 80% more defense genes than *Cyanothece* PCC 8802 or *Roseiflexus* RS-1 (the next highest) with 29% of its genome assigned to defense islands (Makarova et al., 2011). Furthermore, the presence of a large diverse number of CRISPR (clustered regularly interspaced short palindromic repeats) spaces within the *Microcystis* NIES-843 genome suggests this cyanobacterium is frequently exposed to viruses (Kuno et al., 2012).

Conclusions

Toxic blooms of *Microcystis* continue to plague eutrophic waters worldwide, and despite decades of research, many questions remain. The occurrence of toxic blooms of *Microcystis* appears to be expanding, with 108 countries or territories around the world having documented toxic blooms, whereas previous documentation identified fewer than 30 countries (Zurawell et al., 2005). This may be due to increased monitoring efforts, but also illustrates a need for further

efforts to curb eutrophication of freshwater resources. This review highlights the great diversity of microcystins produced by *Microcystis*. Despite several decades of research, the physiological basis for microcystin production in *Microcystis*, and the variables that regulate its biosynthesis, remains a contentious and debated question. Collectively, the studies reviewed herein suggest microcystins might be regulated by multiple variables. They also indicate that the toxin could have several ecological functions for *Microcystis*, or that microcystin may be a regulatory molecule linked to many cellular processes. To date, most studies have been undertaken in the laboratory providing essential knowledge, however, often only one parameter was changed while others were maintained at optimal levels. There is a pressing need for results of laboratory-based studies to be validated in the field and for more multi-parameter investigations. The advent of omics provides an exciting new avenue to explore the genetic basis of toxin synthesis in complex environmental samples. Increased understanding of the regulation of microcystins in the environment may ultimately help in identifying the times of greatest toxicity and health risk.

Evidence is presented suggesting that all *Microcystis* warrant placement into the same species complex as ANI values were above 95%, 16S rRNA identity scores exceeded 99%, and DNA-DNA hybridization was consistently greater than 70%. Genomic analyses of *Microcystis* has provided significant insight into the ecology, physiology and factors influencing toxin production and have revealed the highly dynamic nature of its genome due to the great number of transposons. Challenges still remain due to the highly plastic nature of the *Microcystis* genome and the large portion of predicted genes that remain uncharacterized. Further, targeted and global genomics approaches employed have yet to be standardized leading to difficulty when comparing results. Nutrient loading is regarded as the primary driver of bloom formation. The precise nutrient remediation strategy to limit bloom formation remains the subject of

796 considerable debate. Increasingly, dual (N and P) reduction strategies are being prescribed for
797 eutrophic systems suffering from chronic blooms problems. This review provides evidence from
798 across the globe of the important role that both N and P have in controlling the dynamics of
799 *Microcystis* blooms, as well as the ability of elevated temperatures to promote these events. This
800 review also highlights the ability of *Microcystis* to minimize mortality losses during blooms due
801 to zooplankton, bivalve grazing, and viral lysis and discusses some of the factors facilitating
802 these trends. Studies on the potential impact of viruses on *Microcystis*, however, remain at an
803 effective stand-still and future efforts at bloom control with viruses or virus components will
804 require significant research before they can be realized.

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818

819 **Tables and Figures**

820 **Table 1** Phylogenetic comparisons from a variety of *Microcystis* species exhibit too low genetic diversity to warrant
821 their placement as separate species based on whole genome DNA-DNA hybridization (DDH) values greater than
822 70%, two-way average nucleotide identity (ANI) values greater than 95% or 16S rRNA sequence homology greater
823 than 98.7%.

Genus species	Strain	GenBank Assembly or Accession #	16S Identity (%) NIES-843	Genome ANI (%) NIES-843	ANI Fragments	[†] DNA- DNA (%) NIES-843
<i>Microcystis aeruginosa</i>	NIES-843 ●	NC_010296.1	100.00	100.00	29,201	100.0
<i>Microcystis aeruginosa</i>	NIES-2549	CP011304.1	99.79	95.95	10,491	NA
<i>Microcystis aeruginosa</i>	NIES-44	GCA_000787675.1	99.60	96.35	10,695	NA
<i>Microcystis aeruginosa</i>	DIANCHI-905 ●	NZ_AOC100000000.1	99.59	95.65	10,387	NA
<i>Microcystis aeruginosa</i>	PCC 7005	GCA_000599945.1	99.66	95.90	9,915	NA
<i>Microcystis aeruginosa</i>	PCC 7806 ●	AM778844.1-AM778958.1	99.72	95.64	10,408	NA
<i>Microcystis aeruginosa</i>	PCC 7941 ●	GCA_000312205.1	99.58	95.95	10,912	NA
<i>Microcystis aeruginosa</i>	PCC 9432	GCA_000307995.2	99.73	95.96	10,685	NA
<i>Microcystis aeruginosa</i>	PCC 9443 ●	GCA_000312185.1	99.66	96.16	10,927	NA
<i>Microcystis aeruginosa</i>	PCC 9701	GCA_000312285.1	99.73	96.34	10,539	NA
<i>Microcystis aeruginosa</i>	PCC 9717 ●	GCA_000312165.1	99.80	97.28	12,947	NA
<i>Microcystis aeruginosa</i>	PCC 9806	GCA_000312725.1	99.66	96.18	10,872	NA
<i>Microcystis aeruginosa</i>	PCC 9807 ●	GCA_000312225.1	99.93	95.80	11,503	NA
<i>Microcystis aeruginosa</i>	PCC 9808 ●	GCA_000312245.1	99.73	95.97	10,814	NA
<i>Microcystis aeruginosa</i>	PCC 9809 ●	NZ_CAIO00000000.1	99.73	98.57	15,062	NA
<i>Microcystis aeruginosa</i>	SPC-777 ●	NZ_ASZQ00000000.1	99.66	96.11	10,914	NA
<i>Microcystis aeruginosa</i>	Taihu-98	ANKQ01000001.1	99.46	96.02	11,102	NA
<i>Microcystis</i> sp.	T1-4	NZ_CAIP00000000.1	99.72	95.95	10,594	NA
<i>Microcystis aeruginosa</i>	TAC86 ●	AB012333.1	99.59	NA	NA	75.0
<i>Microcystis flos-aquae</i>	UWOCC C2	AF139328.1	99.65	NA	NA	NA
<i>Microcystis ichthyoblabe</i>	TC24	AB035550.1	99.66	NA	NA	80.7
<i>Microcystis novacekii</i>	BC18	AB012336.1	99.93	NA	NA	74.0
<i>Microcystis panniformis</i>	VN425	AB666076.1	99.58	NA	NA	NA
<i>Microcystis protocystis</i>	VN111	AB666054.1	99.86	NA	NA	NA
<i>Microcystis viridis</i>	CC9	AB035552.1	99.73	NA	NA	91.7
<i>Microcystis wesenbergii</i>	TC7	AB035553.1	99.59	NA	NA	89.7
<i>Microcystis wesenbergii</i>	NIES-107 ●	DQ648028.1	99.72	NA	NA	NA
<i>Aphanocapsa montana</i>	BDHKU210001	NZ_JTJD00000000.1	88.06	80.36	17	NA
<i>Cyanobium gracile</i>	PCC-6307	NC_019675.1	87.62	81.97	19	NA
<i>Gloeocapsa</i> sp.	PCC-7428	GCA_000317555.1	90.18	76.79	52	NA
<i>Gloeocapsa</i> sp.	PCC-73106	GCA_000332035.1	89.74	74.90	95	NA
<i>Synechococcus elongatus</i>	PCC-6301	AP008231.1	89.53	81.33	25	NA

● Denotes microcystin producer ; NA - Not Available ; [†] DNA-DNA hybridization data from Otsuka et al., 2001

Figure 1 The global occurrence of *Microcystis* blooms and microcystin as determined through literature searches for records of *Microcystis* blooms from 257 countries and territories. White indicate no record of bloom or microcystin, blue indicates a record was found for the occurrence of a bloom, and green indicates countries or territories where there was a record of both bloom and microcystin.

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830

831 **Figure 2** A) Satellite image of *Microcystis* bloom in Taihu, China. B) Bloom of *Microcystis* in Lake Agawam, New
832 York, USA, C and D) light microscope images of diverse colony morphologies.

Figure 3 Dot plot matrix comparing whole genome synteny for *Microcystis aeruginosa* (A) relative to five other genera of bacteria known to exhibit different genome rearrangement patterns as described by Novichkov et al., 2009. (B) Complete decay of genome synteny. (C) Absence of rearrangement. (D) Multiple inversions with limited transposition of genes. (E) Lack of inversions but hotspots for recombination, (F) Multiple inversions and transposition of genes/operons.

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